Portable Detection and Quantification of *Karenia brevis* Using NASBA

Erica T. Casper[1], Stacey S. Patterson[1], Andrew S. Farmer[2], David P. Fries[2], Kelly S. Rein[3] and John H. Paul[1]

[1]College of Marine Science, University of South Florida, [2]Center for Ocean Technology, University of South Florida, [3]Florida International University

Abstract

Blooms of *Karenia brevis*, the red tide forming dinoflagellate in the Gulf of Mexico have become an annual event on the West Coast of Florida. Brevetoxins produced by *K. brevis* are responsible for massive fish kills, marine mammal mortalities, and human illness due to ingestion of tainted shellfish or inhalation of aerosolized toxin. Rapid and sensitive detection and quantification of *K. brevis* is critical for the proper management of shellfish bed closures and public notification of red tide affected beaches. Current detection methods rely on cell counts, which are time consuming and require specially trained personnel. In addition, cell counts do not distinguish between live and dead or inactive cells and do not provide information about bloom toxicity. To address these issues, we have developed two nucleic acid sequence-based amplification (NASBA) assays for the detection of *K. brevis*, one to detect viable cells by targeting mRNA of the large sub-unit (*rbcL*) gene of RuBisCO, and the other targeting a putative *K. brevis* polyketide synthase (PKS) gene thought to be involved in brevetoxin production. NASBA amplification of the *rbcL* mRNA occurs in as little as 20 minutes with sensitivity to one cell. This assay has been used to quantify *K. brevis* from bloom samples and has shown agreement with Florida Fish and Wildlife Conservation Commission (FWC) cell counts. Initial results indicate a relationship between *K. brevis* concentration and PKS expression. We are working to determine if a correlation exists between PKS expression and brevetoxin levels. To allow for portable and remote detection, we have developed a simple RNA field extraction protocol coupled with a custom designed and produced handheld NASBA detector (COT, USF). The extraction protocol is a modified version of the Qiagen RNeasy Mini Spin Kit, and requires no specialized training or equipment. Extracted RNA is amplified and detected in the handheld sensor, which generates real-time fluorescence curves of the NASBA amplification. Results indicate 100% recovery of RNA from the field kit versus the traditional lab-based extraction method, with equal sensitivity. Amplification curves generated by the handheld sensor mirror curves from the bench top EasyQ Analyzer (bioMérieux). When used to quantify *K. brevis* in bloom samples, this method produced calculated cell concentrations that fell within an order of magnitude of FWC cell counts. By coupling the NASBA assays to our RNA extraction kit and handheld detector, rapid, accurate and field-based bloom information can be generated.

NASBA Assays

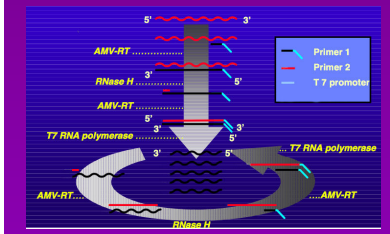


Figure 1. NASBA amplification pathway.

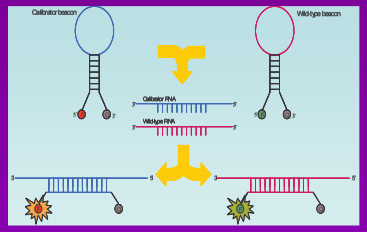


Figure 2. NASBA molecular beacon binding.

NASBA is an isothermal (41ºC) method of RNA amplification in a DNA background (Davey and Malek 1998, Figure 1). Detection of the NASBA amplicon is accomplished by use of molecular beacon probes (Figure 2). Inclusion of internal control RNA (IC-RNA), a synthetic piece of RNA with identical primer sites but an alternate beacon binding site increases the precision of the assay (Patterson et al 2005). The ratio of the time to positivity (TTP) of the target to the TTP of the IC-RNA is used to generate standard curves and to quantify unknowns.

rbcL

Primers were designed to target a 91-bp region of the *K. brevis rbcL* mRNA. This NASBA assay is used to quantify *K. brevis* in unknown samples by the use of standard curves and IC-RNA. Detection with this assay occurs in as little as 20 minutes and is sensitive to one cell. RNA from cultured *K. brevis* cells is extracted and added to the NASBA reaction along with the IC-RNA. The time it takes a sample to fluoresce above background is defined as the time to positivity (TTP). The ratio of target TTP to IC-RNA TTP is calculated and this value used for generation of standard curves and quantification of unknowns.

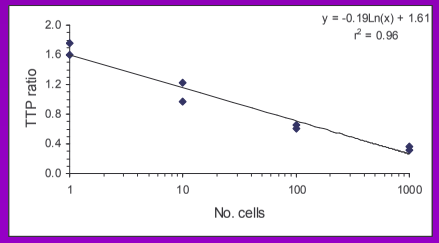


Figure 3. *rbcL* standard curve, based on TTP ratio.

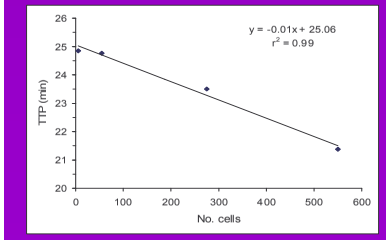
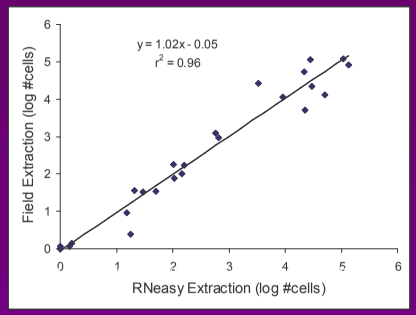


Figure 4. PKS standard curve, based on TTP.

PKS

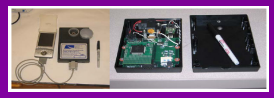
The PKS assay targets a portion of a putative polyketide synthase gene which is thought to be involved with brevetoxin production, and results in a 101-bp amplicon. For all bloom samples, PKS was detected only and in all *rbcL* positive samples. Initial results show a general relationship between cell density and PKS expression. In this figure, both *rbcL* and PKS NASBA assays were performed on cultured cells, and PKS TTP plotted in relation to cell number. Future work will focus on determining a relationship between toxin production and PKS expression, which may not always be related to cell number.

Field RNA Extraction



Bloom samples extracted with the field kit were quantified and compared to FWRI cell counts. Agreement between the methods was good (Figure 6). One potential reason for discrepancies between the numbers include the time delay (typically 1 day) between FWRI cell counting and NASBA analysis.

Handheld NASBA Detector



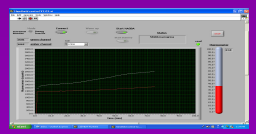


Figure 7. Handheld NASBA Sensor

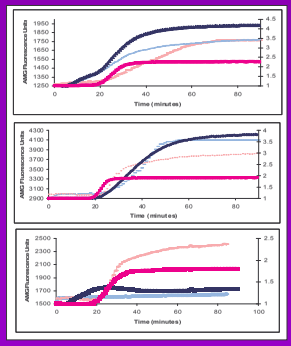


Figure 8. Amplification curves obtained with both the EasyQ and the handheld device. Dark Blue = WT (EasyQ) Light Blue = WT (HH) Dark Pink = IC (EasyQ) Light Pink = IC (HH)

The field extraction kit was developed from the RNeasy Mini Kit (Qiagen, Valencia, CA). A seawater sample (30-50ml) is filtered directly onto an RNeasy column via a 60ml syringe attached by a custom (COT) designed adaptor. The sample is lysed and washed on the column by pulling buffers through the column with a 10ml syringe. Column is dried by pushing out excess liquid with a 60ml syringe at least 5 times until no additional liquid is present. RNA is eluted in 50-100µl DI pushed through column with a clean 60ml syringe. Extraction efficiency of the field extraction kit was compared to RNeasy RNA extraction using both cultured cells and bloom samples. Results show comparable extraction efficiencies between the methods (Figure 5).

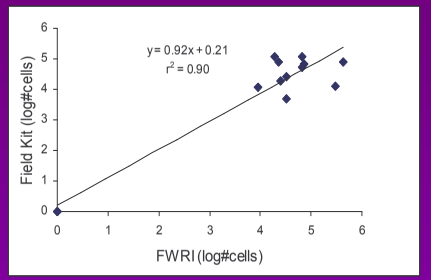


Figure 6. FWRI cell counts vs. field kit extraction and NASBA.

While maintaining the 41°C required for the NASBA reaction, readings of the fluorescence intensity are captured using a *Texas Instruments ADS7808* 12-bit analog to digital converter, processed and routed to the handheld device's serial output. The numeric data are then processed using an in house (COT) developed software (Lab View 6.0 based) to provide a real time fluorescence plotting of the NASBA amplification. Figure 7 illustrates the handheld connected to a palm PC (upper left), the internal configuration (upper right) and a screen shot Lab View 6.0 data output from a NASBA reaction (bottom). In duplicate reactions, the amplification curves generated with the handheld detector closely mirrored the curves generated with the bench top EasyQ detector (bioMérieux) and there was no difference in the sensitivity obtained using the handheld device versus the bench top models (Figure 8).

Conclusions

- NASBA assays developed for *K. brevis rbcL* and PKS
- PKS was detected in all bloom samples contained *K. brevis*, and was not detected in *rbcL* negative samples
- Field extraction kit is equally efficient as the RNeasy kit at RNA extraction
- Bloom samples extracted with the field kit and quantified with NASBA showed general agreement with FWRI cell counts
- Amplification plots generated with the handheld NASBA analyzer showed good agreement with the bench top EasyQ model in terms of quantification and sensitivity
- The field extraction kit, combined with the handheld NASBA analyzer provide a reliable and rapid means to detect *K. brevis* in marine environments

Acknowledgments

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References

Davey, C., and L.T. Malek. 1998. Nucleic acid amplification process. European Patent No. EP0329822.

Patterson, S.S., **E.T. Casper**, L. Garcia-Rubio, M.C. Smith, J.H. Paul. 2005. Increased precision of microbial RNA quantification using NASBA with an internal control (IC-NASBA). J. Microbiol. Methods. 60(3): 343-352.