



Contents lists available at [SciVerse ScienceDirect](http://SciVerse.ScienceDirect)

## Journal of Virological Methods

journal homepage: [www.elsevier.com/locate/jviromet](http://www.elsevier.com/locate/jviromet)



Short communication

# Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions

Yun-Long Tsai<sup>a</sup>, Yu-Chan Lin<sup>a</sup>, Pin-Hsing Chou<sup>a</sup>, Ping-Hua Teng<sup>a</sup>, Pei-Yu Lee<sup>b,\*</sup>

<sup>a</sup> Department of Research and Development, GeneReach Biotechnology Corporation, Taichung, Taiwan

<sup>b</sup> Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Taichung, Taiwan

### A B S T R A C T

#### Article history:

Received 6 October 2011  
Received in revised form 16 January 2012  
Accepted 18 January 2012  
Available online xxx

#### Keywords:

Convective PCR  
Nucleic acid amplification  
Insulated isothermal conditions  
iiPCR  
WSSV

Aiming to develop a rapid, low-cost, and user-friendly system for the diagnosis of white spot syndrome virus (WSSV), a PCR assay performed in capillary tubes under insulated isothermal conditions (iiPCR assay) was established on the basis of Rayleigh–Benard convection. WSSV amplicons were generated reproducibly within 30 min from a target sequence-containing plasmid in an iiPCR device, in which a special polycarbonate capillary tube (R-tube™) was heated isothermally by a copper ring attached to its bottom and shielded by a thermal baffle around its upper half. Furthermore, WSSV-specific amplicons were produced from nucleic acid extracts of WSSV-infected *Penaeus vannamei* in the WSSV iiPCR assay, with sensitivity comparable to that of an OIE-certified commercial nested PCR kit (IQ2000™ WSSV Detection and Prevention System). Specificity of the WSSV iiPCR assay was demonstrated as no amplicons were generated from shrimp genomic DNA, and IHNV, MBV, and HPV DNA. iiPCR has a potential as a low-cost method for sensitive, specific and rapid detection of pathogens.

© 2012 Elsevier B.V. All rights reserved.

White spot syndrome virus (WSSV), a large double-stranded DNA virus, infects a broad range of freshwater and marine crustaceans including shrimp, crabs, crayfish and lobsters (Hameed et al., 2003; Lo et al., 1996a; Syed Musthaq et al., 2006). WSSV infection causes high mortality rates and severe economic losses in the shrimp aquaculture industry worldwide. Pre-screening of WSSV-free broodstock or larvae and regular surveillance of WSSV infection are important strategies to reduce the economic impacts of the disease on shrimp aquaculture. Some nucleic acid-based methods, such as conventional polymerase chain reaction (PCR) (Kasornchandra et al., 1998; Lo et al., 1996b; Vaseeharan et al., 2003), in situ PCR (Jian et al., 2005), real-time PCR (Durand and Lightner, 2002), nested PCR (Lo et al., 1996b; Nunan and Lightner, 2011), and loop-mediated isothermal amplification (LAMP) (Chou et al., 2011a; Kono et al., 2004) have been developed for WSSV detection.

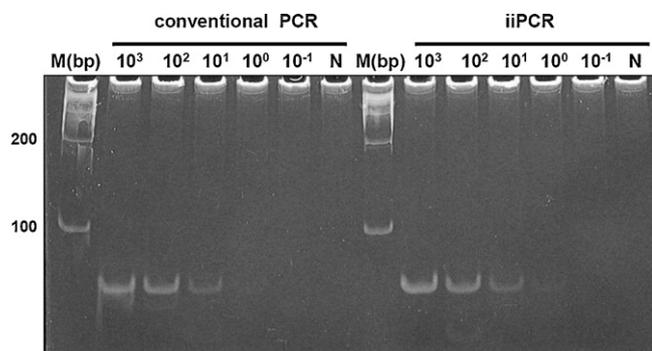
Although PCR offers high degrees of detection sensitivity and specificity, the requirements, such as trained technicians and an expensive thermocycler, have limited the application of PCR-based assays in the aquaculture industry. In Rayleigh–Benard convection, PCR cycles can be completed without the need of a thermocycler. Simple heating sources are able to generate fluid density

gradients to drive laminar convection of the solution in a closed space, carrying the reaction components through sequential temperature zones to facilitate the completion of PCR cycles (Chandrasekhar, 1961; Krishnan et al., 2002). Convection PCR could shorten reaction time from hours to about 30 min in comparison to conventional PCR, providing a method for the development of a fast, low-cost, and user-friendly system for nucleic acid amplification. On the basis of Rayleigh–Benard convection, a recent report demonstrated that amplicons could be generated successfully in glass capillary tubes that were heated at one end (bottom) by a simple heating device (Chou et al., 2011b). However, convection flows in the reaction cell could be affected easily by temperature changes in the surrounding environment (Chou et al., 2011b), leading to erratic termination of the PCR cycles.

In this report, successful convection PCR was carried out in specially designed capillary tubes in an insulated isothermal device (iiPCR) (Chang et al., 2012). A thermal baffle made of aluminum was installed to shield the upper half of reaction tubes and to reduce impacts of environmental temperature fluctuations on the convection in reaction tubes. At the same time, the heating block was covered with insulated material to help reduce the influence of heat radiation on air convection of the surrounding space (Supplementary Fig. 1). As a substitute, heating forks connected to the heating block were positioned to make contact with a 2.5 mm thick copper ring attached at 2 mm above the bottom of each capillary tube (Supplementary Fig. 1). Polycarbonate (PC) capillary tubes (R-tube™, GeneReach) were utilized to substitute for glass tubes or Plexiglas tubes used previously (Chou et al., 2011b; Krishnan et al.,

\* Corresponding author at: Institute of Medical Biotechnology, Central Taiwan University of Science and Technology, Rm1311, No. 11, Buzih Lane, Taichung 406, Taiwan. Tel.: +886 4 2239 1647x6936; fax: +886 4 2239 5474.

E-mail address: [pylee@ctust.edu.tw](mailto:pylee@ctust.edu.tw) (P.-Y. Lee).

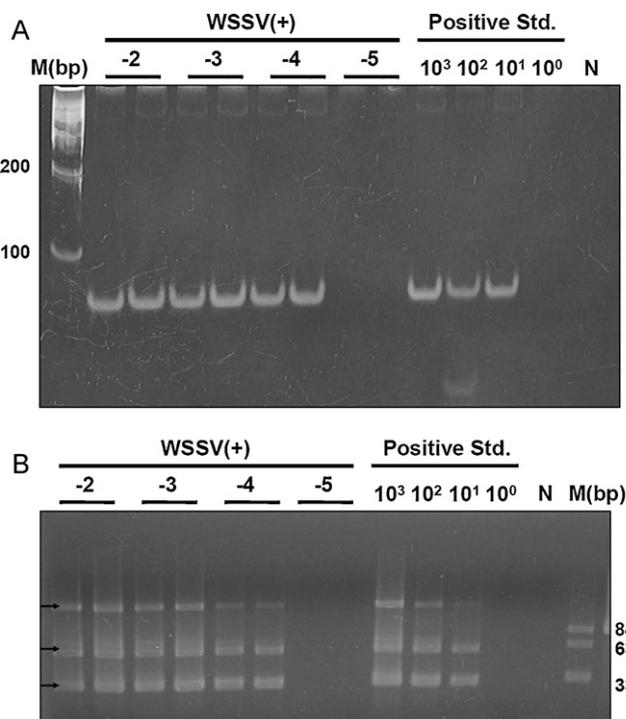


**Fig. 1.** Amplification of pWSSV1 by iiPCR and conventional PCR. Different copies ( $10^3$  to  $10^{-1}$ ) of the pWSSV plasmid were subjected to WSSV iiPCR or conventional PCR assay. Both systems employed the same set of primers. The amplicons were analyzed on a 12% polyacrylamide gel in  $1\times$  TAE buffer. M, DNA size markers; N, non-template control.

2002). Considerable heat conductivity of the copper rings ensured consistent heating at the bottom of the R-tubes. Stable temperature gradients ranged from  $94^\circ\text{C}$  (bottom) to  $50^\circ\text{C}$  (top) could be generated and maintained in the R-tubes in this simple setup when it is placed at room temperature (Chang et al., 2012).

With the aim of developing a rapid WSSV diagnosis system, a WSSV iiPCR was established using this device. First, plasmid pWSSV1 (Chou et al., 2011a) that contains a 1254 bp DNA fragment of the WSSV genome was used as the template to establish the optimal iiPCR conditions. The forward (5'-AATGGTCCCGTCCTCATCTCA-3') and reverse (5'-GCTGCCTTGCCGAAATT-3') primers specific for WSSV were designed by following the criteria described previously for connective PCR (Chou et al., 2011b). The  $50\ \mu\text{l}$  iiPCR mixtures, which contained  $10^3$  copies of pWSSV1,  $0.5\ \mu\text{M}$  forward primer,  $0.5\ \mu\text{M}$  reverse primer,  $0.5\ \text{mM}$  dNTP, 25 units of Taq DNA polymerase (BioMi, Taichung, Taiwan),  $50\ \text{mM}$  Tris-HCl (pH 8.3),  $75\ \text{mM}$  KCl,  $3\ \text{mM}$   $\text{MgCl}_2$ , and  $1\ \text{mM}$  DTT, were assembled in R-tubes and incubated in the device described above for 30 min. Amplicons were analyzed subsequently on a 12% polyacrylamide gel in TAE buffer ( $40\ \text{mM}$  Tris,  $20\ \text{mM}$  acetic acid and  $1\ \text{mM}$  EDTA) and visualized by ethidium bromide staining. For comparison, the same mixtures were subjected to conventional PCR amplification performed in a PCR thermocycler (ABI 2720, Life Technologies, Carlsbad, CA). The program included an incubation period at  $94^\circ\text{C}$  for 2 min, 35 cycles of  $94^\circ\text{C}$  for 20 s,  $50^\circ\text{C}$  for 20 s and  $72^\circ\text{C}$  for 30 s, and one cycle of  $72^\circ\text{C}$  for 30 s and  $20^\circ\text{C}$  for 20 s. The results demonstrate that a 71 bp WSSV-specific product, which was confirmed by DNA sequencing analysis (data not show), was amplified in both iiPCR and conventional PCR. The sensitivity of WSSV iiPCR, at  $10^1$  copies per reaction, was similar to that of the conventional PCR (Fig. 1), suggesting that iiPCR can reach amplification efficiency comparable to that of conventional PCR. Detectable amounts of amplicons were generated consistently from positive samples in 30 min, which was comparable to the 10–30 min range reported in the various convection PCR systems reported so far (Braun, 2004; Chung et al., 2010; Hennig and Braun, 2005; Muddu et al., 2011; Wheeler et al., 2004).

PCR amplification may be interfered by inhibitors present in tissue extracts (Wilson, 1997). In addition, host nucleic acids could also compete with the primers. To test whether iiPCR can amplify WSSV DNA in shrimp samples efficiently, crude nucleic acid extracts of WSSV-infected *P. vannamei* samples were prepared using the Lysis buffer method (BioMi, Taichung, Taiwan) as described previously (Chou et al., 2011a). Ten-fold serial dilutions of the extracts were subjected simultaneously to the WSSV iiPCR assay and the nested PCR-based IQ2000<sup>TM</sup> WSSV Detection and Prevention System (DPS), which was assembled and carried out as

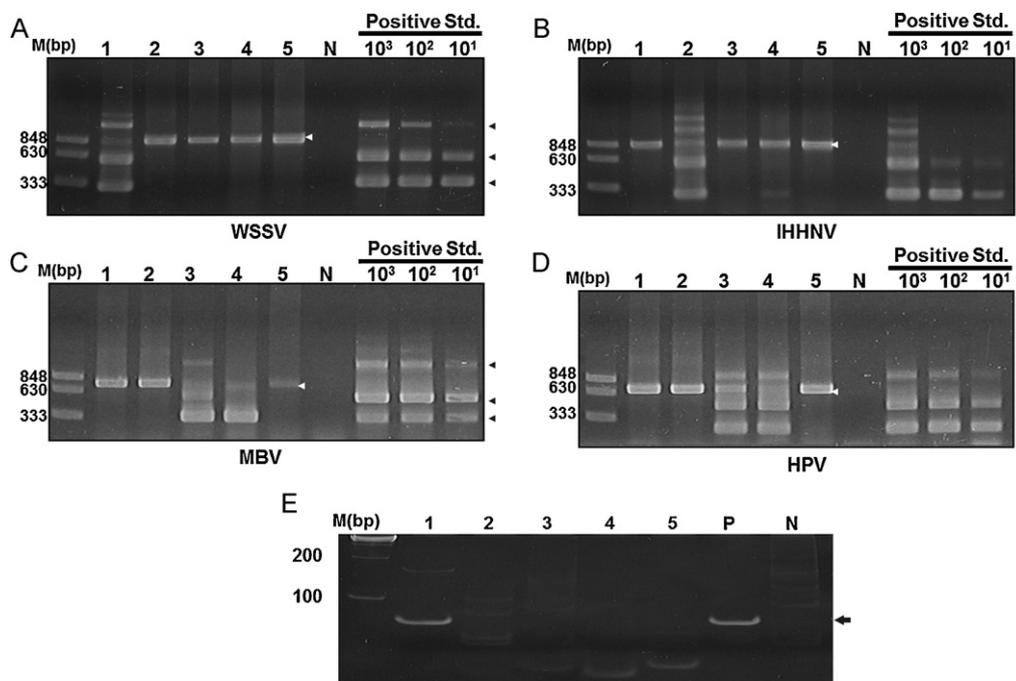


**Fig. 2.** Evaluation of WSSV detection in shrimp samples by WSSV iiPCR. Serial 10-fold dilutions ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$ ) of genomic DNAs extracted from a WSSV-infected shrimp were analyzed by WSSV iiPCR assay (A) or IQ2000<sup>TM</sup> WSSV DPS (B). The IQ2000<sup>TM</sup> WSSV DPS included also serial dilutions ( $10^3$ ,  $10^2$ ,  $10^1$ ,  $10^0$  copies) of a WSSV standard DNA. The kit was designed to generate three amplicons (arrows) from WSSV genome, and the number of the product bands correlates positively with the starting concentrations of target DNA. Each sample was tested in duplicate. Amplicons from iiPCR and IQ2000<sup>TM</sup> WSSV DPS were analyzed on a 12% polyacrylamide gel in  $1\times$  TAE buffer and agarose gel electrophoresis, respectively. M, DNA size markers; N, non-template control.

described by the manufacturer. The endpoints for the detection of WSSV ( $1:10^{-4}$  dilution) were comparable between the two assays (Fig. 2A and B), suggesting that the primers reacted specifically with WSSV DNA with a sensitivity similar to that of IQ2000<sup>TM</sup> WSSV DPS and extracts of shrimp tissues prepared this way were compatible with the iiPCR reaction.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), and hepatopancreatic parvovirus (HPV) are other common DNA virus pathogens found in shrimp (Lightner, 2011). To ensure that the WSSV iiPCR does not cross react with genomes of these viruses, WSSV-, IHHNV, MBV, or HPV-positive *P. vannamei* identified by IQ2000<sup>TM</sup> WSSV DPS (Fig. 3A, lane 1), IQ2000<sup>TM</sup> IHHNV DPS (Fig. 3B, lane 2), IQ2000<sup>TM</sup> MBV DPS (Fig. 3C, lanes 3 and 4), or IQ2000<sup>TM</sup> HPV DPS (Fig. 3D, lanes 3 and 4), respectively, were analyzed by the WSSV iiPCR assay (Fig. 3E). Results of the iiPCR assay demonstrate that amplicons were generated only from WSSV- (Fig. 3E, lane 1), but not from IHHNV-, MBV-, and HPV-positive samples (Fig. 3E, lanes 2–4), indicating the established iiPCR assay could detect WSSV specifically.

Although the established WSSV iiPCR assay demonstrated sensitivity similar to that of a conventional WSSV PCR, several primer sets that were able to generate amplicons in conventional PCR could not produce amplicons efficiently in iiPCR (data not show). Circulation of the reaction in R-tubes allowed relatively short periods of time (only a few seconds) for each iiPCR cycle (Chou et al., 2011b; Krishnan et al., 2004), implying that the optimal parameters (such as primer criteria and buffer compositions) for iiPCR may be different from those for conventional PCR and need to be further investigated. In order to include an internal control



**Fig. 3.** Specificity evaluation of WSSV iiPCR assay. Genomic DNA extracts of WSSV- (lane 1), IHNV- (lane 2), MBV- (lane 3), and HPV-infected (lane 4), and uninfected (lane 5) shrimp were subjected to amplification by (A) IQ2000™ WSSV DPS, (B) IQ2000™ IHNV DPS, (C) IQ2000™ MBV DPS, (D) IQ2000™ HPV DPS and (E) WSSV iiPCR. Positive standard DNAs provided by the IQ2000™ kits were diluted ( $10^3$ ,  $10^2$ ,  $10^1$  copies) and included in the assays. The IQ2000™ kits were designed to generate three amplicons (closed arrow heads) from the target sequences ((A) lane 1; (B) lane 2; (C) lanes 3 and 4; (D) lanes 3 and 4). Samples 3 and 4 appeared to be co-infected by both MBV and HPV. The number of the product bands correlates positively with the starting concentrations of target DNA. In addition, the presence of the internal-control signal (open arrow heads) and the absence of target signals implicate that the samples are target-pathogen free (A–D, lane 5). Arrows, WSSV iiPCR amplicons; M, DNA size markers; N,  $10^3$  copies of a WSSV standard DNA; P, water only.

to ensure that the DNA extraction and the PCR procedures have been implemented properly, development of multiplex iiPCR is in progress. Furthermore, iiPCR amplicons were analyzed by gel electrophoresis, which is labor-intensive, time-consuming and prone to cross-contamination. Different ways of amplicon detection will have to be integrated into the iiPCR device to make the method more user-friendly.

In order to generate a circulatory flow that can transport the reaction components through the critical temperature zones involved in the PCR process, heating mechanisms and geometry of the reaction vessel are major topics in the development of convection PCR. Successful convection PCR has been demonstrated in reactors of cylindrical or looped geometries (Agrawal et al., 2007; Agrawal and Ugaz, 2007; Chen et al., 2004; Chou et al., 2011b; Krishnan et al., 2004; Wheeler et al., 2004). Reactors of looped geometries, which work in closed design, would require manual elimination of air pockets (Chung et al., 2010). Alternatively, successful convective PCR was reported by dipping a constantly heated wire directly into an open vessel (Hennig and Braun, 2005). However, repeated use of the wire might lead to higher risks of cross-contamination from sample to sample. These problems could be largely avoided in the iiPCR method, because all components of the open-designed R-tube™ are inexpensive and disposable. Furthermore, less precaution is required to handle polycarbonate tubes than the glass capillary tubes, which were used by the capillary convection PCR method (Chou et al., 2011b).

The WSSV iiPCR assay could amplify its target DNA specifically, sensitively and reproducibly if carried out in the specially designed R-tube™ in the small iiPCR device designed particularly to stabilize convection PCR. The iiPCR system has a considerable potential to be developed into a fast, user-friendly and portable method for pathogen detection in the aquaculture industry.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jviromet.2012.01.017](https://doi.org/10.1016/j.jviromet.2012.01.017).

## References

- Agrawal, N., Ugaz, V.M., 2007. A buoyancy-driven compact thermocycler for rapid PCR. *Clin. Lab. Med.* 27, 215–223.
- Agrawal, N., Hassan, Y.A., Ugaz, V.M., 2007. A pocket-sized convective PCR thermocycler. *Angew. Chem.* 46, 4316–4319.
- Braun, D., 2004. PCR by thermal convection. *Mod. Phys. Lett.* 18, 775–784.
- Chandrasekhar, S., 1961. *Hydrodynamic and Hydromagnetic Stability*. The Clarendon Press, Oxford, England.
- Chang, H.F.G., Tsai, Y.L., Tsai, C.F., Lin, C.K., Lee, P.Y., Teng, P.H., Su, C., Jeng, C.C., 2012. A thermally baffled device for highly stabilized convective PCR. *Biotechnol. J.* doi:10.1002/biot.201100453.
- Chen, Z., Qian, S., Abrams, W.R., Malamud, D., Bau, H.H., 2004. Thermosiphon-based PCR reactor: experiment and modeling. *Anal. Chem.* 76, 3707–3715.
- Chou, P.H., Lin, Y.C., Teng, P.H., Chen, C.L., Lee, P.Y., 2011a. Real-time target-specific detection of loop-mediated isothermal amplification for white spot syndrome virus using fluorescence energy transfer-based probes. *J. Virol. Methods* 173, 67–74.
- Chou, W.P., Chen, P.H., Miao Jr., M., Kuo, L.S., Yeh, S.H., Chen, P.J., 2011b. Rapid DNA amplification in a capillary tube by natural convection with a single isothermal heater. *Biotechniques* 50, 52–57.
- Chung, K.H., Park, S.H., Choi, Y.H., 2010. A palmtop PCR system with a disposable polymer chip operated by the thermosiphon effect. *Lab Chip* 10, 202–210.
- Durand, S.V., Lightner, D.V., 2002. Quantitative real-time PCR for the measurement of white spot syndrome virus in shrimp. *J. Fish Dis.* 25, 381–389.
- Hameed, A.S., Balasubramanian, G., Musthaq, S.S., Yoganandhan, K., 2003. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). *Dis. Aquat. Organ.* 57, 157–161.
- Hennig, M., Braun, D., 2005. Convective polymerase chain reaction around micro immersion heater. *Appl. Phys. Lett.* 87, 183901–183903.
- Jian, X.F., Lu, L., Chen, Y.G., Chan, S.M., He, J.G., 2005. Comparison of a novel in situ polymerase chain reaction (ISPCR) method to other methods for white spot syndrome virus (WSSV) detection in *Penaeus vannamei*. *Dis. Aquat. Organ.* 67, 171–176.
- Kasornchandra, J., Boonyaratpalin, S., Itami, T., 1998. Detection of white spot syndrome in cultured *Penaeid* shrimp in Asia: microscopic observation and polymerase chain reaction. *Aquaculture* 164, 243–251.

- Kono, T., Savan, R., Sakai, M., Itami, T., 2004. Detection of white spot syndrome virus in shrimp by loop-mediated isothermal amplification. *J. Virol. Methods* 115, 59–65.
- Krishnan, M., Ugaz, V.M., Burns, M.A., 2002. PCR in a Rayleigh-Benard convection cell. *Science* 298, 793.
- Krishnan, M., Agrawal, N., Burns, M.A., Ugaz, V.M., 2004. Reactions and fluidics in miniaturized natural convection systems. *Anal. Chem.* 76, 6254–6265.
- Lightner, D.V., 2011. Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): a review. *J. Invert. Pathol.* 106, 110–130.
- Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996a. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis. Aquat. Organ.* 27, 215–225.
- Lo, C.F., Leu, J.H., Ho, C.H., Chen, C.H., Peng, S.E., Chen, Y.T., Chou, C.M., Yeh, P.Y., Huang, C.J., Chou, H.Y., Wang, C.H., Kou, G.H., 1996b. Detection of baculovirus associated with white spot syndrome (WSBV) in *Penaeid* shrimps using polymerase chain reaction. *Dis. Aquat. Organ.* 25, 133–134.
- Muddu, R., Hassan, Y.A., Ugaz, V.M., 2011. Rapid PCR thermocycling using microscale thermal convection. *J. Vis. Exp.* 49, 1–4.
- Nunan, L.M., Lightner, D.V., 2011. Optimized PCR assay for detection of white spot syndrome virus (WSSV). *J. Virol. Methods* 171, 318–321.
- Syed Musthaq, S., Sudhakaran, R., Balasubramanian, G., Sahul Hameed, A.S., 2006. Experimental transmission and tissue tropism of white spot syndrome virus (WSSV) in two species of lobsters, *Panulirus homarus* and *Panulirus ornatus*. *J. Invert. Pathol.* 93, 75–80.
- Vaseeharan, B., Jayakumar, R., Ramasamy, P., 2003. PCR-based detection of white spot syndrome virus in cultured and captured crustaceans in India. *Lett. Appl. Microbiol.* 37, 443–447.
- Wheeler, E.K., Benett, W., Stratton, P., Richards, J., Chen, A., Christian, A., Ness, K.D., Ortega, J., Li, L.G., Weisgraber, T.H., Goodson, K., Milanovich, F., 2004. Conectively driven polymerase chain reaction thermal cyler. *Anal. Chem.* 76, 4011–4016.
- Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63, 3741–3751.