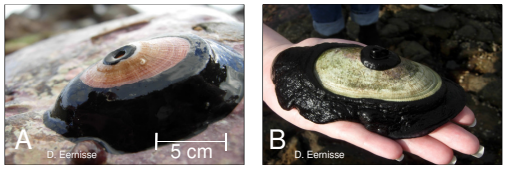
When is a keyhole limpet a giant?: A rapid and inexpensive assay to verify *Megathura crenulata* hemocyanin

Eernisse, Douglas J.[1], Smith, James M.[1], McCune, Kenneth S.[1], and Lincicum, R. Brandon[2]

[1] Department of Biological Science, California State University Fullerton & [2] Stellar Biotechnologies, Inc., Port Hueneme, CA

Introduction

- Molluscan hemocyanins are proteins that transport oxygen throughout the bodies of gastropods such as the giant keyhole limpet.[1]
- Keyhole limpet hemocyanin (KLH) is used extensively as a carrier protein for antibody production in biotech research and therapeutic applications.[2]
- Recent preclinical data demonstrates that source and form of KLH impacts immunogenicity. [3]
- KLH is a blood product of one species: *Megathura crenulata*, giant keyhole limpet, common in the shallow subtidal of southern California.
- Giant keyhole limpets are sustainably cultivated by aquaculture and their hemolymph (blood) is extracted non-lethally at periodic intervals.
- Our PCR-based assay can verify whether the source of hemolymph product is from a giant keyhole limpet or from some other gastropod.



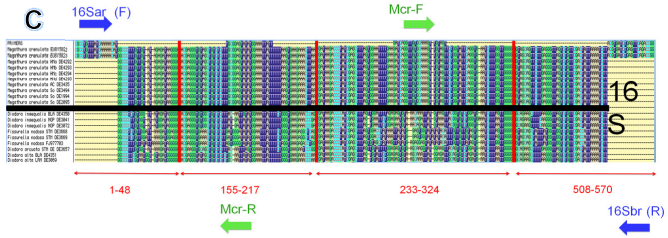


Figure 1. A.-B. Giant keyhole limpets, from southern California to northern Baja California.[4] C. Portions of a multiple sequence alignment across several Fissurellidae 16S sequences including 10 from *M. crenulata*. Blue arrows represent the universal primer binding sites (16Sar, 16Sbr). Green arrows represent *M. crenulata* specific primer binding sites (Mcr-F, Mcr-R).

Objectives

- To design a combination of 16S ribosomal DNA (16S) primers that can be used for species specific PCR-based verification of *M. crenulata*, without sequencing.
- To provide users of KLH in the biomedical community and aquaculture facilities with a cost effective method for positively verifying the authenticity of KLH as derived from *M. crenulata*.

- *Megathura crenulata* and other Fissurellidae tissue samples were obtained from positively identified field-collected specimens; *M. crenulata* blood was obtained from animals raised in Stellar Biotechnologies' aquaculture facility.
- Genomic DNA was extracted using the GeneJET DNA Purification kit (Thermo Scientific).
- We preformed PCR using the protocol provided with the HotStar Taq kit (Fermentas).
- PCR was unconventional, using four primers, including two universal primers[5], 16Sar and 16Sbr, and two species specific primers designed based on a sequence alignment of Fissurellidae 16S.

Results

- As predicted, our species specific primers only amplified with a unique pattern with *M. crenulata* genomic DNA (Fig. 2).
- All *M. crenulata* genomic DNA templates yield two bands: ~200 & 300 bp (16Sar–Mcr-R & Mcr-F–16Sbr); Other Fissurellidae yield one band: 570 bp (16Sar–16Sbr).

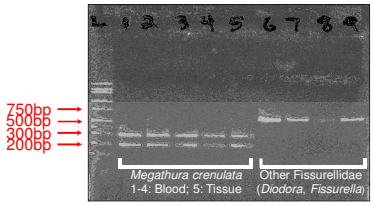


Figure 2. Amplified 16S from Fissurellidae. Lanes 1-4 from *M. crenulata* blood; 5 from *M. crenulata* tissue, 6-9 from selected other Fissurellidae corresponding to the sequences in Figure 1. Standard molecular ladder is on the left.

Discussion

- PCR with our four-primer combination produces only two short amplified products if genomic DNA comes from the giant keyhole limpet, whereas a single standard 16S product amplified when we used other Fissurellidae DNA.
- No large product is amplified for *M. crenulata*, probably because of the preferential amplification of the two smaller products.
- As a natural commodity, KLH is subject to counterfeiting with blood from other Fissurellidae/gastropods. Our method provides a rapid and inexpensive method of product verification.

Literature Cited

- 1-Markl, J. (2013). Evolution of Molluscan Hemocyanin Structures. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*.
- 2-Wirguin, I., Suturkova-Milosević, L., Briani, C., & Latov, N. (1995). Keyhole limpet hemocyanin contains Gal (β1–3)-GalNAc determinants that are cross-reactive with the T antigen. *Cancer Immunology, Immunotherapy*, *40*(5), 307-310.
- 3-Lebrec, H. and nine coauthors. 2013. T-cell-dependent antibody responses in the rat: Forms and sources of keyhole limpet hemocyanin matter. *Journal of Immunotoxicology*, posted online on 20 Aug 2013.
- 4-McLean, J. H. (1969). *Marine shells of southern California* (No. 11). Los Angeles County Museum of Natural History.
- 5-Palumbi, S. (2002). The simple fool's guide to PCR Version 2.0. (http://palumbi.stanford.edu/SimpleFoolsMaster.pdf)

Acknowledgements

This study was supported by a CSUF faculty-student research fellowship to DJE and JMS and by Stellar Biotechnologies, Inc. We thank Dr. Erik Pilgrim, U.S. EPA, for help in obtaining the16S sequences of Fissurellidae used for study design.