IQ2000™ YHV/GAV Instruction Manual

Yellow Head Virus/Gill Associated Virus (YHV/GAV)

in-vitro use only

No contagious materials included

Manufacturer:
Farming IntelliGene Tech. Corp.
NO.2-1, 7th Rd., Taichung Industry Park, Taichung 407, Taiwan, ROC
TEL: 886-4-23580768 **FAX:** 886-4-23580769
E-mail: sales@iq2000kit.com http://www.iq2000kit.com

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I. Introduction

In 1992, Yellow-head virus (YHV) was first discovered in Thailand on black tiger prawn (*Penaeus monodon*) though it is now known to have caused extensive losses to the east coast of the Gulf of Thailand early in 1991 (Flegel et al. 1995). This disease affects juvenile to sub-adult prawn and is usually characterized by a pale to yellowish coloration of the cephalothorax and gills, and erratic swimming of infected animal near the surface at the pond edge. Gill associated virus (GAV), which morphologically resembles YHV, has also been reported in *Penaeus monodon* from Australia, and has been associated with sporadic outbreaks of disease since 1996 (Spann et al., 1997).

The IQ2000^{TM} YHV/GAV Detection and Typing System for penaeid shrimps is a commercialized molecular biology system for direct detection of these two similar viruses. It can detect and differentiate the prototype of these two viruses and provide semi-quantitative results plus build-in internal control system. The knowledge about the nature of YHV and GAV and their RNA sequences which the system was based upon were originally developed by CSIRO, Australia, and BIOTEC, Thailand. A licensing agreement was signed in 2001 between Farming IntelliGene and CSIRO/BIOTEC to commercialize this technology for the manufacturing of YHV/GAV detection system.

According to the recent research results, other YHV/GAV related viruses have also been found in some Asian countries. Due to the lack of sequence information, IQ2000^{TM} YHV/GAV Detection and Typing System may not be able to detect these YHV/GAV related viruses, or may cross-react with these viruses. The impact and severity of these viruses on shrimp farming and their relationship with YHV/GAV is still under investigation.

II. System Components: **stored at -20℃**

(1) RNA Extraction Solution 100 ml/bottle, **stored at 4℃**

(2) YHV-GAV Specific Sequence Amplification kit (stored at -20℃)

RT-PCR PreMix	4 vials	420 µl/vial, includes reaction buffer, dNTPs, and YHV/GAV specific primers
Nested PCR PreMix	4 vials	840 µl/vial, includes reaction buffer, dNTPs, and YHV/GAV specific primers
YHV P(+) Standard	1 vial	100 µl/vial, 10^4 copies/µl
GAV P(+) Standard	1 vial	100 µl/vial, 10^4 copies/µl
Yeast tRNA	1 vial	500 µl/vial, 40 ng/µl
IQzyme DNA polymerase	1 vial	2U/µl, 360 µl/vial
RT Enzyme Mix	1 vial	120 µl/vial
6X Loading Dye	1 vial	1500 µl/vial
DNA Marker	1 vial	100 µl/vial, 848 bp, 630 bp & 333 bp
DEPC ddH_2O	100 ml/bottle	

III. Equipment and Reagents Required but not provided

1. Thermal cycler
2. High-speed bench top microcentrifuge (12000 rpm, d=5 to 8 cm)
3. Electrophoresis apparatus
4. UV transilluminator
5. Vortex mixer
6. Heating block
7. Micropipette
8. Polaroid camera or digital photo system
9. Chloroform
10. 95% ethanol
11. Ethidium bromide
12. TAE or TBE electrophoresis buffer
13. Agarose
14. Isopropanol (2-propanol)

IV. Detection Limit and Sensitivity

This detection system generates different levels of detection limit and sensitivity according to the different sources of sample tested. The table below lists some common samples tested. Based on the knowledge of viral distribution in shrimp, gill sample is highly recommended for juvenile and adult shrimps screening.

Specimen	Test Quantity	Detection limit	Equivalent of Sensitivity
YHV/GAV DNA plasmid	2 copies	2 copies/reaction	2 copies/µl plasmid sample
In vitro transcribed RNA	20 copies	20 copies/reaction	20 copies/µl *in-vitro* transcribed RNA
< PL12	10 PLs	20 copies/reaction	500 copies/PL
PL12 to PL20	5 PLs	20 copies/reaction	1000 copies/PL shrimp
Gills from >PL20 to 5g-shrimp	All gills	20 copies/reaction	2000 copies/sample
Gills from 5g-shrimp to broodstock	2 to 3 pieces	20 copies/reaction	2000 copies/sample
Gill from broodstock	half piece	20 copies/reaction	2000 copies/sample

Base on the above table, users have to know that a “negative” test result indicates that the specimen was either not infected or that the infected level was lower than the detection limit. All the test results listed in the table were tested according to the standard procedure and reagents described in this manual. We do not guarantee RNA extracted by other manufacturers’ RNA extraction reagents will comply with our detection system.

V. Sample preparation and RNA extraction

1. RNA Extraction Solution: 200 reactions (100 ml/bottle), stored at 4℃

2. RNA extraction procedure

   a. Put sample into a 1.5 ml tube with 500 µl RNA Extraction Solution.
   b. Grind the sample in the tube with a disposable grinder, stand in room temperature for 5 minutes.
   c. Add 100 µl of $CHCl_3$ then vortex 20 seconds. Sit in room temperature for 3 minutes, then centrifuge it at 12000g (12000 rpm r=6 cm) for 15 minutes.
   d. Transfer 200 µl of the upper clear aqueous phase to a fresh 0.5 ml tube with 200 µl 2-propanol (isopropanol)
   e. Vortex briefly, centrifuge at 12000g for 10 minutes, then decant the isopropanol.
   f. Wash the pellet with 0.5 ml 75% ethanol, then spin down 5 minutes by 7500 g (9000 rpm r=6 cm) to recover RNA pellet, then decant the ethanol and dry the pellet.
   g. Dissolve the pellet with DEPC ddH_2O

3. RNA Dissolution

   As different sample sources may contain different RNA concentration, the final RNA concentration needs to be adjusted by dissolving the RNA pellet in different volume of DEPC ddH_2O.

SAMPLE SOURCE	VOLUME
PLs	500 µl
Gill	200 µl

VI. Amplification Protocol

The following amplification conditions apply to 0.2 ml thin-wall tube or 96-well plate.

1. Reagents preparation:

   a. RT-PCR reaction reagent: 8 µl/reaction

RT-PCR PreMix	7.0 µl
IQzyme DNA polymerase 2 units/µl	0.5 µl
RT Enzyme Mix	0.5 µl

   b. Nested PCR reaction reagent: 15 µl/reaction

Nested PCR PreMix	14 µl
IQzyme DNA polymerase 2 units/µl	1 µl

2. Reaction condition: (Uni-IQ RT-PCR profile)

   a. RT-PCR reaction profile:

   42℃ 30 min; 94℃ 2min; then

   94℃ 20 sec; 62℃ 20 sec; 72℃ 30 sec, repeat 15 cycles, then add

   72℃ 30 sec; 20℃ 30 sec at the end of the final cycle.

   b. Nested PCR reaction profile:

   94℃ 20 sec; 62℃ 20sec; 72℃ 30 sec, repeat 30 cycles, then add

   72℃ 30 sec; 20℃ 30 sec at the end of the final cycle.

3. Reaction Procedure:

a. Pipette 8 µl of RT-PCR reaction reagent into each 0.2 ml reaction tube with proper label.
b. Add 2 µl of the extracted sample RNA or standard* into each reaction mixture.
c. Perform RT-PCR reaction profile.
d. Add 15 µl Nested PCR reaction reagent into each tube after RT-PCR reaction was completed.
e. Perform Nested PCR reaction profile.
f. After Nested PCR reaction is completed, add 5 µl 6X Loading Dye to each reaction tube and mix well.
g. After mixing, sample is ready for electrophoresis.

* 10 copies/µl standard is highly recommended for every batch of experiment to monitor the sensitivity of reactions. We also recommend Yeast tRNA to be used as diluents for dilution of positive standards. Under this condition, the standard can be kept at –20℃ for a week.

VII. Electrophoresis

1. Agarose gel preparation

a. First, decide a buffer system of electrophoresis between TAE and TBE. Then, dilute the selected buffer to 1X operation concentration to produce agarose gel and process electrophoresis. Note that the buffer for processing electrophoresis and producing agarose gel must be the same system.

b. A 2% agarose gel is recommended for electrophoresis. To prepare 2% agarose gel, add 2g of agarose into a glass-made wide mouth bottle or flask with 100ml of electrophoresis buffer.

c. Heat the mixture until it becomes hyaline without any gel particle. Heating process can be done by using alcohol lamp, gas lamp, heat plate or microwave oven. To avoid the boiled gel from slopping over, a bigger glass-made container (twice the solution volume) is recommended.

d. Cool down the clear agarose gel solution under room temperature to around 50℃, then slowly pour the gel into the gel box. The volume of the gel required varies depending on the size of the gel box. Generally speaking, the level of agarose gel needs to be higher than the bottom of plastic comb for about 0.3~0.5 cm, and the gel thickness is suggested to be less than 0.8 cm.

e. Carefully remove the gel comb and blockers at both sides of the gel box when agarose gel is completely coagulated. This agarose gel, then, is able to process electrophoresis. The finished agarose gel shouldn't be exposed under room temperature for longer than 4 hours.

2. Electrophoresis

a. Lay the coagulated agarose gel inside the gel box. During the electrophoresis, DNA molecular will swift toward (+) because DNA molecular is negatively charged.

b. Add 1X electrophoresis buffer into the gel box until the buffer is just cover the gel.

c. Load 5~10 µl each of the "PCR product/ loading dye mixture" into each well. The mixture will sink to the bottom of the well because its density is higher than buffer. This procedure should be operated with extra care to avoid contamination.

d. DNA marker is required for every electrophoresis. About 5 µl of DNA marker is recommended. The DNA molecular weight marker is served as size reference for PCR products

e. When all the samples are loaded, connect the gel box with power supply before switching on. Check electrodes again and use 100~150 voltage (DO NOT RUN OVER 150 voltage) to run the electrophoresis.

f. The loading dye in the kit contains 2 colourants: Bromphenol Blue will form deep blue colour; Xylene Cyanol will form light blue colour. When the dark blue dye approaches 1/2 to 2/3 of the gel, stop the electrophoresis. Then, remove the gel from the gel box to proceed with the EtBr staining procedure.

g. To avoid contamination, DO NOT re-use the gel electrophoresis buffer, unless several gels will be used in the same day, and when electrophoresis is finished, wash the gel box with plenty of water.

3. Gel staining and data assay

a. Ethidium Bromide (EtBr) is usually prepared to 10mg/ml stock solution. This solution should be stored in an amber bottle because EtBr is a light degradable chemical. Note that EtBr is a known carcinogen, protective suit, gloves, and goggles are highly recommended.

b. Dilute the 10mg/ml stock solution 20,000 times, i.e. add 5µl of the above stock solution into 100ml of distilled water to prepare the staining solution.

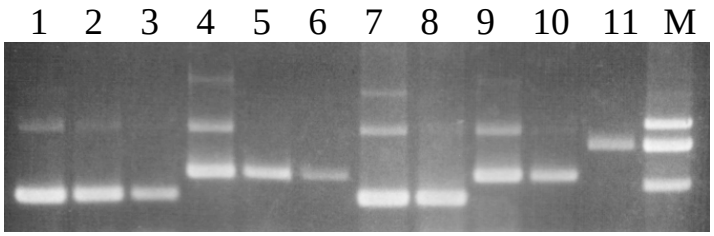
c. Pour the above staining solution into the plastic tray or zip-lock bag with electrophoresis-finished gel. The solution must cover the whole gel.

d. Shake lightly at room temperature for 10 minutes. Then, de-stain the gel in another plastic tray with distilled water for another 10 minutes to eliminate the background.

e. Lay the gel on a UV transilluminator to read the final result.

VIII. Diagnosis

1. Positive samples and standards will show the following patterns on gel:



Lane 1: YHV positive standard, 2000 copies/reaction
Lane 2: YHV positive standard, 200 copies/reaction
Lane 3: YHV positive standard, 20 copies/reaction
Lane 4: GAV positive standard, 2000 copies/reaction
Lane 5: GAV positive standard, 200 copies/reaction
Lane 6: GAV positive standard, 20 copies/reaction
Lane 7: Severe infected YHV sample
Lane 8: Light infected YHV sample
Lane 9: Severe infected GAV sample
Lane 10: Light infected GAV sample
Lane 11: YHV/GAV negative sample
Lane M: molecular weight marker, 848 bp, 630 bp, 333 bp

2. Negative samples will show only one band at 680 bp, which is the product of house keeping gene, β -actin mRNA of shrimp.

3. Diagnostic procedure:

   a. Bands formed at 277 bp and 777 bp: severe YHV P(+)

   b. Bands formed at 277 bp only: light YHV P(+)

   c. Bands formed at 406 bp and 777 bp: severe GAV P(+)

   d. Band formed at 406 bp only: light GAV P(+)

   e. Band formed only at 680 bp: N(-)

4. Each experiment requires positive and negative controls, if the 10^3 positive standard did not result a band at 277 or 406bp, it means RT and/or PCR reaction has failed. On the other hand, if negative control resulted a band at 277 or 406 bp, it means contamination has occurred.

IX. Trouble-shooting

Problem or Symptoms	Possible causes	Recommendations
Faint bands or No bands resulted after staining	1. EtBr degraded.	1. Prepare new EtBr or extent staining time.
	2. UV light was not turn on.	2. Check UV table.
	3. Background was too strong.	3. Soak gel in clean water at 4C for another 30 minutes.
	4. Agarose gel was too thick.	4. Check thickness of gel if gel is more than 0.8 cm. Prepare a thinner gel and run the electrophoresis again.
Positive standard shows normal bands but maker band did not show up	Marker was degraded or under loaded.	Change marker or increase loading volume.
Marker shows normal bands but P(+) has no band.	1. RT and/or PCR failed.	1. Check reagent mixture preparation record and PCR cycle profile setting.
	2. Enzyme was not added.	2. Add enzyme.
	3. P(+) was degraded.	3. Prepare new P(+).
High P(+) (10^3) shows band but low positive has no band.	1. P(+) was degraded.	1. Prepare new P(+).
	2. 10^4 standard was degraded.	2. Replace 10^4 standard
	3. Low enzyme activity.	3. Check expiration date and storage condition of enzyme, or replace enzyme

Problem or Symptoms	Possible causes	Recommendations
Negative (-) control shows positive band	1. Micropipette contamination	1. Disassemble pipette and do clean up. We recommend using aerosol free tip. Also, a separate pipette should be used for PCR product pipetting
	2. Reagent contamination	2. Re-run reactions with new opened reagents
	3. Lab contamination	3. Consult with Farming IntelliGene for lab clean up
P(+) control and N(-) shows normal banding, but known-infected sample has no band	1. RNA extraction failed	1. Check RNA extraction procedure
	2. Bad RNA quality or DNA concentration too high.	2. Check OD 260/280 ratio. Normally, this ration should be 1.6 to 1.8
	3. PCR inhibitor	3. Spike 10^3 P(+) standard for a parallel PCR reaction. If the one with 10^3 P(+) shows normal band, then inhibition was rule out. If 10^3 P(+) has no band then there is inhibition. Users need to prepare another RNA extraction.

X. Reference:

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Jeff A. Cowley, Christine M. Dimmock, Chainarong Wongteerasupaya, Vichai Boonsaeng, Sakol Panyim, Peter J. Walker (1999) Yellow head virus from Thailand and gill associated virus from Australia are closely related but distinct prawn viruses. Dis Aquat Org 36: 153-157.