IQ2000™ IHHNV Detection and Prevention System

Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV)

in-vitro use only
No contagious materials included

Instruction Manual

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I. Introduction

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) has been wildly reported as one of the major viral diseases in shrimp culture industry. It is known to be transmitted both vertically and horizontally. This single strand, negative sense DNA virus can cause mass mortality for *P. stylirostris* and runt deformity syndrome (RDS) for *L. vannamei*. Recent research findings pointed out that there is a close correlation between IHHNV and muscle whitening syndrome. Although IHHNV may not affect the growth of *P. monodon* significantly, however, recent research results also pointed out that it will influence the survival rate of the *monodon* broodstocks. Thus, the economic loss cause by this virus has been underestimated for a long time.

IQ2000™ IHHNV Detection and Prevention System is a single step PCR system. But same as our IQ2000™ serial products, this PCR based product also includes internal control, quantified positive standard, and semi-quantitative capability to provide the best quality diagnostic results. Besides, the design of IQ2000™ IHHNV System is also able to detect the known IHHNV in South East Asia and America.

II. System Components:

1. DNA extraction reagents*: storage condition as per label

DNA Extraction Kit: 200 reactions/kit, including

- DTAB Solution	125ml/btl	store at room temperature
- CTAB Solution	25ml/btl	store at room temperature
- Dissolving Solution	30ml/btl	store at 4℃
Lysis buffer (200 reactions/kit)	100ml/btl	store at room temperature

* Lysis buffer is the default item with the test kit, but DNA Extraction Kit is only available upon request.

2. IHHNV Amplification kit: 200 reactions/kit; store at -20℃

Pre-mixed reagent	4 vials	750 µl/vial, includes reaction buffer, dNTPs, and IHHNV specific primers
Positive control	1 vial	100 µl/vial, 10^5 copies/ul. Contains IHHNV DNA plasmid standard
Yeast tRNA	1 vial	500 µl/vial, 40 ng/µl
IQzyme DNA polymerase	1 vial	2U/µl, 125 µl/vial
6X loading dye	1 vial	1500 µl/vial
DNA molecular weight marker	1 vial	100 µl/vial, 848 bp, 630 bp & 333 bp

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

IV. Detection Limit and Sensitivity

This detection system generates different detection limit and sensitivity according to the different sources of samples tested. The table below lists some common samples tested.

Specimen	Test Quantity	Detection Limit (copies /reaction)	Equivalent of Sensitivity
IHHNV DNA plasmid	50 copies/µl	100	50 copies/µl plasmid standard
< PL 12	~ 30 PLs	100	300000 copies/PL shrimp
> PL 12	Tail (not including hepatopancrea)	100	10000 copies/half shrimp
Pleopod	Single leg	100	10000 copies/foot
Gill	Single piece	100	10000 copies/piece

Base on the above table, users have to know that a “negative” test result indicates that the specimen is either not infected or that the infected level is lower than the detection limit. But from the experimental data, we know that the infected virus concentration is at least 10~100 fold of detection limitation. Therefore, a negative result should indicate that the sample or culture environment at time of testing is free of IHHNV epidemic. Furthermore, there is no significant economic loss caused by low IHHNV infection in both *monodon* and *vannamei.* The phenomenon of runt deformity syndrome (RDS) in *vannamei* and survival threat to adult *monodon* will only occur when it is severely infected.

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 DNA extracted by other manufactures’ DNA extraction reagents will comply with our detection kit.

V. Sample preparation and DNA extraction

1. DTAB-CTAB DNA extraction procedures

a. Place sample (PL, pleopod, gill, or 50 µl haemolymph sample) into a 1.5 to 2 ml tube that contains 0.6 ml DTAB solution.
b. Grind the sample in the tube by a disposable grinder.
c. Incubate the prepared sample at 75℃ for 5 minutes, then cool down to room temperature.
d. Vortex briefly and spin down the mixture, then add 0.7 ml of chloroform, vortex for another 20 seconds and centrifuge at 12000g (12000 rpm r=5~7cm) for 5 minutes.
e. Transfer the upper aqueous phase to a new 1.5 to 2 ml tube , add 100 ul of CTAB Solution and 900 µl ddH_2O, vortex briefly, then incubate at 75℃ for 5 minutes.
f. Cool down to room temperature and centrifuge at 12000g for 10 minutes.
g. Carefully decant the supernatant, resuspend the pellet with 150 µl Dissolving Solution, incubate at 75℃ for 5 minutes then cool down to room temperature.
h. Spin at 12000g for 5 minutes. Transfer the clear solution to a fresh 0.5ml tube with 300 µl 95% ethanol
i. Vortex briefly, centrifuge at 12000g for 5 minutes, then wash the pellet with 200 µl of 70% ethanol, spin down, dry the pellet and resuspend in TE buffer. Refer to 3. DNA dissolution for volume of TE buffer to be used.

2. DNA extracted by Lysis Buffer (for pleopod, gill, or < PL12 samples only)

a. Put shrimp sample in a 1.5ml tube.
b. Add 500 µl Lysis Buffer to the tube and grind well.
c. Incubate the prepared sample at 95℃ for 10 minutes, then centrifuge at 12000g (12000 rpm r=5~7cm) for 10 minutes.
d. Transfer 200 µl of the upper clear solution to a fresh 1.5ml tube with 400 µl 95% ethanol
e. Vortex briefly, centrifuge at 12000g for 5 minutes, then decant the ethanol and dry the pellet.
f. Dissolve the pellet by ddH_2O or TE buffer.

3. DNA Dissolution

The optimized DNA concentration is 200 ng/reaction for the IQ2000™ Series products. Concentration adjustment by OD_{260} measurement is highly recommended, however, the acceptable concentration range is from 10 ng to 1000 ng. Generally, in the case without OD measurement, 200 µl ddH_2O or TE buffer is recommended to dissolve the DNA pellet. However, the bigger pellet may use more ddH_2O or TE buffer to adjust the final DNA concentration, and vice versa. If sample needed to be preserved for longer period, TE buffer is recommended. Sample can be stored in -20℃ for at least six months one year.

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

1. Reagents preparation: 13 µl/reaction. Mix the following:

Pre-mixed reagent		12.5µl
IQzyme DNA polymerase	2U/µl	0.5 µl

2. Reaction condition:

94°C 20 seconds; 70°C 20 seconds; repeat 10 cycles, then

94°C 20 seconds; 56°C 20 seconds; 72°C 30 seconds, repeat 35 cycles, then add 72°C 30 seconds; 20°C 30 seconds at the end of the final cycle.

3. Reaction procedure:

a. Prepare reaction mixtures required according the sample number. For each reaction mixture preparation, user also needs to take into account 3 positive standards (10^4, 10^3 and 10^2) and 1 negative control (ddH_2O or Yeast tRNA).

b. Pipette 13 µl of mixed reagent (with Taq DNA polymerase) into each 0.2 ml reaction tube with proper label.

c. Add 2 µl of the extracted sample DNA or standard into each reaction mixture.

d. Cover each reaction mixture with 20 µl of mineral oil unless your thermal cycler is equipped with oil-free design.

e. Perform reaction.

f. When reaction is completed, add 3µl of 6X loading dye to each reaction tube and mix well. After mixing, sample is ready for electrophoresis.

VII. Electrophoresis

1. Agarose gel preparation

a. First, decide a buffer system of electrophoresis between TAE and TBE. Then, dilute the buffer to 1X operation concentration to process electrophoresis and produce agarose gel. 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 agarose into a glass-made wide mouth bottle or flask with 100ml electrophoresis buffer.

c. Heat the mixture until it becomes hyaline without any gel particle. Heating can be done by using alcohol lamp, gas lamp or heat plate to heat, microwave oven is able too heat as well. 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 under room temperature until the temperature is around 50℃ and slowly pour the gel into the gel box. The volume of the gel varies from the size of the gel box. Generally speaking, the height of agarose gel only has to go above the bottom of the gel comb for about 0.3~0.5 cm, and thickness is suggested to be no less than 0.8 cm.

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

2. Electrophoresis

a. Lay 2% of the coagulated agarose gel inside the gel box. DNA molecular will swift towards (+) because DNA molecular is negative charged.

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

c. Add 5~10 ml “amplification production-loading dye mixture” into each well. The mixture will sink to the bottom of the wells because its density is higher than buffer. This step should be carefully handled in order 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 reference for PCR product size.

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 OVER 150 voltage) to run the electrophoresis.

f. The loading dye in the kit contains 2 colorants: Bromphenol Blue forms deep blue colour; Xylene Cyanol forms 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 procedures.

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

3. Gel staining and data assay

a. Ethidium Bromide (EtBr) is usually prepared for 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 10 mg/ml stock solution 20,000 times (i.e. add 5 µl of the above stock solution into 100 ml distilled water to prepare the staining solution.)

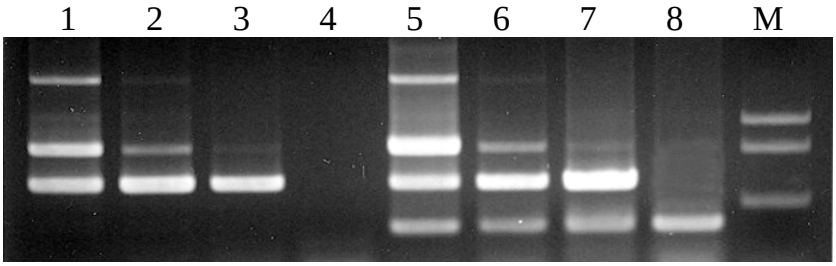
c. Pour the above staining solution into the plastic tray or zip-lock bag with electrophoresis-finished gel. The solution must cover the whole gel.

d. Shake lightly at room temperature for 10 minutes. Then, distain 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: standard 1, 20000 copies/reaction
Lane 2: standard 2, 2000 copies/reaction
Lane 3: standard 3, 200 copies/reaction
Lane 4: ddH_2O
Lane 5: sample of severe IHHNV infection
Lane 6: sample of moderate IHHNV infection
Lane 7: sample of light IHHNV infection
Lane 8: IHHNV negative sample
Lane M: molecular weight marker, 848 bp, 630 bp, 333 bp

2. Negative samples will show only one band, 243 bp, which is the product of house keeping gene.

3. Diagnostic procedure:
   a. Band formed at 438 bp and/or 644 bp: P(+)
   b. Band formed only at 243 bp: N(-)
   c. No band show on gel: bad DNA quality

4. Each experiment requires positive and negative controls. If the 10^2 positive standard did not result a band at 438 bp, it means that the PCR reaction has failed. On the other hand, if the negative control resulted a band at 438 bp, it means that contamination has occurred.

IX. Troubleshooting

Problem or Symptoms	Possible causes	Comments
Faint bands or No bands resulted after staining	1. EtBr degraded.	1. Prepare new EtBr or extent staining time.
	2. UV light was not turned on.	2. Check UV transilluminator.
	3. Background too strong.	3. Soak gel in clean water at 4℃ for another 30 minutes.
	4. Agarose gel 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 show normal bands but maker band did not show up	Marker was degraded or under load.	Change marker or increase loading volume.
Marker shows normal bands but P(+) has no band.	1. 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(+) (10^4) shows band but low positive has no band.	1. P(+) was degraded.	1. Prepare new P(+)
	2. P(+) dilution was done by using H2O instead of tRNA.	2. Prepare new P(+) using tRNA as diluent.
	3. 10^5 standard was degraded.	3. replace 10^5 standard
	4. Low enzyme activity.	4. Check expiration date and storage condition of enzyme, or replace enzyme

Negative (-) control shows band at 438bp.	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. Replace reagent
	3. Lab contamination	3. Consult with FITC for lab clean up
P(+) control and N(-) shows normal banding, but known-infected sample has no band	1. DNA extraction failed	1. Check DNA extraction procedure
	2. Bad DNA quality or DNA concentration too high.	2. Check OD 260/280 ratio. Normally, this ratio 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. User need to prepare another DNA extraction.

X. Reference:

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