Grouper Forensics - 91-10-12000/0
Final Project Report
Gulf and South Atlantic Fisheries Foundation
Project Title: In Support of Grouper Forensics
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University of South Florida
February 2011 - November 2013

Work Accomplishments:

The proposed work for this project consisted of two primary goals: 1) Develop and assess the molecular RT-NASBA assay for grouper forensics using commercial (bench-top) instrumentation and RNA purification technologies, and 2) develop a handheld NASBA sensor (QuadPyre) which employs the molecular assay developed in the first goal, allowing for *in situ* analysis of fish tissue. Goal 1 was successfully completed and the work is detailed in the peer-reviewed journal article accompanying this report. The article, titled "Ensuring seafood identity: Grouper identification by real-time nucleic acid sequence-based amplification (RT-NASBA)" was published in *Food Control* in 2013 (Ulrich et al., 2103) and G&SAFF was cited as one of the funding sources supporting the research. We are currently preparing a manuscript detailing the development and evaluation of the QuadPyre remote RT-NASBA assay (Goal 2) which we hope to have published by early 2014. We have also been notified that our US Patent Application on this technology will be issued December 24th 2013 as patent #8,614,062.

Goal 1: Development and evaluation of the grouper RT-NASBA assay using commercial instrumentation

Summary of accomplishments:
The USFDA recognized 56 species of fish that can carry the market name "grouper" for both domestic and international commerce at the time of publication. We were able to obtain tissue samples from 35 of these species for RT-NASBA validation. Using a commercial, bench-top NucliSENS EasyQ® analyzer (bio-Mérieux) we positively detected 29 of the 35 species (83%), and there is bioinformatic evidence that we would be able to detect 19 other species that we were unable to obtain tissues from (two species have no 16S rDNA sequence information in GenBank). Given the combined data, the assay developed for this project can positively identify 86% of the FDA grouper species as being grouper in a single RT-NASBA reaction. The assay was also specific against 14 non-target fish species commonly used as surrogate grouper by the seafood industry. Moreover, 100% of the Florida groupers are discernible from imposter fish using said assay. We also present evidence that the assay can be successfully performed on some cooked grouper tissues.

Goal 2: Development of a remote RNA purification method and handheld NASBA sensor

Remote RNA extraction and purification from fish tissue:
All data from the *Food Control* journal article was derived using a commercially available RNA purification kit (RNeasy®, Qiagen) which requires the use of a bulky bench-top centrifuge that cannot be easily transported for remote testing of fish tissue. Therefore, we have devised a remote RNA purification protocol which, in conjunction with the QuadPyre, enables the user to

perform the forensic analysis in its entirety while outside the laboratory. This protocol will be fully detailed in the latest manuscript; however we provide a summary description below.

The remote RNA purification method employs several constituents included in the RNeasy® Mini Kit, only our method negates the necessity of a centrifuge required in the commercial protocol. Briefly, the user begins by excising a small (approx. 20 mg) piece of fish tissue using a sterile disposable biopsy punch which is typically used for clinical procedures. The tissue is then incubated in a cell lysis solution for a short period of time before progressing to the purification stages (Fig. 1). Our method still requires the use of the RNeasy® purification columns as well as some accompanying wash solutions. However, remote RNA purification is carried out using disposable plastic syringes to provide the required air pressure forces to drive the various solutions through the purification column, essentially replacing the role of a centrifuge (Fig. 2). Following the wash procedures, purified RNA is collected from the purification column using additional syringes and inexpensive disposable luer adapters (Fig. 3).

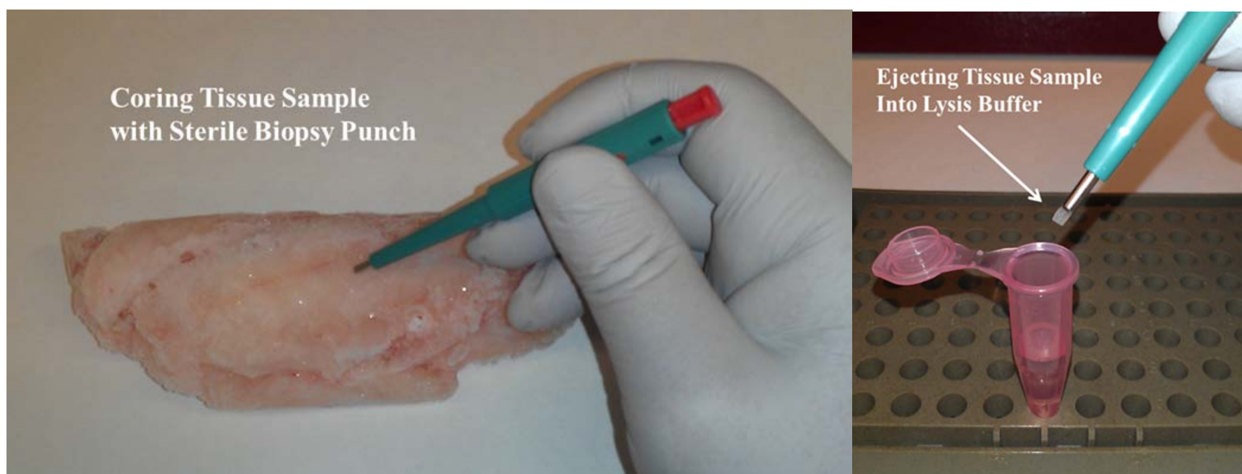

Figure 1. The use of a disposable biopsy punch allows for the sterile transfer of uniform tissue samples from the interrogated specimen to the lysis solution.

We thoroughly evaluated the performance of the remote RNA purification method by comparing it with the standard RNeasy® Mini Kit protocol. We found no significant difference in efficiencies between the two methods when testing a large number of replicate tissue samples using RT-NASBA. Moreover, the remote protocol does not alter the specificity of the RT-NASBA assay nor does it add a significant amount of time to the overall analysis. Both the RNeasy® and remote purification methods can be performed in less than 20 minutes by inexperienced users.

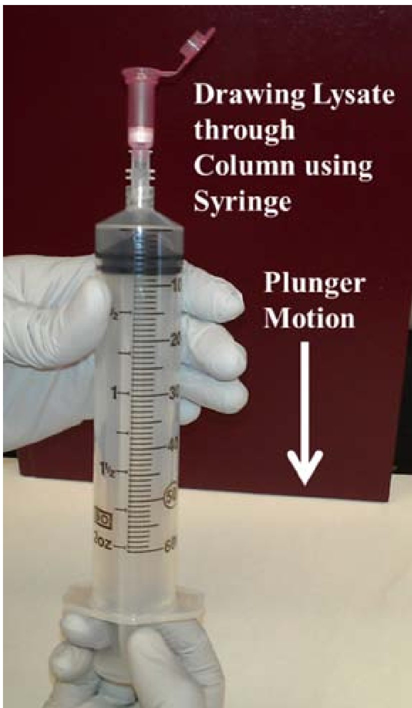

Figure 2. Syringe and column apparatus used to draw fluids through the purification column.

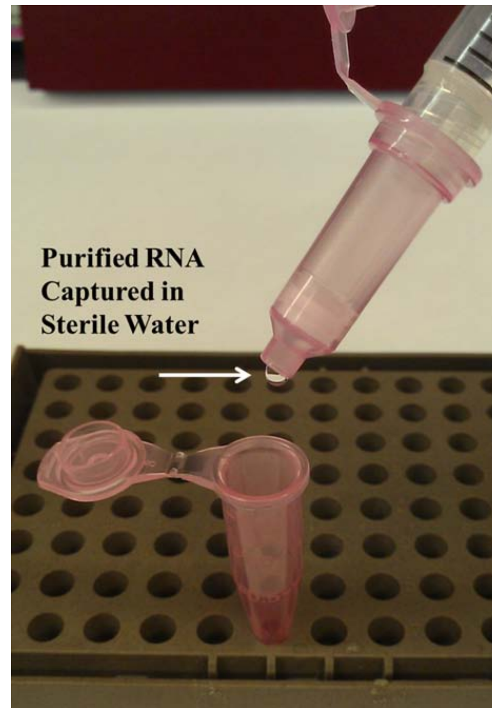

Figure 3. Capturing purified RNA to be analyzed using the RT-PCR QuadPyre assay

Development of the handheld NASBA sensor:

Hardware:

We have completed development of the four-chamber QuadPyre RT-NASBA analyzer to prototype specifications (Fig. 4). Rigorous evaluation of the sensitivity and reproducibility of the prototype sensor has been performed using commercial instrumentation as a benchmark of optimal performance. We have determined that the QuadPyre is generally less sensitive than the commercial NucliSENS EasyQ® analyzer, however the time-to-positivities (TTP, time at which a positive grouper signal reaches the detection threshold) of replicate RT-NASBA reactions assayed on both instruments are generally within 5 minutes of each other. We have also determined that there is a high level of reproducibility between the 4 QuadPyre reaction chambers when assaying replicate RT-NASBA reactions, providing low levels of inter-assay variation (TTPs typically within 3 minutes of each other) when testing multiple samples at once (Fig. 5).

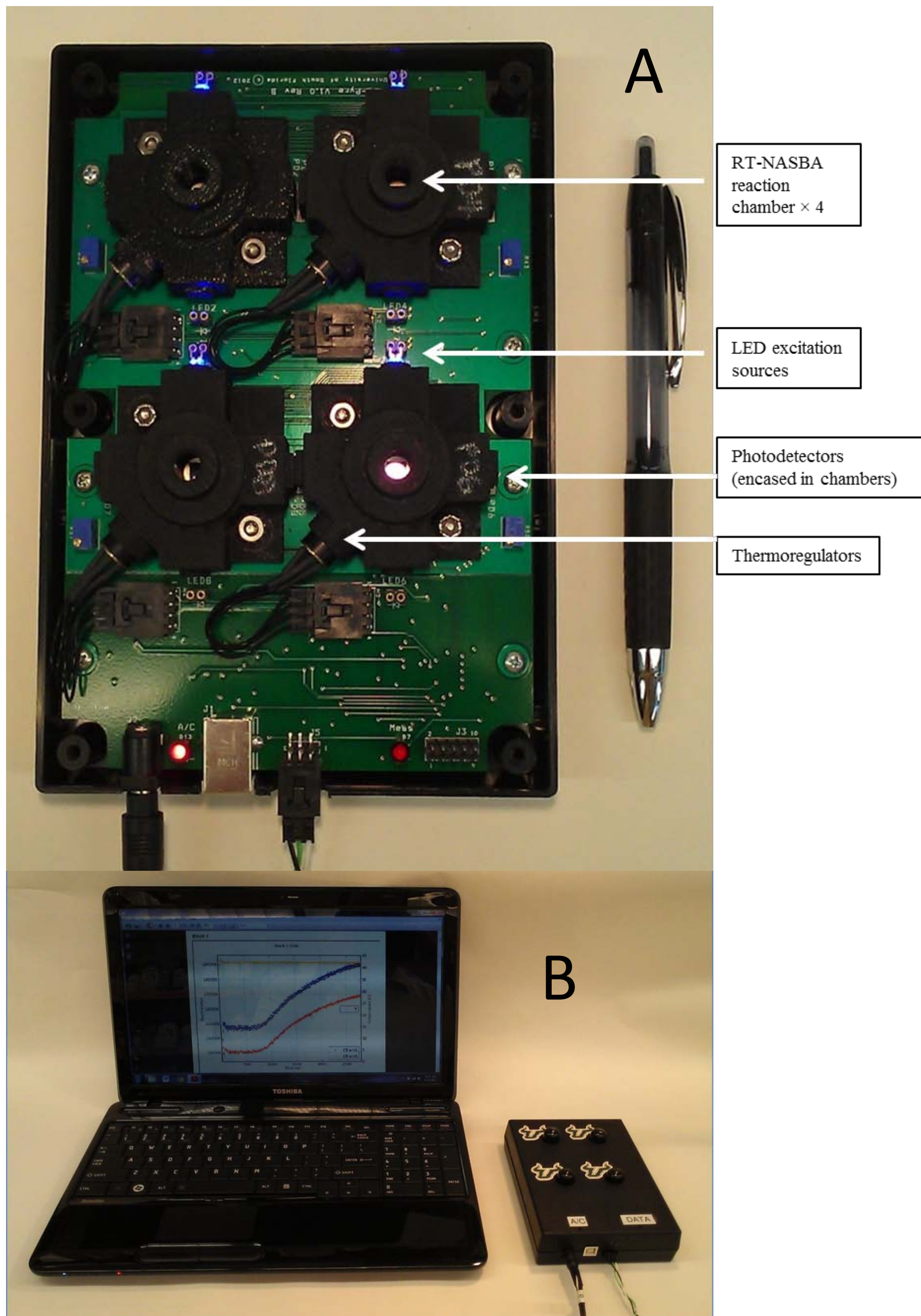

Figure 4. A) Prototype QuadPyre four-chamber RT-NASBA analyzer (protective cover removed). This isothermal (41°C) handheld fluorometer functions similar to the large commercial NucliSENS EasyQ® analyzer, only in a remotely operated laptop-based format (B).

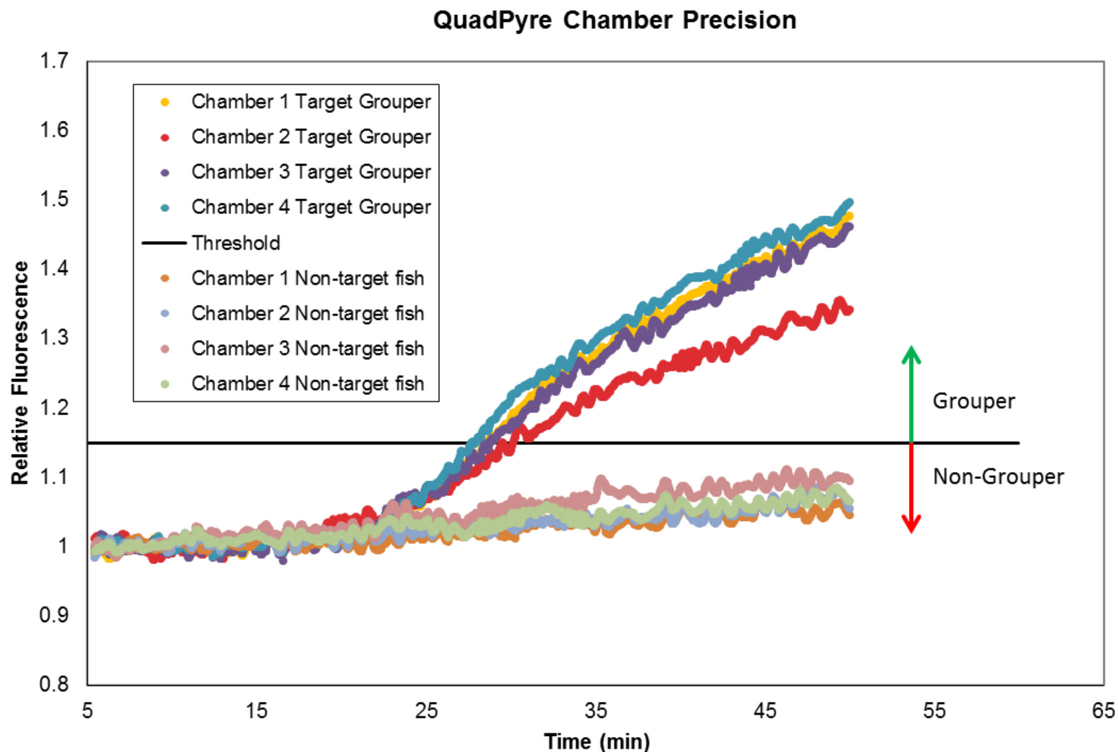

Figure 5. Replication efficiencies between the 4 QuadPyre reaction chambers when testing replicate target grouper (Gag) and non-target fish (Tilapia). The positive threshold is typically set at 1.15 relative fluorescence units and is determined empirically for each assay set.

Software:
The QuadPyre device outputs the integrated readings of voltages across a photo-detector. That data is streamed using a standard serial connection to a personal computer, where it is logged and processed by a novel set of analytical functions written using Java™ mathematics. By utilizing Java™, we have modernized the graphical interface of the QuadPyre allowing the instrument to be controlled by either Microsoft® or Apple® based operating systems. A Hamming filter is first applied to the data to smooth incidental noise at the detectors, the data is then analyzed to find an initial stable period, and the fluorescent reading at that stable period is used to normalize the data so that each photo-detector's reading is analyzed with regard to a baseline. This allows the software to consider the relative amplification of fluorescence, which is the indication of a successful amplification reaction. This graphical interface allows the user to identify the TTPs in a running process (real-time) during the execution of a set of RT-NASBA reactions.

Validation of the QuadPyre remote RT-NASBA assay:
The assay requires three molecular beacon variants (multiplex) in a single RT-NASBA reaction to compensate for gene sequence heterogeneity between target grouper species. Therefore, there are some acceptable nucleotide differences between each of the three beacons and their respective target species. We have determined that at least one of the three beacon variants cannot have more than three nucleotide mismatches to a target grouper species (raw tissue) for it to be detectable. Given these limitations, only 6 USFDA grouper species were not detectable by RT-NASBA using the NucliSENS EasyQ® analyzer (Ulrich et al., 2013). Moreover, all but one

(*Diplectrum formosum*, Sand Perch) of these species is endemic to the Indo-Pacific region and are not a significant source of imported grouper to the U.S. The Sand Perch, although present in Florida waters, is considerably smaller (max length 30 cm) than most other adult groupers and its inclusion in the USFDA's saleable list is somewhat controversial among some taxonomists and seafood regulators.

Provided that the QuadPyre tends to be less sensitive than the commercial instrumentation, we aimed to determine if using the remote assay would reduce the total number of detectable grouper species. We performed the remote QuadPyre RT-NASBA assay (including remote RNA purification protocol) on 14 uncooked grouper species for which we also had EasyQ® detection results (Table 1). Using the QuadPyre, we were able to detect 9 grouper species that were also detectable using the EasyQ® instrument, all of which had no more than 2 nucleotide mismatches to any one beacon variant. Two of the species tested were not detectable using either the remote or bench-top instrumentation. Incidentally, 3 species that were detectable using the EasyQ® were not detectable using the QuadPyre sensor, and all of these groupers had no less than 3 nucleotide mismatches to any beacon variant. Thus, there is evidence that the reduced sensitivity of the QuadPyre decreases the beacon nucleotide mismatch threshold from 3 to 2 when compared with the EasyQ® instrument. However, this sensitivity limitation only reduces the total number of detectable USFDA grouper species from 86% (EasyQ®) to 80% (QuadPyre), not including the two species for which there is no sequence information. Furthermore, the remote RT-NASBA assay still remains 100% efficient in detecting grouper species commonly found in Florida waters.

Another goal of ours was to evaluate the efficiency of the remote RT-NASBA assay when testing cooked grouper tissues. We obtained 7 species of grouper with varying levels of beacon heterogeneity and cooked multiple specimens of each species using the frying protocol described in the Ulrich et al. article. RNA from the cooked tissue was purified using the remote extraction protocol described above, and RT-NASBA was performed for each species using the QuadPyre platform. A general decrease in the fluorescence signal intensity was observed for each cooked specimen when compared with raw tissues from respective specimens (Fig. 6). The data suggests that this decrease in signal is not large enough to cause species with most beacon sequence homogeneity to become undetectable when cooked. We were able to detect cooked tissues from all replicate specimens (13/13) from species with at least one beacon variant having zero nucleotide mismatches (Table 2). However, when testing cooked tissues from Black Grouper and Yellowfin Grouper, we were only able to detect 2/3 and 1/2 specimens from each, respectively. Both of these species have a slightly higher level of beacon heterogeneity (all beacon variants have at least 1 nucleotide mismatch) which may describe why there is intermittent detection when testing cooked tissues. There appears to be an aggregate effect in signal reduction when species with lower beacon homogeneity are cooked, which further reduces the signal intensity. Never the less, we believe that the greater utility of the remote RT-NASBA assay will be at the time of wholesaling and not at the point of consumption (i.e. after cooking).

Table 1. RT-NASBA results testing uncooked tissues from target and non-target species

Latin Binary Name	Common Name	Molecular Beacon Nucleotide Mismatches			NucliSENS EasyQ® Detection	QuadPyre Remote Detection
		A	B	C		
Epinephelus acanthistius	Gulf Coney	0	1	1	+	NA
Epinephelus areolatus	Persian Grouper	0	1	1	+	NA
Epinephelus coioides	Orange-spotted Grouper	1	2	0	+	NA
Epinephelus diacanthus	Spinycheek Grouper	0	1	1	+	+
Epinephelus fasciatus	Blacktip Grouper	1	0	2	+	NA
Epinephelus flavolimbatus	Yellowedge Grouper	0	1	1	+	+
Epinephelus guttatus	Red Hind	0	1	1	+	NA
Epinephelus hexagonatus	Starspotted Grouper	1	2	2	+	NA
Epinephelus itajara	Goliath	1	2	0	+	+
Epinephelus lanceolatus	Giant Grouper	1	2	0	+	NA
Epinephelus macrospilos	Snubnose Grouper	0	1	1	+	NA
Epinephelus malabaricus	Malabar Grouper	1	2	0	+	NA
Epinephelus marginatus	Dusky Grouper	1	0	2	+	NA
Epinephelus morio	Red Grouper	0	1	1	+	+
Epinephelus nigritus	Warsaw Grouper	0	1	1	+	NA
Epinephelus niveatus	Snowy Grouper	0	1	1	+	NA
Epinephelus polyphekadion	Camouflage Grouper	2	3	1	+	NA
Epinephelus striatus	Nassau Grouper	2	3	1	+	NA
Mycteroperca bonaci	Black Grouper	1	2	2	+	+
Mycteroperca interstitialis	Yellowmouth Grouper	1	0	2	+	+
Mycteroperca jordani	Gulf Grouper	0	1	1	+	NA
Mycteroperca microlepis	Gag	0	1	1	+	+
Mycteroperca tigris	Tiger Grouper	2	1	3	+	NA
Mycteroperca venenosa	Yellowfin Grouper	1	2	2	+	+
Mycteroperca xenarcha	Broomtail Grouper	1	0	2	+	NA
Cephalopholis argus	Purplespotted Grouper	5	4	5	−	NA
Cephalopholis cruentata	Graysby	4	3	4	+	−
Cephalopholis fulva	Coney	4	3	4	+	−
Cephalopholis miniata	Coral Hind	5	4	5	−	NA
Cephalopholis taeniops	Spotted Grouper	4	3	4	+	−
Cephalopholis urodeta	Chevron Tailed Grouper	5	4	5	−	−
Anyperodon leucogrammicus	Slender Grouper	3	2	4	+	+
Plectropomus areolatus	Squaretail Coralgrouper	8	7	8	−	NA
Variola louti	Yellow-edged Lyretail	6	7	7	−	NA
Diplectrum formosum	Sand Perch	6	5	6	−	−

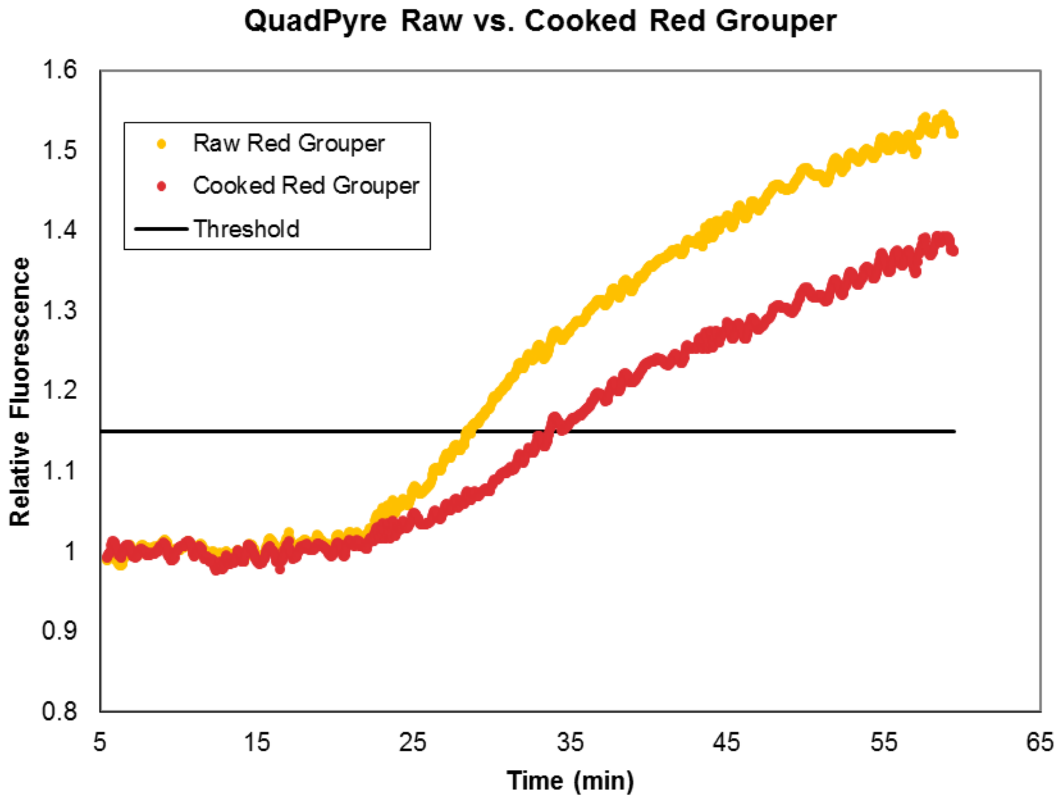

Figure 6. Example of the typical decrease in fluorescence signal intensity when testing cooked grouper tissue.

Table 2. Testing cooked grouper tissue using the QuadPyre

Latin Binary Name	Common Name	Raw Tissue Detection	Cooked Tissue Detection
Epinephelus diacanthus	Spinycheek Grouper	3/3	3/3
Epinephelus flavolimbatus	Yellowedge Grouper	2/2	2/2
Epinephelus morio	Red Grouper	3/3	3/3
Mycteroperca bonaci	Black Grouper	3/3	2/3
Mycteroperca interstitialis	Yellowmouth Grouper	2/2	2/2
Mycteroperca microlepis	Gag	3/3	3/3
Mycteroperca venenosa	Yellowfin Grouper	2/2	1/2

Future Directions: What's Next?

The ultimate goal of th**is project is to bring this technology to market for use in seafood authentication.** The first task would be a telephone survey of the seafood industry to ensure this technology is viable and meets needs of the industry. Are there any changes or upgrades in the technology that would make it easier to operate by unskilled users? We will then move forward in presenting this technology to the end users in various locales throughout Florida and at any appropriate trade shows.

We estimate that an additional level of funding of $75K for two years would be necessary to bring this exciting new technology to market and complete the project.

Expenditures:
There were no additional expenditures for the period of 03/31/2013 through 12/01/2013.

References:

Ulrich RM, John DE, Barton GW, Hendrick GS, Fries DP, Paul JH (2013) Ensuring seafood identity: Grouper identification by real-time nucleic acid sequence-based amplification (RT-NASBA). Food Control 31 (2):337-344. doi:DOI 10.1016/j.foodcont.2012.11.012

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ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 181 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

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