

IQ2000™  
**WSSV**  
Instruction Manual

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*White Spot Syndrome Virus (WSSV)*

*In-vitro use only*

No contagious materials included



Validated and certified by the OIE  
as fit for the purposes defined in  
this kit insert provided with this kit.  
Registration 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. japonicus* 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™ 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™ 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™ 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™ Systems. Under this reaction profile, different kinds of shrimp viral diseases can be screened at the same batch of PCR reaction.

The validation data for this kit have been certified by the OIE, based on expert review, as fit for:

- 1) To certify freedom from infection (<10 virions/sample) in individual animals or products for trade/movement purposes;
- 2) To confirm diagnosis of suspect or clinical cases (confirmation of a diagnosis by histopathology or clinical signs);
- 3) To estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control).



### 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°C 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,  $r = 5\sim 7$  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<sub>2</sub>O. Vortex briefly, then incubate at 75°C 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°C 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<sub>2</sub>O 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°C for 10 minutes, then centrifuge at 12000g (12000 rpm  $r=5\sim 7$ cm) 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<sub>2</sub>O 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<sub>2</sub>O or TE buffer.

<u>SAMPLE SOURCE</u>	<u>VOLUME</u>
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°C for one year.
- c. Please fine tune the volume of ddH<sub>2</sub>O 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°C 30 seconds; 62°C 30 seconds; 72°C 30 seconds, repeat 5 cycles,  
then

94°C 15 seconds; 62°C 15 seconds; 72°C 20 seconds, repeat 15 cycles,  
then

add 72°C 30 seconds; 20°C 30 seconds at the end of the final cycle.

- b. Nested PCR reaction profile:

94°C 20 seconds; 62°C 20seconds; 72°C 30 seconds, repeat 25 cycles,  
add 72°C 30 seconds; 20°C 30 seconds at the end of the final cycle.

\* IQ2000™ 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°C 30min; 94°C 2min; then

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

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

b. Nested PCR reaction profile:

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

72°C 30 sec; 20°C 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<sub>2</sub>O 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°C 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°C 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.

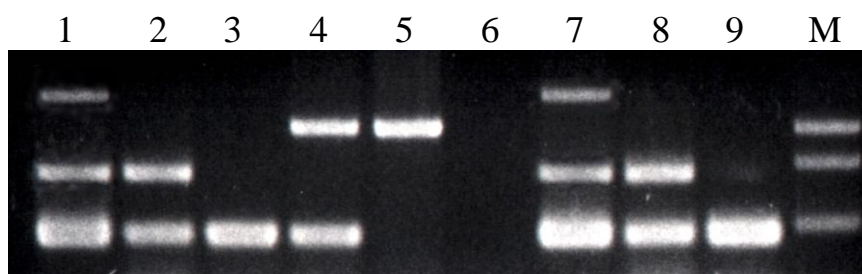
### 3. Gel staining and data assay

- 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<sub>2</sub>O)  
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<sup>2</sup> 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. 2. UV light was not turned on. 3. Background too strong. 4. Agarose gel too thick.	1. Prepare new EtBr or extend staining time. 2. Check UV transilluminator. 3. Soak gel in clean water at 4 °C for another 30 minutes. 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. 2. Enzyme was not added. 3. P(+) was degraded.	1. Check reagent mixture preparation record and PCR cycle profile setting. 2. Add enzyme. 3. Prepare new P(+).
High P(+) ( $10^3$ ) shows band but low positive has no band.	1. P(+) was degraded. 2. P(+) dilution was done by using H <sub>2</sub> O instead of tRNA. 3. $10^3$ standard was degraded. 4. Low enzyme activity.	1. Prepare new P(+). 2. Prepare new P(+) using tRNA as diluent. 3. Replace $10^3$ standard. 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.	<ol style="list-style-type: none"> <li>1. Micropipette contamination.</li> <li>2. Reagent contamination.</li> <li>3. Lab contamination.</li> </ol>	<ol style="list-style-type: none"> <li>1. Disassemble pipette and do clean up. We recommend using aerosol free tip. Also, a separate pipette should be used for PCR product pipetting.</li> <li>2. Replace reagent.</li> <li>3. Consult with GeneReach for lab clean up</li> </ol>
P(+) control and N(-) shows normal banding, but known-infected sample has no band.	<ol style="list-style-type: none"> <li>1. DNA extraction failed.</li> <li>2. Bad DNA quality or DNA concentration too high.</li> <li>3. PCR inhibitor.</li> </ol>	<ol style="list-style-type: none"> <li>1. Check DNA extraction procedure</li> <li>2. Check OD 260/280 ratio. Normally, this ratio should be 1.6 to 1.8.</li> <li>3. Spike <math>10^3</math> P(+) standard for a parallel PCR reaction. If the one with <math>10^3</math> P(+) shows normal band, then inhibition was rule out. If <math>10^3</math> P(+) has no band then there is inhibition. User need to prepare another DNA extraction.</li> </ol>

## X. Reference:

1. Chang PS, Lo CF, Wang YC, Kou GH (1996) Identification of white spot syndrome associated baculovirus (WSSV) target organs in shrimp, *Penaeus monodon* by *in situ* hybridization. *Dis. Aquat. Org.* 27:131–139.
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6. Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSSV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis. Aquat. Org.* 30:53–72.
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9. Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn

A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21: 69-77.

## XI. Appendix



### OIE Procedure for Registration of Diagnostic Kits

#### Abstract sheet

<p><b>Name of the diagnostic kit:</b> IQ 2000™ WSSV Detection and Prevention System <b>Manufacturer:</b> GeneReach Biotechnology Corporation <b>OIE Approval number:</b> 20080304 <b>Date of Registration:</b> May 2008</p>
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**Disease:** White Spot Disease

**Pathogen Agent:** White spot syndrome virus (WSSV) found in different penaeid shrimp species including *P. monodon*, *P. japonicus* and *L. vannamei* as well as other crustaceans, such as crab and crayfish.

**Type of Assay:** The IQ 2000™ WSSV Detection and Prevention System is a PCR test.

**Purpose of Assay:** Certified by the OIE in May 2008 as fit for the diagnosis of white spot syndrome in crustaceans and for the following purposes:

1. To certify freedom from infection (<10 virions/sample) in individual animals or products for trade/movement purposes;
2. To confirm diagnosis of suspect or clinical cases (confirmation of a diagnosis by histopathology or clinical signs);
3. To estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control).

**Species and Specimen:** Validated in shrimp but may be useful for detection of WSSV in other crustaceans.

#### 1. Information on the kit

Please refer to the kit insert available on the OIE Registry web page or contact manufacturer at:

GeneReach Biotechnology Corporation

TEL: 886-4-24639869

FAX: 886-4-24638255

Email: [sales@genereach.com](mailto:sales@genereach.com)

No. 19, Keyuan Second Road. Central Taiwan Science Park, Taichung, Taiwan, 407

## 2. Summary of validation studies

### Analytical characteristics

*Analytical sensitivity* - The analytical sensitivity data were done by comparing the sensitivity of IQ2000™ WSSV Detection and Prevention System and the PCR diagnostic method listed by OIE (Lo, et al., 1996). DNA extracted from WSSV infected *L. vannamei* was 10 fold serial diluted, and subsequently taken as DNA template for both detection methods. The dilutants are ddH<sub>2</sub>O or DNA extract of SPF *L. vannamei*. No apparent difference on sensitivity between the 2 detection methods was observed.

*Analytical specificity* - SPF *L. vannamei*, Monodon baculovirus or Baculovirus Penai infected *P. monodon*, as well as WSSV infected shrimp collected from different geographic areas were acquired from OIE reference laboratories. Results of all SPF samples and MBV or BP infected samples have shown no detectable non-specific band; while all WSSV infected samples can be detected by IQ2000™ WSSV Detection and Prevention System.

*Repeatability data* - The repeatability study was carried out using pleopods of *L. vannamei* with 3 production batches in triplicates. The data were consistent.

### Diagnostic Characteristics

Two evaluation studies were carried out for the estimation of the diagnostic sensitivity and specificity:

- In one study, the OIE recommended nested PCR method (Lo, et al., 1996; Chapter 4.1.2. of the *Manual of Diagnostic Tests for Aquatic Animals*, 2007, available on the OIE website) was applied to classify certain populations of shrimp as either negative or positive. After screening, 300 white spot syndrome negative samples and 300 white spot syndrome positive samples were used for this study.

- In a second study, 100 un-defined samples collected from market were used to compare IQ2000™ WSSV Detection and Prevention System with the OIE recommended method.

*Diagnostic sensitivity (DSn) and specificity (DSp) estimates with 95% confidence limits (CI)*

Tests performed with virus negative and positive animals:

	<b>WSS virus positive by reference method</b>	<b>WSS virus negative by reference method</b>
<b>Test Positive by IQ2000™</b>	289	0
<b>Test Negative by IQ2000™</b>	11	300
Relative Diagnostic Sensitivity: 96.33%, CI [93.53 – 98.16%] Relative Diagnostic Specificity: 100.00%, CI [98.78 – 100.00%]		

Tests performed with undefined samples collected from market:

	<b>WSS virus positive by reference method</b>	<b>WSS virus negative by reference method</b>
<b>Test Positive by IQ2000™</b>	51	0
<b>Test Negative by IQ2000™</b>	0	49
Relative Diagnostic Sensitivity: 100.00%, CI [92.89 – 100.00%]		
Relative Diagnostic Specificity: 100.00%, CI [92.89 – 100.00%]		

#### *Agreement between tests*

The results presented above reveal that the consistency between IQ2000™ WSSV Detection and Prevention System and the OIE recommended method is very good.

#### Reproducibility

Different lots of IQ2000™ WSSV Detection and Prevention System were sent to three different laboratories in Chinese Taipei (including one OIE reference laboratory) to be tested. 35 shrimp samples (*L. vannamei*) were collected and sent to all 3 laboratories. Each sample was analysed with 3 different lots of IQ2000™ WSSV Detection and Prevention System. Results of the 3 independent laboratories were collected and the data were analysed statistically

The reproducibility data indicates that there is no significant difference in assay performance between laboratories. Furthermore, good reproducibility was demonstrated using three different lots of kits, suggesting that lot to lot variability is minimal.

#### Applications

The IQ2000™ WSSV Detection and Prevention System kit is currently used by several laboratories worldwide as an official test.

#### References

1. LO CF, LEU JH, HO CH, CHEN CH, PENG SE, CHEN YT, CHOU CM, YEH PY, HUANG CJ, CHOU HY, WANG CH, AND KOU GH (1996 a) Detection of baculovirus associated with white spot syndrome (WSSV) in penaeid shrimps using polymerase chain reaction. *Dis Aquat Org* 25: 133-141.
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6. Chapter 2.2.6., White spot disease; *Manual of Diagnostic Tests for Aquatic Animals*, 2009, OIE, pp. 177-190.

**Manufacturer:**  
GeneReach Biotechnology Corp.  
No.19, Keyuan 2nd Road, Central Taiwan Science Park, Taichung 407, TAIWAN  
**TEL:** 886-4-2463-9869 **FAX:** 886-4-2463-8255  
**E-mail:** sales@iq2000kit.com <http://www.iq2000kit.com>