MBPCR254 Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit

Description

EHP (Enterocytozoon hepatopenaei) and WSSV (white spot syndrome virus) infections are amongst the most significant threat diseases in shrimp aquaculture. EHP is an obligate, intracellular, spore-forming parasite, which mainly infects the gastrointestinal tract of shrimps. Whereas WSSV is the lone virus of the genus *Whispovirus* (white spot) causing white spots (ranging from 0.5–3.0 mm in diameter) on the exoskeleton, appendages and inside the epidermis of the shrimps. Both infections cause high mortality in shrimps and outbreaks of which leads to major economic losses. Thus, early diagnosis with rapid and reliable detection of these infections is essential for quick surveillance and control. Hi-PCR® EHP and WSSV detection and quantitation multiplex probe PCR kit will aid as a reliable molecular tool for early detection of shrimp infections, thereby prevention of disease outbreaks and economic losses.

NOTE: Hi-PCR® EHP and WSSV detection and quantitation multiplex probe PCR kit is for *in-vitro* use only.

Intended Use

Recommended for sensitive, specific and simultaneous detection and quantitation of EHP and WSSV infections in shrimp samples.

Principle

Real-time polymerase chain reaction, also called quantitative Polymerase Chain Reaction (qPCR) or kinetic Polymerase Chain Reaction, is a laboratory technique based on the principle of PCR. This technique is used to amplify a targeted DNA sequence by use of hydrolysis probes that are short oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. Hi-PCR® EHP and WSSV detection and quantitation multiplex probe PCR kit is designed to detect the target gene of EHP, WSSV and Internal Control in FAM, TexRed and JOE channel respectively. The kit allows sensitive, specific and simultaneous detection and quantitation of EHP and WSSV in a single tube reaction.

Positive control

This is a control reaction using a known template (target pathogen). A positive control is usually used to check that the primers have been designed properly and the PCR conditions have been set up correctly.

Internal Control

This is a control sequence which is amplified in the same reaction tube along with the target sequence (target species) but detected with a different primer (i.e. Multiplex PCR). An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified.

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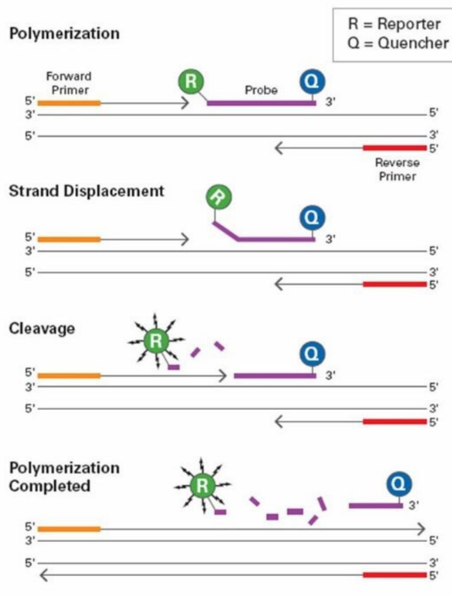
Diagrammatic representation of preferential binding of probe specific to DNA fragments in Real-time PCR



Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' end of the probe respectively

Strand displacement: When the probe is intact, the report dye emission is quenched.

Cleavage: During each extension cycle, the DNA polymerae cleaves the reporter dye from the probe

Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). The probes are designed such that they anneal within a DNA region amplified by a specific set of primers. During PCR amplification, these probes will hybridize to the target sequences located in the amplicon i.e. the DNA. As the *Taq* DNA polymerase replicates the template with the bound probe, the 5'- nuclease activity of the polymerase enzyme cleaves the fluorescent probe. The end result in cleavage of the probe is separation of the reporter dye from the quencher dye and increasing the reporter dye signal. As the probe is removed from the target strand, primer extension continues to the end of the template strand. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Thus, inclusion of the probe does not inhibit the overall PCR process.

Features

- Fast and simple
- Good sensitivity and specific results
- Guaranteed reproducible results

Types of Specimen: Shrimps.

Specimen collection and Handling

Follow appropriate techniques for handling specimens; after use, contaminated materials must be sterilized by autoclaving before discarding. Standard precautions as per established guidelines should be followed while handling contaminated samples. Safety guidelines may be referred in individual safety data sheets.

Storage and Shelf life

The provided kit has a claimed shelf-life of 12 months when stored between -10°C to -20°C. Repeated thawing and freezing of PCR reagents should be avoided, as this may reduce the sensitivity. If the reagents are to be used multiple times, we recommend storing reagents as aliquots to avoid repeated freeze and thaw. Degradation of sample DNA specimens can also reduce the sensitivity of the assay. HiMedia Laboratories does not recommend using the kit after the expiry date stated on pack.

Kit Contents: The provided PCR kit contains:

Components	Product code	Reagents provided for (reactions)* (µL)	
		25R	50R
EWI Master Mix	DS1585	270	540
EWI Primer-Probe Mix	DS1353	54	108
EWI Quantitative Standard 1	DS1589	20	40
EWI Quantitative Standard 2	DS1590	20	40
EWI Quantitative Standard 3	DS1591	20	40
EWI Quantitative Standard 4	DS1592	20	40
Water	DS0440	20	40

Materials needed but not provided

- PCR tubes (Product code PW1255) or PCR Strips (Product code: PR17) or PCR Plates (Product code: PR2 / PR3 / PR19) & Sealing film (PR18)
- Insta Q Real Time PCR System (Product Code: LA1012 / LA1023 / LA1024 / LA1073 / LA1074)
- Barrier Micropipette Tips (Product Code: LA749 / LA749A / LA751 / LA751A / LA750 / LA750A / LA859 / LA859A)
- Micropipettes
- For Shrimp samples: HiPurA® Shrimp DNA Purification Kit (MB581)

General Preparation Instructions

- Before use all PCR components should be completely thawed on ice (4°C).
- Perform the amplification reactions in a clean area, preferably in a biosafety cabinet.
- Use of aerosol barrier pipette tips is recommended to reduce contamination risks from extraneous DNA templates.
- Extract and store positive control sample (if used) separately from all other reagents to avoid contamination and add it to the reaction mix in a separate area.

Protocol for PCR Reaction Mix Preparation

1. In the “Master mix Preparation” area, thaw all components from the kit on ice, mix by inverting the tubes and centrifuge the reagents for several seconds. Keep on ice for later use.
2. Based on the number of specimens to be tested (N), including the quantitative standards and NC, calculate the volume of the components to be added as N* volume of 1X

Components	Product code	Volume (µL) to be added for 1R (for a 20 µL reaction)
EWI Master Mix	DS1585	10
EWI Primer-Probe Mix	DS1353	2
Template DNA/Quantitative standard/Negative Control	-	8
Total volume	-	20

*NOTE: (Optional) – The user can also set up an additional PCR reaction containing 8 µL Positive Control for PCR in a separate tube.

3. Use 1.5 mL Nuclease free centrifuge tube(s) for the preparation of the reaction system. After all the reagents are added, mix them thoroughly and centrifuge for several seconds.
4. Load 12 µL of master mix into the 0.1/0.2 mL PCR reaction tube/plate/strips, compatible to the instrument to be used; add 8 µL Nuclease free water to the negative control.

5. In the “Nucleic acid handling” area, add 8 µL of EWI Quantitative Standard Control and extracted test DNA into the plate/strip to respective wells.
6. Tightly cap the tubes/strips or seal the plate using an optically clear adhesive film.
7. Briefly, spin the strips/tubes to settle the reagent to the bottom of the tube.
8. Place the plate/strips/tubes in Real-time PCR machine and set the PCR program.

Recommended PCR program

	Step	Condition	Cycles
1.	Initial denaturation:	95°C for 10 minutes	No. of cycles: 01
2.	Denaturation:	95°C for 15 seconds	No. of cycles: 40
3.	Annealing:	62°C for 30 seconds Sampling: FAM/TexRed/JOE	

Data Analysis

The following conditions should be meet for a valid diagnostic test:

Control	Detection channels		
	FAM (EHP)	TexRed (WSSV)	JOE (Internal Control)
Positive Template Control	+	+	+
Negative Template Control	-	-	-

Data Interpretation

Detection Channels			Result Interpretation
FAM (EHP)	TexRed (WSSV)	JOE (Internal Control)	
≤ 37 cycles		15 – 35 cycles*	Positive for EHP
-	≤ 34 cycles	15 – 35 cycles*	Positive for WSSV
≤ 37 cycles	≤ 34 cycles	15 – 35 cycles*	Positive for EHP and WSSV
-	-	15 – 35 cycles	Negative for EHP and WSSV
-	-	-	PCR inhibition or reagent failure. Repeat PCR or repeat extraction from original sample

*The presence or absence of a signal in the JOE channel is not relevant for the validity of the test run due to competition between the test template and Internal Control template.

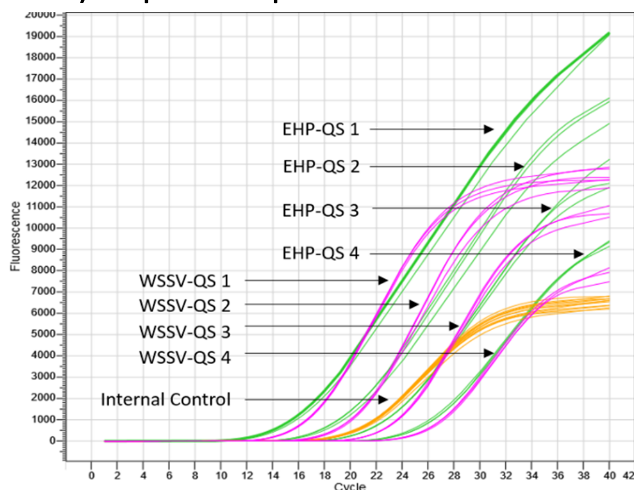
Limit of Detection (LoD) - Analytical Sensitivity

Sensitivity for the Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit was conducted on HiMedia, InstaQ96® Real Time PCR system, Applied Biosystems, Quant Studio™ 5 and Bio-Rad, CFX96™ C1000 Real Time PCR systems. The detectable limit of the HiMedia’s HiPCR® EHP and WSSV multiplex Probe PCR Kit on all instruments was determined to be 2.82 copies/µL and 141 copies/µL with 100% and 95% probability for EHP and WSSV respectively.

Standard Curve Analysis

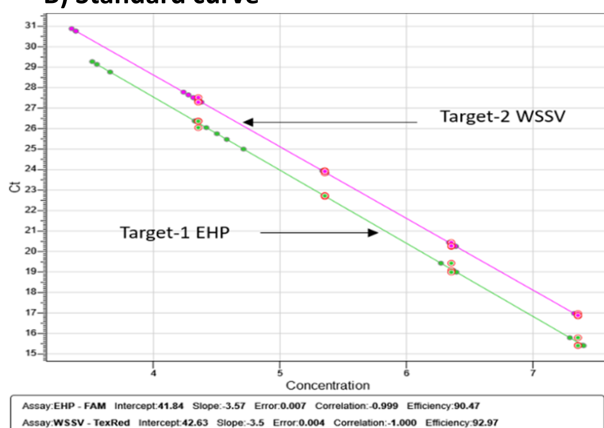
To generate a standard curve, all 4 quantitation standards should be used and defined in the plate setup as standards with the specified concentrations (see the below table for copy number). After completion of the run the device will build standard curve. Take the values for unknown samples, only if the following conditions of standard curve are met: (1) R^2 >0.98; (2) Slope of the quantitative standards is between -3.0 to -3.7; (3) PCR efficiency is between 85%-115%; and (4) No amplification any channel of negative control.

A) Amplification plot



Sr. No.	Sample	C_t value
1	EHP QS 1 ($2.26x10^7$copies/reaction)	15.54
2	EHP QS 2 ($2.26x10^6$copies/reaction)	19.37
3	EHP QS 3 ($2.26x10^5$copies/reaction)	22.71
4	EHP QS 4 ($2.26x10^4$copies/reaction)	26.26
5	WSSV QS 1 ($2.26x10^7$copies/reaction)	16.9
6	WSSV QS 2 ($2.26x10^6$copies/reaction)	20.32
7	WSSV QS 3 ($2.26x10^5$copies/reaction)	23.9
8	WSSV QS 4 ($2.26x10^4$copies/reaction)	27.38
9	Internal Control	21.10
10	Negative control	NA

B) Standard curve



Standard Curve	Target 1 EHP	Target 2 WSSV
Slope	-3.57	-3.5
Correlation coefficient (R^2)	-0.99	-1.00
Efficiency	90.47%	92.97%
Y-Intercept	41.84	42.63

Image representing A) amplification plot and B) standard curve generated using HiMedia's Hi-PCR® EHP and WSSV detection and quantitation multiplex probe PCR kit on Insta Q96® Plus RT-PCR machine.

Quantitation

The quantitation standards are defined as copies/reaction. The following equation has to be applied to convert the unknown samples values estimated using the standard curve (copies/reaction) into copies/ mg of shrimp tissue:

$$\textbf{Copies/mg of shrimp tissue} = \frac{\textbf{Estimated copies per reaction x Elution Volume } (\mu L)}{\textbf{Template volume } (\mu L) \textbf{ x Weight of Initial sample (mg)}}$$

Whereas, 1) Estimated copies/reaction= Number of copies extrapolated by machine from the standard plot.
2) Elution Volume (µL) = Total volume in which extracted DNA is eluted.
3) Template volume (µL) = Eluted DNA volume added to the PCR reaction tube.
4) Quantity of Initial sample (mg)= Amount of shrimp tissue used for DNA extraction

*Note: As a matter of principle, the initial sample weight should be entered in the equation above only when the complete volume of tissue lysis solution (Tissue lysed in lysis buffer) is used for extraction. Otherwise, consider the amount of tissue present in the tissue lysis solution volume used for extraction.

Kit Compatibility with Real-Time PCR systems:

Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit

Real-Time PCR system	Company	Dye 1 (EHP)	Dye 2 (IC)	Dye 3 (WSSV)
Insta Q96® - 6.0/Insta Q96® Plus/Insta Q48®	HiMedia Laboratories Pvt. Ltd.	FAM	JOE	Texas Red
Quant Studio™ 5	Applied Biosystems	FAM	VIC	Texas Red
Bio-Rad CFX Opus 96/CFX96	Bio-Rad Laboratories, Inc.	FAM	HEX/VIC	Texas Red

Note: The kit is practically tested on the mentioned Real Time PCR systems, the kit can be compatible with other Real Time PCR systems as well. Ensure that the Real-Time PCR system is calibrated for dyes and is maintained according to the manufacturer ́s instructions and recommendations.

Warning and Precautions
Read the procedure carefully before beginning the protocol. Wear protective gloves/protective clothing/eye protection/face protection. Follow good laboratory practices while handling samples. Standard precautions should be followed as per established guidelines. Safety guidelines may be referred in safety data sheets of the product.

Limitations
Although rare, mutations within the highly conserved regions of the targets genes covered by the kit's primers and/or probe may result in under quantitation or failure to detect the presence of the target regions in these cases. Validity and performance of the assay design are revised at regular intervals.

Performance and Evaluation
Each lot of Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Quality Control
Each lot of Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit is assayed for contaminating endonuclease, exonuclease and non-specific DNase activities. Functionally tested in DNA amplification.

Troubleshooting Guide

Sr. No.	Problem	Cause	Solution
1.	No amplification	Degraded samples	Check the integrity of DNA using agarose gel electrophoresis.
			Use freshly prepared DNA to ensure the availability of intact template sequence for efficient amplification.
		Error in protocol setup	Verify that the correct reagent volumes, dilutions and storage conditions have been used.

2.	Variability between replicates	Error in reaction set-up	Prepare a large volume master mix, vortex thoroughly and aliquot into reaction tubes.
		Air bubbles in reaction mix	Briefly centrifuge reaction samples/plate prior to running on a real-time PCR instrument.
		Pipetting error	C_t values of replicates can show increased variation due to poor laboratory technique or imprecise pipettes.
3.	Amplification in negative control	Reagents contaminated	Replace all critical solutions. Repeat the analysis of all tests with fresh aliquots of critical reagents.
4.	No signal with positive controls	Incorrect programming of the temperature profile of the thermocycler	Compare the temperature profile to the manual.

Safety Information

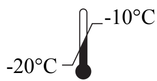
Hi-PCR® EHP and WSSV Detection and Quantitation Multiplex Probe PCR Kit is for laboratory use only, not for drug, household or other uses. Take appropriate laboratory safety measures and wear gloves when handling.

Disposal

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques.

Technical Assistance

At HiMedia, we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com.



Storage temperature

Do not use if package is damaged

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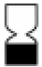

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