

PrimerDesign™ Ltd

Quantification of Grass Carp Reovirus genomes

L2 inner capsid protein VP2

For general laboratory and research use only

Standard Kit





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Introduction to Grass Carp Reovirus

Hemorrhagic virus of grass carp (GCRV) was the first fish virus isolated in China (Chen & Jiang 1983), and about 10 additional isolations have been reported since then. The virions are ether and chloroform resistant, and non-sensitive to acid (pH 3) and alkaline (pH 10) treatment. (Mao et al. 1989, Ke 1990). They are 60 to 80nm in diameter and consist of a double layered capsid containing a genome composed of 11 segments of double-stranded (ds) RNA and belong to the genus Aquareovirus (Francki et al. 1991) in the family Reoviridae. The aquareoviruses comprise one of the most rapidly growing groups of finfish viruses (Hetrick & Hedrick 1993) and many of them are not associated with clinical disease. However, GCRV can cause a contagious hemorrhagic disease with high mortalities in grass carp *Ctenopharyngodon idellus*, black carp *Mylopharyngodon piceus*, topmouth gudgeon *Pseudorasbora parva* (Ding et al. 1991) and rareminnow *Gobiocypris rarus* (Wang et al. 1994). It also can replicate in silver carp *Hypophthalmichthys molitrix* and one kind of Chinese minnow, *Hemiculter bleekeri*, although no symptoms are evident (Ding et al. 1991). The virus causes large losses in fresh water fish culture facilities in China. A sensitive diagnosis method is essential if dissemination of the virus is to be controlled since no effective vaccines currently exist for its prevention. Several methods have been developed for GCRV detection, some of which utilize immunoenzyme staining (Ye et al. 1989), staphylococcal coagglutination (Yang et al. 1991), and immunofluorescence and enzyme-linked immunosorbent assays.

Specificity

The *PrimerDesign™ Quantification Kit for Grass Carp Reovirus (GCRV) Genomes* is designed for the *in vitro* quantification of all GCRV subtypes. There is very little sequence data available for these viruses at this time, but primers have 100% homology with both reference sequences currently in the NCBI database. The primers also have 96% homology to Golden shiner reovirus and are highly likely to detect this closely related strain. However, due to the inherent instability of RNA viral genomes, it is not possible to guarantee quantification of all clinical isolates.

Accession numbers for detected GCRV isolates
AF284502, AF260512



Kit Contents

- Pathogen specific primer/probe mix (150 reactions **BROWN**)
- Pathogen positive control template (for Standard curve **RED**)
- Pathogen RT primer mix (150 reactions **GREEN**)
- RNase/DNase free water

Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x *Precision*[™] Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

Kit storage and stability

This kit is stable at room temperature. Once the lyophilised components have been re-suspended the kit should be stored at -20°C. Unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity. Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between 1×10^8 and 1×10^2 copies of target template.



Principles of the test

Real-time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan[®] principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR cabinet. Filter tips are recommended for all pipetting steps.

1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

2. Reconstitute the kit components according to the table below

To ensure complete resuspension, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
Pre-PCR box	
Pathogen Primer/Probe mix (BROWN)	165 µl
Pathogen RT primer mix (GREEN)	165 µl
Post PCR bottle	
Positive Control Template (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.



Reverse Transcription

The protocols below are for PrimerDesign *Precision*[™] Reverse Transcription kit. If you are using an alternative RT kit then follow the manufacturer's instruction.

1. For each RNA sample prepare a reaction mix according to the table below:

Component	1 reaction
RNA template	X μ l
Pathogen RT primer mix (GREEN)	1.0 μ l
dNTP mix 10mM of each (ORANGE)	1.0 μ l
RNAse/DNAse free water (WHITE)	X μ l
Final volume	10 μl

2. Heat samples to 65°C for 5 minutes.

This can be achieved using a thermostatically controlled “hot block” or heated water bath. Do not heat the RNA to a higher temperature as this can lead to RNA degradation.

3. Immediately cool the tubes in an ice water bath.

It is important that the samples are transferred directly from 65°C to the ice. Do not allow the samples to cool prior to cooling on ice.

4. For each RNA sample prepare a reaction mix according to the table below:

Component	1 reaction
MMLV 5Xbuffer (CLEAR)	4.0 μ l
RNA/DNAse free water (WHITE)	5.2 μ l
MMLV enzyme (CLEAR)	0.8 μ l
Final volume	10 μl

5. Add 10 μ l of this mix to each of the samples on ice.

6. Apply a lid to each sample, mix tubes by briefly vortexing and incubate at 42°C for 60 minutes.

This can be achieved using a thermostatically controlled “hot-block” or heated water bath

7. Store cDNA samples at -20°C until use.



Real-time PCR detection

1. Prepare a reaction mix according to the tables below:

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

Pathogen detection mix

Component	1 reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix (BROWN)	1.0 µl
RNase/DNase Free water	4.0 µl
Final volume	15 µl

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample cDNA templates for each of your samples (suggested concentration 5ng/µl) in RNase/DNase free water.

If the concentration of RNA that was used to make the cDNA is not known, then dilute your RT reaction mix 1:20 (10µl of sample DNA and 90µl of water)

4. Pipette 5µl of diluted cDNA template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl



5. Preparation of standard curve dilution series

- 1) Pipette 900 μ l of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100 μ l of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100 μ l from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2×10^7 per μ l
Tube 2	2×10^6 per μ l
Tube 3	2×10^5 per μ l
Tube 4	2×10^4 per μ l
Tube 5	2×10^3 per μ l
Tube 6	2×10^2 per μ l
Tube 7	20 per μ l
Tube 8	2 per μ l

6. Pipette 5 μ l of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20 μ l



Amplification Protocol

Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	DATA COLLECTION*	60s	60°C

* Fluorogenic data for the control DNA should be collected during this step through the FAM channel

** Required if your mastermix includes UNG to prevent PCR carryover contamination

*** Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

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