Laboratoire Environnement Ressource des Pertuis charentais, Ifremer, La Tremblade
Laboratoire de Génétique et Pathologie, Ifremer, La Tremblade

Jean-François Pépin

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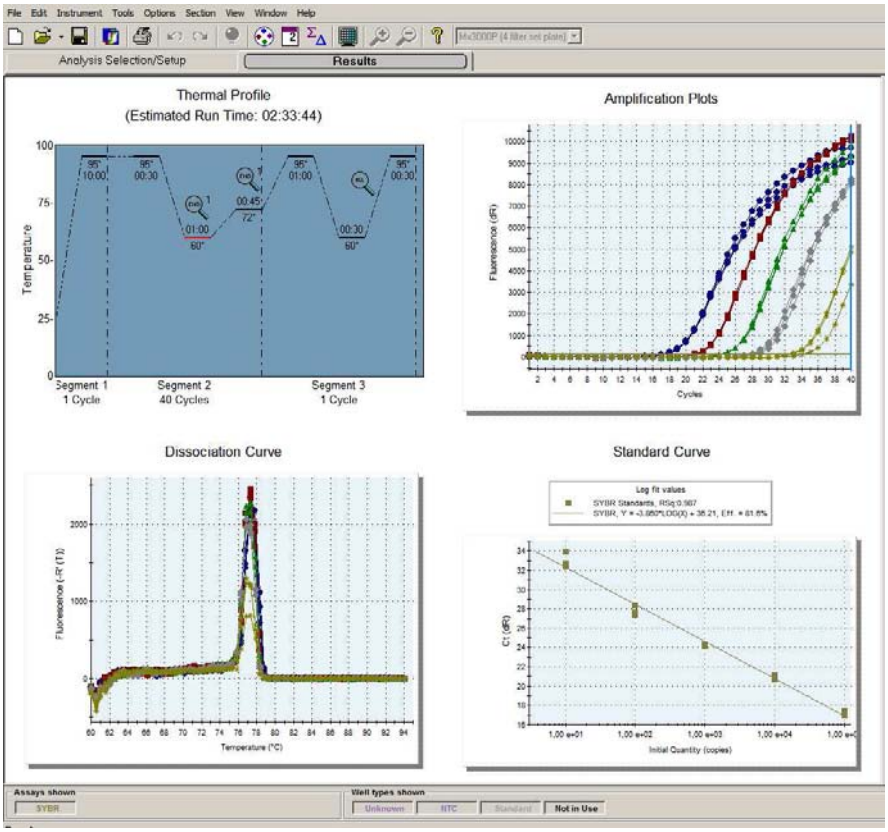
SHORT TECHNICAL REPORT
for
OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction using OsHV-1 DNA polymerase sequence



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The present report completes a previously described procedure routinely used for the detection and quantification of OsHV-1 by Sybr green Real Time Polymerase Chain Reaction targeting the OsHV-1 DNA polymerase sequence.

This report can be considered as an appendix of the Standard Operating Procedure (SOP) "OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction using OsHV-1 DNA polymerase sequence".

Fiche documentaire

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Contributeur(s) : Jean-François Pépin, Delphine Tourbiez	Laboratoire Environnement Ressource des Pertuis charentais, Ifremer, La Tremblade Laboratoire de Génétique et Pathologie, Ifremer, La Tremblade
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Résumé

Abstract : The present report completes a previously described procedure routinely used for the detection and quantification of OsHV-1 by Sybr green Real Time Polymerase Chain Reaction targeting the OsHV-1 DNA polymerase sequence.

More particularly, this document brings information about analytical sensitivity, quantitation limit, assay precision, reproducibility of the technique.

Mots-clés : PCR quantitative HDP-F/R, virus OsHV-1, diagnostic, DNA polymerase, validation

Keys words: real-time PCR HDP-F/R, OsHV-1 virus, diagnostic, DNA polymerase, validation

The present report completes a previously described procedure routinely used for the detection and quantification of OsHV-1 by Sybr green Real Time Polymerase Chain Reaction assay targeting the OsHV-1 DNA polymerase sequence.

This report can be considered as an appendix of the Standard Operating Procedure (SOP) “OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction using OsHV-1 DNA polymerase sequence” (available on the website of the EURL for mollusc diseases).

This appendix presents unpublished information about analytical sensitivity, quantitation limit, assay precision, reproducibility of the technique.

Ifremer, Laboratoire de Génétique et Pathologie, Av. de Mus de Loup, 17390 La Tremblade, France .
http://www.eurl-mollusc.eu/

OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction (*1st edition, March 2011*)
http://www.eurl-mollusc.eu/content/download/42545/578238/file/OsHV-1%20RTPCR_1.pdf

Technical appendix of to the Standard Operating Procedure (SOP) for OsHV-1 detection and quantification by Real Time Polymerase Chain Reaction targeting the OsHV-1 DNA polymerase sequence

Preamble:
Sybr green Real Time Polymerase Chain Reaction targeting the OsHV-1 DNA polymerase sequence.

Additional specific assays were carried out in order to define the analytical sensitivity, quantitation limit, assay precision, reproducibility of the Sybr green Real Time PCR assay targeting the OsHV-1 DNA polymerase sequence using primers HDPF - HDPRFR (forward, HVDP-F 5'ATTGATGATGTGGATAATCTGTG 3', reverse, HVDP-R 5' GGTAAATACCATTGGTCTTGTTCC 3').

In the context of this work, fluorescence threshold was determined based on the background noise by selecting the **Background-based threshold** option in our setup software.

C**ycle threshold** (CT) values were calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of the baseline of the background signal).

1- Determination of the detection limit, LOD:

Quantitation limit of the Real Time PCR assay was determined according to the following standards: experimental standard **XPT 90-471** for *Legionnella* DNA PCR detection and/or **XP U47-600-1** «Requirements and recommendations for the implementation of PCR in animal health».

The **detection limit**, **LOD** or **Analytical sensitivity,** was defined as the lowest number of GU, genomic unit, generating a positive result (amplification); in 9 out of 10 cases related to XPT 90-471, or in 8 out of 8 related to XP U47-600-1.

In order to determine the LOD, 2 independent assays were performed as follows: serial dilutions of OsHV-1 genomic DNA from 1000 to 1 **DNA copies/**µL were tested in 10 replicates.
Results are presented in Table 1: 9 positive amplification reactions were obtained from 10 replicates for the dilution corresponding to 10 DNA copies/µL while less than 50% positive detection was observed for dilution corresponding to 5 DNA copies/µL. In our conditions, the **limit of detection** of the method was thus estimated to be **10 DNA copies/µL**.

Table 1: Results of the Real Time PCR tests of serial dilutions of OsHV-1 genomic DNA

	Serial dilution of OsHV-1 genomic viral DNA (concentration = copies/µL)					
	1000	**100**	**10**	**5**	**2,5**	**1,0**
replicate #	Ct value	Ct value	Ct value	Ct value	Ct value	Ct value
1	25,89	29,39	No Ct	35,07	32,91	No Ct
2	25,97	29,89	33,48	33,87	No Ct	No Ct
3	26,12	30,84	35,21	No Ct	No Ct	No Ct
4	23,74	27,14	30,68	34,67	No Ct	35,11
5	23,82	27,65	30,96	No Ct	No Ct	32,60
6	24,19	26,91	30,42	No Ct	33,74	No Ct
7	24,93	29,19	33,78	No Ct	32,76	No Ct
8	24,92	28,25	35,08	30,94	30,85	No Ct
9	25,14	28,76	33,33	No Ct	32,08	No Ct
10	24,59	27,88	32,56	/	/	No Ct
Mean Ct value	24,93	28,59	**32,83**	33,64	32,47	/
SD (+/- Ct)	0,87	1,26	**1,81**	1,87	1,08	/
[a]CI (+/- Ct)	0,54	0,78	**1,12**	1,16	0,67	/
[b]CV (%)	3,48	4,39	**5,52**	5,55	3,33	/
max. Ct value (mean + CI)	25,47	29,37	**33,96**	34,79	33,14	/
total number of reactions	**10**	**10**	**10**	**9**	**9**	**10**
number of Positive reactions	**10**	**10**	**9**	**4**	**5**	**2**
proportion of Positive results	**1,00**	**1,00**	**0,90**	**0,44**	**0,56**	**0,20**

[a]CI: Confidence Interval, calculated from Ct values to determine confidence limits using: alpha-risk is 5%, standard deviation, n =10.

[b]CV: Coefficient of Variation, calculated from Ct values, dimensionless measure of variability (relative standard deviation) defined as standard deviation divided by the arithmetic mean, reported as a percentage.

2- Determination of the quantitation limit, LOQ:

The quantitation limit, LOQ, was defined as the lowest number of GU, genomic unit that can be reliably quantified with an acceptable level of precision and accuracy.
PCR efficiency (E) was calculated from standard curves as the percentage of template molecules that was doubled during each cycle ([10(−1/slope) −1]×100), with requirements that it fell into the range 95–105% and that the coefficient of determination (R^2) was >0,98.

To determine the analytical sensitivity of the assay, serial dilutions of viral genomic DNA were tested in the context of six different experiments including triplicates for each dilution (data not shown). A linear relationship was observed between the input copy number of viral DNA template (X) and the associated Ct values (Y) (Fig. 1; Y = -3,333*LOG(X) + 34,01; E = 99,5%; R^2 = 0,995). Accurate quantification of 10 DNA copies/µL was obtained associated with a mean CT value ~ 32,0 +/- CI 5% = 0,97 and an acceptable coefficient of variation CV = 4,8%.

The dynamic range for the real-time PCR was estimated from several standard curve assays. A linear relationship was obtained between input copy number of the viral DNA template and CT value for over 5 log10 dilutions. It was possible to quantify OsHV-1 DNA copy numbers at least from 10 to 1×10^5 copies/µL (Fig. 1).
Quantitation limit of the technique in our conditions is thus **10 DNA copies/µL**

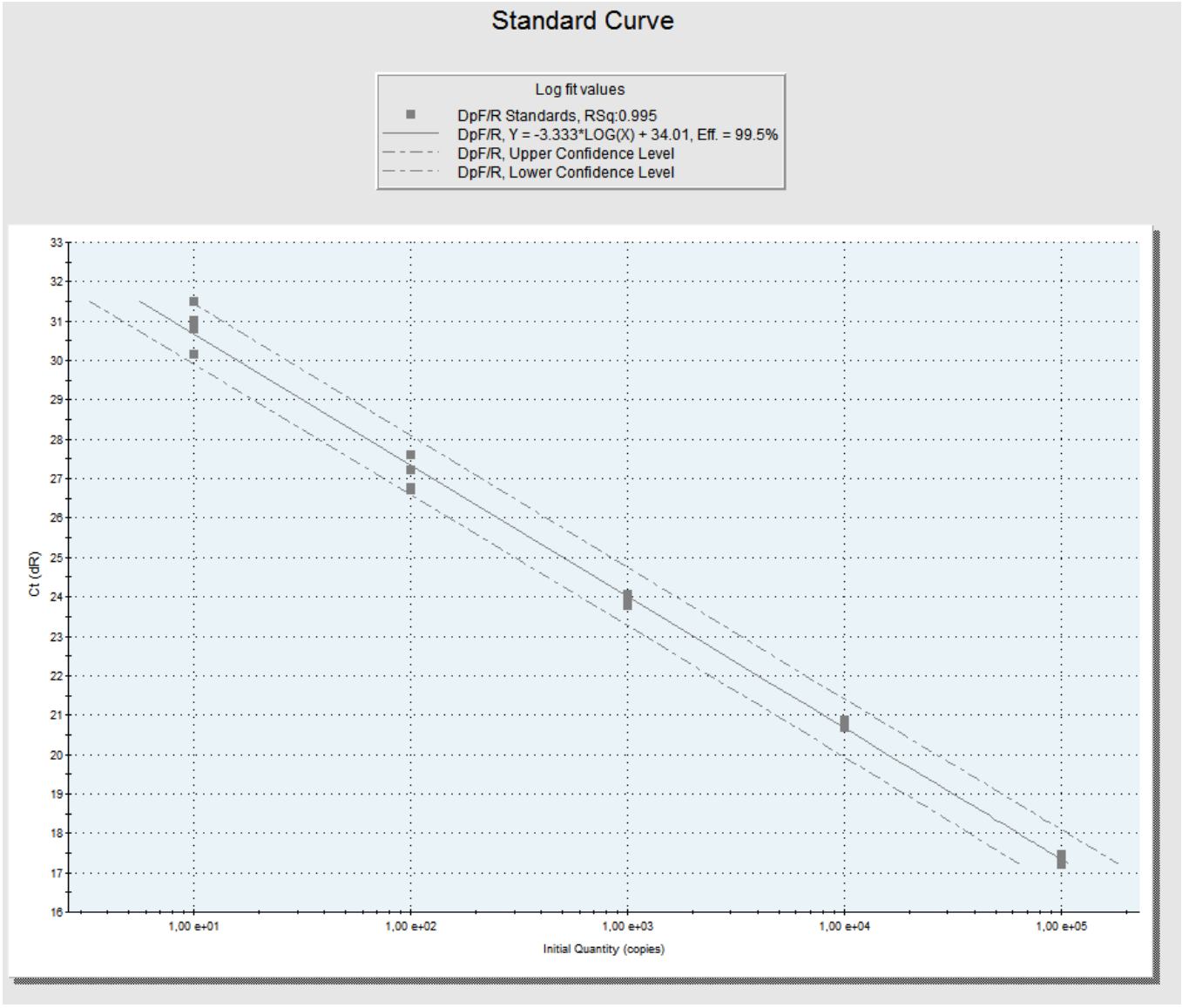

Figure 1: Linearity and sensitivity of SYBR® Green real-time PCR using HDPF -HDPR primer pair and dilutions of viral genomic DNA. A linear regression plot of the SYBR® Green assay standard curve is shown (*X*, log10 copy number; *Y*, CT value) for the standard curve.

3- Specificity of the assay

In order to determine the ability of the assay to amplify only OsHV-1 DNA, the Tm value of amplified products from the melt curve was systematically checked. In our conditions (device, settings, reagents), standard and positive samples gave a specific peak at Tm= 77.2 ±0.4 °C. Samples yielding non-specific amplicons were considered to be negative. The NTCs (no template control) had Ct values over 37 cycles or 'No Ct' and no specific peak was observed. The size of real-time PCR amplicons was checked by agarose gel electrophoresis, and bands were observed at the expected size (197 bp).

In addition, OsHV1 infected samples from various locations and different hosts were tested in duplicate to compare the specificity of the present Real Time PCR assay with previously published data obtained using other primer sets (C9/C10, Barbosa-Solomieu *et al***,** 2004 **;** B3/B4, Arzul *et al*, 2001; Gp7/Gp4, Pepin *et al*. 2008; 206/356A, Burge *et al*. 2008; HDPF/HDPR, Webb *et al*, 2007).

All tested samples showed concordant results whatever the primer set was (Table2).

Table 2 : Comparison of results obtained for infected samples of different origin and host using different primer sets (1st column)

	Sample name	Japan I3	Japan Jp 177	Japan Jp 215	China E50 40,000cp	China QD 18cp	V+2 raw, not infect. oyster	France Z-AC 77 125,000cp	California CHH 1500 cp	California 07-CB 63,000cp	OSHV-1 32 cp	OSHV-1 20,000cp
	Samples courtesy of:	K. Reece	K. Reece	K. Reece	K. Reece	K. Reece	J.F. Pepin	C. Sauvage	C. Burge	C. Burge	T. Renault	T. Renault
	Origin	Japan	Japan, Ariaki Bay	Japan, Ariaki Bay	China	China	France	France	USA California	USA California	France	France
	Date of sampling	2003	2003	2003	2002	?	2007	2006	2006	2007	1995	1995
Primer set	Host species	*C. ariakensis*	*C. sikamea*	*C. sikamea*	*C. hongkongensis*	*C. hongkongensis*	*C. gigas*	*C. gigas*	*C. gigas*	*C. gigas*	*C. gigas*	*C. gigas*
206 /356 A	Ct (dR)	No Ct	No Ct	No Ct	21,66	34,31	32,56	23,37	nd	nd	32,22	22,34
	Tm Prod	60,47	60,47	60,47	73,38	73,38	69,12	73,38	nd	nd	73,38	73,38
	Qualification	NN	NN	NN	PP	PP	NN	PP	nd	nd	PP	PP
C9/ C10	Ct (dR)	No Ct	No Ct	35,92	17,76	28,8	32,47	19,49	22,38	17,06	28,3	18,47
	Tm Prod	65,78	61,43	75,72	75,72	75,72	72,95	75,72	74,85	75,25	75,25	75,25
	Qualification	NN	NN	NP	PP	PP	NN	PP	PP	PP	PP	PP
B3 /B4	Ct (dR)	35,17	No Ct	No Ct	18,65	30,17	27,99	19,04	19,49	17,74	29,05	19,55
	Tm Prod	79,97	60,53	74,35	79,52	79,52	75,3	79,52	79,08	79,52	79,52	79,52
	Qualification	PN	NN	NN	PP	PP	NN	PP	PP	PP	PP	PP
HDPF/HDPR	Ct (dR)	No Ct	No Ct	No Ct	19,1	29,99	27,97	20,97	19,31	17,97	29,56	20,21
	Tm Prod	61,55	62,48	61,55	**78,15**	**77,68**	69,22	**77,68**	**77,23**	**77,7**	**77,7**	**77,23**
	Qualification	NN	NN	NN	**PP**	**PP**	NN	**PP**	**PP**	**PP**	**PP**	**PP**
Gp7 /Gp4	Ct (dR)	No Ct	36,47	No Ct	19,98	31,26	33,29	21,24	21,6	18,88	30,64	21,55
	Tm Prod	60,75	75,53	60,75	75,53	75,53	75,03	75,53	74,5	75,03	75,05	75,05
	Qualification	NN	PN	NN	PP	PP	NN	PP	PP	PP	PP	PP

N: reaction/well with Negative Tm
P: reaction/well with Positive Tm
Tm : melting temperature from dissociation curve
No Ct : no available fluorescence signal
nd : not done

4- Assay precision

Intra-assay variability or **repeatability**, was estimated by testing five dilutions of viral DNA in five replicates simultaneously in the same plate (Table 3). The mean intra assay coefficient of variation calculated from the 5 replicate Ct values was 1,7% ± 0,8, which is acceptable and corresponds to high accuracy (<2,5%) (Reed al., 2002).

Inter-assay variability was estimated by testing five dilutions of viral DNA in 3 replicates in the context of four experiments carried out on different days (Table 4). The mean inter assay coefficient of variation calculated from the 4 experiments was 4,0 ± 0,7%, which is acceptable (<5%).

Table 3 :
Intra-assay variability calculated from 5 replicates of a same OsHV-1 viral DNA dilution on the same plate. SD : Standard deviation. CV: Coefficient of Variation

DNA	CT values								Intra-assay variability
copies/µL	1	2	3	4	5	Mean (CT)	SD	CV %	Mean±S.D. of CV%
1,0E+05	17,86	18,18	17,83	17,59	17,87	17,87	0,21	1,2	
1,0E+04	21,3	21,02	21,22	20,78	20,61	20,99	0,29	1,4	
1,0E+03	24,56	24,93	24,8	24,28	23,97	24,51	0,39	1,6	1,70 +/- 0,8
1,0E+02	28,34	28,32	28,38	27,55	27,96	28,11	0,36	1,3	
1,0E+01	31,92	33,9	31,41	31,62	31,84	32,14	1,00	3,1	

Table 4 :
Inter-assay variability calculated from four independent OsHV-1 viral DNA dilution series

DNA copies/µL	Test A	Test B	Test C	Test D	Mean CT	SD	CV%	Intra-assay variability
	Mean CT	Mean CT	Mean CT	Mean CT				Mean±S.D. (CV%)
1,0E+05	17,68	18,12	19,02	17,29	18,03	0,74	4,1	
1,0E+04	20,67	21,14	22,28	20,74	21,21	0,75	3,5	4,06+/- 0,75
1,0E+03	23,92	25,00	25,47	23,89	24,57	0,79	3,2	
1,0E+02	27,23	28,73	29,52	27,03	28,13	1,20	4,3	
1,0E+01	30,69	34,06	32,98	30,81	32,13	1,66	5,2	

5- Reproducibility: Real Time Polymerase Chain Reaction targeting the OsHV-1 DNA polymerase sequence

In 2011, the National reference laboratory for mollusc diseases in France (LGPMM, Ifremer La Tremblade) organised an interlaboratory comparison (ILC) to test the competency of fourteen French laboratories to detect OsHV-1 by The Real Time PCR assay targeting the OsHV-1 DNA polymerase sequence (Garcia et al., 2012). . The test included thirty oyster tissue samples displaying various controlled levels of viral infections: non-infected, weakly and strong infected samples. Participants had to test samples in duplicate and had to report the status of each sample regarding the presence of viral DNA detection: detected, not detected or indeterminate (qualitative results).

Table 6 presents percentages of 'good results' obtained by participants. Specificity and sensitivity were estimated globally and for weak or strong level of infections.

The fourteen laboratories had a 100% median of good results demonstrating the strong reproducibility of the method.

Table 6: Percentages of 'good results' for the ILC organised in 2011 by the French NRL for Mollusc diseases.

Mean	99,8%
Minimum	96,7%
First quartile	100%
Median	100%
Third quartile	100%
Maximum	100%
Specificity	100%
Sensitivity at strong level of infection	100%
Sensitivity at weak level of infection	98,8%

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