European Union Reference
Laboratory for Crustacean Diseases
Cefas Weymouth Laboratory, Barrack Road, Weymouth, Dorset DT4 8UB, United Kingdom

PROFICIENCY TEST 2013

Detection of White Spot Syndrome Virus in Lenticules

EURL Ring Trial Reference Number: EURL13001

Distribution Date:	**31/08/13**
Report Date:	**23/08/13**
Report compiled by:	**K. Bateman**

Introduction

This scheme is intended to provide proficiency testing samples for National Reference Laboratories (NRLs) undertaking examination of crustacean tissues for the presence/absence of White Spot Syndrome Virus (WSSV) in accordance with EC Directive 2006/88. The invitation to participate in this year's proficiency test was sent to 21 NRL's in 19 Member States. Samples were sent to 16 NRL's in 14 Member States, 5 NRL's declined to take part in this trial.

This Proficiency test was organised by the European Union Reference laboratory (EURL) for Crustacean Diseases.

Further information can be obtained via the EURL website (www.crustaceancrl.eu)

Sample Preparation

Viral inoculates of WSSV were originally obtained from the OIE reference laboratory at the University of Arizona, USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *L. vannamei* from an original outbreak in *F. chinensis* in China in 1995. Subsequent passages of this isolate into naïve *L. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued infectivity of this isolate.

There are currently no crustacean cell lines available; WSSV infected shrimp carcasses were prepared by direct intramuscular injection of WSSV inoculum into specific pathogen free (SPF) *L. vannamei* at a rate of 10 µl g^1 shrimp weight. Water temperature was held constant at 24˚C. Shrimp were monitored throughout the day for five days, dead and moribund shrimp were removed from the experimental tanks. SPF shrimp provided tissues for WSSV negative samples. Shrimp were confirmed as WSSV positive and WSSV negative (SPF) by nested PCR techniques. These shrimp were homogenised and the homogenate used to inoculate a lenticulating fluid at various dilutions. This fluid was then aliquoted into 25µl drops which formed the lenticule discs.

Prior to distribution the EURL tested 10% of lenticule discs produced to ensure a satisfactory titre in the tissue and homogeneity of content of sample. Tissues were prepared and tested according to PCR protocols accredited under ISO 17025 standards.

Methods

The dessication filter was removed from the tubes containing the Lenticules and 1ml of molecular grade water was added and left for 5 minutes at room temperature. The sample was vortexed until the lenticule was fully dissolved and 50µl of this was added to 140µl G2 buffer and 10µl proteinase K. The samples were incubated for 4

hrs at 56ºC. Total DNA was extracted from the samples using an EZ1 DNA tissue kit and EZ1 Advanced XL BioRobot® (Qiagen) following manufactures' instructions.

Separate first and second round (Nested) PCR reactions were performed on each DNA extract using the OIE recommended WSSV primer sets (Lo *et al.* 1996a and b) Table 1. Reactions were performed in 50µl reaction mix consisting of 1 X Green Go Taq buffer, 2.5mM $MgCl_2$, 0.25mM dNTPs, 100 pmol each of the forward and reverse primer, 0.25 units Go Taq Flexi (Promega), and 2.5µl extracted nucleic acid. Amplifications were performed using the following WSSV thermal cycler program on a Peltier PTC-225 thermal cycler: 94ºC x 2 minutes followed by 29 cycles of 94ºC x 30 seconds, 62ºC x 30 seconds and 72ºC x 30 seconds, followed by 72ºC x 2 minutes and held at 4ºC. A 1447bp product should be seen for positive samples in the first round PCR and a 941bp product in the second round.

Amplification products were resolved on 2% agarose gels stained with ethidium bromide and visualised using a UV illuminator.

Table 1. WSSV PCR primers (Lo *et al*., 1996)

Primer name	Sequence
WSSV 146 F1	ACTACTAACTTCAGCCTATCTAG
WSSV 146 R1	TAATGCGGGTGTAATGTTCTTACGA
WSSV 146 F2	GTAACTGCCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

A nested PCR reaction was not completed for the neat samples as these produced a bright product of the correct size in the 1st round PCR.

The five batches of 20 samples were also tested using the OIE recommended QPCR protocol for the detection of WSSV (Durand *et al*. 2002). Each QPCR reaction contained 100nM of WSSV probe, 300nM of WSSV QPCR forward primer, 300nM of WSSV QPCR reverse primer (Table 2), 10µl of Taqman master mix (Applied Biosystems) and 2.5µl of each sample and the total volume of 20µl was made up with molecular grade water.

To make the standard curve 4µl of WSSV plasmid (1.6×10^9 copies/µl) was added to 156µl of molecular grade water and vortexed to give 4×10^7 copies/µl. Five microlitres of this was added to a microfuge tube containing 45µl molecular grade water vortexed to mix and subsequent serial ten fold dilutions were made to give concentrations from 4×10^6 to 4×10^1 copies/µl. Each of the ten fold serial dilutions (4×10^7 to 4×10^1copies/µl) were vortexed and 2.5µl of each dilution was added in triplicate to the QPCR plate containing 17.5µl of the master mix to give a total volume of 20µl. This gave final concentrations in the standard curve of: 1×10^8 to 1×10^1 copies.

Table 2. WSSV QPCR primers and probes (Durand & Lightner, 2002)

Primer and probe name	Sequence
WSSV probe	6FAM AGCCATGAAGAATGCCGTCTATCACACA Tamra
WSSV forward primer 1011F	TGGTCCCGTCCTCATCTCAG
WSSV reverse primer 1079R	GCTGCCTTGCCGGAAATTA

Three QPCR plates were run using the Applied Biosystems Stepone Plus Real Time QPCR machine using the following program parameters:

50ºC for 2 minutes
95ºC for 10 minutes
95ºC for 15 seconds
60ºC for 1 minute
For 40 cycles.

Quality Control

The 20 replicate lenticules of each batch (negative control and dilutions of WSSV) produced consistent results in both the PCR, where products amplified were of similar brightness, and the QPCR, where CT values between replicates of the same dilution were no more than a log different from each other.

Distribution

Samples were sent to 16 NRL's in 14 Member States. The test was sent out according to current international regulations for shipment of diagnostic specimens UN 3373, "Biological substance, Category B". All proficiency tests were handled by courier and were delivered to all participants within three days.

All NRLs received lenticule discs from the same batch.

Expected Results

Participants were asked to identify the content of each tube by the method used in their laboratory.

The table below highlights the expected results:

Lenticule Reference	Expected Results	
	Nested PCR[1]	Real-time PCR[2]
RA13001-1	WSSV Positive	WSSV Positive Ct 28
RA13001 - 2	WSSV Negative	WSSV Negative
RA13001 - 3	WSSV Positive	WSSV Positive Ct 23
RA13001 - 4	WSSV Positive	WSSV Positive Ct 31
RA13001 - 5	WSSV Positive	WSSV Positive Ct 30
RA13001 - 6	WSSV Negative	WSSV Negative
RA13001 - 7	WSSV Positive	WSSV Positive Ct 28
RA13001 - 8	WSSV Positive	WSSV Positive Ct 23
RA13001 - 9	WSSV Positive	WSSV Positive Ct 23
RA13001 - 10	WSSV Positive	WSSV Positive Ct 28
RA13001 - 11	WSSV Positive	WSSV Positive Ct 31
RA13001 - 12	WSSV Positive	WSSV Positive Ct 31
RA13001 - 13	WSSV Positive	WSSV Positive Ct 32
RA13001 - 14	WSSV Positive	WSSV Positive Ct 30
RA13001 - 15	WSSV Positive	WSSV Positive Ct 23
RA13001 - 16	WSSV Negative	WSSV Negative
RA13001 - 17	WSSV Positive	WSSV Positive Ct 30
RA13001 - 18	WSSV Negative	WSSV Negative
RA13001 - 19	WSSV Positive	WSSV Positive Ct 30
RA13001 - 20	WSSV Positive	WSSV Positive Ct 28

[1] OIE recommended technique (Lo *et al*., 1996)

[2] Durand and Lightner, 2002

Actual Results

- 15 laboratories correctly identified all samples, 20/20 (100%).
- 1 laboratory correctly identified 16/19 samples (84%), one sample being lost during shipping.

The following methods were used by the participants:

- 11 laboratories used nested PCR
- 5 laboratories used real-time PCR

General Comments

EURL offered to send fresh ring trial material to the NRL which did not correctly diagnose all materials; NRL was unable to repeat ring trial due to staff shortages.

The results presented in this report will be presented and discussed at the 5th Annual Meeting of National Reference Laboratories for Crustacean Diseases, 31st August 2013 in Tampere, Finland.

Kelly Bateman
European Union Reference laboratory for Crustacean diseases
23rd August 2013