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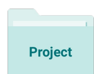

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High-throughput isolation of oyster DNA facilitates diagnosis of "Dermo" disease

The parasite Perkinsus marinus*, the causative agent of "Dermo" disease, has been linked with mass mortalities of the eastern oyster* Crassostrea virginica *along the Atlantic and Gulf Coasts of the United States. Here we describe a method for efficient high-throughput DNA isolation from oysters using the QIAGEN® DNeasy 96 Tissue Kit, and a sensitive, species-specific, PCR-based method for the diagnosis of* P. marinus*.*

José A. F. Robledo, Wolf T. Pecher, and Gerardo R. Vasta

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, MD, USA

"Dermo" disease which has caused heavy mortalities in many oyster populations in the Northeastern United States, is caused by infection of oysters by the parasite *Perkinsus marinus*. Other *Perkinsus* species in Europe and Australasia have also been associated with disease in commercially relevant mollusk species including abalones, scallops, and clams. We have developed a sensitive, species-specific PCR-based assay for the detection of *P. marinus*, which can detect a single *P. marinus* cell spiked in approximately 30 mg of oyster tissue (1, 2). The non-transcribed spacer (NTS) of the rRNA gene cluster was selected as the target DNA sequence that would be unique to this parasite. The species specificity of the assay was confirmed by testing other known *Perkinsus* species. We have subsequently developed PCR assays for the detection of other *Perkinsus* species, including the European species *P. atlanticus* (3). In every case, the initial step in assay development was the selection and optimization of a rapid and reliable method for routine isolation of genomic DNA from bivalve tissues. Tissues were routinely processed using the QIAGEN DNeasy Tissue Kit. However, the increasing number of samples processed in our laboratory for experimental purposes, added to those received from oyster producers for certification of parasite-free oyster seed, required a higher throughput assay platform. This study was designed to test a 96-sample format for the DNA purification from bivalves using *C. virginica/P. marinus* as a model.

Materials and methods

Isolation of DNA

DNA isolation from *Crassostrea virginica* was based on the Mouse Tail Protocol in the *DNeasy 96 Tissue Kit Handbook* with modifications as follows. DNA was purified from various tissues including mantle, gills, and rectum (Figure 1). Tissue samples of 20, 40, and 60 mg were lysed overnight. No RNase A treatment was carried out in this particular experiment. Following the addition of

Figure 1 *The Eastern oyster Crassostrea virginica*

Buffer AL/E, the samples containing the digested tissues were vigorously mixed, and loaded into the wells of a DNeasy 96 plate with the aid of an electronic multichannel pipette. The plate was centrifuged at 6000 rpm for 20 min. The membrane was washed and dried following the provided protocol and DNA was eluted with 100 µl of sterile water. Nucleic acid concentrations were determined by A_{260} measurements, and DNA quality was analyzed by agarose gel electrophoresis. DNA was also isolated from oyster hearts and cultured *P. marinus* using the DNeasy 96 Tissue Kit and the protocol described above. ▶

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PCR

PCR primers (5′-CAC TTG TAT TGT GAA GCA CCC-3′) and (5′-TTG GTG ACA TCT CCA AAT GAC-3′) that amplify a 307 bp target region within the NTS domain located between the 5S and SSU rRNA genes from *P. marinus* were used for detection of the parasite (1, 2). PCR assays contained 1 µg sample DNA in reaction buffer, 1 µM each of the PCR primers, 200 µM each dNTP, and 1.5 units of *Taq* DNA polymerase in a total volume of 25 µl. Positive control reactions contained 50 ng purified *P. marinus* DNA as a template. In negative controls template DNA was substituted by distilled water. PCR products were resolved in a 1.5% agarose gel in TAE buffer.

P. marinus Detected in Oyster Tissues

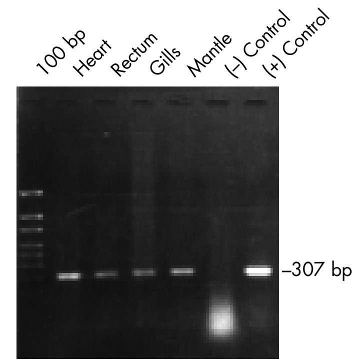

Figure 3 Agarose gel analysis of PCR products obtained using P. marinus specific NTS primers in a PCR assay with 1 µg DNA isolated from the indicated oyster tissue. The detection of a 307 bp fragment is indicative of P. marinus infection.

Nucleic Acid Yields from Oyster Tissues

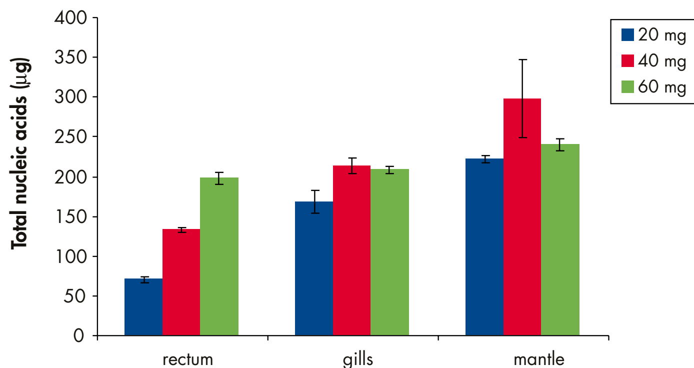

Figure 2 Bar chart showing total nucleic acids isolated from the indicated amounts of oyster tissue. Nucleic acids quantitated by A_{260} measurements.

Results

The use of the QIAGEN DNeasy 96 Tissue Kit and the modified procedure described above delivered high-quality genomic DNA in high yields, from oyster heart, mantle, gills, and rectum. For rectum and gill tissue, an increase of the sample size up to 60 mg resulted in a proportional increase of nucleic acid yield. This trend was less apparent when isolating DNA from mantle tissue, where the system probably became saturated (Figure 2). Although in tissue samples larger than 40 mg DNA yields were increased, a reduction of the A_{260}/A_{280} ratio was observed (data not shown). Variability of yield was higher for DNA purified from larger amounts of tissue. For routine applications, we typically use 30 mg of oyster tissue, although it should be noted that in a study using mouse tissues, optimal results were obtained using tissue samples of 20 mg or less (4). DNA isolated from all oyster tissues was suitable for downstream applications. In infected oysters, *Perkinsus marinus* DNA could be detected in all tissues tested, by PCR amplification using parasite-specific primers (Figure 3).

Discussion

Most lifeforms inhabiting the oceans can be affected by disease. Recently, reports of mass mortalities due to disease outbreaks affecting marine organisms have been increasing (5). Both climate changes and human activities may have accelerated global transport of species, bringing together pathogens and previously unexposed host populations. Hence, in order to effectively monitor the health of the oceans, it is of paramount importance to develop novel diagnostic technologies, or to adapt methods developed for other systems, that can be easily implemented in the marine environment. The 96-well format method for the purification of DNA, in conjunction with the PCR reaction, currently enables us to obtain diagnostic results on 190 oyster samples within a single working day. Although the dissection of the bivalves is the lengthiest step in the diagnostic process, the standardized DNA extraction methodology in a 96-well format, has enabled us to significantly increase sample throughput. In addition

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to oysters, we have now applied the DNeasy Tissue Kit to DNA isolation from a variety of marine organisms, with minimal modifications (disruption of the cells with glass beads and increasing the amount of proteinase K and/or incubation time) to the supplied protocol. These organisms include clams, mussels, and most recently, marine phytoplankton (for detection of the toxic dinoflagellate *Pfiesteria piscicida*). Furthermore, because "Dermo" disease has been reported in oyster beds since the early 1950's (6), the testing of archival samples is critical for the understanding of how outbreaks have spread. We have recently obtained promising results using the DNeasy Tissue Kit for purification of DNA from fixed and paraffin-embedded oyster tissue samples, and plans for the near future include a thorough study of archival sample collections at several institutions.

Acknowledgements

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Table 1. List of host organisms and target species from which genomic DNA is routinely isolated in our lab using the DNeasy Tissue system.

Host organism	Target species
Marine phytoplankton	*Pfiesteria piscicida*
Eastern oyster (*Crassostrea viriginica*)	*Perkinsus marinus*
European flat oyster (*Ostrea edulis*)	*Bonamia ostreae*
Australian blood cockle (*Andara trapezia*)	*Perkinsus sp.*
Baltic clam (*Macoma balthica*)	*Perkinsus sp.*
Carpet shell clam (*Ruditapes decussatus*)	*Perkinsus atlanticus*
Hardshell clam (*Mercenaria merceneria*)	*Perkinsus sp.*

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Product	Contents	Cat. No.	Price (US$)
DNeasy 96 Tissue Kit (4)*†	For 4 x 96 DNA preps: 4 DNeasy 96 plates, Proteinase K, Buffers, Square-Well blocks, Tape Pads, Collection Microtubes (1.2 ml), Caps	69581	785.00
DNeasy Tissue Kit (50)*	50 DNeasy Spin Columns, Proteinase K, Buffers, Square-Well blocks, Tape Pads, Collection Tubes (2 ml)	69504	92.00
Centrifuge 4-15C			
(120-V)	Universal laboratory centrifuge with brushless motor (120 V, 60 Hz)	81010	7,995.00
Plate rotor 2 x 96	Rotor for 2 QIAGEN 96-well plates for use with QIAGEN Centrifuges‡	81031	1,759.00

* *Other kit sizes available; please inquire.*
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‡ *The Plate Rotor 2 x 96 is available exclusively from QIAGEN and its distributors. Under the current liability and warranty conditions, the rotor may only be used in Centrifuges 4-15C, and 4K15C from QIAGEN, and freely programmable models of centrifuges 4-15, 4K15, 6-10, 6K10, 6-15, and 6K15 from Sigma Laborzentrifugen GmbH.*

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