

# Development of rapid molecular tests and cell culture procedures for the detection and identification of iridoviruses

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By Edna Gias, Investigation and Diagnostic Centre, Wallaceville, MAF  
Biosecurity New Zealand

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Requests for further copies should be directed to:

Strategic Science Team  
Policy and Risk Directorate  
MAF Biosecurity New Zealand  
P O Box 2526  
WELLINGTON

Telephone: 0800 00 83 33  
Facsimile: 04-894 0300

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# Abstract

There are various pathways by which exotic aquatic animal diseases can be introduced into the environment, and this includes the importation of ornamental fish. New Zealand is vulnerable to the introduction of exotic aquatic animal pathogens as over one million live ornamental fish are imported into the country each year. The introduction of these exotic pathogens into our environment could be detrimental to our aquatic organisms and impact on our economic, environmental and cultural values. It is crucial that MAFBNZ possesses the ability to detect exotic aquatic animal pathogens, both from the perspective of fisheries/aquaculture sustainability and protection, and compliance with Import Health Standards. The development of molecular based assays to identify key aquatic animal pathogens of interest will increase the rapid, specific diagnostic capabilities of MAFBNZ and thus help in the mitigation of any risk associated with the importation of live ornamental fish.

A two year operational research-funded project was conducted at the Animal Health Laboratory at the Investigation and Diagnostic Centre, Wallaceville. The project “Development of rapid molecular tests and cell culture procedures for the detection and identification of iridovirus” involved the development and validation of real time PCR assays to identify iridoviruses of high risk to New Zealand. The iridoviruses of concern are ranaviruses and megalocytiviruses, with a particular interest in gourami iridovirus. The project also involved the validation of cell culture procedures for the isolation of target iridoviruses.

Findings from this project show that the real time PCR assays developed and validated are specific, sensitive and efficient for the detection and identification of ranaviruses, megalocytiviruses and gourami iridoviruses. The cell culture procedures for the isolation and propagation of ranaviruses have also been validated. All BF2 (Blue Gill Fry), EPC (Epithelioma Populosum Cyprini), ChSE (Chinook Salmon Embryo), ChSE-214 (Chinook Salmon Embryo) and FHM (Fat Head Minnow) cell lines were found to be sensitive to ranaviruses at higher incubation temperature, with BF2 being the most sensitive. A preliminary field survey which ran both the gold standard (virus isolation) and ranavirus real time PCR assay in parallel revealed that all imported *Poecilia reticulata* (guppy fish) tested were free from ranavirus. With the development of these real time PCR assays, MAFBNZ has increased its diagnostic capability in aquatic disease to include diagnostic testing for exotic iridoviral diseases.

## Keywords

Iridovirus, ranavirus, megalocytivirus, ornamental fish, real time PCR, virus isolation

# 1. Introduction

Ornamental fish have been recognised as vectors of a variety of pathogens. International trade of ornamental fish facilitates the spread of diseases globally including those that are caused by iridoviruses. Iridoviruses affect various species of fish, reptiles and amphibians. Most of the serious infections have been attributed to the members of the *Ranavirus* and *Megalocytiavirus* genera and therefore they present a high risk to New Zealand. Infections caused by ranaviruses and red sea bream iridovirus (RSIV), a member of the *Megalocytiavirus* genus, are listed by the OIE. Fish species known to be susceptible to iridoviruses are among those permitted to enter New Zealand, and a number of susceptible aquatic and amphibian hosts are present in the native environment. Introduction of iridoviruses into the environment may pose a risk to the fisheries/aquaculture industries, native fish and amphibian populations.

The new Import Health Standard (IHS) on importation of ornamental fish will require testing for iridoviral diseases in high risk species showing mortalities or clinical signs of the disease. Currently MAF has capabilities in histopathological, virological and biochemical analysis for aquatic animal pathogens but this can be costly and time consuming. There is therefore the need for the development and implementation of molecular-based tests that offer increased specificity, efficiency and cost effectiveness. Additionally, there is also a need to validate the cell culture procedures for isolation of iridoviruses, which will function as a gold standard test for comparison with the developed molecular based tests.

## Project objective(s)

1. To develop and validate real-time molecular-based diagnostic assays to detect iridoviruses that have the potential to impact upon native fish and amphibian populations and fisheries/aquaculture industries.
  - a) To review historical and current literature on high risk iridoviruses and the methods used for their identification
  - b) To develop and validate molecular-based assays to detect ranaviruses, megalocytiaviruses and gourami iridoviruses.
- Validate assays in accordance with OIE guidelines  
[http://www.oie.int/eng/normes/fmanual/A\\_00012.htm](http://www.oie.int/eng/normes/fmanual/A_00012.htm)
  - Chapter 1.1.4. [Principles of validation of diagnostic assays for infectious diseases](#)
  - Chapter 1.1.5. [Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases](#)
- c) Preliminary field survey of targeted iridoviruses in imported ornamental fish.
2. To validate virus isolation procedures for iridoviruses in cell line(s)
  - a) To review historical and current literature on procedures used for isolation of high risk iridoviruses.

b) To validate virus isolation procedures for selected iridoviruses

- Validate assays in accordance with OIE guidelines  
[http://www.oie.int/eng/normes/fmanual/A\\_00012.htm](http://www.oie.int/eng/normes/fmanual/A_00012.htm)

## 1.1. BACKGROUND INFORMATION ON TARGET IRIDOVIRUSES

Of the iridovirus genera, *Ranavirus* and *Megalocytiavirus* are important to New Zealand and have been selected as targets for the molecular diagnostic tests. These viruses are exotic to New Zealand.

### 1.1.1 Ranaviruses

Ranaviruses have a broad host range, infecting various species of marine and freshwater fish, reptiles and amphibians. They are systemic, causing disease in organs such as kidney and spleen (characterised by cell necrosis), and skin ulceration in some amphibian species (Cunningham et al. 1996). Members of the *Ranavirus* genus are genetically diverse and are classified into different species. Virus species that are of high risk to New Zealand are guppy virus 6 (GV6), doctor fish iridovirus (DFV), epizootic haematopoietic necrosis virus (EHNV) and bohle iridovirus (BIV).

### 1.1.2 Guppy virus 6 and Doctor fish iridovirus

GV6 and DFV were first isolated from freshwater ornamental fishes imported into the United States from Southeast Asia. These viruses naturally infect *Poecilia reticulata* (guppy) and *Labroides dimidiatus* (bluestreak cleaner wrasse), respectively, and were shown to experimentally infect rainbow trout (*Oncorhynchus mykiss*) (Hedrick & McDowell. 1995). They have been isolated and propagated in a variety of fish cell lines at various incubation temperatures ranging from 20 to 25°C (Hedrick & McDowell. 1995, Hedrick, personal communication)

### 1.1.3 Epizootic haematopoietic necrosis virus (EHNV)

EHNV is endemic in Australia and causes severe systemic necrotic disease in redfin perch (*Perca fluviatilis*) and rainbow trout. EHNV was responsible for a high mortality outbreak among redfin perch in Australia in 1986 (Langdon et al. 1986; Langdon & Humphrey. 1987; Langdon et al. 1988). Other host species that are experimentally susceptible to EHNV include Macquarie perch (*Macquaria australasica*), mosquito fish (*Gambusia affinis*), silver perch (*Bidyanus bidyanus*) and mountain galaxias (*Galaxias olidus*). EHNV has been isolated in a broad range of fish cell lines at incubation temperature ranging from 15 to 25°C (Ahne et al. 1998; Hyatt et al. 2000; Crane et al. 2005).

### 1.1.4 Bohle iridovirus (BIV)

BIV was first isolated from metamorphs of the ornate burrowing frog *Lymnodynastes ornatus* in Australia (Speare & Smith. 1992). The virus experimentally infected various frog species, *Litoria caerulea*, *L. alboguttata*, *Cyclorana brevipes* and *Pseudophryne coriacea*, and fish species, *Lates calcarifer* (barramundi) and *Oreochromis mossambicus* (tilapia) (Hengstberger et al. 1993; Cullen et al. 1995; Ariel & Owens. 1997). BIV grew readily in various mammalian cell lines at 25°C and fish cell lines at temperature ranging from 23 to 25°C (Speare & Smith. 1992).

### 1.1.5 Megalocytiaviruses

Megalocytiaviruses cause systemic disease in a wide variety of marine and freshwater fish. The disease is characterised by cell hypertrophy in affected organs, commonly kidney and spleen.

Megalocytiviruses have been responsible for mass mortality outbreaks in aquaculture settings in many countries including Denmark, China, Japan, Singapore, Korea and Taiwan, resulting in significant economic losses (Bloch & Larsen. 1993; Chua et al. 1994; Nakajima et al. 1995; Chao & Pang. 1997; Chi et al. 1997; Chou et al. 1998; Nakajima et al. 1998; He et al. 2000; Jung & Oh. 2000; Wang et al. 2003). It is also pathogenic for several ornamental fish species, and has been reported to affect various species of gouramis including three-spot gourami (*Trichogaster trichopterus*), pearl gourami (*T. leeri*) and silver gourami (*T. microlepis*), dwarf gourami (*Colisa lalia*) and thick-lipped gourami (*C. labiosa*), African lampeye (*Aplocheilichthys normani*), angelfish (*Pterophyllum scalare*) and swordtails (*Xiphophorus hellerii*) (Fraser et al. 1993; Rodger et al. 1997; Paperna et al. 2001; Gibson-Kueh et al. 2003; Go & Whittington. 2006; Jeong et al. 2008). In contrast to ranaviruses, members of the *Megalocytivirus* genus share extensive DNA sequence identity, albeit with some nucleotide differences. Most megalocytiviruses are difficult to isolate and propagate in cell culture and only a few strains, such as RSIV and gourami iridovirus, can grow in certain fish cell lines. Megalocytivirus strains that are of high risk to New Zealand are gourami iridovirus, dwarf gourami iridovirus (DGIV) and African lampeye iridovirus (ALIV). All these viruses are genetically similar and they most likely represent strains of infectious spleen and kidney necrosis virus (ISKNV), the type species of the *Megalocytivirus* genus (Go et al. 2006).

#### 1.1.6 Gourami iridovirus

Gourami iridovirus was first isolated from farm-raised three-spot gouramis (*T. trichopterus*) in Florida, USA in 1993. Infected fish have extensive necrosis and abnormally enlarged cells, predominantly in the spleen and kidney (Fraser et al. 1993). Gourami iridovirus does not grow in many common fish cell lines and, to date, it can only be isolated in tilapia heart (TH) cells (Fraser et al. 1993).

#### 1.1.7 Dwarf gourami iridovirus and African lamp-eye iridovirus

Infection with DGIV was reported in imported dwarf gouramis (*Colisa lalia*) in Australia and Japan (Anderson et al. 1993; Sudthongkong et al. 2002). ALIV, another megalocytivirus strain genetically similar to DGIV, was isolated from African lamp-eye imported into Japan (Sudthongkong et al. 2002). In 2003, DGIV was responsible for death among dwarf gouramis in Sydney pet shops. Molecular and pathogenesis studies have linked DGIV with a high mortality outbreak among farmed Murray cod (*Maccullochella peelii peelii*) (Go et al. 2006) in Australia in 2003. Isolation and propagation of DGIV and ALIV in many common fish cell lines is difficult and the viruses have been isolated successfully only in Grunt Fin (GF) cell lines (Sudthongkong et al. 2002; Gibson-Kueh et al. 2003).

## 2. Methods

### 2.1. VIRUSES AND AQUATIC BACTERIA

All viruses and aquatic bacteria used in this project are detailed in Appendix 1 and 2.

The EHNV, DFV and GV6 virus stocks were prepared by propagating these viruses in EPC or ChSE cell lines at 22°C in the PC3 containment laboratory. Sourcing of megalocytiviruses for positive controls proved difficult. Live virus isolates were not available as they have not been successfully isolated from cell cultures and the DNA material available for testing was limited due to difficulty of sourcing them from overseas laboratories.

### 2.2. CELL LINES

All fish cell lines used for validation of iridoviruses isolation are detailed in Appendix 3. The cell lines were kindly provided for this project by the IDC Virology team.

### 2.3. FISH CELL LINES SENSITIVITY TO EHNV, DFV AND GV6

EHNV, DFV and GV6 have been successfully isolated and propagated in a number of fish cell lines at temperatures ranging from 15 to 25°C. The sensitivity of a number of fish cell lines (Appendix 3) to these viruses was evaluated at incubation temperatures of 15 and 22°C. These temperatures were selected as they were readily available in the PC3 containment laboratory. The cell monolayers were infected with each virus at multiplicity of infection or MOI of 1, adsorbed for 30 minutes at room temperature and, after the addition of fish media, the cultures were incubated at 15 and 22°C. The cultures were examined every 2 to 3 days for virus cytopathic effect (CPE) and were harvested when 80 to 100 percent CPE were observed.

The sensitivity of a cell line to virus was assessed by establishing the concentration of virus produced after growth in the cell line. This concentration of virus or viral titre is expressed as 50 percent tissue culture infective dose per ml or TCID<sub>50</sub>/ml. To determine the viral titre the harvested virus cultures were diluted ten-fold in fish media from neat to 10<sup>-8</sup>, inoculated onto cell monolayers, adsorbed for 30 minutes at room temperature and after the addition of fish media, the cultures were incubated at 15 and 22°C for 14 days. The TCID<sub>50</sub>/ml of each virus grown in different cell lines at different incubation temperatures were calculated using the Karber method.

### 2.4. SELECTION OF TARGET GENE

The level of knowledge of the molecular characteristics of the iridovirus genomes is limited. Only a few genes such MCP, ATPase and DNA polymerase have been characterised for vertebrate iridoviruses. The gene target selected for the molecular assays was the major capsid protein (MCP) which has been frequently used for detection of iridoviruses (Marsh et al. 2002; Caipang et al. 2003; Go et al. 2006; Pallister et al. 2007). MCP is the predominant structural component of the virus particles but whose function is yet to be elucidated. It is highly conserved and has been shown to be stable in various strains of ranaviruses isolated from different animals at different locations and time (Tidona et al. 1998; Hyatt et al. 2000).

## 2.5. PRIMERS AND PROBE DESIGN

MCP sequences for all known ranaviruses and megalocytiviruses in GenBank were obtained via the National Center for Biotechnology Information website (<http://www.ncbi.nih.gov>) (NCBI) (Wheeler D.L. et al. 2003). Target gene sequences were analysed using Beacon Designer version 7 (PREMIER Biosoft International, USA) and primers and probes were selected to identify target species (Table 1). All selected primers, probes and amplicons were checked for possible similarity to lymphocystis, a ubiquitous fish iridovirus as well as other sequences present in the GenBank database.

Real time PCR based on SYBR Green chemistry was chosen for the identification of ranaviruses and megalocytiviruses. Primer pairs were designed within the conserved regions of each virus MCP gene to target all high risk virus species/strains described previously. However for the ranavirus assay, preliminary specificity testing revealed problems with DFV and GV6. The target DNA of these viruses were not amplified, although they were shown to have 100 percent homology with other target ranavirus sequences. Another potential gene, ATPase, was investigated, however, this was not practical as this gene has not been characterised for DFV and GV6. Unpublished results by Dr Alex Hyatt (personal communication) have shown that the primer pair used for identification of EHNV in the OIE protocol was able to amplify all targeted virus species including DFV and GV6, and therefore was selected to specifically identify ranaviruses.

For identification of gourami iridovirus, molecular beacon based probe chemistry was chosen to give increased specificity in the real time PCR assay. The probe bears a single nucleotide polymorphism (SNP) within its sequence that allows specific hybridization with gourami iridovirus DNA.

Table 1. Primers and probes nucleotide sequences.

Primer/ Probe name	Assay	Nucleotide sequence (5'-3')	Amplicon size
M151	Ranavirus	5'-AACCCGGCTTTCGGGCAGCA-3'	321
M152	Ranavirus	5'-CGGGGCGGGGTTGATGAGAT-3'	
Mega1	Megalocytivirus/ Gourami iridovirus	5'-GTGTGGCTGCGTGTTAAG-3'	214
Mega2	Megalocytivirus	5'-TGCCAATCATCTTGTTGTAGC-3'	
Mega3	Gourami iridovirus	5'- GAGGAACTCACTGGTCAGG-3'	NA
GTT-MB*	Gourami iridovirus	5' d FAM-CGCGATC (ACATCCGCTGGTGTGACAATCTGATG) GATCGCG-BHQ-1 3'	NA

\* Molecular beacon probe; Red=Probe sequence; Blue=stem sequence, FAM= fluorescent dye; BHQ-1= quencher; NA= Not applicable

## 2.6. DNA EXTRACTION

DNA was extracted from virus cultures using a commercial kit (QIAamp DNA Mini Kit, QIAGEN) according to the manufacturer's protocol. DNA from clarified spleen and kidney tissue homogenates were extracted using Vx Viral DNA/RNA extraction kit in an automated X-tractor gene (Corbett Life Science, Australia) according to the manufacturer's protocol. The DNA elutes were stored at -20°C until required for analysis.

## 2.7. OPTIMIZATION OF REAL TIME PCR ASSAYS

Each real time PCR assay was assessed for optimal reagent concentrations and cycling parameters. The PCR amplification was performed with a Rotor-Gene 3000 real time thermocycler (Corbett Research, Australia). The optimal concentrations and cycling parameters were determined from the earliest Ct values produced.

## 2.8. SYBR GREEN AMPLICON ANALYSIS

When considering SYBR Green based real time PCR assay, due to the ability of the SYBR Green dye to bind any double stranded DNA, the amplification of a target virus needs to be confirmed by the presence of the specific melting temperature (T<sub>m</sub>) of the amplicon. The amplicon T<sub>m</sub> was determined by performing a melt curve analysis over a range of increasing temperature at the end of each PCR run. To determine the range of T<sub>m</sub>, the T<sub>m</sub> of amplicons from different target viruses were assessed. The amplification was carried out in triplicate and the run was repeated two to three times.

Analytical specificity

The analytical specificity of each assay was assessed by analysing various species/strains of iridoviruses, DNA viruses and aquatic bacteria (Appendix 1 and 2).

## 2.9. ANALYTICAL SENSITIVITY

### 2.9.1 Purified DNA

The analytical sensitivity or detection limit was determined using purified DNA. In addition, the detection limit of the ranavirus real time PCR assay was also determined using cultured virus. For megalocytivirus and gourami iridovirus real time PCR assay, gourami iridovirus DNA eluted from the FTA card (Whatman, UK) was used. Ten-fold serial dilutions were performed on viral DNA of known concentration using nuclease free water as diluent from neat to 10<sup>-6</sup>. Amplification of each dilution was carried out in duplicate and these experiments were repeated twice. The detection limit was determined from the lowest concentration of DNA that could be detected on the Rotor-Gene 3000.

### 2.9.2 Virus titre

The analytical sensitivity of real time PCR was determined from cell culture derived virus titres, and due to the lack of viral isolates, this experiment was carried out for the ranavirus assay only, using EHNV as the reference virus. EHNV stock with a titre of 4.74 x 10<sup>5</sup> TCID<sub>50</sub>/ml was diluted ten-fold in fish media from neat to 10<sup>-6</sup>. DNA was extracted from each virus dilution and amplification was carried out in duplicates. These experiments were repeated three times. The detection limit was determined as described previously and the highest virus dilution showing amplification was then correlated with the titres of virus that were used in the assay.

### 2.9.3 Real time PCR vs. virus isolation

The sensitivity of ranavirus real time PCR assay was also compared with the gold standard method of virus isolation. BF2 cell line has been shown to be most sensitive to EHNV and therefore was selected for use in this comparative experiment. The same EHNV stock as above was diluted ten-fold in fish media from neat to 10<sup>-6</sup> dilutions. One hundred microlitres of each virus diluent was inoculated in duplicates onto 24 hour confluent BF2 cell lines, adsorbed for 30 minutes at room temperature, and after the addition of fish media, incubated at 22°C. The cultures were examined every 2 to 3 days for virus cytopathic effect (CPE) for 14 days. DNA from each virus diluent was extracted as described previously and then subjected to ranavirus real time PCR assay. This experiment was repeated three times. The detection limit was determined for both real time PCR assay and virus isolation as described previously and the highest virus dilution showing amplification or 100 percent CPE in cell cultures was then correlated with the titres of virus that were used in the assay.

## 2.10. REPEATABILITY

To assess the variability within (intra-run) and between runs (inter-run), assay repeatability was carried out. Assay repeatability is determined by establishing the co-efficient of variation of the cycle threshold (Ct) or CV of the assay. To determine the intra-run CV %, each assay was performed on 1ng of the same DNA in duplicates. This experiment was repeated three times. To determine the inter-run CV %, each assay was performed by three individuals on a series of 10-fold dilution of the same DNA. These experiments were repeated three times.

## 2.11. PRELIMINARY SURVEY

To assess the presence of ranaviruses and megalocytiviruses in imported ornamental fish, two high risk fish species were selected as the representative host of each virus. Guppy fish (*P. reticulata*) and dwarf gourami (*C. lalia*) are known host of GV6 and DGIV respectively. The fish were imported from an exporter in Singapore and the shipment was facilitated by a New Zealand-based ornamental fish importer. However, screening could only be carried out for GV6 as the entire shipment of dwarf gourami was dead on arrival.

The fish were euthanized using fish anaesthetic (clove oil). The kidneys and spleens were aseptically removed and pooled from 10 fish. The tissues were then homogenised using pestle followed by vigorous vortexing in 10 percent (w/v) of fish transport medium (FTM) and incubated at 4°C overnight. The tissue debris was pelleted by centrifugation at 2500g for 10 minutes at 4°C, after which DNA was extracted from the clarified homogenate as described previously and subjected to ranavirus real time PCR assay. The tissue homogenates were also subjected to virus isolation as it is considered the gold standard for the detection of iridoviruses. The homogenates were inoculated onto BF2 cell line which has been shown to be most sensitive to GV6. Another fish cell line, EPC, which is also sensitive to GV6, was included as an additional cell line in the virus isolation procedure. The tissue samples were passaged twice in both cell lines and incubated at 25°C for a total of 21 days. This temperature was selected as it was readily available in the PC2 laboratory. In addition, ranaviruses have been isolated at 25°C as described in a number of published papers (Speare & Smith. 1992; Hengstberger et al. 1993; Cullen et al. 1995; Ariel & Owens. 1997; Ahne et al. 1998; Hyatt et al. 2000; Crane et al. 2005).

### 3. Results

#### 3.1. SENSITIVITY OF FISH CELL LINES TO EHNV, GV6 AND DFV

The sensitivity of five fish cell lines to ranaviruses was evaluated at two different incubation temperatures (Table 2). Generally, all cell lines were sensitive to EHNV, GV6 and DFV and the sensitivity increased at a higher incubation temperature of 22°C. The viruses grew well in all cell lines yielding good viral titres, although at 15°C, the growth of DFV and GV6 were slow, yielding considerably lower titres than that at 22°C. Of the cell lines, BF<sub>2</sub> was found to be most sensitive to all three viruses.

Table 2. Fish cell lines sensitivity to ranaviruses.

Cell line	EHNV		DFV		GV6	
	TCID <sub>50</sub> /ml (log <sub>10</sub> )		TCID <sub>50</sub> /ml (log <sub>10</sub> )		TCID <sub>50</sub> /ml (log <sub>10</sub> )	
	15°C	22°C	15°C	22°C	15°C	22°C
BF2	7.52± 7.16	7.80± 0	5.87±5.17	8.12±7.43	5.78 ± 5.53	8.49 ± 7.80
EPC	6.62± 5.92	7.80± 0	NA	5.24±5.11	NA	5.62 ± 4.92
ChSE	5.49± 5.36	6.24± 5.55	0.63±0.77	5.78±5.54	1.07 ± 0.67	5.79 ± 5.50
ChSE-214	6.92± 0	6.30± 0	1.44±1.04	7.30±0	2.82 ± 2.42	7.62 ± 6.92
FHM	6.68± 0	6.99± 6.29	1.80±0	6.97±6.91	2.12 ± 1.43	6.44 ± 6.04

NA= virus titres were not able to be determined as the EPC cells died after 14 days of incubation.

#### 3.2. OPTIMISED PARAMETERS FOR REAL TIME PCR ASSAYS

The real time PCR parameters optimized included primer (0.1-0.5 µM), magnesium chloride (1.5-7.0mM) and probe (0.4-1.0 µM) concentrations, annealing temperature and cycling parameters. For SYBR Green based assays, the reaction contained 1x SYBR<sup>®</sup> Green JumpStart<sup>™</sup> Taq ReadyMix<sup>™</sup> (Sigma-Aldrich, USA), 0.1 µM or 0.2 µM of each primer for megalocytivirus and ranavirus assay respectively, 1-10 ng/µl DNA and nuclease free water was added to a final volume of 25µl. For probe based assay, the reaction contained 1x JumpStart<sup>™</sup> Taq ReadyMix<sup>™</sup> (Sigma-Aldrich, USA), additional 3.0 mM MgCl<sub>2</sub>, 0.1 µM of each primer, 0.4 µM of the molecular beacon, 1-10 ng/µl DNA and nuclease free water was added to a final volume of 25µl.

The cycling parameters varied for different assays to assure increased specificity and sensitivity. The ranavirus real time PCR was conducted with an incubation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 65°C for 30 seconds and 72°C for 30 seconds with fluorescence acquisition. The point of dissociation of the double-stranded DNA (the melt) was determined by raising the temperature from 60 to 95°C, holding for 5 seconds at each temperature step during fluorescence acquisition. The megalocytivirus real time PCR followed the same cycling protocol except that the annealing temperature was reduced to 60°C. The gourami iridovirus real time PCR was conducted with an incubation at 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 62°C for 45 seconds with fluorescence acquisition, and 72°C for 45 seconds.

### 3.3. SYBR GREEN AMPLICON ANALYSIS

The specific  $T_m$  of each target virus was determined from multiple runs with replicated samples in each run. The melting temperatures ( $T_m$ ) of the amplicon for each target virus species varied, with  $T_m$  ranging from 89.5°C to 91°C for ranavirus assay and from 88°C to 89.5°C for megalocytivirus assay (Table 3). Based on these results, the specific  $T_m$  of  $90 \pm 1^\circ\text{C}$  and of  $88.75 \pm 0.75^\circ\text{C}$  will be used to confirm the presence of a ranavirus and megalocytivirus respectively.

Table 3. Melting temperatures ( $T_m$ ) of target virus amplicons.

Assay	Target virus	$T_m$ (°C)
Ranavirus	EHNV	90.3 - 91.0
	DFV	89.5 – 89.7
	GV6	89.5 – 89.7
	BIV	90.2 – 90.3
	FV3	90.3 – 90.5
	ESV	90.3 – 90.7
	Tssetsudo hermanni ranavirus	90.2 – 90.5
	Gecko ranavirus	90 – 90.2
Megalocytivirus	Gourami iridovirus	88.2 - 89.5
	RSIV	88
	PGIV	88.5

### 3.4. ANALYTICAL SPECIFICITY

The analytical specificity of the real time PCR assay was determined by testing different species of iridoviruses, reptilian DNA viruses and aquatic bacterial pathogens (Appendix 1 and 2). All assays were found to have 100 percent analytical specificity (Table 4). Each assay did not amplify any aquatic bacterial pathogens and non-target viruses including lymphocystis virus, a ubiquitous fish iridovirus.

Table 4 Analytical specificity of real time PCR assays. The number of target, non-target virus and aquatic bacteria tested for each real time PCR assay.

Assay	% of positive target virus	% of positive non-target virus	% of positive non-target aquatic bacteria
Ranavirus	100 (n=8)	0 (n=8)	0 (n=25)
Megalocytivirus	100 (n=3)	0 (n=13)	0 (n=25)
Gourami iridovirus	100 (n=1)	0 (n=15)	0 (n=25)

### 3.5. ANALYTICAL SENSITIVITY

All assays were found to be highly sensitive, with detection limits within the femtogram range using purified DNA as the template (Table 5). The sensitivity of the ranavirus real time PCR assay was also tested for virus culture and the detection limit was determined as 47.4 TCID<sub>50</sub>/ml (equivalent to 0.047 TCID<sub>50</sub> per amplification reaction). Comparatively, the ranavirus real time assay was as sensitive as virus isolation (Table 5).

Table 5: Analytical sensitivity of real time PCR assays and comparison of ranavirus real time assay with virus isolation.

Assay	Real time PCR		Virus isolation
	DNA ( $\pm$ SD fg)	Virus titre ( $\pm$ SD TCID <sub>50</sub> /ml)	Virus titre ( $\pm$ SD TCID <sub>50</sub> /ml)
Ranavirus	100 $\pm$ 0	47.4 $\pm$ 0	47.4 $\pm$ 0
Megalocytivirus	10 $\pm$ 0	NT	NT
Gourami iridovirus	100 $\pm$ 0	NT	NT

fg, femtogram; SD, standard deviation;

NT – not tested as virus isolates were unable to be sourced.

### 3.6. REPEATABILITY

The repeatability of the real time PCR assays was evaluated from multiple runs with replicated samples in each run. The intra-run and inter-run CV for each assay (Table 6) were well within the acceptable variation published in the literature indicating that the assays are robust and easily repeatable (Wang et al. 2006).

Table 6: Intra-run and inter-run CV of real time PCR assays.

Assay	<sup>a</sup> Intra-run CV (%)	<sup>b</sup> Inter-run CV (%)
	n=2	n=3
Ranavirus	0 - 0.71	1.80
Megalocytivirus	0.16 - 0.32	1.82
Gourami iridovirus	1.93 - 2.97	2.76

a=duplicates of DNA sample were amplified in the same PCR run and repeated 3 times.

b= mean of average triplicates for 3 separate runs

### 3.7. PRELIMINARY SURVEY

A preliminary survey was conducted on imported *P. reticulata* for the presence of GV6. All fish tested negative for GV6 using the ranavirus real time PCR assay. This was consistent with the negative results for virus isolation in the BF<sub>2</sub> and EPC cell lines (Table 7).

Table 7: Preliminary survey of GV6 in imported *Poecilia reticulata* (n=100).

<sup>a</sup> Pool number	Ranavirus assay	<sup>b</sup> Virus isolation
1	Negative	Negative
2	Negative	Negative
3	Negative	Negative
4	Negative	Negative
5	Negative	Negative
6	Negative	Negative
7	Negative	Negative
8	Negative	Negative
9	Negative	Negative
10	Negative	Negative

a= pool of 10 fishes

b= virus isolation results from BF<sub>2</sub> and EPC cell lines.

## 4. Discussion

For isolation of EHNV, DFV and GV6 it is recommended that BF<sub>2</sub> cell lines be used at higher incubation temperatures, between 22°C to 25°C as this cell line produced the highest titre of virus within the shortest timeframe. Other cell lines such as EPC, ChSE or ChSE-214 are recommended for use when additional cell lines are required in the isolation procedure. These cell lines can be used to isolate ranavirus from PCR positive samples if further characterization is required or if the isolates are to be used as reference strains for other aquatic molecular diagnostic test development. BF<sub>2</sub> cells can also be used to produce high titre virus stocks for preparation of real time PCR positive controls.

Three real time PCR have been developed, optimised and validated for the identification of ranaviruses, megalocytiviruses and gourami iridovirus. The generic ranavirus and megalocytivirus real time PCR assays were designed for identification of various known species or strains of ranavirus and megalocytivirus, respectively, and they have been shown to be highly specific and sensitive. Specific identification of gourami iridovirus was achieved using a probe based real time PCR and the assay has been shown to be highly specific to the target virus, capable of differentiating gourami iridovirus from other megalocytiviruses tested. The variability within replicates and between runs of each assay was low with coefficient of variation values below 3 percent.

The ranavirus PCR assay detected all high risk viruses and was as sensitive as the gold standard method of virus isolation. In addition to ornamental fish ranaviruses, the assay also detected ranaviruses isolated from reptiles and amphibians. In preliminary survey on field samples, the assay was used to screen for GV6 in imported *P. reticulata* and the results were in agreement with that of the gold standard method of virus isolation.

The megalocytivirus PCR assay was capable of detecting the OIE-listed RSIV, an iridovirus which is important in marine aquaculture, as well as two strains of ornamental fish megalocytivirus. Although the material available for analytical specificity testing was limited, the PCR developed in this project was predicted to amplify DNA from other ornamental fish megalocytiviruses including DGIV, ALIV and ISKNV. Review of published literature has shown that megalocytiviruses isolated from ornamental fish represent various virus quasispecies which are genetically very similar to each other (Go et al. 2006; Jeong et al. 2008). Nucleotide sequence analysis of MCP gene of all known megalocytivirus available in the GenBank database shows that the primer sequences were conserved among the ornamental fish viruses. In addition, the nucleotide sequence of PGIV tested in this project has been shown to be identical to DGIV, ALIV and ISKNV (Go et al. 2006; Jeong et al. 2008). Future specificity testing is possible as there is a potential collaboration with a laboratory in Australia to further assess the assay specificity on their collection of ornamental fish megalocytivirus.

In conclusion, the real time PCR assays developed in this project were highly specific and sensitive. They are also accurate, robust and easily repeatable. These assays can be used in population or batch testing of high risk imported ornamental fish species, or in diagnostic testing of suspected iridoviral disease or mortalities in transitional facilities. Other potential application includes surveillance study of imported high risk ornamental fish and diagnostic testing of mortality in fin-fish and amphibians.

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## 6. References

- Ahne, W., Bearzotti, M., Bremont, M., Essbauer, S., 1998. Comparison of European systemic piscine and amphibian iridoviruses with epizootic haematopoietic necrosis virus and frog virus 3. *Journal of Veterinary Medicine Series B* 45, 373-383.
- Ahne, W., Ogawa, M., Schlotfeldt, H.J., 1990. Fish viruses: transmission and pathogenicity of an icosahedral cytoplasmic deoxyribovirus isolated from sheatfish (*Silurus glanis*). *Journal of Veterinary Medicine Series B* 37, 187-190.
- Anderson, I.G., Prior, H.C., Rodwell, B.J., Harris, G.O., 1993. Iridovirus-like virions in imported dwarf gourami (*Colisa lalia*) with systemic amoebiasis. *Aust. Vet. J.* 70, 66-67.
- Ariel, E., Owens, L., 1997. Epizootic mortalities in tilapia *Oreochromis mossambicus*. *Dis. Aquat. Org.* 29, 1-6.
- Bloch, B., Larsen, J.L., 1993. An iridovirus-like agent associated with systemic infection in cultured turbot *Scophthalmus maximus* fry in Denmark. *Dis. Aquat. Org.* 15, 235-240.
- Caipang, C.M., Hirono, I., Aoki, T., 2003. Development of a real-time PCR assay for the detection and quantification of red seabream iridovirus (RSIV). *Gyobyu Kenkyu = Fish Pathology* 38,1-7.
- Chao, C.B., Pang, V.F., 1997. An outbreak of an iridovirus-like infection in cultured grouper (*Epinephelus* spp.) in Taiwan. *Journal of the Chinese Society of Veterinary Science* 23, 411-422.
- Chi, S. and Council of Agriculture, Taipei (Taiwan Province of China). 1997. The investigation of viral disease among cultured groupers in Southern Taiwan.
- Chou, H.Y., Hsu, C.C., Peng, T.Y., 1998. Isolation and characterization of a pathogenic iridovirus from cultured grouper (*Epinephelus* sp.) in Taiwan. *Gyobyu Kenkyu = Fish Pathology* 33, 201-206.
- Chua, F.H.C., Ng, M.L., Ng, K.L., Loo, J.J., Wee, J.Y., 1994. Investigation of outbreaks of a novel disease, 'Sleepy Grouper Disease', affecting the brown-spotted grouper, *Epinephelus tauvina* Forskal. *J Fish Dis* 17, 417-427.
- Crane, M.S., Young, J., Williams, L.M., 2005. Epizootic haematopoietic necrosis virus (EHNV): Growth in fish cell lines at different temperatures. *Bull. Eur. Assoc. Fish. Pathol.* 25, 228-231.
- Cullen, B.R., Owens, L., Whittington, R.J., 1995. Experimental infection of Australian anurans (*Limnodynastes terraereginae* and *Litoria latopalmata*) with Bohle iridovirus. *Dis. Aquat. Org.* 23, 83-92.
- Cunningham, A.A., Langton, T.E., Bennett, P.M., Lewin, J.F., Drury, S.E., Gough, R.E., Macgregor, S.K., 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). *Philos. Trans. R. Soc. Lond. B. Biol.Sci.* 351,1539-1557.
- Fraser, W.A., Keefe, T.J., Bolon, B., 1993. Isolation of an iridovirus from farm-raised gouramis (*Trichogaster trichopterus*) with fatal disease. *J Vet Diagn Invest* 5, 250-253.
- Gibson-Kueh, S., Netto, P., Ngoh-Lim, G.H., Chang, S.F., Ho, L.L., Qin, Q.W., Chua, F.H., Ng, M.L., Ferguson, H.W., 2003. *The pathology of systemic iridoviral disease in fish*. *J. Comp. Pathol.* 129, 111-119.
- Go, J., Lancaster, M., Deece, K., Dhungyel, O., Whittington, R., 2006. The molecular epidemiology of iridovirus in Murray cod (*Maccullochella peelii peelii*) and dwarf gourami (*Colisa lalia*) from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. *Mol Cell Probes* 20, 212-222.
- Go, J., Whittington, R., 2006. Experimental transmission and virulence of a megalocytivirus (Family Iridoviridae) of dwarf gourami (*Colisa lalia*) from Asia in Murray cod (*Maccullochella peelii peelii*) in Australia. *Aquaculture* 258, 140-149.

- Granoff, A., Came, P.E., Breeze, D.C., 1966. Viruses and renal carcinoma of *Rana pipiens* I. The isolation and properties of virus from normal and tumor tissue. *Virology* 29:133-148.
- He, J.G., Wang, S.P., Zeng, K., Huang, Z.J., Chan, S., 2000. Systemic disease caused by an iridovirus-like agent in cultured mandarin fish, *Siniperca chuatsi* (Basilewsky), in China. *J. Fish. Dis.* 23, 219-222.
- Hedrick, R.P., McDowell, T.S., 1995. Properties of iridoviruses from ornamental fish. *Vet. Res.* 26, 423-427.
- Hengstberger, S.G., Hyatt, A.D., Speare, R., Coupar, B.E.H., 1993. Comparison of epizootic haematopoietic necrosis and Bohle iridoviruses, recently isolated Australian iridoviruses. *Dis. Aquat. Org.* 15, 93-107.
- Hyatt, A., Gould, A.R., Zupanovic, Z., Cunningham, A.A., Hengstberger, S., Whittington, R.J., Kattenbelt, J., Coupar, B.E.H., 2000. Comparative studies of piscine and amphibian iridoviruses. *Arch. Virol.* 145, 301-331.
- Jeong, J.B., Kim, H.Y., Jun, L.J., Lyu, J.H., Park, N.G., Kim, J.K., Jeong, H.D., 2008. Outbreaks and risks of infectious spleen and kidney necrosis virus disease in freshwater ornamental fishes. 78, 209-215.
- Jung, S.J., Oh, M.J., 2000. Iridovirus-like infection associated with high mortalities of striped beakperch, *Oplegnathus fasciatus* (Temminck et Schlegel), in southern coastal areas of the Korean peninsula. *J. Fish. Dis.* 23, 223-226.
- Kurita, J., Nakajima, K., Hirono, I., Aoki, T., 1998. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). *Fish Pathol.* 33, 17-23.
- Langdon, J.S., Humphrey, J.D., Williams, L.M., 1988. Outbreaks of an EHNV-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. *J. Fish. Dis.* 11:93-96.
- Langdon, J.S., Humphrey, J.D., 1987. Epizootic haematopoietic necrosis, a new viral disease in redfin perch, *Perca fluviatilis* L., in Australia. *J. Fish. Dis.* 10, 289-297.
- Langdon, J.S., Humphrey, J.D., Williams, L.M., Hyatt, A.D., Westbury, H.A., 1986. First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. *J. Fish. Dis.* 9, 263-268.
- Marschang, R.E., Braun, S., Becher, P., 2005. Isolation of a ranavirus from a gecko (*Uroplatus fimbriatus*). *J. Zoo. Wildl. Med.* 36, 295-300.
- Marschang, R.E., Becher, P., Posthaus, H., Wild, P., Thiel, H.J., Muller-Doblies, U., Kalet, E.F., Bacciarini, L.N., 1999. Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudo hermanni*). *Arch. Virol.* 144, 1909-1922.
- Marsh, I.B., Whittington, R.J., O'Rourke, B., Hyatt, A.D., Chisholm, O., 2002. Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. *Mol Cell Probes* 16, 137-151.
- Nakajima, K., Inouye, K., Sorimachi, M., 1998. Viral diseases in cultured marine fish in Japan. *Gyobyo Kenkyu = Fish Pathology* 33, 181-188.
- Nakajima, K., Maeno, Y., Fukudome, M., Fukuda, Y., Tanaka, S., Matsuoka, S., Sorimachi, M., 1995. Immunofluorescence test for the rapid diagnosis of red sea bream iridovirus infection using monoclonal antibody. *Gyobyo Kenkyu = Fish Pathology* 30, 115-119.
- Pallister, J., Gould, A., Harrison, D., Hyatt, A., Jancovich, J., Heine, H., 2007. Development of real-time PCR assays for the detection and differentiation of Australian and European ranaviruses. *J. Fish. Dis.* 30, 427-438.
- Paperna, I., Vilenkin, M., de Matos, A.P., 2001. Iridovirus infections in farm-reared tropical ornamental fish. *Dis. Aquat. Organ.* 48, 17-25.
- Rodger, H.D., Kobs, M., Macartney, A., Frerichs, G.N., 1997. Systemic iridovirus infection in freshwater angelfish, *Pterophyllum scalare* (Lichtenstein). *J. Fish. Dis.* 20, 69-72.
- Speare R., Smith, J.R., 1992. An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. *Dis. Aquat. Org.* 14:51-57.

- Sudthongkong, C., Miyata, M., Miyazaki, T., 2002. Iridovirus disease in two ornamental tropical freshwater fishes: African lampeye and dwarf gourami. *Dis. Aquat. Org.* 48,163-173.
- Teifke, J.P., Löhr, C.V., Marschang, R.E., Osterrieder, N., Posthaus, H., 2000. Detection of chelonid herpesvirus DNA by nonradioactive in situ hybridization in tissues from tortoises suffering from stomatitis-rhinitis complex in Europe and North America. *Veterinary pathology* 37, 377-385.
- Tidona, C.A., Schnitzler, P., Kehm, R., Darai, G., 1998. Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? *Virus Genes* 16, 59-66.
- Wang, C.S., Shih, H.H., Ku, C.C., Chen, S.N. 2003. Studies on epizootic iridovirus infection among red sea bream, *Pagrus major* (Temminck & Schlegel), cultured in Taiwan. *J. Fish. Dis.* 26,127-133.
- Wang, X.W., Ao, J.Q., Li, Q.G., Chen, X.H. 2006. Quantitative detection of a marine fish iridovirus isolated from large yellow croaker, *Pseudosciaena crocea*, using a molecular beacon. *J. Virol. Methods* 133, 76-81.
- Weinmann, N., Papp, T., Pedro Alves de Matos A, Teifke, J.P., Marschang, R.E. 2007. Experimental infection of crickets (*Gryllus bimaculatus*) with an invertebrate iridovirus isolated from a high-casqued chameleon (*Chamaeleo hoehnelii*). *J. Vet. Diag. Invest.* 19, 674-679.
- Wheeler, D.L., Church, D.M., Federhen, S., Lash, A.E., Madden, T.L., et al., 2003. Database resources of the national center for biotechnology. *Nucleic Acids Res.* 31, 28-33.

## 7. Appendices

### Appendix 1

Table 1. Viruses used to assess the analytical specificity of real time PCR assays.

Virus	Abbreviation/Strain	Country of origin	Reference
Epizootic haematopoietic necrosis virus	EHN strain 86/8774	Australia	(Langdon et al. 1988; Marsh et al. 2002)
Bohle iridovirus	BIV	Australia	(Speare & Smith. 1992; Marsh et al. 2002)
Doctor fish iridovirus	DFV	USA, imported from Southeast Asia	(Hedrick & McDowell. 1995)
Guppy iridovirus	GV6	USA, imported from Southeast Asia	(Hedrick & McDowell. 1995; Hyatt et al. 2000)
Frog virus 3	FV3	USA	(Granoff et al. 1966)
European sheatfish virus	ESV	Germany	(Ahne et al. 1990)
Tortoise ranavirus	CH8/96	Switzerland	(Marschang et al. 1999)
Gecko ranavirus	2000/99	NA	(Marschang et al. 2005)
Invertebrate iridovirus	IIV strain 100/01	NA	(Weinmann et al. 2007)
Tortoise herpesvirus	4295/7R/95	Germany	(Teifke et al. 2000)
Snake adenovirus	IBD27/00	NA	NA
Red sea bream iridovirus	RSIV	Japan	(Kurita et al. 1998)
Gourami iridovirus	-	USA	(Fraser et al. 1993)
Pearl gourami iridovirus	PGIV	NA	(Jeong et al. 2008)
Lymphocystis virus	-	NA	NA
Chanel catfish virus	CCV	NA	NA

NA= not available

## Appendix 2

### Abbreviations for strain references:

ATCC - American Type Culture Collection

IDC - Investigation and Diagnostic Centre,

TCFB - Tasmanian Collection of Fish Bacteria

Table 2. Bacterial strains used to assess the analytical specificity of real time PCR assays.

Strain	Reference no.
<i>Aeromonas salmonicida salmonicida</i>	ATCC 33658
<i>Aeromonas. salmonicida</i> [Goldfish biovar] (Atypical)	TCFB 673
<i>Aeromonas salmonicida biovar Acheron</i> (Atypical)	TCFB 799
<i>Aeromonas hydrophila</i>	ATCC 7965
<i>Aeromonas sobria</i>	ATCC 9071
<i>Aeromonas caviae</i>	ATCC 15468
<i>Streptococcus iniae</i>	ATCC 29178
<i>Edwardsiella tarda</i>	ATCC 15947
<i>Edwardsiella ictaluri</i>	TCFB 2157
<i>Yersinia ruckeri</i>	ATCC 29473
<i>Lactococcus piscium</i>	TCFB 213
<i>Lactococcus garvieae</i>	TCFB 453
<i>Vagococcus salmoninarum</i>	ATCC 51200
<i>Carnobacterium maltaromaticum</i>	ATCC 35586
<i>Vibrio anguillarum</i>	TCFB 1406.
<i>Vibrio parahaemolyticus</i>	ATCC 17802
<i>Vibrio fischeri</i>	ATCC 7744
<i>Vibrio harveyi</i>	ATCC 14126
<i>Photobacterium damsela</i> ssp. <i>damsela</i>	TCFB 874
<i>Photobacterium damsela</i> ssp. <i>piscicida</i>	TCFB 1273
<i>Flavobacterium psychrophilum</i>	ATCC 49511
<i>Flavobacterium columnare</i>	TCFB 383
<i>Mycobacterium fortuitum</i>	IDC T3-F40
<i>Mycobacterium salmoniphilum</i>	IDC T5-F1
<i>Vagococcus salmoninarum</i>	ATCC 51200

## Appendix 3

Table 3. Fish cell lines used to assess sensitivity to ranaviruses.

Cell lines	Abbreviation	Origin	Source
Blue Gill Fry cells	BF <sub>2</sub>	<i>Lepomis macrochirus</i>	ATCC
Epithelioma Populosum Cyprini	EPC	<i>Pimephales promelas</i>	California University
Chinook Salmon Embryo	ChSE	<i>Oncorhynchus tshawytscha</i>	California University
Chinook Salmon Embryo	ChSE-214	<i>Oncorhynchus tshawytscha</i>	ATCC
Fat Head Minnow	FHM	<i>Pimephales promelas</i>	ATCC

## Appendix 4

**Tissue Culture Infective Dose (TCID<sub>50</sub>/ml)** – the concentration of virus which will produce a cytopathic effect in 50 percent of the cell cultures inoculated

**Karber method** – formula for calculation of TCID<sub>50</sub>/ml

$$\Sigma T_{50} = t^* + \Delta t$$

$t^*$  = the highest virus dilution demonstrating 100 percent CPE in the wells inoculated.

$\Delta t$  = number of infected wells at  $t^*$  plus the number infected above  $t^*$ , divided by the number of wells inoculated, minus  $\frac{1}{2}$ .

**Multiplicity of infection (MOI)** – the average number of virus particles that infect a single cell

**Cytopathic effect (CPE)** - degenerative changes in cell cultures due to multiplication of virus

**Analytical specificity** - defined as the ability of an assay to distinguish the target agent from other non-target agents

**Analytical sensitivity (limit of detection)** - defined as the lowest concentration of DNA or TCID<sub>50</sub>/ml of the target agent that can be detected and distinguished from a zero result.

**Repeatability** - measured as both the amount of agreement between replicates within the same run or between replicates tested in different runs

## Appendix 5

Project timetable	Date	Status
Brief approved		
<b>Start “Plan” phase</b>	July 2007	Achieved
Combined Business Case & PMP approved		
<b>Start “Deliver” phase</b>		
Deadlines		
<ul style="list-style-type: none"> <li>• Complete and submit project plan</li> </ul>	March 2008	Achieved
<ul style="list-style-type: none"> <li>• Complete a literature review on iridoviruses</li> </ul>	March 2008	Achieved
<ul style="list-style-type: none"> <li>• Complete validation of VI procedures for iridoviruses in the selected cell line(s) to a minimum of OIE stage 2 (Detailed milestones in project plan)</li> </ul>	October 2008	Achieved
<ul style="list-style-type: none"> <li>• Complete validation of the molecular techniques to a minimum of OIE stage 2 (Detailed milestones in project plan)</li> </ul>	March 2009	Achieved
<ul style="list-style-type: none"> <li>• Submit final report</li> </ul>	June 2009	Achieved
Key stage/milestone date(s)		
<ul style="list-style-type: none"> <li>• Plan for obtaining reference material and samples for test validation</li> </ul>	February 2008	Achieved
<ul style="list-style-type: none"> <li>• Initiate contact with overseas reference laboratories for import of iridoviruses</li> </ul>	March 2008	Achieved
<ul style="list-style-type: none"> <li>• Obtain new cell line(s)</li> </ul>	March 2008	Achieved
<ul style="list-style-type: none"> <li>• Develop molecular techniques for detection of selected iridoviruses</li> </ul>	May 2009	Achieved
Acceptance of all project deliverables	June 2009	
<b>Start “Close” phase</b>	March 2009	Achieved
Close report approved	July 2009	
<b>Completion date</b>	July 2009	