Performance of the Freedom4 for detection of *Pseudo-nitzschia australis*

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Introduction

Algal blooms cause serious problems but when these blooms consist of harmful algae their effects can be devastating. Harmful algae are responsible for human illness, fish kills, marine mammal strandings and negatively impact recreation, tourism and fishery economics[1]. One harmful algae species is the diatom *Pseudo-nitzschia* spp. These organisms produce a neurotoxin called domoic acid that can lead to seizures and death[2].

Holly Bowers and the team at MBARI study algal blooms to find out what triggers them, how they spread and the best approaches for managing them when they occur. One tool the team uses is the Polymerase Chain Reaction (PCR) to identify harmful algal species within a bloom. Current protocols require that samples collected at sea are processed on shore. A mobile qPCR platform would enable sample processing and analysis while the team is still at sea. This would allow real time assessment of an algal bloom leading to communication of potential risk to authorities more quickly and the better use of MBARI's deployed resources, e.g. through implementation of informed sample collection strategies while boats are still at sea.

The Freedom4 is a fully mobile qPCR platform that would meet the needs of the MBARI team. In the example given here a PCR assay used to detect *Pseudo-nitzschia australis*, the most toxic species in Monterey Bay[3], is shown to be compatible with the Freedom4.

Materials and Methods

qPCR instruments

All reactions were run in parallel on the Freedom4 (Ubiquitome, New Zealand) and the LightCycler 480 (Roche, Switzerland)

Template

Both positive (Paus) and negative (NTC) standards were supplied by Holly Bowers from MBARI. The two positive standards were supplied at concentrations of 10^7 and 10^5 copies of the target sequence. Serial dilutions of these standards were made by diluting samples with PCR quality water.

qPCR Assay for *Pseudo-nitzschia australis*

The qPCR assay developed by Kendra Negrey[4] at MBARI targets the rDNA ITS 1-5.8S ITS2 region of the *Pseudo-nitzschia australis* genome. The primer sequences are: PauF1 – CTTGCTTTTAAAGCTCTCCG; PauR2 – CTTGACAAGACAGGTTGAGG. These primers produce an amplicon of 477bp. The thermal program used in this work was a 5 to 10 minute enzyme activation step at 95°C followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A 1 minute extension step followed temperature cycling. No attempt was made to shorten or optimize the cycling parameters in this series of experiments.

qPCR amplification chemistry

Two qPCR amplification chemistries were compared in this work. These were the JumpStart Taq DNA Polymerase system using EVAgreen reporter dye (Sigma-Aldrich Cat. No. D9307) and the LightCycler 480 SYBR Green I Master Mix (Roche Cat No. 04 707 516 001).

Each JumpStart reaction consisted of 1x JS Buffer, 4mM $MgCl_2$, 1x PCR enhancer, 0.2mM each dNTP, 0.52x EVA Green, 0.05 U/µl JumpStart Taq, and 0.2µM primer in a final volume of 25µl. The reaction mix for each SYBR Green I Master Mix assay consisted of 1x SYBR Green I Master Mix and 0.2µM primer in a final reaction volume of 25µl. 5µl of template, or water for the NTC, was added to each reaction.

Agarose Gel Electrophoresis

The EGel system from Invitrogen (USA) was used for gel electrophoresis as per the manufacturer's instructions. Gels were 2% Agarose with SYBR Safe (Invitrogen Cat No. G521802)

Results and Discussion

Figure 1 shows amplification of the Paus and NTC 10^7 standards on the Freedom4 and the LightCycler 480 using two amplification chemistries. The Cq for each positive standard was similar for both instruments:

Freedom 4: JumpStart PAUS 10^7= 15.0; SYBR Master Paus 10^7= 19.5

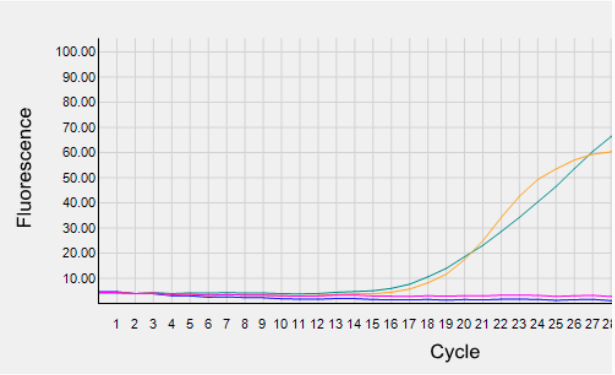
LightCycler 480: JumpStart Paus 10^7 = 16.8; SYBR Master PAUS 10^7 = 16.5.

An agarose gel was run to demonstrate that each qPCR reaction amplified a single product for the positive reactions and that no amplicon was present in the NTCs. As expected, single products of the expected size (477bp) were detected only in reactions where *Pseudo-nitzschia australis* standards were present. Results were identical for both the Freedom4 and the LightCycler 480.

Interestingly, the amplification characteristics differed between the JumpStart and SYBR Green qPCR amplification systems. The SYBR Green chemistry presented a characteristic sigmoidal curve and plateaued earlier than the JumpStart amplification mix. This was independent of the qPCR instrument used to amplify reactions.

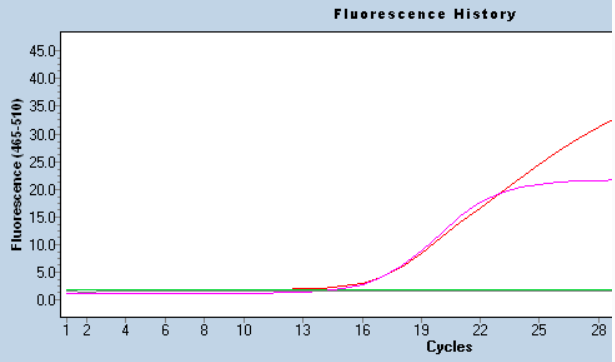
Limit of detection was investigated for this assay using the SYBR Green I Master Mix chemistry in combination with the *Pseudo-nitzschia australis* qPCR assay (Figure 2). Ten fold serial dilutions of the 10^6 *Pseudo-nitzschia australis* standard were made. 5µl of each dilution was then amplified using either a single run on the

Figure 1: Comparison of two qPCR amplification chemistries on the Freedom4 and LightCycler 480



Paus 10^7 copies JS
Paus 10^7 copies SYBR

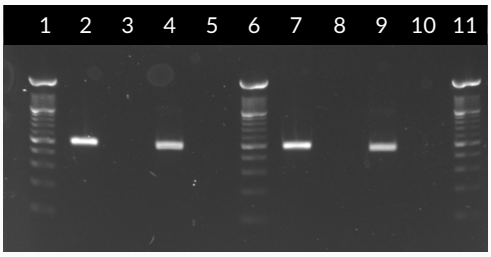
NTC, JS and SYBR



Paus 10^7 copies JS

Paus 10^7 copies SYBR

NTC, JS and SYBR



Key			
NTC	No Template Control	Paus	Pseudo-nitzschia positive control
JS	Jump Start qPCR system with EVAgreen reporter (Sigma)	SYBR	SYBR 2x Master Mix with SYBR reporter (Roche)

2% Agarose E Gel			
10µl PCR Product, 5µl XIV size ladder			
Freedom4		LightCycler 480	
1	XIV Ladder	7	Paus 10^7 copies Jump Start
2	Paus 10^7 copies Jump Start	8	NTC Jump Start
3	NTC Jump Start	9	Paus 10^7 copies SYBR
4	Paus 10^7 copies SYBR	10	NTC SYBR
5	NTC SYBR	11	XIV Ladder
6	XIV Ladder		

LightCycler 480 or two runs on the Freedom4. Both the Freedom4 and the LightCycler 480 were able to detect 10,000 copies of the target sequence.

These experiments show that the Freedom4 is able to perform the *Pseudo-nitzschia australis* qPCR to a comparable standard as a larger laboratory based instrument. Therefore it is possible to incorporate the Freedom4 into on-ocean operations.

References

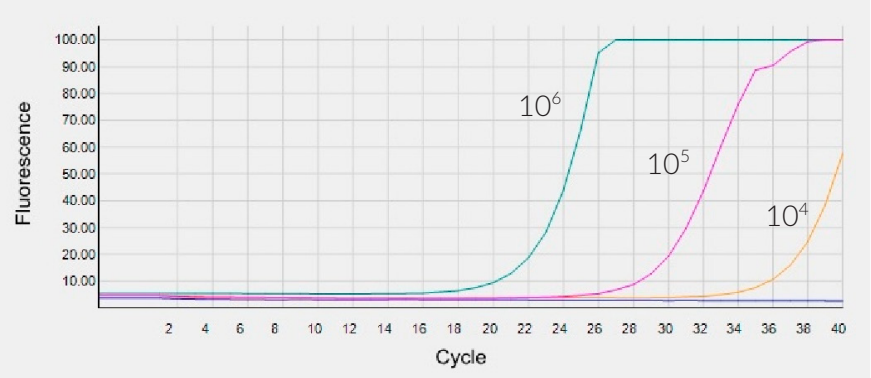
(1) Seltenrich N. (2014). Keeping Tabs on HABs: New Tools for Detecting, Monitoring, and Preventing Harmful Algal Blooms. *Environ Health Perspect*. 122(8):A206-A213. doi:10.1289/ehp.122-a206.

(2) Scholin, C. a, Gulland, F., Doucette, G. J., Benson, S., Busman, M., Chavez, F. P., and others. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature, 403(January), 80–84. http://doi.org/10.1038/47481

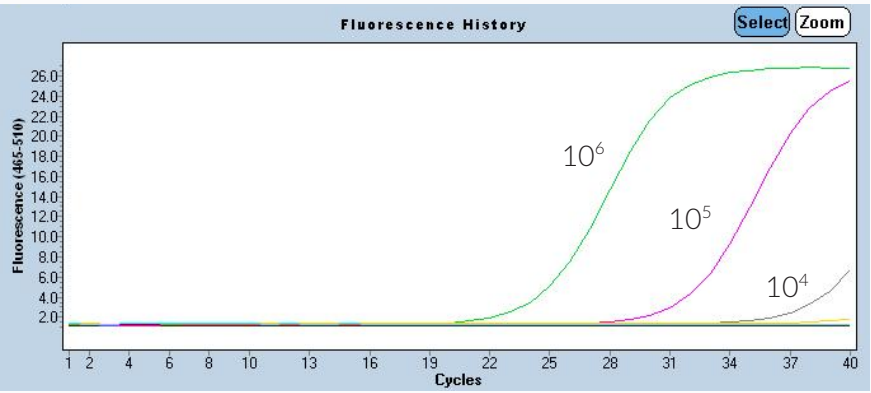
(3) Bargu S, Goldstein T, Roberts K, Li C, Gulland F. (2011). Pseudo-nitzschia blooms, domoic acid, and related California sea lion strandings in Monterey Bay, California. *Marine Mammal Science*. 28(2):237-253. doi:10.1111/j.1748-7692.2011.00480.x.

(4) Hayashi K., Smith G.J., and Welschmeyer N. A. (2007). Long term, temporal variability in Pseudo-nitzschia population dynamics and domoic acid toxicity in Monterey Bay, CA. *Fourth Symposium on harmful algae in the U.S.* Woods Hole, Massachusetts, USA.

Figure 2: Limit of Detection for the Pseudo-nitzschia australis qPCR assay



Freedom4	
Template copy number	**Cq**
10^6	22.9
10^5	31.7
10^4	39



LightCycler 480	
Template copy number	**Cq**
10^6	24.5
10^5	30.4
10^4	39

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