Viruses of salmonids: Virus isolation in fish cell lines

Mark St J Crane
AAHL Fish Diseases Laboratory
Australian Animal Health Laboratory
CSIRO Livestock Industries
Private Bag 24
Geelong VIC 3220
mark.crane@csiro.au

Lynette M Williams
AAHL Fish Diseases Laboratory
Australian Animal Health Laboratory
CSIRO Livestock Industries
Private Bag 24
Geelong VIC 3220
lynette.williams@csiro.au

SUMMARY

Salmonids are susceptible to infection with a range of viruses including Atlantic salmon reovirus (TSRV), infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV), infectious haematopoietic necrosis virus (IHNV), epizootic haematopoietic necrosis virus (EHNV), *Oncorhynchus masou* virus (OMV) and infectious salmon anaemia virus (ISAV). Apart from TSRV each of these viruses can cause mass mortalities in farmed salmonids and thus diagnosis is of critical importance.

A range of fish cell lines that can sustain growth of these viruses has been progressively developed. This has resulted in the most sensitive technique for diagnosis being a two-step procedure of isolation and growth of the virus in cell culture followed by identification of the virus by use of an immunocytochemical assay with specific monoclonal or polyclonal antibodies or by a nucleic acid-based assay such as in situ hybridisation (ISH) or polymerase chain reaction (PCR).

This document provides standards and guidelines for the isolation/detection of viruses, infectious for salmonid species, as part of i) a disease investigation, ii) health certification for export purposes, iii) health surveillance activities.

The information provided includes:

- details of the fish cell lines available
- the maintenance of fish cell lines used for isolation and replication of known salmonid viruses some of which are exotic to Australia and New Zealand, others are endemic
- testing the susceptibility of the fish cells to infection by salmonid viruses (QA)
- information, host range and geographic distribution, on the salmonid viruses of interest
- sample collection and processing details
- detailed procedure for the isolation of salmonid viruses using fish cell lines
- a series of appendices with detailed supporting information on the specific cell lines used, their virus susceptibility and culture conditions

Status of Australia and New Zealand: EHN is the only salmonid viral disease currently listed in the OIE *Aquatic Animal Health Code* that is present in parts of Australia.

Introduction

Australia and New Zealand are fortunate to be free from many of the serious viral diseases of salmonids, such as infectious pancreatic necrosis (IPN), infectious haematopoietic necrosis (IHN) and viral haemorrhagic septicaemia (VHS). These diseases are enzootic in some countries in the northern hemisphere and diminish aquaculture production. Viral diseases and infections that are enzootic in salmonids in Australia have restricted host and geographical ranges[1,2] and, accordingly, individual States have developed diagnostic capability commensurate with local needs. For those diseases that are exotic to Australia, however, all research and development of diagnostic procedures that requires the use of live virus is centralised at the Fish Diseases Laboratory at the Australian Animal Health Laboratory, CSIRO Livestock Industries, Geelong, Victoria.

With the aim of assuring the sanitary safety of international trade in aquatic animals and their products, the OIE lists a number of aquatic animal diseases in the *Aquatic Animal Health Code*.[3] Viral diseases of salmonids currently listed by the OIE include epizootic haematopoietic necrosis (EHN), infectious haematopoietic necrosis (IHN), viral haemorrhagic septicaemia (VHS) and infectious salmon anaemia (ISA).

EHN is the only viral disease of salmonids currently listed by the OIE that is present in parts of Australia.[4] Other viruses isolated from salmonids in Australia, natural or farmed, include Atlantic salmon (*Salmo salar*) reovirus from farmed Tasmanian Atlantic salmon,[5] and aquatic birnavirus.[2]

The ability to diagnose accurately those viral diseases that are exotic to Australia is of utmost importance not only to protect natural and farmed fisheries but also to maintain the confidence of our trading partners. Due to the increase in international trade and travel in recent years it is even more critical to develop accurate and reliable diagnostic procedures to ensure that the risk of introduction of exotic disease to Australia remains low.

The exotic nature of the major viral diseases of salmon requires that research and development of diagnostic techniques is undertaken at the microbiologically secure facilities at the AAHL Fish Diseases Laboratory (AFDL). Representative strains of VHSV, IHNV, OMV, ISAV and IPNV have been imported into AAHL to be used specifically for the development/establishment of diagnostic procedures.

This document describes the methods currently used at the AAHL Fish Diseases Laboratory for isolation of the salmonid viruses: Atlantic salmon reovirus, IPNV, VHSV, IHNV, EHNV, ISAV and *Oncorhynchus masou* virus (OMV). The methods are based on those outlined in the OIE *Manual of Diagnostic Tests for Aquatic Animals*.[6]

Table 1. Geographic distribution of salmonid viruses

	DISTRIBUTION	
VIRUS	AUSTRALIA	OVERSEAS
IPNV IPN-like viruses	Absent[1]	United States, Canada, Japan, South Korea, Taiwan, Thailand, China, United Kingdom, continental Europe, Iceland, Chile, South Africa
IHNV	Absent	United States, Canada, Japan, China, Taiwan, Italy, France, Belgium,
VHSV	Absent	United States, continental Europe, United Kingdom
OMV	Absent	Japan
Atlantic salmon reovirus	Tasmania	Other aquareoviruses have been isolated from a range of aquatic animals overseas (United States, Canada, Japan, China, Singapore, Thailand, Malaysia, France, Germany, Spain, United Kingdom). Since many of these are not associated with disease information is scant.
EHNV and other viruses of the genus *Ranavirus*	Victoria, New South Wales, South Australia	Related *Ranavirus* spp have been isolated from aquatic animals in Germany, France, Italy
ISAV	Absent	Norway, Scotland, Canada, United States

[1] In 1998, an aquatic birnavirus was isolated for the first time from Atlantic salmon farmed in Macquarie Harbour, Tasmania. Similarly, an aquatic birnavirus has been isolated from salmonids in New Zealand. The viruses are of low pathogenicity and are not considered IPNV.

Virus isolation on fish cell lines is the most sensitive technique for the detection of these important pathogens of salmonid species, and its use is of fundamental importance to the control of disease spread.[6] The technique is used in health surveillance programs, for virus exclusion, and in disease diagnosis.

Distribution (in Australia or overseas)

The geographic distribution of these viruses is shown in Table 1. The salmonid viruses, IPNV, IHNV, VHSV, OMV and ISAV are exotic to Australia, while EHNV is enzootic to parts of southeast Australia.[4] An aquatic birnavirus (non-pathogenic IPN-like virus) was isolated for the first time from Atlantic salmon farmed in Macquarie Harbour, Tasmania in 1998.[2] To date, its occurrence is restricted to Macquarie Harbour. Atlantic salmon reovirus was first isolated from farmed Atlantic salmon from southeast Tasmania in 1990 and continues to be isolated on a regular basis. In 2006, an orthomyxovirus (not ISAV) was isolated from farmed Atlantic salmon from Macquarie Harbour, Tasmania (Crane *et al*., unpublished results). Due to the recent nature of the isolation this virus has not been fully characterised. The other viruses are exotic to Australia.

The Viruses

This document describes salmonid virus isolation in fish cell culture and is not specific for any one virus. Detailed descriptions of diseases caused by the viruses discussed here is not appropriate but can be obtained from various texts.[7,8]

Table 2. The known host range for each of the specified viruses

VIRUS	HOST RANGE
IPNV IPN-like viruses	Wide host range including species from the families Clupeidae, Anguillidae, Esocidae, Salmonidae, Cyprinidae, Channidae, Cichlidae, Percidae, Percichthyidae, Poeciliidae, Gadidae, Pleuronectidae as well as several aquatic invertebrate species
IHNV	Several species of the genera *Oncorhynchus*, *Salmo*, *Salvelinus*
VHSV	Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O kisutch*), golden trout (*S aguabonita*), grayling (*Thymallus thymallus*), lake trout (*Salvelinus namaycush*), Pacific cod (*G macrocephalus*), Pacific herring (*Clupea harengus pallasi*), pike (*Esox lucius*), rainbow trout (*O mykiss*), sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), whitefish (*Coregonus* sp).
OMV	Species of the genus *Oncorhynchus* including kokanee salmon (*O nerka*), masou salmon (*O masou*), chum salmon (*O keta*), coho salmon (*O bismuth*) and rainbow trout (*O mykiss*).
Atlantic salmon reovirus	Atlantic salmon (*Salmo salar*). A large number of aquareoviruses have been isolated from finfish, molluscs and crustaceans.
EHNV and other *Ranavirus* spp	Natural infections in redfin perch (*Perca fluviatilis*) and rainbow trout (*O mykiss*). Macquarie perch (*Macquaria australasica*), mosquito fish (*Gambusa affinis*), silver perch (*Bidyanus bidyanus*) and mountain galaxias (*Galaxias olidus*) are susceptible by experimental infection. Similar iridoviruses isolated from sheatfish (*Silurus glanis*), catfish (*Ictalurus melas*) and from amphibian species.
ISAV	Natural outbreaks of ISA have been recorded in farmed Atlantic salmon only. Subclinical infections have been detected in wild Atlantic salmon, brown trout and sea trout (*S trutta*). ISAV has also been detected in two marine species, pollock (*Pollachius virens*) and cod (*Gadus morhua*)

The known host range for each of the specified viruses is shown in Table 2. The known host range for IPNV and IPN-like viruses is very broad and, as further information is obtained, is expanding.[9,10,11,12] In Australia, an aquatic birnavirus has been isolated from several fish species within Macquarie Harbour[2], including farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), wild flounder (*Rhombosolea tapirina*), cod (*Pseudophycis* sp), spiked dogfish (*Squalus megalops*) and ling (*Genypterus blacodes*).

In Australia, natural infections with EHNV have occurred in redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*). Macquarie perch (*Macquaria australasica*), mosquito fish (*Gambusa affinis*), silver perch (*Bidyanus bidyanus*) and mountain galaxias (*Galaxias olidus*) are susceptible to experimental infection by immersion.[13] Similar iridoviruses[14,15] have been isolated from sheatfish (*Silurus glanis*) in Germany,[16] catfish (*Ictalurus melas*) in France[17] and Italy, and from various amphibian species.[18]

Diagnostic Test

Fish cell lines

Isolation of fish viruses has been made possible by the development of standard fish cell lines derived from a number of fish species.[20] Each of the cell lines, some of which have been in use since the mid-1960s,[21,22] have been characterised with respect to their growth characteristics and other properties including their susceptibility to a wide range of viruses.[23] Appendix 1 lists the more common fish cell

lines used for isolation of finfish viruses, and summarises their properties. Appendix 2 lists finfish viruses and conditions for their isolation in fish cell cultures.

Many of these cell lines are used worldwide by laboratories involved in fish disease diagnosis and research. In addition, several of the lines have been nominated by importing countries as the required lines to be used for testing for freedom from specific diseases for export certification purposes. Thus virus isolation in fish cell lines is a fundamental technique used in health surveillance programs, virus exclusion tests as well as disease diagnosis. Appendix 3 lists fish cell lines recommended by various international authorities for use in virus isolation.

Selection of specimens

Specimens, whether they are for a disease investigation, export certification, or health surveillance, should be selected according to international standards. Guidelines are available from OIE[3] and the National Aquatic Animal Health Technical Working Group (NAAH-TWG).[24]

Collection of tissues for virus isolation

Collection of tissue samples should be supervised by the State fish pathologist and conform to international protocols,[3,25,26,27] and is summarised below.
Tissues or fluids from 5 or 10 fish (depending on the specific situation) may be pooled in one container containing transport medium (Appendix 4) at a ratio of 1 part tissue (weighing a minimum of 0.5 g) to 5 parts medium, representing one pooled sample. Pooled tissues in transport medium may be stored on ice but not frozen during transportation.

Storage of samples

Samples should not be frozen before processing but should be maintained between 4 and 10^{O}C (for example, shipped in a styrofoam shipping container with sufficient wet ice or ice bricks to maintain the samples at the required temperature for the total travel time). To maximise sensitivity, samples should be processed and assayed within 24 h of sampling but when this is not possible they must be processed within 72 h of sampling, during which storage must be at 2-5^{O}C. While it is not recommended, samples to be assayed after 72 h after collection should be frozen in the temperature range -70^{O}C to -80^{O}C.

Table 3. Sampling Regimens

# Fish in population	# Fish required in sample at a 2% infection level of the population	# Fish required in sample at a 5% infection level of the population
50	50	35
100	75	45
250	110	50
500	130	55
1000	140	55
2500-10000	145	60
≥100,000	150	60

Sample size

For disease diagnosis or virus exclusion, at least 10 moribund fish or fish exhibiting clinical signs of disease should be collected.

For health surveillance programs, Table 3 shows the number of fish that must be sampled in populations of various sizes to give a 95% confidence level of detecting a virus with an assumed minimum incidence of infection of 2% or 5%.[3,24] If the population size is estimated to be between two levels, then sampling must be carried out at the higher level. Because of the statistical nature of the sampling, failure to detect the presence of the specified viruses tested for in the sample does not guarantee absence of those viruses in the specimen examined or in the population from which the sample originated.

Sampling should be undertaken on a random basis but should include any freshly dead fish and any moribund fish.

Tissues to be examined

Tissues to be examined will be dependent on the size of fish in the population being tested, the time of year and whether sampling is for disease diagnosis, virus exclusion or health surveillance.[3,24] During spawning, reproductive fluids (preferably ovarian fluid but sometimes milt) should be used for testing, especially for health surveillance. Tissue samples obtained during non-spawning season will be either whole fry (for the current year class) or selected fish tissues (from older fish of previous year classes), collected aseptically. Samples for testing could include any of the following.

Fish size (length)	Tissue sample
< 4 cm	entire fish (remove yolk sac if present)
4-6 cm	entire viscera including kidney
> 6 cm	kidney, liver, spleen, encephalon, heart and gill filaments
sexually mature	ovarian fluids, kidney, liver, spleen, encephalon, heart and gill filaments

Design of a laboratory for isolation of viruses in fish cell lines

Ideally, there should be three separate work areas. One area or laboratory would be used to maintain stock cultures of fish cell lines in the absence of any viruses to ensure that the cell lines are not contaminated with any adventitious agent. Another laboratory would be used to maintain virus stocks and to check the susceptibility of cell lines to viruses. A third area would be used to carry out the actual testing. By keeping the testing area separate, there is no risk of obtaining false positive results. All areas should be maintained according to Australian Standard AS 2243.3.[28]

Maintenance of fish cell lines

Standard tissue culture methods are used in the routine maintenance of fish cell lines. A modern tissue culture facility providing a stable physical environment (adequate temperature, humidity and pH control) is required to maintain the cell lines in optimal condition. In addition, proficient aseptic technique, essential in a diagnostic facility, is required to ensure freedom from contamination by adventitious infectious agents.[29,30]

Tissue culture media developed for mammalian cell lines appear to provide the essential nutrients for the maintenance and growth of the standard fish cell lines. As is the case with mammalian cell lines, high quality foetal bovine serum (FBS), pretested for its ability to support the growth of specific cell lines, is an essential supplement to the medium. For more information, see Appendix 5.

Cryopreservation of fish cell lines

Cryopreservation techniques for the stable storage of fish cell lines in liquid nitrogen follow those developed for other vertebrate cell lines.[29]

Virus susceptibility testing of fish cell lines

Routine susceptibility testing is undertaken to ensure that the fish cell lines in current use are susceptible to the range of viruses of interest. This testing should show their susceptibility to infection and their ability to support virus replication as indicated by development of cytopathic effect (CPE) detected by light microscopic examination. Susceptibility checking is carried out by titrating stock viruses in the fish cell lines as described in Appendix 6. Stock viruses are maintained as detailed in Appendix 7.

Virus isolation

Detailed procedure for the isolation of viruses using fish cell lines are described in Appendix 8.

Briefly, pooled fish tissue samples are homogenised, clarified, diluted and inoculated onto duplicate monolayer cultures of fish cell lines prepared in 24-well cluster plates. Inoculated cultures are incubated in a controlled atmosphere at 15^{O}C for 7-10 days with monitoring by light microscopy. Following this initial incubation period, the samples are sub-cultured onto fresh cultures and incubated at 15^{O}C for a further time period so that the total incubation period is 21 days. The cultures are examined by light microscopy every 2-3 days for the appearance of viral CPE, such as plaque or syncytia formation. If CPE develops further investigation is required, such as examination by electron microscopy (EM) for the presence of viral particles. Further sub-culturing may be required. If EM examination demonstrates presence of virus particles, virus identification is required. New isolates should be stored in liquid nitrogen and at -80^{O}C. Procedures for identification of specific viruses will be covered by other ANZSDPs. Examples of viral CPE are shown in Figures 2-8.

Figures

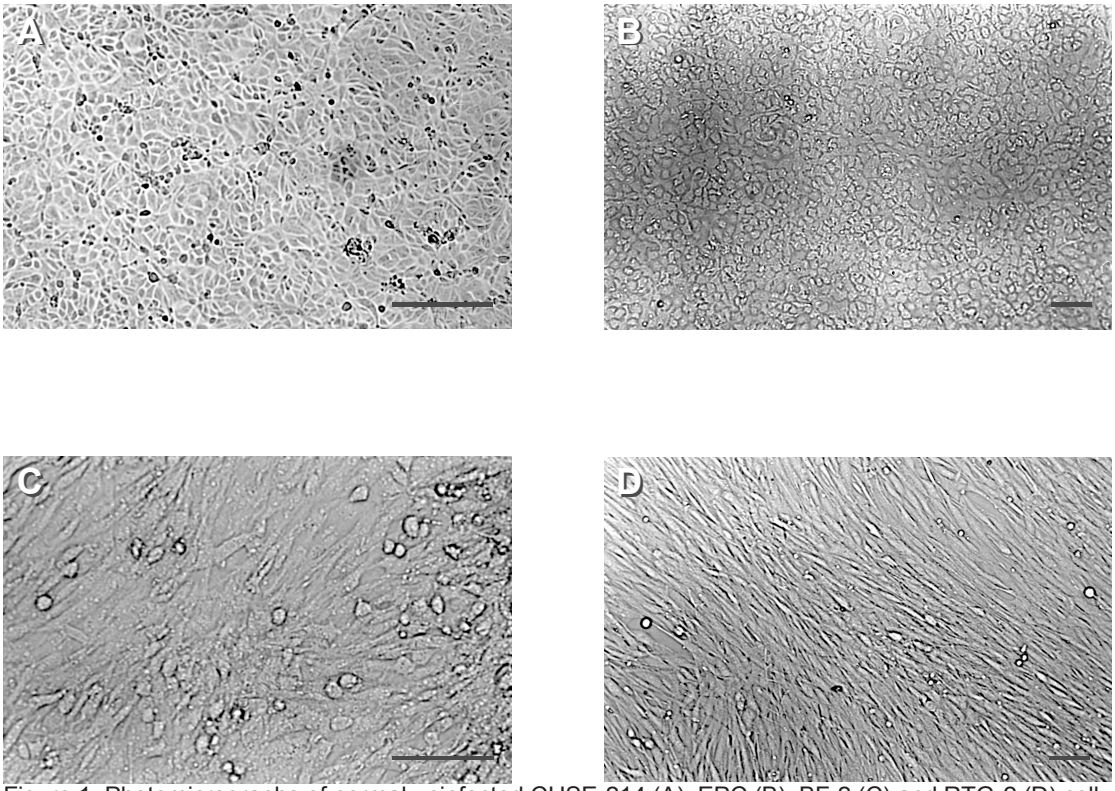


Figure 1. Photomicrographs of normal uninfected CHSE-214 (A), EPC (B), BF-2 (C) and RTG-2 (D) cell cultures after 3-7 days incubation at 15^{O}C. (Scale bars = 100 µm).

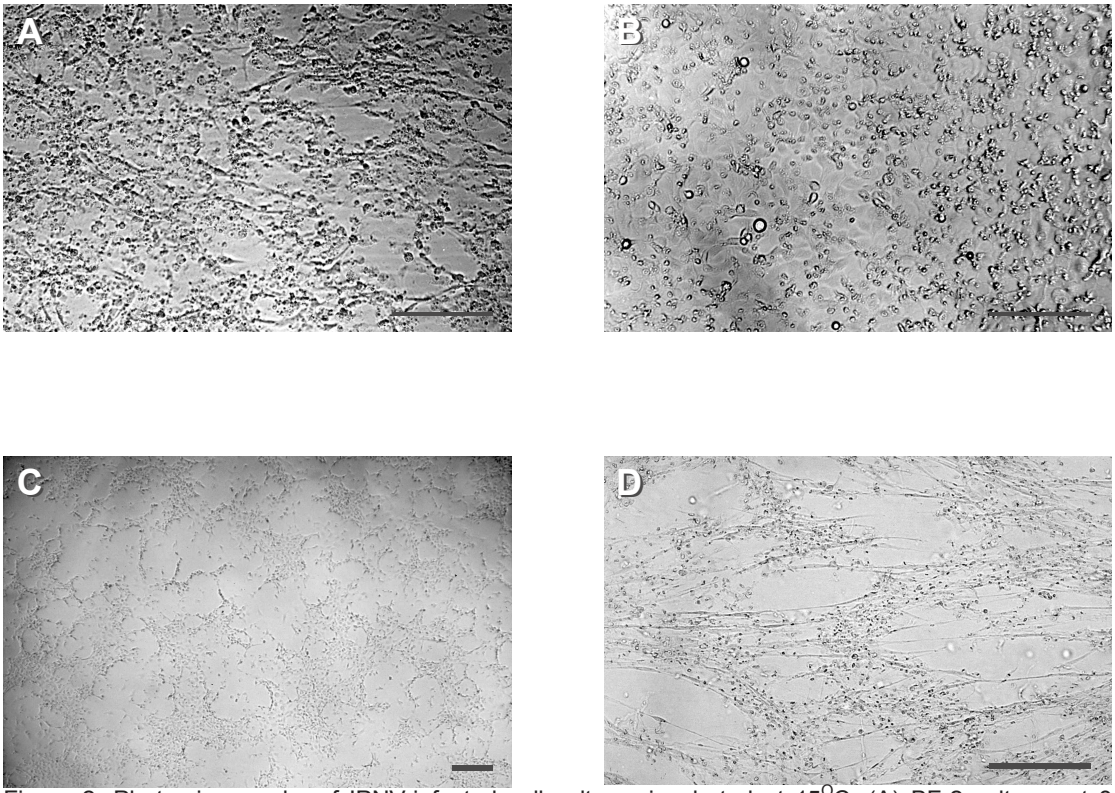


Figure 2. Photomicrographs of IPNV-infected cell cultures incubated at 15^{O}C: (A) BF-2 cultures at 8 days post-infection (dpi), (B) CHSE-214 cultures at 3 dpi, (C) EPC cultures at 2 dpi and (D) RTG-2 cell cultures at 6 dpi. (Scale bars = 100 µm).

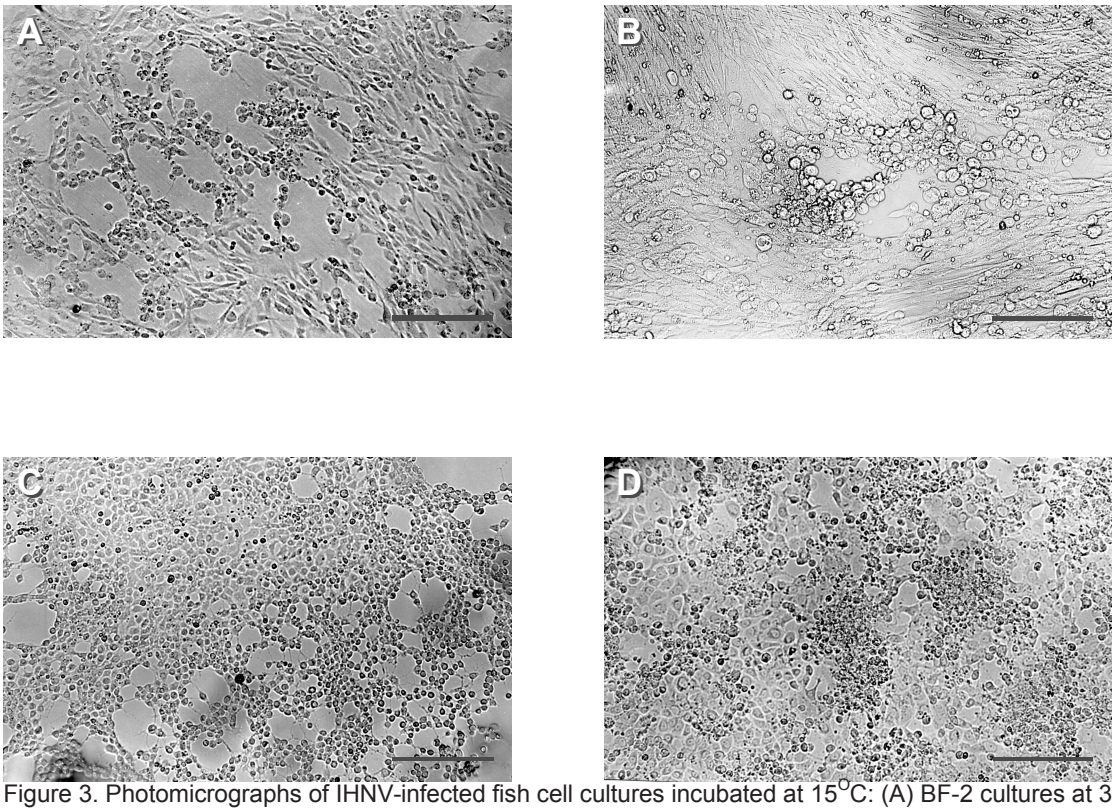


Figure 3. Photomicrographs of IHNV-infected fish cell cultures incubated at 15^{O}C: (A) BF-2 cultures at 3 dpi, (B) RTG-2 cultures at 4 dpi, (C) EPC cultures at 6 dpi and (D) CHSE-214 cultures at 6 dpi. (Scale bars = 100 µm).

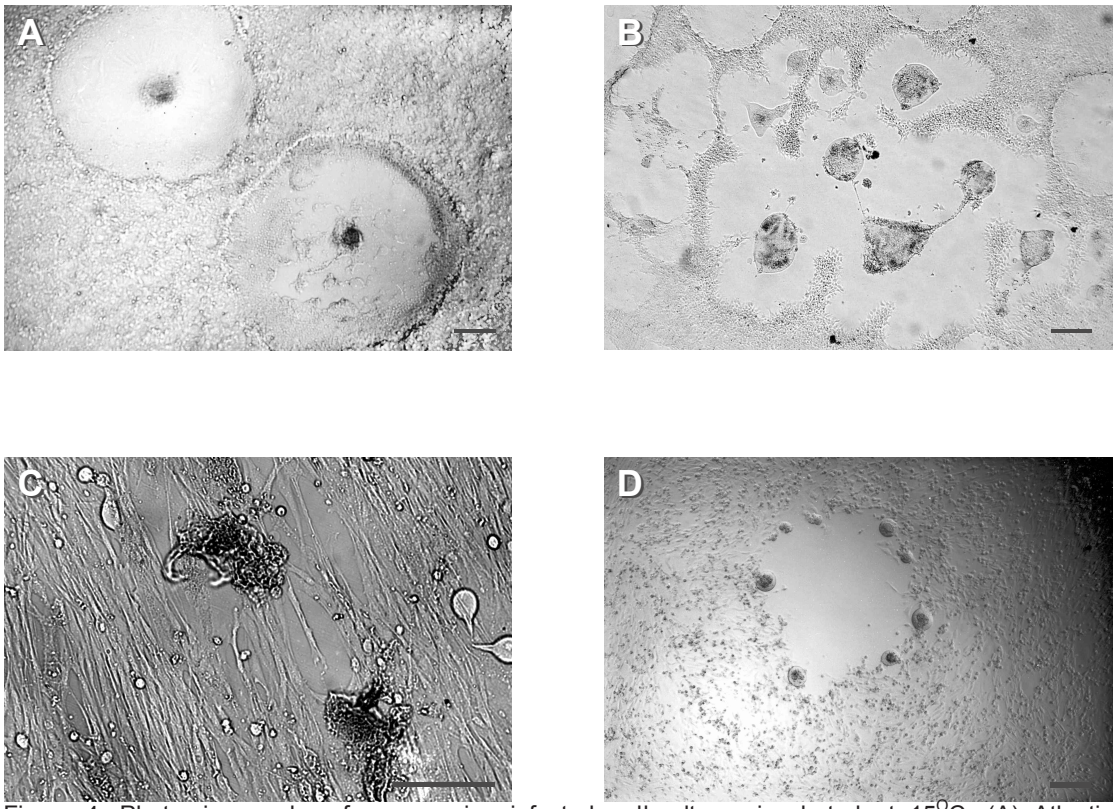


Figure 4. Photomicrographs of aquareovirus-infected cell cultures incubated at 15^{O}C: (A) Atlantic salmon reovirus-infected CHSE-214 cultures at 4 dpi, (B) Atlantic salmon reovirus-infected EPC cultures at 5 dpi, (C) Atlantic salmon reovirus-infected RTG-2 cultures at 6 dpi and (D) Learmonth reovirus-infected BF-2 cultures at 10 dpi. (Scale bars = 100 µm).

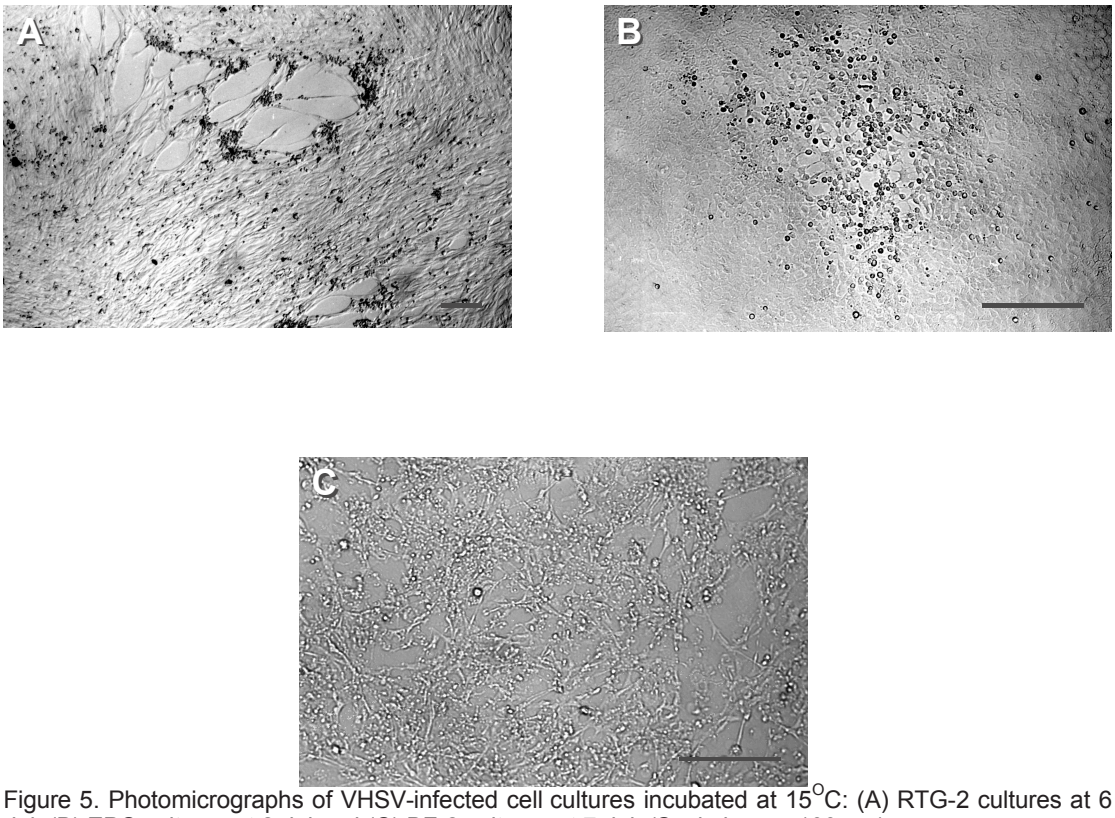


Figure 5. Photomicrographs of VHSV-infected cell cultures incubated at 15^{O}C: (A) RTG-2 cultures at 6 dpi, (B) EPC cultures at 2 dpi and (C) BF-2 cultures at 7 dpi. (Scale bars = 100 µm).

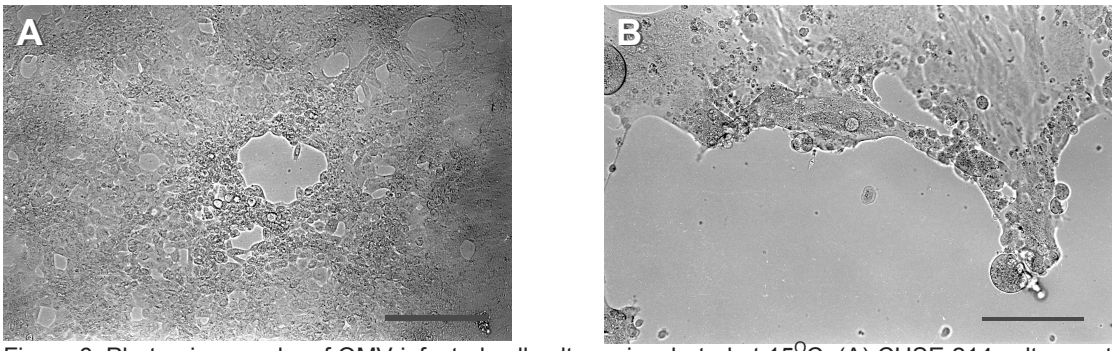
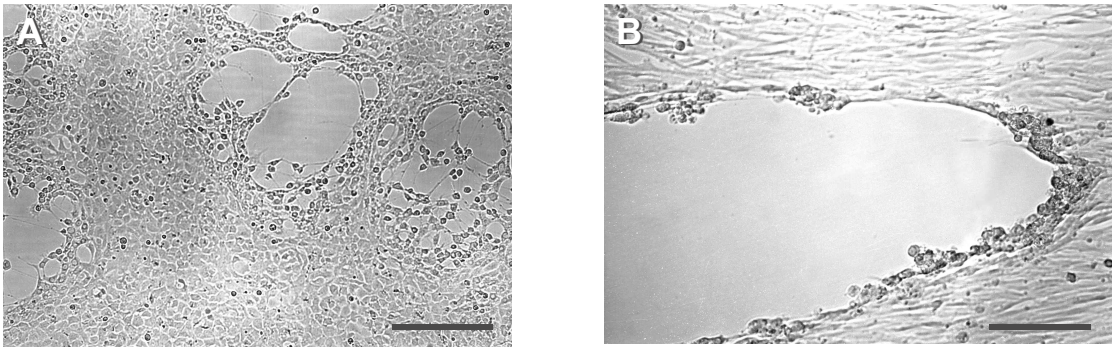


Figure 6. Photomicrographs of OMV-infected cell cultures incubated at 15^{O}C: (A) CHSE-214 cultures at 9 dpi and (B) RTG-2 cultures at 7 dpi. (Scale bars = 100 µm).



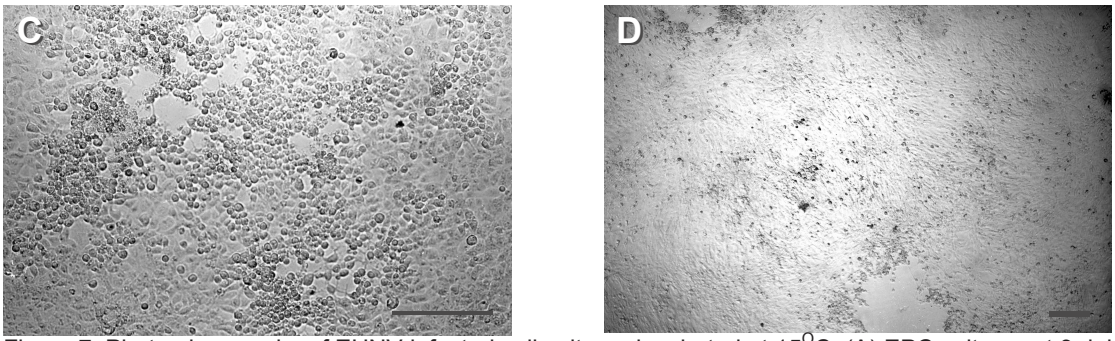


Figure 7. Photomicrographs of EHNV-infected cell cultures incubated at 15^{O}C: (A) EPC cultures at 3 dpi and (B) RTG-2 cultures at 7 dpi, (C) CHSE-214 cultures at 6 dpi and (D) BF-2 cultures at 7 dpi. (Scale bars = 100 µm).

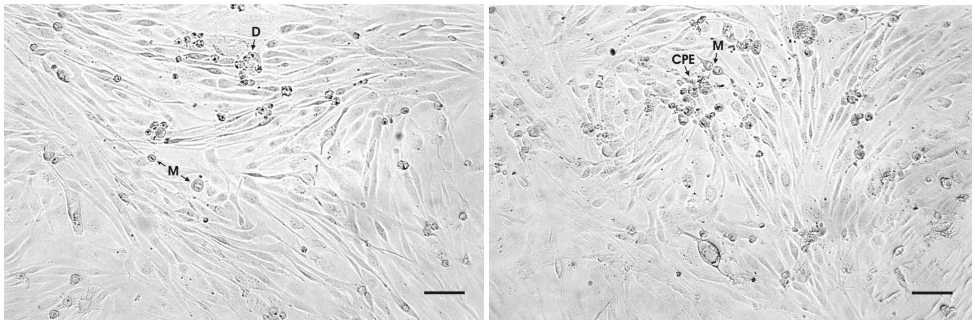


Figure 8A Figure 8B

Figure 8. Photomicrographs of cultures of SHK-1 cell line (Scale bars = 100 µm). Uninfected normal control cultures (Figure 8A) contain actively growing cells with mitotic figures (M). Such cultures contain some dead cells (D), which are transferred following trypsinisation. These cells can be confused with viral cytopathic effect (CPE) but, unlike CPE, which continues to develop as the culture ages, these cells tend to break down and disappear from the culture. In cultures infected with ISAV (Figure 8B) host cells eventually die as indicated by the cells rounding up and producing CPE. The virus tends to develop quite slowly and healthy cells continue to divide (M) so that the cultures may survive for prolonged periods following infection. Figure 8B demonstrates relatively early stages of CPE.

Reporting requirements in Australia and New Zealand

Each Australian State and Territory maintains a list of aquatic animal diseases that are 'notifiable' to the relevant State/Territory authorities within that jurisdiction. Australia also has a National List of Reportable Diseases of Aquatic Animals [see http://www.daff.gov.au/animal-plant-health/aquatic/reporting]. Suspected or confirmed cases of diseases (on both the national and relevant state/territory list) should be reported to the relevant state/territory authority. In turn, these findings will be reported by the state/territory authorities to the Australian Government, as appropriate.

For New Zealand, suspected or confirmed cases of diseases should be reported to the relevant authority (see http://www.biosecurity.govt.nz/pest-and-disease-response/surveillance-risk-response-and-management/marine).

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Appendices

Appendix 1. Properties of Fish Cell Lines in Common Use

CELL LINE	BF-2	SHK-1	CHSE-214	EPC	FHM	RTG-2
Name	Bluegill fry	Atlantic salmon head kidney	Chinook salmon embryo	Epithelioma Papulosum Cyprini	Fat Head Minnow	Rainbow trout gonad
Scientific Name	*Lepomis macrochirus*	*Salmo salar*	*Oncorhynchus tshawytscha*	*Cyprinus carpio*	*Pimephales promelas*	*O mykiss*
Common Name	Bluegill	Atlantic salmon	Chinook salmon	Carp	Fat Head minnow	Rainbow trout
Family Name	Centrarchidae	Salmonidae	Salmonidae	Cyprinidae	Cyprinidae	Salmonidae
Tissue	N-caudal trunk	N-head kidney	N-embryo	A-epithelium	N-caudal trunk	N-gonads
Cell morphology	F	F	E	E	E	F
Temperature range	15-30°C	15-20°C	4-27°C	8-33°C	0-36°C	4-26°C
Optimum temperature	25°C	20°C	21°C	30°C	34°C	20°C
Virus range	EHNV IHNV IPNV TSRV VHSV	IPNV ISAV TSRV	EHNV HS IHNV IPNV OMV TSRV	EHNV IHNV IPNV* TSRV VHSV	CSPV CSRV EHNV IHNV IPNV* VHSV	EHNV HS IHNV IPNV OMV TSRV VHSV

LEGEND:

CSPV: Chinook salmon paramyxovirus
CSRV: Chum salmon reovirus
EHNV: Epizootic haematopoietic necrosis virus
HS: *Herpesvirus salmonis*
IHNV: Infectious haematopoietic necrosis virus
IPNV: Infectious pancreatic necrosis virus
ISAV: Infectious salmon anaemia virus
OMV: *Oncorhynchus masou* virus
TSRV: Tasmanian Atlantic salmon reovirus
VHSV: Viral haemorrhagic septicaemia virus
*: not IPNV-Ab
F/E: Fibroblastic/epithelioid
N/A: Normal/abnormal

Appendix 2. Salmonid Virus Temperature Ranges and Susceptible Cell Lines

VIRUS	SUSCEPTIBLE CELLS	OPTIMUM TEMP (^{o}C)
Atlantic salmon reovirus (TSRV) - reovirus	EPC, CHSE-214, SHK-1	15
Herpesvirus salmonis (HS) - herpesvirus	CHSE-214, RTG-2	10
infectious haematopoietic necrosis virus (IHNV) - Rhabdovirus	CHSE-214, EPC, FHM, RTG-2	15
infectious pancreatic necrosis virus (IPNV) - Birnavirus	BF-2, CHSE-214, EPC, RTG-2	15
infectious salmon anaemia virus (ISAV) - orthomyxovirus	SHK-1, CHSE-214	15
Oncorhynchus masou virus (OMV) - herpesvirus	CHSE-214, RTG-2	10-15
epizootic haematopoietic necrosis virus (EHNV) - Iridovirus	FHM, BF-2, RTG-2	15-24
viral haemorrhagic septicaemia virus (VHSV) - rhabdovirus	BF-2, CHSE-214, EPC, FHM, RTG-2	15
chinook salmon paramyxovirus (CSPV) - paramyxovirus	CHSE-214	15-20
chum salmon reovirus (CSRV) - reovirus	CHSE-214	15

Appendix 3. Recommended Fish Cell Lines for Isolation of Salmonid Viral Pathogens

Introduction

Some cell lines are more susceptible to virus infection and support growth and development of some viruses better than other cell lines. Thus protocols from the EU, UK, USA, Canada and OIE specify certain fish cell lines that are recommended for virus isolation studies. Based on this information, AAHL Fish Diseases Laboratory has identified two fish cell lines for each importing authority and one/two back-up cell line(s) to be used for export certification purposes.

For export certification testing, every effort is taken to keep the number of cell lines and total cell cultures/cell line required to a minimum while ensuring that international protocols for the importing country/ies and/or regions are adhered to.

Summary of International Recommendations

The following Table summarises information from international protocols.

Country/ Region/ Organisation	**Virus**	**Recommended Cell Line**	**References**
UK	All	BF-2 and EPC RTG-2 as back-up	Hill BJ 1976. MAFF 1980a, 1980b.
EU	VHSV and IHNV	BF-2 (or RTG-2) EPC (or FHM)	European Union 1992. European Union 1996.
USA	OMV IHNV IPNV VHSV Rhabdoviruses OMV/IPNV	CHSE-214, RTG-2 EPC, CHSE-214, FHM BF-2, CHSE-214, RTG-2 CHSE-214, EPC, FHM, RTG-2 EPC CHSE-214	Blanchard B 1993. Thoesen JC 1994.
Canada	All	Two of: either RTG-2, CHSE-214 or FHM	DFO 1984
OIE	EHNV IHNV OMV SVCV VHSV IPNV ISAV	BF-2 or FHM EPC or BF-2 CHSE-214 or RTG-2 EPC or FHM BF-2 or EPC or RTG-2 BF-2 or CHSE-214 SHK-1 or ASK	OIE 2006.

References

Blanchard B. Salmonid Importation Regulations. Fish Health. U.S. Fish & Wildlife Service. Division of Fish Hatcheries. *Federal Register* Vol. 58. No. 213. November 5, 1993.

DFO. Fish Health Protection Regulations: manual of compliance. *Fish Mar Serv Misc Spec Publ 31 (Revised)*. Department of Fisheries and Oceans (Canada), Ottawa 1984. 32pp.

European Union. Commission Decision of 19 November 1992 laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases (92/532/EEC). *Official J Eur Communities* No. L. 337/18, 21 November 1992.

European Union. Commission Decision of 5 February 1996 amending Decision 92/532/EEC laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases (96/240/EEC). *Official J Eur Communities* No. L. 79/19, 29 March 1996.

Hill BJ. Procedures for the isolation and identification of IPN, VHS, IHN and SVC viruses from diseased fish. *Fisheries Research Technical Report Number 27*. Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research, Lowestoft, U.K. 1976.

MAFF. *Test requirements for health certification of live cultivated fresh-water fish imports*. DOF 5. Ministry of Agriculture, Fisheries and Food, London 1980a. 5p.

MAFF. *Test requirements for health certification of fish egg imports. DOF 6*. Ministry of Agriculture, Fisheries and Food, London. 1980b. 6p.

OIE. *Manual of Diagnostic Tests for Aquatic Animals.* Fifth Edition. World Organisation for Animal Health, Paris, 2006, 469 pp.

Thoesen JC, Editor. *Suggested procedures for the detection and identification of certain finfish and shellfish pathogens*. 4th edition, version 1. Fish Health Section, American Fisheries Society, Bethesda, Maryland, 1994.

Appendix 4. Transportation Medium

This medium is used for transportation of samples from the field to the testing laboratory and should be made up within one month of use and stored at 2-5^{O}C until use. It is also used for homogenisation of samples.

Materials

400 mL Hank's Balance Salt Solution (HBSS)
200 IU penicillin/200 µg streptomycin/mL (final dilution)
2% (v/v) foetal bovine serum (FBS).

Method

- Using aseptic technique, add 8 mL FBS to 400 mL HBSS.
- Thaw the antibiotic solution.
- Aseptically add antibiotic solution to the HBSS/FBS and dispense into convenient volumes for transportation of tissue samples.

Appendix 5. Maintenance of Fish Cell Lines and Preparation of Cultures for Virus Isolation

5.1 BF-2 (caudal trunk, bluegill fry, *Lepomis macrochirus*)

General Information

ATCC catalogue number: CCL 91
Temperature range 15-33^{O}C. Temperature for optimum cell growth is 23^{O}C.
Incubation temperature for routine maintenance at AAHL: 22^{O}C.

Materials

Growth Medium
Eagle's minimum essential medium with Earle's salts and non-essential amino acids (EMEM)
10 mM HEPES buffer
2 mM Glutamine
100 IU penicillin/100µg streptomycin/mL
10% (v/v) foetal bovine serum (FBS).

Trypsin-Versene (T/V) 10X solution
Sterile phosphate buffered saline pH 7.4 without Ca^{2+}/Mg^{2+} (PBSA)
Tissue culture flasks or plates

Method

All manipulations must be undertaken in a safety cabinet and using aseptic techniques

1. Bring medium to near room temperature.

2. In safety cabinet, decant old medium from cell cultures into discard vessel.

3. Rinse cell monolayer with PBSA and decant fluid into discard vessel.

4. Add T/V solution [add solution (3 mL for 75cm^2 flask or 5 mL for 150 cm^2 flask) to flask on opposite side to cell monolayer].

   Incubate cells (time depends on age of monolayer) at room temperature, until monolayer detaches (gentle knocking of the culture vessel will encourage cells to detach).

5. Resuspend detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5^{o}C at 100 x *g* for 5 min.

6. After centrifugation, *carefully* decant the supernatant medium and T/V solution mixture into discard vessel leaving cell pellet in the tube.

7. Resuspend cell pellet in a known volume (for example, 10 mL) of growth medium and count the cells using a haemocytometer.

Determine cell density (number of cells per mL) of suspension and seed a 75 cm^2 flask at 4.5 million cells in approximately 20 mL growth medium (which should produce a 100% confluent monolayer in 5 days at 22^{O}C).

Incubate cell cultures at 22^{O}C.

Cultures for Virus Isolation

Twenty-four-well cluster plate cultures are used for virus isolation. Generally, plates are seeded with cells approximately 24 h before use.

Procedure:

All manipulations must be undertaken in a safety cabinet and using aseptic techniques

Plates are seeded at a cell density of 6 million cells/plate (250,000 cells/1.5 mL/well).

1. Using a cell suspension prepared by the above method and, based on the cell count, determine the volume of cells required. Note that 40 mL of cell suspension is required to seed one 24-well plate.

2. Add required volume of cell suspension to required volume of growth medium to achieve a final cell density of 6 million cells/40 mL/plate.

3. Gently swirl to mix the suspension and using a sterile pipette dispense 1.5 mL cell suspension to each well.

4. Replace plate lids and incubate, in a plastic container, at approximately 22^{o}C in an atmosphere of 5% CO_2/95% air until required.

Reference

Hay R *et al.* 1988. *ATCC catalogue of cell lines and hybridomas*. 6th Edition.

5.2 CHSE-214 (normal salmon embryo, *Oncorhynchus tshawytscha*)

General Information

ATCC catalogue number: CRL 1681
Temperature range: 4-27°C. Incubation temperature for optimum cell growth: 21-23°C.
Incubation temperature at AAHL: 22°C.

Materials

Growth medium:

Eagle's minimum essential medium with Earle's salts and non-essential amino acids (EMEM).
20 mM HEPES buffer
2 mM Glutamine
100 IU penicillin/100µg streptomycin (Pen/Strep)/mL
10% (v/v) foetal bovine serum (FBS).

Trypsin-Versene (T/V) 10X solution
Sterile phosphate buffered saline pH 7.4 without Ca^{2+}/Mg^{2+} (PBSA)
Tissue culture flasks or plates

Method

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

1. Bring medium to near room temperature.

2. In safety cabinet, decant old medium from cell cultures into discard vessel.

3. Rinse the cell monolayer with PBSA and decant the fluid into discard vessel.

4. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to flask on opposite side to cell monolayer].

   Incubate cells (the time depends on age of monolayer) at room temperature, until monolayer detaches (gentle knocking of the culture vessel will encourage cells to detach).

5. Resuspend detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5°C at 100 *g* for 5 min.

6. After centrifugation, *carefully* decant supernatant medium and T/V solution mixture into discard vessel leaving cell pellet in the tube.

7. Resuspend cell pellet in a known volume (for example, 10 mL) of growth medium and count the cells using a haemocytometer.

8. Determine cell density (number of cells per mL) of suspension and seed a 75 cm^2 flask at 4.5 million cells in approximately 20 mL of growth medium (which should produce a 100% confluent monolayer in 5 days at 22 °C).

9. Incubate cell cultures at 22°C.

Cultures for Virus Isolation

Twenty-four-well cluster plate cultures are used for virus isolation. Generally, plates are seeded with cells approximately 24 h before use.

Procedure:

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

Plates are seeded at a cell density of 6 million cells/plate (250,000 cells/1.5 mL/well).

1. Using a cell suspension prepared by the above method and, based on the cell count, determine the volume of cells required. Note that 40 mL cell suspension is required to seed one 24-well plate.

2. Add required volume of cell suspension to required volume of growth medium to achieve a final cell density of 6 million cells/40 mL/plate.

3. Gently swirl to mix the suspension and using a sterile pipette dispense 1.5 mL cell suspension to each well.

4. Replace plate lids and incubate, in a plastic container, at approximately 22^{o}C in an atmosphere of 5% CO_2/95% air until required.

Reference

Hay R *et al.* 1988. *ATCC catalogue of cell lines and hybridomas*. 6th edition.

5.3 EPC (*epithelioma papulosum cyprini*)

General Information

Origin**:** Cells were received from The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK
Temperature range: 4-35^{o}C. Temperature for optimum cell growth: 30^{o}C.
Incubation temperature at AAHL: 22^{o}C or 30^{o}C.

Materials

Growth medium:
L-15 Leibovitz medium
2 mM Glutamine
100 IU penicillin/100μg streptomycin/mL
10% (v/v) foetal bovine serum, (FBS).

Trypsin-Versene (T/V) 10X solution
Sterile phosphate buffered saline pH 7.4 without Ca^{2+}/Mg^{2+} (PBSA)
Tissue culture flasks or plates

Method

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

1. Bring medium to near room temperature.

2. In safety cabinet, decant old medium from cell cultures into discard vessel.

3. Rinse cell monolayer with PBSA and decant fluid into discard vessel.

4. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to flask on opposite side to cell monolayer]:

   Incubate cells (time depends on age of monolayer) at room temperature, until monolayer detaches (gentle knocking of the culture vessel will encourage cells to detach).

5. Resuspend detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5^{o}C at 100 *g* for 5 min.

6. After centrifugation, *carefully* decant supernatant medium and T/V solution mixture into discard vessel leaving cell pellet in the tube.

7. Resuspend cell pellet in a known volume (for example 10mL) of growth medium and count the cells using a haemocytometer.

8. Determine cell density (number of cells per mL) of suspension and seed a 75 cm^2 flask at 4.5 million cells in approximately 20 mL growth medium (which should produce a 100% confluent monolayer in 5 days at 22^{O}C).

9. Incubate cell cultures at 22^{O}C in a normal atmosphere (100% air).

Cultures for Virus Isolation

Twenty-four-well cluster plate cultures are used for virus isolation. Generally, plates are seeded with cells approximately 24 h before use.

Procedure:

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

Plates are seeded at a cell density of 12 million cells/plate (500,000 cells/1.5 mL/well).

1. Using a cell suspension prepared by the above method and, based on the cell count, determine the volume of cells required. Note that 40 mL of cell suspension is required to seed one 24-well plate.

2. Add required volume of cell suspension to required volume of growth medium to achieve a final cell density of 12 million cells/40 mL/plate.

3. Gently swirl to mix suspension and using a sterile pipette dispense 1.5 mL cell suspension to each well.

4. Replace plate lids and incubate, in a plastic container with lid, at approximately 22^{o}C in a normal atmosphere of 100% air until required.

Reference

Fijan N, Sulimanovic D, Bearzotti M et al. Some properties of the *Epithelioma Papulosum Cyprini* (EPC) cell line from carp (*Cyprinus carpio). Ann Virol (Inst. Pasteur)* 1983;134E:207-220.

5.4 RTG-2 (gonadal tissue, rainbow trout)

General Information

ATCC catalogue number: CCL 55
Temperature range 4-24^{O}C. Maximum cell growth occurs at 22^{O}C
Incubation temperature for routine maintenance at AAHL: 22^{O}C.

Materials

Growth Medium:

Eagle's minimum essential medium with Earle's salts and non-essential amino acids (EMEM)
20 mM HEPES buffer
2 mM Glutamine
100 IU penicillin/100µg streptomycin (Pen/Strep)/mL
10% (v/v) foetal bovine serum (FBS).

Trypsin-Versene (T/V) 10X solution
Sterile phosphate buffered saline pH 7.4 without Ca^{2+}/Mg^{2+} ions (PBSA)
Tissue culture flasks or plates

Method

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

1. Bring medium to near room temperature.

2. In safety cabinet, decant old medium from cell cultures into discard vessel.

3. Rinse cell monolayer with PBSA and decant fluid into discard vessel.

4. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to flask on opposite side to cell monolayer.

   Incubate cells (time depends on age of monolayer) at room temperature, until monolayer detaches (gentle knocking of the culture vessel will encourage cells to detach).

5. Resuspend detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5^{o}C at 100 *g* for 5 min.

6. After centrifugation, *carefully* decant supernatant medium and T/V solution mixture into discard vessel leaving cell pellet in the tube.

7. Resuspend the cell pellet in a known volume (for example, 10 mL) of growth medium and count the cells using a haemocytometer.

8. Determine cell density (number of cells per mL) of suspension and seed a 75 cm^2 flask at 4.5 million cells in approximately 20 mL growth medium (which should produce a 100% confluent monolayer in 5 days at 22^{O}C).

9. Incubate cell cultures at 22^{O}C.

Cultures for Virus Isolation

Twenty-four-well cluster plate cultures are used for virus isolation. Generally, plates are seeded with cells approximately 24 h before use.

Procedure:

All manipulations must be undertaken in a safety cabinet and using aseptic techniques

Plates are seeded at a cell density of 6 million cells/plate (250,000 cells/1.5 mL/well).

1. Using a cell suspension prepared by the above method and, based on the cell count performed above, determine volume of cells required. Note that 40 mL cell suspension is required to seed one 24-well plate.

2. Add required volume of cell suspension to required volume of growth medium to achieve a final cell density of 6 million cells/40 mL/plate.

3. Gently swirl to mix the suspension and using a sterile pipette dispense 1.5 mL of the cell suspension to each well.

4. Replace plate lids and incubate, in a plastic container, at approximately 22^{o}C in an atmosphere of 5% CO_2/95% air until required.

Reference

Hay R *et al.* 1988. ATCC catalogue of cell lines and hybridomas. 6th edition.

5.5 SHK-1 (salmon head kidney)

General Information

Origin: Cells were supplied by Central Veterinary Laboratory, Oslo, Norway.
Temperature range: 4-27^{O}C. Temperature for optimum cell growth: 15-20^{O}C.
Incubation temperature at AAHL: 20^{O}C for growth of cell cultures and15^{O}C for maintenance of cell cultures during culture for ISAV isolation and growth.

Materials

Growth medium:
L-15 Leibovitz medium
4mM glutamine
100IU penicillin/100µg streptomycin per mL
40µM 2-mercaptoethanol
5% (v/v) foetal bovine serum

Trypsin-Versene (T/V) 10X solution
Sterile phosphate buffered saline pH 7.4 without Ca^{2+}/Mg^{2+} (PBSA)
Tissue culture flasks or plates

Method

All manipulations must be undertaken in a safety cabinet and using aseptic technique.

1. Bring medium to near room temperature.

2. In safety cabinet, decant old medium from cell cultures into discard vessel.

3. Rinse the cell monolayer with PBS-A and decant the fluid into glass discard bottle.

4. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to the flask on the opposite side to the monolayer]. Wash over cells then immediately remove excess.

   Rock the flask for a minute or two at room temperature. Look for the monolayer becoming translucent and patchy then tap the flask firmly against your hand to assist cell detachment.

5. Resuspend the detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5^{O}C at 100 *g* for 5 min.

6. After centrifugation, decant the supernatant medium-T/V solution mixture into the discard vessel leaving the cell pellet in the tube.

7. Resuspend the cell pellet in a known volume (for example 3 mL) of growth medium and count the cells using a haemocytometer.

8. Determine cell density (number of cells per mL) of the suspension and seed culture flasks or plates as shown in table below.

Vessel	Total cells	Cells per well	Volume of medium
25 cm^2 flask	600,000		10-15 mL
75 cm^2 flask	2 million		20-30 mL
150 cm^2 flask	6 million		50-70 mL
24-well plate	3 million in 40ml	120,000	1.5 mL/well
96-well plate	2 million in 11ml	18,000	100 µL/well

Flasks seeded at these rates should produce a 100% confluent monolayer in 5 days at 20^{O}C). Multiwell plates should be 80% to 95% confluent after overnight incubation at 20^{O}C.

9. Incubate cell cultures at 20^{O}C in a normal atmosphere (100% air).

Cultures for virus isolation

Twenty-four-well cluster plate cultures are used for virus isolation. Generally, plates are seeded with cells approximately 24 hours prior to use.

Procedure:

All manipulations must be undertaken in a safety cabinet and using aseptic technique.

Plates are seeded at a cell density of approximately 1.5 million cells/plate (approximately 60,000 cells/1.5 ml/well).

1. Using a cell suspension prepared by the above method and, based on the cell count, determine the volume of cells required. Note that approximately 40 mL of cell suspension is required to seed one 24-well plate.

2. Add the required volume of cell suspension to the required volume of growth medium to achieve a final cell density of 1.5 million cells/40 mL/plate.

3. Gently swirl to mix the suspension and using a sterile pipette dispense 1.5 mL of the cell suspension to each well.

4. Replace plate lids and incubate, in a plastic container, at approximately 20^{O}C in an atmosphere of 100% air until required.

References

Dannivig BH, Brudeseth BE, Gjøen T, Rode M, Wergeland HI, Evensen Ø, Press CMcL. Characterization of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol* 1997;7: 213-226.

Dannevig BH, Falk K, Namork E. Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. *J Gen Virol* 1995;76:1353-1359.

Appendix 6. Virus Susceptibility Testing of Fish Cell Lines

To ensure cell lines, used on a routine basis, are susceptible to the viruses of concern, virus titrations on each of the cell lines should be carried out on a regular basis (every 3-6 months preferably just prior to the onset of export certification testing).

Method

All manipulations must be carried out in a safety cabinet and using aseptic technique.

1. For BF-2, CHSE-214 and RTG-2 cell lines, prepare a cell suspension of each with a cell density of 4 million cells in 11 mL appropriate growth medium (provides sufficient suspension for one 96-well culture plate). For EPC cell line, a cell suspension of 8 million cells in 11 mL appropriate growth medium per 96-well culture plate is required. See Appendix 5 for method to prepare cell suspensions from stock cell cultures.

2. Pour the prepared cell suspension into a sterile reservoir and with constant gently mixing (to ensure an even suspension), use a multi-stepper pipette and sterile tips to dispense 100 µL cell suspension into each well of the 96-well microtitre culture plates.

   For each virus titration, one 96-well-microtitre culture plate per cell line is required.

3. Replace plate lid and incubate cultures in a sealed, plastic container at approximately 22°C in an atmosphere of 5% CO_2/95% air (CHSE-214, BF-2, RTG-2 cell lines in EMEM medium or 100% air (EPC cell line in L-15 medium) for 24 hours.

4. On the following day, check microscopically, that cultures are suitable for inoculation (they should be 75-90% confluent with mitotic figures evident and cell density and morphology should be relatively consistent among all wells).

5. Using a multi-stepper pipette dispense 50 µL appropriate maintenance medium for each cell line, into each sample well of the cell culture plates (all wells of columns 1 to 10) and 100 µL into each well of the two control (no virus) columns (columns 11 and 12).

6. Prepare a ten-fold dilution series of each virus from 10^{-1} to 10^{-10} (using 0.9 mL growth medium and 0.1 mL stock virus for each dilution) in sterile 1.5 mL tubes. Discard pipette tips after each dilution to prevent carry over of virus, which makes dilutions inaccurate.

7. Using sterile tips and a micro-pipette, dispense 50 µL of each virus dilution into all wells of the appropriate column starting with the 10^{-10} virus dilution into column 10, 10^{-9} dilution into column 9……....10^{-1} into column 1. There is no need to change pipette tips between each dilution of the series if the highest dilution (10^{-10}) of virus is dispensed first.

8. Gently and briefly agitate culture plate to mix contents of wells.

9. Seal plate with a MYLAR cover, place in a sealed plastic container and incubate at approximately 15°C.

10. Examine plates at days 3, 7 and 10 after inoculation noting development of viral CPE (uninfected control cultures should appear normal throughout the incubation period).

Results

Results are expressed as $TCID_{50}$/50 µL (Reed & Muench 1938).

Reference

Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493-497.

Appendix 7. Maintenance of Exotic Virus Stocks

Exotic virus stocks are maintained at AAHL in accordance with Section 5 of AAHL Microsecurity and Safety Manual.[1]

Reference strains of fish viruses are stabilised in both liquid nitrogen and –80^{O}C freezers.

In general, virus strains are obtained as tissue culture supernatants from infected cell cultures. This protocol describes the procedure for maintaining virus stocks subsequent to receipt by AAHL.

Methods

Following plaque-purification, if required, and confirmation of virus identity, stock cultures of virus need to be expanded, titrated, dispensed in convenient volumes and stored until further use.

Procedure:

All manipulations must be carried out in a Class II safety cabinet and using aseptic technique.

1. Examine, using an inverted light microscope, the fish cell culture to ensure that it is not contaminated, is of the correct confluency (approximately 95%) and pH, is actively replicating (exhibits mitotic figures) and contains cells with cellular morphology typical of the fish cell line from which it is derived.

2. Decant culture medium from the cell culture into the discard bottle and replace with 15-20 mL maintenance medium.

3. Based on the approximate cell number estimated (from the number of cells seeded on the previous day) for the cell culture and the estimate of the virus titre (from previous experiments or from the provider), inoculate the cell culture at a multiplicity of infection of approximately 0.01-0.001 or that recommended by the providers of the virus strain.

4. Incubate the inoculated cell culture at the recommended temperature for optimum virus growth (usually 15^{O}C for salmonid viruses).

5. Monitor (at intervals of 1-3 days), by microscopic examination, the cell culture for contamination but, more particularly, for the development of virus-specific cytopathic effect (CPE).

6. When viral CPE has developed fully (100% of the cell monolayer is affected), the cell culture supernatant is harvested, centrifuged at 5^{O}C at 100 *g* for 15 min, stored at 4^{O}C and a sample titrated on a known susceptible cell line.

   Appendix 6, Virus Susceptibility Testing of Fish Cell Lines, describes the method used to titrate fish viruses.

7. Following titration, sterile 1.5 mL vials are labelled and convenient volumes of the virus-containing cell culture supernatant are placed into each vial for cryopreservation.

Reference

Anon. *AAHL Microsecurity and Safety Manual*. Geelong: CSIRO Livestock Industries. For information, contact: Communication Manager, CSIRO Livestock Industries (Tel. 03 5227 5000).

Appendix 8. Procedure for Isolation of Salmonid Viruses on Cell Lines

Preparation of cell cultures

All manipulations must be undertaken in a safety cabinet and using aseptic techniques.

On the day prior to receipt of a sample, prepare sufficient 24-well cultures of the appropriate fish cell lines. Ensure that the cells are derived from stocks that have been tested and shown to be susceptible to the viruses of interest within the previous 6 months (see Appendix 6 for appropriate method). One 24-well plate is sufficient to undertake testing of 5 tissue sample pools (two dilutions of each homogenised sample inoculated onto duplicate cultures requiring 4 wells per pooled sample) plus one set of four uninoculated negative control cultures/wells.

Appendix 5 describes methods to establish fish cell cultures for virus isolation. The cell cultures should not be older than 48 h, should be actively replicating and 80-90% confluent on the day of inoculation of the sample.

Plates are seeded at a cell density of 6-12 million cells/plate (250,000-500,000 cells/1.5 mL/well) depending on the cell lines used.

1. In safety cabinet, decant old medium from stock cell culture into a discard vessel.

2. Rinse the cell monolayer with PBSA and decant the fluid into a discard vessel.

3. Add T/V solution [add solution (3 mL for 75 cm^2 flask or 5 mL for 150 cm^2 flask) to the flask on the opposite side to the monolayer and then allow the solution to bathe the cell monolayer]:

   Incubate cells (the time depends on the age of monolayer) at room temperature, until the monolayer detaches (gentle knocking of the culture vessel will encourage cells to detach).

4. Resuspend the detached cells in growth medium (using three times the volume of T/V solution used), transfer to a sterile centrifuge tube and centrifuge at 5-10^{O}C at 100 *g* for 5 min.

5. After centrifugation, decant the supernatant medium and T/V solution mixture into the discard vessel leaving the cell pellet in the tube.

6. Resuspend the cell pellet in a known volume (for example 10 mL) of growth medium and perform a cell count using a haemocytometer.

7. Determine the cell density (number of cells per mL) of the suspension.

8. Using a cell suspension prepared by the above method and, based on the cell count, determine the volume of cells required. Note that 40 mL of cell suspension is required to seed one 24-well plate (1.5 mL/well).

9. Add the required volume of cell suspension to the required volume of growth medium to achieve a final cell density of 6 million or 12 million cells/40 mL/plate.

10. Gently swirl to mix the suspension and, using a sterile pipette, dispense 1.5 mL of the cell suspension to each well.

11. Replace plate lids and incubate, in a plastic container, at approximately 22^{O}C in a normal (100% air) atmosphere (for EPC cell cultures) until required. Cultures of RTG-2, BF-2 and CHSE-214 cell lines are placed in a CO_2 incubator at 22^{O}C and an atmosphere of 5% CO_2/95% air. Alternatively, an air-tight gassing chamber can be used in which the atmosphere has been exchanged to achieve 5% CO_2/95% air and then placed in a non-CO_2 incubator at 22^{O}C.

Inoculation of samples onto cell cultures

On receipt, samples should be checked to ensure that they correspond to information submitted on submission document. The submission should be recorded according to standard protocols. All samples must be kept chilled (4-10^{O}C) until inoculation onto fish cell lines.

On the day of inoculation of the samples, examine cell cultures to be used in the test, by inverted light microscopy. Ensure that they are at the correct confluency, free from overt microbial contamination, mitotic figures are visible (that is, they are actively dividing), and cell morphology is typical of the specific fish cell line. Any problems should be noted and, if necessary, fresh cultures prepared for use on the next day.

1. Switch on refrigerated centrifuge to cool to approximately 4^{O}C.

2. Switch on safety cabinet and ensure that it is operating according to the manufacturer's specifications.

3. Weigh a sample container with a submitted, pooled tissue sample and subtract the weight of an empty sample container to obtain an estimate of the weight of the tissue sample pool.

4. Repeat with 4 other submitted samples from the same source and calculate an approximate mean weight for all the tissue sample pools for the current submission.

5. Prepare two sets of sterile centrifuge tubes. Using an indelible marker, label one set of tubes with the submission identification (ID) number, sample number and dilution, that is, "1/10". Using an indelible marker, label the second set of tubes with the ID number, sample number, date and dilution, that is, "1/100".

6. Operating within the safety cabinet and using 10 mL sterile pipettes, dispense 4.5 mL supplemented HBSS/transportation medium (Appendix 4) into each "1/100" tube. Place all tubes in a test tube rack that is sitting in an ice-water slurry.

7. Operating within the safety cabinet, remove one pooled tissue sample and place in a frozen, sterile mortar and pestle (previously stored at –20^{O}C). Grind the tissue sample pool to a homogeneous paste.

8. Resuspend homogenate in extra supplemented HBSS so that the ratio tissue weight (in grams): supplemented HBSS volume (in mL) is 1:10.

9. Pipette/pour approximately 10 mL resuspended homogenate into a sterile centrifuge tube labelled with its ID number, sample identification and "1/10" and replace cap.

10. Repeat 6 to 9 for each tissue sample pool.

11. Centrifuge all sample-filled centrifuge tubes at 2-5^{O}C at 2000-4000 *g* for 15 min.

12. Following centrifugation, retrieve and return centrifuge tubes to test-tube rack (in ice/water slurry) and return the rack to the safety cabinet.

13. Pipette 0.5 mL of the supernatant from each 1/10 tissue sample dilution into the corresponding test tubes labelled "1/100" for each sample.

14. Operating within the safety cabinet, decant and discard cell culture medium from the 24-well cell cultures for up to 5 pooled samples at any one time (one tissue culture plate – see Table 1).

Table 1. Example of use of a 24-well cluster plate

Sample 1 1/10	Sample 2 1/10	Sample 3 1/10	Sample 4 1/10	Sample 5 1/10	Negative Control Culture
Sample 1 1/10	Sample 2 1/10	Sample 3 1/10	Sample 4 1/10	Sample 5 1/10	Negative Control Culture
Sample 1 1/100	Sample 2 1/100	Sample 3 1/100	Sample 4 1/100	Sample 5 1/100	Negative Control Culture
Sample 1 1/100	Sample 2 1/100	Sample 3 1/100	Sample 4 1/100	Sample 5 1/100	Negative Control Culture

15. One set (column) of four well-cultures on each 24-well cluster plate should be used as negative controls, which are treated as test cultures except that they are inoculated with 150 uL supplemented HBSS only, rather than diluted tissue sample. An example of a typical culture-plate set-up is illustrated in Table 1.

16. Inoculate duplicate cultures of each cell line with 150 uL of each sample dilution (1/10 and 1/100) as illustrated above.

17. Place plates in plastic containers and, using appropriate culture conditions, incubate the cultures at 15^{O}C for 1 h to allow any virus particles to adsorb onto the cell monolayer.

18. Following adsorption, add 1.5 mL appropriate cell culture maintenance medium [growth medium containing 2% (v/v) FBS instead of 10%] to each culture (negative controls first) yielding final sample dilutions of 1/100 and 1/1000. Return culture plates to 15^{O}C incubator. Appendix 5 describes culture conditions for each cell line.

Monitoring inoculated cell cultures

On the day following inoculation, and every 1-3 days thereafter, examine the cultures, by inverted light microscopy, for any microbial contamination, tissue sample cytotoxicity and viral cytopathic effect (CPE). Record observations.

Sub-culturing

At 8-12 days after inoculation (pi), the test cultures should be passaged. For each tissue sample, the contents of each of the four replicate cultures, irrespective of dilution, are pooled into sterile centrifuge tubes that have been labelled with the sample ID number, sample number, date and respective cell line identification.

If tissue sample cytotoxicity or bacterial/fungal contamination has been observed during the initial culture period for each pool, the pooled contents should be filtered, using 0.45 μm or 0.22 μm filter, into the sterile centrifuge tube. A 0.45 μm is often preferred to reduce the likelihood of eliminating viruses. Alternatively, samples could be microcentrifuged at 10000 x *g* for 10 minutes.

A sample is used to make a 1/10 dilution of each pool (for example, 0.5 mL added to 4.5 mL cell culture maintenance medium in tubes labelled with the sample ID number, sample number, cell line identification and "1/10" as described previously).

Without decanting the cell culture medium, inoculate two sets of duplicate fresh cell cultures in 24-well culture plates prepared on the previous day (Appendix 5), one set with samples (150 uL) of the pooled supernatant and the other set with 150 uL samples of the 1/10 dilution.

Place the cell culture plates in plastic containers and incubate cultures at 15^{O}C (see Appendix 5 for culture conditions).

Observe these cultures for at least 9-13 days and record observations.

Irrespective of the time at which the passage occurred, cell cultures should be observed for a total of at least 21 days for completion of the assay.

Interpretation (including limitations)

Reading

Microscopic examination of the cultures is undertaken using an appropriate inverted light microscope equipped with 4X, 10X and 40X objectives.

The cultures are examined for tissue sample cytotoxicity, bacterial/fungal contamination, viral CPE and any other abnormalities compared with the negative control cultures.

Record observations.

Interpretation

The test is valid if the uninoculated negative control cultures retain normal cellular morphology for the full period of incubation.

The test sample is NEGATIVE if the inoculated cell cultures retain normal cellular morphology similar to the negative control cultures and the cellular monolayer retains normal integrity, that is, it does not demonstrate viral CPE such as plaque or syncytia formation.

If any of the cell cultures inoculated with test samples demonstrate any abnormalities, such as plaque or syncytia formation, further investigation is required, such as examination by electron microscopy and/or further sub-culturing. If EM examination demonstrates presence of virus particles, virus identification is required. Procedures for identification of specific viruses will be covered by other ANZSDPs.

Appendix 9. Acronyms and abbreviations

ACRONYM/ ABBREVIATION	DEFINITION
AAHL	Australian Animal Health Laboratory
AFDL	AAHL Fish Diseases Laboratory
ANZSDP(s)	Australian and New Zealand Standard Diagnostic Procedure(s)
ATCC	American Type Culture Collection
BF-2	bluegill fry cell line
CEFAS	The Centre for Environment, Fisheries and Aquaculture Science, UK
CSPV	chinook salmon paramyxovirus
CSRV	chum salmon reovirus
CHSE-214	chinook salmon embryo cell line
CPE	cytopathic effect
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAFF	Australian Government Department of Agriculture, Fisheries and Forestry
DFO	Department of Fisheries and Oceans, Canada
EHN(V)	epizootic haematopoietic necrosis (virus)
EM	electron microscopy
EMEM	Eagle's minimal essential medium with Earle's salts
EPC	*epithelioma papulosum cyprini* cell line
EU	European Union
FBS	foetal bovine serum
FHM	fathead minnow cell line
g	gravitational force
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl] piperazine-N'-[ethanesulphonic acid]
HS	*Herpesvirus salmonis*
ID	identification
IHN(V)	infectious haematopoietic necrosis (virus)
IPN(V)	infectious pancreatic necrosis (virus)
ISA(V)	infectious salmon anaemia (virus)
IU	international unit
L	Litre
L-15	Leibovitz's L-15 medium
MAFF	Ministry of Agriculture, Fisheries and Food, UK
mL	millilitre
mM	millimolar
OIE	World Organisation for Animal Health (formerly known as Office International des Epizooties)
OMV(D)	*Oncorhynchus masou* virus (disease)
PBSA	phosphate-buffered saline (pH 7.4) without Ca^{2+} and Mg^{2+} ions
pi	post-infection or post-inoculation
RTG-2	rainbow trout gonad cell line
T/V	trypsin/versene
ug	microgram
uL	microlitre
UK	United Kingdom
USA	United States of America
VHS(V)	viral haemorrhagic septicaemia (virus)
v/v	volume/volume