IQ2000™ WSSV

Instruction Manual

White Spot Syndrome Virus (WSSV)

in-vitro use only

No contagious materials included

Fitness for purpose validated and certified by OIE

Register number: 20080304

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I. Introduction

White Spot Syndrome Virus (WSSV) is a major shrimp disease, which has caused high mortality rate and economic losses to the major shrimp farming countries in South East Asia, Central America and Southern USA. It is a pathogen found in different penaeid shrimp species including *P. monodon*, *P. japonicu*s and *L. vannamei* as well as other crustaceans, such as crab and crayfish.

Various molecular methods, such as microtomy, immuno-assay, hybridization, and PCR (Polymerase Chain Reaction) for pathogen detection have been developed for shrimp diseases. At present, nested PCR method is recognised to be the most effective diagnostic tool for this pathogen. For the development of effective diagnostic tools, a WSSV genomic library has been constructed and analysed. In addition, a very conserved segment of WSSV has also been selected as the target for PCR diagnosis.

Working in collaboration with Prof. Guang-Hsiung Kou and Prof. Chu-Fang Lo from National Taiwan University, GeneReach has successfully developed IQ2000^{TM} WSSV Detection and Prevention System which can differentiate infected shrimps into 4 different levels of infection: very light, light, medium, and severe. The diagnostic results produced by IQ2000^{TM} Systems are very helpful for shrimp disease control program in the shrimp farming industry.

To provide a more convenient reaction condition and time saving procedure for multi-viral diagnosis, IQ2000^{TM} WSSV Detection and Prevention System can be performed not only by its own PCR reaction condition but also by the Uni-IQ Program, a universal PCR profile which can be used for all IQ2000^{TM} Systems. Under this reaction profile, different kinds of shrimp viral diseases can be screened at the same batch of PCR reaction.

II. System Components

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

   DNA Extraction Kit (200 reactions/kit), including

   - DTAB Solution 125 ml/btl store at room temperature
   - CTAB Solution 25 ml/btl store at room temperature
   - Dissolving Solution 30 ml/btl store at 4℃

   Lysis Buffer (200 reactions/kit):
   100 ml/btl store at room temperature

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

2. WSSV specific sequence amplification kit (200 reactions/kit):
   store at –20℃

   - **First PCR PreMix** 4 vials 450 ul/vial
     includes reaction buffer, dNTPs, and WSSV specific primers
   - **Nested PCR PreMix** 4 vials 840 ul/vial
     includes reaction buffer, dNTPs, and WSSV specific primers
   - **P(+) standard** 1 vial 100 ul/vial
     10^4 copies/ul plasmids containing WSSV partial sequence
   - **Yeast tRNA (40 ng/ul)** 1 vial 500 ul/vial
   - **IQzyme DNA polymerase (2 U/ul)** 1 vial 360 ul/vial
   - **6X loading dye** 1 vial 1500 ul/vial
   - **DNA molecular weight marker** 1 vial 100 ul/vial
     848 bp, 630 bp & 333 bp

III. Equipment and reagents required but NOT provided

1. Thermal cycler with sample block which fits 0.2ml thin-walled tube
2. High-speed benchtop 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. 75% ethanol
12. Ethidium bromide
13. TAE or TBE electrophoresis buffer
14. Agarose

IV. Detection Limit and Sensitivity

This detection system results different detection limits and levels of sensitivity according to the different sources of sample tested. The following table lists some common samples tested.

Specimen	Test Quantity	Detection limit (copies/reaction)
WSSV DNA plasmid	5 copies/ul	10
Eye stalk of broodstock	Single eye stalk	20
< PL12	25 - 50 PLs	20
PL12 to 30	Tail (without hepatopancreata)	20
Pleopod, Periopod, or gill	2 pieces (or 20 mg)	20
Muscle	20 mg	20

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. We learn from the present publication that the infected viral concentration has to reach 10~100 fold of the detection limit for a white spot syndrome to occur. Therefore, a negative result can indicate that the culture environment at the time of testing is free from white spot syndrome epidemic.

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 manufacturers' DNA extraction reagents will comply with our detection system.

V. Sample preparation and DNA extraction

1. Sample preparation (For DTAB-CTAB method)

a. Broodstock eye stalk

(i) Rinse the cut eye stalk with clean water.

(ii) Put eye stalk into a 2ml tube that contains 0.6 ml DTAB solution.

(iii) Grind the eye stalk in the tube with a disposable grinder.

b. Larvae, PL or Juvenile

(i) Place about 20 mg specimen into a 2ml tube containing 0.6 ml DTAB solution (The test requires at least 50 pieces for larvae; 30 pieces for <PL12; for PL12-30, only half tail should be used. Do not sample the hepatopancrea or head.).

(ii) Grind the sample in the tube with a disposable grinder.

c. Pleopod, pereopod, or gill of adult shrimp

(i) Place 2 pieces into a 2ml tube containing 0.6 ml DTAB solution.

(ii) Grind the sample in the tube with a disposable grinder.

d. Tail or muscle of adult shrimp

(i) Place a tail or about 20 mg muscle into a 2ml tube containing 0.6 ml DTAB solution.

(ii) Grind the sample in the tube with a disposable grinder.

2. DTAB-CTAB DNA extraction procedure

a. Incubate the prepared sample at 75℃ for 5 minutes, then cool down to room temperature.

b. 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 = 5~7 cm) for 5 minutes.

c. Transfer 200 ul of the upper aqueous phase to a new 1.5ml tube. Add 100 ul of CTAB Solution and 900 ul ddH_2O. Vortex briefly, then incubate at 75℃ for 5 minutes.
d. Cool down to room temperature and centrifuge at 12000g for 10 minutes.
e. Carefully decant the supernatant, resuspend the pellet with 150 ul Dissolving Solution, incubate at 75℃ for 5 minutes then cool down to room temperature.
f. Spin at 12000g for 5 minutes. Transfer the clear solution to a fresh 1.5ml tube with 300 ul of 95% ethanol.
g. Vortex briefly, centrifuge at 12000g for 5 minutes, then wash the pellet with 200 ul of 75% ethanol, spin down, dry the pellet and dissolve in ddH_2O or TE buffer. Refer to **4. DNA dissolution** for volume of TE buffer to be used.

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

a. Add 500 ul Lysis Buffer in a 1.5ml tube.
b. Put shrimp sample into the tube and grind it with a disposable grinder.
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 ul of the upper clear solution to a fresh 1.5ml tube with 400 ul 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. Refer to **4. DNA dissolution** for volume of TE buffer to be used.

4. DNA dissolution

a. The concentration of DNA are different from different sample sources, therefore the concentration of DNA needs to be adjusted by dissolving the DNA pellet in different volume of ddH_2O or TE buffer.

SAMPLE SOURCE	VOLUME
Eye stalk of brood stock	100 ul
PL	200 ul
Pleopod or periopod	200 ul
Gill	50 ul

b. If sample needed to be preserved for longer period, TE buffer is recommended. Sample can be stored in -20℃ for one year.

c. Please fine tune the volume of ddH_2O or TE buffer according to the real recovery efficiency.

VI. Amplification Protocol

The following amplification conditions apply to 0.2ml thin-wall tube or 96-well plate. Before executing the following PCR procedures, please confirm hot start function of the machine has already been shut down.

1. Reagents preparation:
   a. First PCR reaction reagent mixture: 8 ul/reaction
      Mix the following:

First PCR PreMix	7.5 ul
IQzyme DNA Polymerase (2 U/ul)	0.5 ul

   b. Nested PCR reaction reagent mixture: 15ul/reaction
      Mix the following:

Nested PCR PreMix	14 ul
IQzyme DNA Polymerase (2 U/ul)	1 ul

2. Reaction condition:
   a. First PCR reaction profile:
      94℃ 30 seconds; 62℃ 30 seconds; 72℃ 30 seconds, repeat 5 cycles, then 94℃ 15 seconds; 62℃ 15 seconds; 72℃ 20 seconds, repeat 15 cycles, then add 72℃ 30 seconds; 20℃ 30 seconds at the end of the final cycle.
   b. Nested PCR reaction profile:
      94℃ 20 seconds; 62℃ 20seconds; 72℃ 30 seconds, repeat 25 cycles, add 72℃ 30 seconds; 20℃ 30 seconds at the end of the final cycle.

* IQ2000^{TM} WSSV Detection and Preservation System can also be performed by the Uni-IQ Program. The reaction condition of Uni-IQ Program is:

a. First PCR reaction profile:
   42℃ 30min; 94℃ 2min; then
   94℃ 20 sec; 62℃ 20sec; 72℃ 30sec, 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. Prepare first and nested PCR reaction reagent mixtures required according to the sample number. For each reaction mixture preparation, user also needs to take into account 3 positive standards (10^3, 10^2 and 10^1) and 1 negative control (ddH_2O or Yeast tRNA).
b. Pipette 8 ul of first PCR reaction reagent mixture into each 0.2ml reaction tube with proper label.
c. Add 2 ul of the extracted sample DNA or standard into each reaction mixture.
d. Cover each reaction mixture with 20 ul of mineral oil unless your thermal cycler is equipped with oil-free design.
e. Perform first PCR reaction.
f. Add 15 ul of nested PCR reaction reagent mixture to each tube after first PCR was completed. Be sure that the reagents go through oil overlay.
g. Perform nested PCR reaction.
h. After nested reaction is completed, add 5 ul of 6X loading dye to each

reaction tube and mix well.

i. After mixing, sample is ready for electrophoresis.

* 10 copies/ul 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 diluting 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 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 2 g 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 particles. Heating can be done by using alcohol lamp, gas lamp or heat plate to heat, microwave oven is able to 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 at room temperature for longer than 4 hours.

2. Electrophoresis

a. Lay the coagulated agarose gel inside the gel box. DNA molecules will swift toward (+) 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. Load 5 ul each of the "PCR product-loading dye mixture" into each well. The mixture will sink to the bottom of the wells because its density is heavier than buffer. This step should be carefully handled in order to avoid contamination.

d. DNA marker is required for every electrophoresis. About 5 ul of DNA marker is recommended. The DNA molecular weight marker is a reference for PCR product size.

e. When all the samples are loaded, connect the gel box to the power supply before switching on. Constant voltage between 100 V ~ 150 V is recommended for electrophoresis.

f. The loading dye in the kit contains 2 colorants: Bromphenol Blue gives deep blue color; Xylene Cyanol gives light blue color. 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 ul of the above stock solution into 100 ml distilled water to prepare the staining solution.).

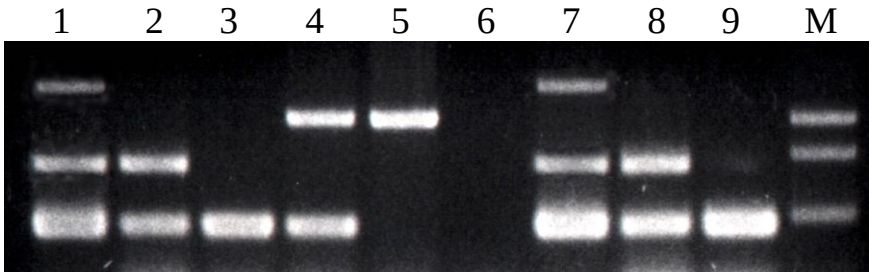
   c. Pour the above staining solution into the plastic tray or zip-lock bag with electrophoresis-finished gel. The solution must cover the whole gel.

   d. Shake lightly at room temperature for 10 minutes. Then, destain 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: Sample of severe WSSV infection
Lane 2: Sample of moderate WSSV infection
Lane 3: Sample of light WSSV infection
Lane 4: Sample of very light WSSV infection
Lane 5: WSSV negative sample
Lane 6: Negative control (Yeast tRNA or ddH_2O)
Lane 7: WSSV P(+) standard, 2000 copies/reaction
Lane 8: WSSV P(+) standard, 200 copies/reaction
Lane 9: WSSV P(+) standard, 20 copies/reaction
Lane M: Molecular weight marker, 848 bp, 630 bp, 333 bp

2. Negative samples will show only one band at 848 bp, which is a PCR product of house keeping gene as a internal control.

3. Diagnostic procedure:
   a. Band formed at 296 bp and/or 550 bp: P(+)
   b. Band formed only at 848 bp: N(-)

4. Each experiment requires positive and negative controls. If the 10^2 positive standard did not result a band at 296 bp, it could be due to the failed PCR reaction or other possibilities. On the other hand, if the result of negative control shows a band at 296 bp, it means that contamination has occurred. For more information, please refer to troubleshooting on the following pages or contact GeneReach Biotechnology Corp.

IX. Troubleshooting

Observation or Problems	Possible causes	Comments or solution
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 marker 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(+) (10^3) shows band but low positive has no band.	1. P(+) was degraded.	1. Prepare new P(+).
	2. P(+) dilution was done by using H_2O instead of tRNA.	2. Prepare new P(+) using tRNA as diluent.
	3. 10^3 standard was degraded.	3. Replace 10^3 standard.
	4. Low enzyme activity.	4. Check expiration date and storage condition of enzyme, or replace enzyme.

Observation or Problems	Possible causes	Comments or solution
Negative (-) control shows band at 296 bp.	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 GeneReach 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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