Validated and certified by the OIE as fit for the purposes defined in this kit insert provided with this kit. Registration number 20130108

IQ Plus™ WSSV Kit with POCKIT System

For White Spot Syndrome Virus Detection

User Manual

***in vitro* use only**

2014/11

Manufacturer : GeneReach Biotechnology Corporation
Address : No. 19, Keyuan 2nd Road, Central Taiwan Science Park, Taichung City 407, Taiwan
TEL : 886-4-24639869
FAX : 886-4-24638255
Web Site : www.iq2000kit.com

INTENDED USE

IQ Plus™ WSSV Kit with POCKIT System uses insulated isothermal polymerase chain reaction (iiPCR) technology to detect the DNA of white spot syndrome virus (WSSV) (Chang et al., 2012; Tsai et al., 2012). This detection kit is specially designed to be used on an iiPCR-compatible instrument, **POCKIT™** Nucleic Acid Analyzer. The intended users of this detection kit are aquaculture technicians who have basic laboratory skills.

This detection kit is intended for *in vitro* use only.

SUMMARY AND EXPLANATION

White spot syndrome virus (WSSV) is a major shrimp disease which has caused high mortality rate and economic losses to major shrimp farming countries. 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.

WSSV is highly lethal and contagious. Within only few days of WSSV infection, mass mortalities occur and can affect the entire populations of many shrimp farms, resulting in large economic losses to the shrimp farming industry. Since there is no effective therapeutic method available, the only way to prevent WSSV infection is through screening. Various molecular methods, such as microtomy, immuo-assay, hybridization, and PCR for WSSV detection have been developed. At present, PCR method is recognized to be the most effective diagnostic tool for WSSV.

GeneReach has developed **IQ Plus™** WSSV Kit with POCKIT

System based on iiPCR technology, which is highly sensitive and specific for WSSV detection. **IQ Plus™** WSSV Kit with POCKIT System is specially designed for on-site viral detection in the farm. The assay has been simplified for easy and fast operation with the use of compact and portable equipment for WSSV detection at pond-side.

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/reaction) 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).

PRINCIPLE OF THE PROCEDURE

The assay is based on multiplex iiPCR for qualitative detection of white spot syndrome virus (WSSV). Fluorogenic probe hydrolysis chemistry is used to generate fluorescent signal when a specific DNA sequence of WSSV is amplified. The primers and probe target specific sequences of WSSV, and do not react with nucleic acids of other pathogens. In addition, internal control (IC) primers and probe are used to target a house-keeping gene of Penaeid shrimps.

PRODUCT DESCRIPTION

A. Materials Provided

1) **IQ Plus™** WSSV Kit with POCKIT System (48 tests/kit)

Component	Contents	Amount
WSSV Premix Pack	■ Vials with lyophilized pellet containing dNTPs, WSSV specific primers, fluorescent probes, and enzyme. ■ Desiccating agent pack.	6 individually sealed zip-lock packs (8 vials/pack)
Premix Buffer B	■ Reaction buffer to re-dissolve the lyophilized pellet.	2 vials (1.3 ml/vial)
WSSV P(+) Control	■ Dried plasmid pellet containing WSSV partial sequence as positive control.	1 vial
P(+) Control Buffer	■ Reaction buffer to re-dissolve the WSSV P(+) Control.	1 vial (110 μl/vial)
Inoculating Loops		3 packs (20 pieces/pack)
User Manual		1 copy
Operation Guide		1 copy

2) **IQ Plus™** Extraction Kit (50 tests/kit)*

***Please refer to the IQ Plus™ Extraction Kit user manual for details.**

3) R-tube (48 tubes/pack)

B. Materials and Equipment Required, but Not Provided

1) **POCKIT™** Nucleic Acid Analyzer: the iiPCR-compatible instrument for **IQ Plus™** Detection Kit

2) **cubee™** Mini-Centrifuge

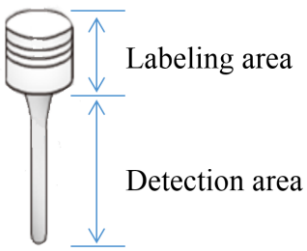
3) Micropipette and filter tips

C. Storage and Stability

1) The kit should be stored at 4℃ and is stable until the expiration date stated on the label.
2) Store Premix vials in sealed Premix Pack to avoid hydration of lyophilized components.
3) Reconstituted P(+) Control is stable for 6 months at 4℃. Aliquot reconstituted P(+) Control to avoid degradation of nucleic acid.

PRECAUTIONS

1) Do not open R-tube(s) after reaction to prevent any carryover contamination.
2) Perform extraction and amplification in two independent spaces to minimize contamination.
3) Bring Premix Pack to room temperature before use.
4) Do not reuse R-tube and Premix.
5) Include the P(+) Control to:
   - Ensure **POCKIT™** Nucleic Acid Analyzer is working.
   - Ensure detection kit performance after storage.
6) To get optimal fluorescence detection.
   - Wear powder-free gloves to handle R-tubes.
   - Do not label in the detection area of R-tube.



LIMITATION

1) **IQ Plus™** Extraction Kit or **taco™ mini** Automatic Nucleic Acid Extraction System is recommended for nucleic acid extraction.

2) The test should only be used for testing nucleic acid extracts. Do not add specimens directly into the Premix.

3) Any deviation from the recommended procedures may lead to sub-optimal results. Performance of the modified protocol should be validated by the users.

4) It is strongly recommended to use freshly prepared nucleic acids (within 1 hour after extraction) to achieve optimal results with **IQ Plus™** WSSV Kit with POCKIT System.

SAMPLE TYPE

This detection kit is intended for analyzing nucleic acids extracted from shrimp tissue as below:

- Pleopod of broodstocks or juvenile shrimps.
- Post larvae (PL).

OPERATION PROCEDURE

- **NOTE: Before preparing the reactions for iiPCR testing, turn on POCKIT™ Nucleic Acid Analyzer to initiate the calibration for the instrument. The device will complete self-test within 5 minutes. Please refer to the user manual of POCKIT™ Nucleic Acid Analyzer for further details.**
- **NOTE: Before using for the first time, add 100 µl P(+) Control Buffer to P(+) Control. Store reconstituted P(+) Control at 4°C.**

1) Label R-tube(s) in the labeling area.
2) Prepare one Premix for each sample. (Premix tubes are in Premix Pack. Each Premix Pack contains eight Premix tubes.)
   - **NOTE: When the pellet is not found at the bottom of the tube, spin tube briefly to bring it down.**
3) Add 50 µl Premix Buffer B to each Premix tube.
4) Use the inoculating loop, take nucleic acid extracts or dissolved P(+) Control into each Premix tube. Spin Premix tubes briefly in a mini centrifuge (such as **cubee™** Mini-Centrifuge).
   - **NOTE: Please repeatedly dip the inoculating loop into solution three times to collect the correct solution volume.**

5) Transfer 50 µl Premix/sample mixture into R-tube.

6) Seal top of each R-tube with a cap. Make sure R-tube is capped tightly.

7) Place R-tube into the holder of **POCKIT™** Nucleic Acid Analyzer.

8) Spin tube/holder set briefly in **cubee™** Mini-Centrifuge to make sure all solution is collected at the bottom of R-tube.

   - **NOTE: Make sure there are no bubbles in the solution.**
   - **NOTE: Start reaction within 1 hour to prevent nucleic acid degradation (to prevent nucleic acid degradation and non-specific reaction).**

9) **POCKIT™** Nucleic Acid Analyzer reaction:

   a) Select "520 nm + 550 nm".

   b) When "System READY" is displayed, place the holder with R-tube(s) into the reaction chamber.

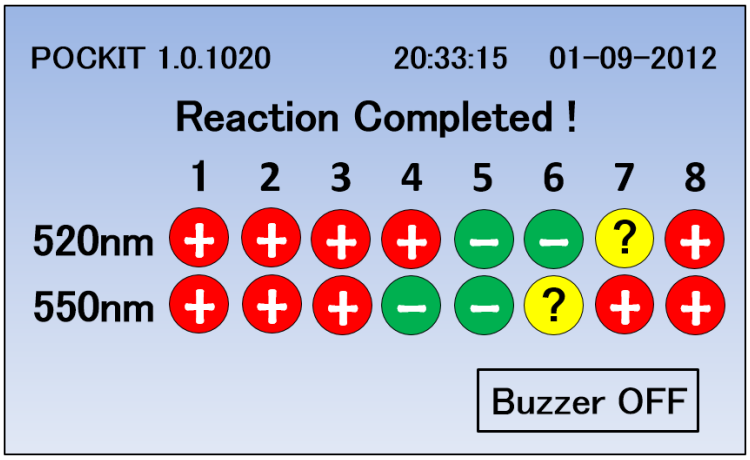
   c) Tap cap of each R-tube to make sure the tube is positioned properly.

10) Close lid and press "Run" to start reaction program.

11) Test results are shown on the monitor after the reaction is complete.

DATA INTERPRETATION

- 520-nm fluorescent signal is used to detect nucleic acid sequence of virus; 550-nm fluorescent signal as the internal control (IC) is used to target a house-keeping gene of Penaeid shrimps.
- The following example is iiPCR reaction results shown on the monitor.



520 nm	550 nm	Interpretation
+	+	WSSV positive
+	−	WSSV positive
−	+	WSSV negative
−	−	■ For shrimp tissue, please recheck (see Troubleshooting). ■ For non-shrimp tissue (such as pond dirt), the sample shows WSSV-negative result.
+	?	WSSV positive
?	+	Repeat reaction with freshly prepared nucleic acid.
−	?	Repeat reaction with freshly prepared nucleic acid.
?	−	Repeat reaction with freshly prepared nucleic acid.
?	?	Repeat reaction with freshly prepared nucleic acid.

ANALYTICAL SENSITIVITY

1) The detection limit of **IQ Plus™** WSSV Kit with POCKIT System is up to 10 copies/reaction.

2) The sensitivity of IC of **IQ Plus™** WSSV Kit with POCKIT System is 20 ng genomic nucleic acid/reaction.

TROUBLESHOOTING

Problems	Possible causes	Comments or solution
Negative in 550 nm and 520 nm (internal control is negative)	1) Poor nucleic acid quality.	■ Please see the Troubleshooting section in the **IQ Plus™** Extraction Kit manual.
	2) Premix pellet was not reconstituted to correct volume.	■ Check the preparation procedure of the Premix.
	3) No nucleic acid added	■ Please repeat the test.
	4) Deterioration of the reagents.	■ Check the expiration date and storage condition. ■ Check to make sure the Premix pellet is dry, and has not been rehydrated before use.
	5) PCR inhibition	■ Do not add too much nucleic acid. Please follow the recommendation in this user manual. ■ Spike 5 µl nucleic acid sample into a positive control reaction for a parallel PCR reaction. Negative results indicate the presence of inhibitors in the nucleic acid. In that case, prepare another nucleic acid extract.
No template control showed positive result	1) Micropipette contaminated.	■ We recommend using aerosol free tips.
	2) Reagent contaminated.	■ Replace reagent(s).
	3) Lab contaminated.	■ Consult with **GeneReach** for lab clean up

Problems	**Possible causes**	**Comments or solution**
Reaction solution or other objects found in the reaction chamber of the **POCKIT™** Nucleic Acid Analyzer.	1) R-tube broken or solution spilled in the reaction chamber of the **POCKIT™** Nucleic Acid Analyzer.	■ Please contact local distributor for further assistance.

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APPENDIX

OIE Procedure for Registration of Diagnostic Kits

Abstract sheet

Name of the diagnostic kit: IQ Plus™ WSSV Kit with POCKIT System **Manufacturer**: Genereach Biotechnology Corporation **OIE Approval number**: 20130108 **Date of Registration**: May 2013

Disease: White Spot Disease
Pathogen Agent: White Spot Syndrome Virus (WSSV)
Type of Assay: Insulated isothermal Polymerase Chain Reaction
Purpose of Assay: Certified by the OIE in May 2013 as fit for the detection of white spot disease in target tissues (Shrimp tissue of ectodermal and mesodermal origin) of *Litopenaeus vannamei* and for the following purposes:

1) To certify freedom from infection (<10 virions/reaction) 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: *Litopenaeus vannamei*; Shrimp tissue of ectodermal and mesodermal origin.

A. Information on the kit

Information can be found by emailing: sales@genereach.com or by visiting: http://www.iq2000kit.com/products_2.php?bgid=3&gid=6&sgid=34.

In summary, IQ Plus™ WSSV Kit with POCKIT System was designed for qualitative detection of WSSV DNA based on multiplex insulated isothermal PCR technology (iiPCR; Chang *et. al.*, 2012; Tsai *et. al.*, 2012a; Tsai *et. al.*, 2012b). IQ Plus™ WSSV Kit with POCKIT System is designed to be used with a compact and portable iiPCRcompatible instrument, POCKIT™ Nucleic Acid Analyzer (POCKIT™). IQ Plus™ WSSV Kit with POCKIT System is highly sensitive and specific for WSSV DNA detection from aquaculture specimen and suitable for onsite viral DNA detection. Specific primers and probe (520-nm fluorescent signal) are designed to detect WSSV DNA in samples. In addition, internal control (IC) primers and probe (550-nm fluorescent signal) are used to target a house-keeping gene of *Penaeid* shrimps. The assay has been simplified for easy and fast operation in POCKIT™ for pond-site WSSV DNA detection.

B. Summary of validation studies

1) Analytical characteristics

■ ***Repeatability*:**

Various types including negative and positive WSSV-infected shrimps (*L. vannamei*) were selected and three production batches were tested. Each sample was tested in quadruplicates per run. The data showed 100% agreement among the test results.

■ ***Analytical specificity*:**

WSSV-, Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)-, or Hepatopancreatic Parvovirus (HPV)-infected *L. vannamei* were used to test the specificity of IQ Plus™ WSSV Kit with POCKIT System. The IHHNV- and HPV-infected samples were confirmed to be WSSV-negative by IQ2000™ WSSV Detection and Prevention System (DPS). Signals were generated from only WSSV-infected, not from IHHNV- and HPV-infected samples in IQ Plus™ WSSV Kit with POCKIT System.

■ ***Analytical sensitivity*:**

Analysis using standard plasmid (pWSSV1) and purified WSSV genomic DNA of known copy numbers shows that the ≥ 95% detection rate of IQ Plus™ WSSV Kit with POCKIT System was 23.7 and 16.9 copies pWSSV1 and WSSV DNA, respectively, per reaction. Furthermore, analysis of DNA extracted from WSSV-infected *L. vannamei* serially diluted with ddH2O or DNA extracts of SPF *L. vannamei* shows that the detection endpoint (10^4 dilution) of IQ Plus™ WSSV Kit with POCKIT System was similar to that of IQ2000™ WSSV DPS.

2) Diagnostic Characteristics

■ ***Test Cut-off Determination:***

IQ Plus™ WSSV Kit with POCKIT System, based on iiPCR and fluorescent probe detection principles, is designed to work in an iiPCR-compatible instrument, POCKIT™. The cut-off for POCKIT™ device were determined on the basis of fluorescent signal of numerous NTC and positive reactions of iiPCR assays developed for various targets at GeneReach (confidential data).

Readouts of the results are determined as follows:

- When "+" is displayed on POCKIT™, the sample is classified as WSSV positive.
- When "-" is displayed on POCKIT™, the sample is classified as WSSV negative.
- When "?" is displayed on POCKIT™, the test result is indeterminate and should be repeated.

■ ***Diagnostic sensitivity (DSn) and specificity (DSp) estimates***

Diagnostic sensitivity was evaluated by comparing the test results of IQ Plus™ WSSV reaction of positive reference animals which were identified by IQ2000™ WSSV DPS. Pleopods of 400 WSSV-positive

samples were sampled and tested in this study. Negative reference animals selected by IQ2000™ WSSV DPS were also subjected to analysis by IQ Plus™ WSSV Kit with POCKIT System.

		IQ2000™ WSSV DPS	
		Positive	Negative
IQ Plus™ WSSV Kit with POCKIT System	Positive	374	9
	Negative	26	291

In summary, this validation testing was conducted on 700 samples. The results are: sensitivity: 93.5% [95% confidence interval (CI): 90.61–95.56%], specificity: 97.0% [95% CI: 94.31–98.50%].

■ ***Comparative performance***

See "*Diagnostic sensitivity (DSn) and specificity (DSp) estimates".*

■ ***Agreement and discrepancies***

The results revealed that when compared to IQ2000™ WSSV DPS, with defined reference animals, the diagnostic sensitivity of IQ Plus™ WSSV Kit with POCKIT System was 93.5% with a 95% CI of 90.61% - 95.56%, and the diagnostic specificity was 97.0% with a 95% CI of 94.31% - 98.50%. In addition, with un-defined reference animal, IQ Plus™ WSSV Kit with POCKIT System showed 100% agreement (100/100) for both sensitivity and specificity. For this experiment, pleopods of 100 un-defined shrimps obtained randomly from a local farm were sampled and examined by both IQ2000™ WSSV DPS and IQ Plus™ WSSV Kit with POCKIT System. Statistical analysis using one-tailed binomial test suggested that results from these two experiments (defined reference animals and un-defined animals) agreed with each other.

3) Reproducibility

Different lots of IQ Plus™ WSSV Kit with POCKIT System were sent

to three different labs located in Chinese Taipei and USA (including two OIE Reference Laboratories) to be tested. Trunk muscles of total of 64 *L. vannamei* samples were aliquoted, preserved in 95% ethanol, and sent to all three laboratories. Each sample was analysed with 3 batches of IQ Plus™ WSSV Kit with POCKIT System. Chi-square test for homogeneity was conducted to analyse the experimental results generated from the three labs.

The results showed that there was no difference among different laboratories with three batches of IQ Plus™ WSSV Kit with POCKIT System.

4) Applications

The kit is being used worldwide by different laboratories (Private and Public)

C. References

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