1. Procedure summary

This procedure summarizes the process of qPCR for detecting pest DNA.

1.1. Related Procedures

Predator qPCR Primer Design	LC-06-001-008
Pond sampling	LC-01-003-001

1.2. Procedure impacts and concerns

Safety	NA	
Quality	NA	
Delivery	NA	
Environmental	NA	
Cost		
Compliance	Compliance with OSHA's Hazardous Waste Operations and Response, and Hazardous Communication Standard in addition to the Sapphire Energy, Inc. Chemical Hygiene Plan is required. See 29 CFR 1910.120 and 1200. An authorized user list, MSDS's and label information will be available for easy reference in a binder in the administration building.	
Anomaly Reporting	The initial discoverer of an anomaly should remediate the anomaly as long as such can be performed safely and with little or no additional resources. After remediation of the anomaly, the initial discoverer must document the anomaly and disposition using form XXX (Incidental Anomaly Reporting). If additional resources, equipment, and/or personnel are required then the initial discoverer of the anomaly must proceed in accordance with the Anomaly Reporting and Disposition Procedure (Doc Number).	An anomaly is any event or observation that is not a part of the normal procedure or expected operation. This includes human failure as well as unexpected events/outcomes.

1.3. Responsibilities and owners

Document Owner	Manage contentment and distribution	Micheal Burnett
Process Owner	Responsible for content and process validation	Phil Lee
Plant Manager	Responsible for implementation and conformance	Bryn Davis

2. Process

2.1.

I. PURPOSE

The purpose of this SOP is to describe procedures used for monitoring pest DNA levels by qPCR.

II. HEALTH, SAFETY AND ENVIRONMENTAL COMPLIANCE

This SOP does not involve the use of hazardous chemicals or potentially dangerous equipment and is not subject to environmental regulation.

III. REFERENCE DOCUMENTS/INFORMATION:

Relevant reference documents include the manuals for the Bio-Rad MyiQ2 Two-Color Real-Time PCR Detection System and/or the Bio-Rad CFX96 Real Time Detection System, the Bio-Rad iQ5 and/or the Bio-Rad CFX Manager 2.1 software packages, Microsoft Excel, and the Bio-Rad SsoFast EvaGreen SuperMix (Cat#172-5204).

IV. PREREQUISITES:

A basic understanding of molecular biology techniques is necessary to perform the procedures outlined below.

V. INSTRUCTIONS:

Complete the steps outlined below in the order described. Name the Data file by Pond ID(s), date and time (Pro Pond X,Y,Z Data 201x-xx-xx (time)) and save the data file and the output in Excel format to N:\NM LAB\Molecular Biology folder.

VI. PROCEDURE/PROTOCOL

I. Lysate Preparation

a. Pipet 50 microliters directly from pond sample and place in 96-well plate (Bio-Rad Cat # MLP-9601) or 8-tube Strip (Bio-Rad Cat # TBS0201)

b. Add 50 microliters of 0.25x Lysis Buffer (1x Lysis Buffer = 50 mM Tris-HCl pH8, 200mM NaCl, 20 mM EDTA pH8, 1.0% SDS) to each sample.

c. Place plate or strip into MJ Research PTC-200 or Bio-Rad DNA Engine PCR machine, launch the Algaelys protocol (10 minutes 95C, 5 minutes 25C, repeat 1x, 4C indefinitely) in the Main menu, select appropriate vessel (plate or strip) and reaction volume and begin the program.

d. Dilute lysates 1:20 in ddH_2O.

II. Reaction Set-up

a. Each qPCR reaction consists of the following:

i. For the CFX Real Time System: 5 microliters Bio-Rad SsoFast EvaGreen SuperMix (Cat#172-5204), 2.6 microliters diluted lysate, 2.4 microliters of oligonucleotide set diluted as determined during qPCR primer optimization. Reactions should be run in triplicate.

ii. For the MyiQ2 Real time System: 10 microliters Bio-Rad SsoFast EvaGreen SuperMix (Cat#172-5204), 5.2 microliters diluted lysate, 4.8 microliters of oligonucleotide set diluted as determined during qPCR primer optimization. Reactions

should be run in triplicate.

b. A plate control should be run with every plate. The plate control consists of a purified plasmid template and primer set that, when used together, will result in a consistent and reproducible Critical Threshold value. The plate control should be run in triplicate and replicates should be loaded into the same wells for each plate.

c. Transfer reactions to a 96-well plate (Bio-Rad Cat # MLP-9601 for MyiQ2 Two Color Real-Time PCR Detection System, Bio-Rad Cat # MLL-9601 for the Bio-Rad CFX96 Real-Time System) taking care not to produce bubbles and to ensure that all of the reaction volume is at the bottom of the well.

III. qPCR Machine Set-up

a. Launch appropriate software package—Bio-Rad iQ5 for the MyiQ2 Two Color Real-Time PCR Detection System or CFX Manager for the Bio-Rad CFX96 Real-Time System.

b. Select appropriate qPCR Protocol and Plate format. For CFX Manager, this is done by left clicking on “New”, selecting “Run” in the pull down menu. This opens a new window with tabs for “Protocol”, “Plate” and “Start”.

i. To select the qPCR Protocol, left click on the “Protocol” tab, navigate to the “CFX Files” folder on the desktop, and launch the appropriate protocol. For normal qPCR with optimized gene-specific primers, select the “qPCR without Melt Curve”. For qPCR with Universal primers, select “qPCR with Melt Curve”. When optimizing new primers, select “qPCR with Melt Curve and Gradient”.

ii. To select the appropriate plate format, left click on the “Plate” tab, navigate to the “CFX Files” folder and select the 96-well Format FAM Only file. This plate format works for all runs.

iii. To start a run, click on the “Start” tab, click on the “Start Run” button in the lower right corner, name the new data fill using the “Pro Pond X,Y,Z Data 201x-xx-xx (time)” format, and determine where to save the file. The “CFX Files” folder is the default destination, and

click on “Save”. The run should begin automatically.

c. Select appropriate qPCR Protocol and Plate format. For iQ5 Software , this is done by left clicking on “Workshop” button in the upper left of the main window.

i. To select the qPCR protocol, click on the “Protocol” button, navigate to the “MyiQ5 Files” folder and selecting the appropriate protocol. For normal qPCR with optimized gene-specific primers, select the “qPCR without Melt Curve”. For qPCR with Universal primers, select “qPCR with Melt Curve”. When optimizing new primers, select “qPCR with Melt Curve and Gradient”.

ii. To select the plate format, click on the “Plate” button, navigate to the “MyiQ5 Files” folder and select the “96-well FAM” format. This format works for all runs.

iii. To start the run, click on the “Run” button in the upper right corner of the Workshop window, and navigate to the appropriate folder to save the file. The “MyiQ5 Files” folder is the default destination, and click on “Save”. The run should begin automatically. If the run is beginning normally, the qPCR machine should make a mechanical whine for a few seconds as the heated lid lowers onto the qPCR plate.

IV. Data Analysis

a. At the conclusion of the run, the Data File should be generated automatically. In both versions of the software, check the Ct values of the wells that were loaded with the plate control reaction. Determine the average Ct of these wells, and then adjust the plate Ct to a position that adjusts the plate Ct to a value as close as possible to the expected value for the control.

i. For data files generated by the CFX Manager software, the plate Ct is adjusted by clicking on the “Quantification” tab (upper left) and then left clicking and holding on the horizontal Ct bar (the only completely horizontal like in the Ct Graph window). After each adjustment, re-calculate the average Ct value of the plate

control wells. Once the optimal position of the Ct bar is determined, re-save the data file.

ii. For data files generated by the iQ5 software, click on the Data Analysis tab (middle left) left clicking and holding on the horizontal Ct bar (the horizontal light green bar After each adjustment, re-calculate the average Ct value of the plate control wells. Once the optimal position of the Ct bar is determined, re-save the data file.

b. Export Ct data into Microsoft Excel.

i. For the CFX Manager Software, click on the "Quantification Data" tab, left click anywhere within the resulting spreadsheet, and select the "Export to Excel" option. Save the Excel file in the same location as the Data file, using the same name (the extension will automatically be set to ".xls").

ii. For the iQ5 software, left click anywhere in the spreadsheet portion of the data file (bottom of the window) and select the "Export to Excel" option. Save the Excel file in the same location as the Data file, changing the extension from ".opd" to ".xls".

c. In the Excel file determine the average Ct values and standard deviations for each sample. Ct's above 36 are considered negative. Ct's below 16 are the result of aberrant reactions. Standard deviations greater than 0.5 should be evaluated carefully to determine if one of the replicates was the result of a pipetting error or due to excessive evaporation resulting from an improperly sealed plate.

d. After the run, the qPCR plate can be discarded in regular trash.

VII. STORAGE

The qPCR machines are located in the lab (building 200) on the "Molecular Bench" in the east end of the main lab room. qPCR plates (all types) are stored in the drawer beneath the Mini-hood. Plate seals are on the shelf behind the Mini-Hood. SsoFast Evagreen Master Mix and primers are stored in the "Molecular Freezer".

VIII. WASTE

All qPCR waste can be discarded in the regular trash stream.

Required documents

Input documents

Predator DNA Sequencing Report

3. Output documents

3.1. Document control

TBD

3.2. Revision history

R0 – Initial Release –	TBD
R1 – <Editor name>	

4.

Document approval

4.1. <Name>

02-16-2012
<Date>

Document reviewers

<Name>
<Name>

4.2.

Risk analysis
<Risk name>

<Approval date>

4.3.

<Last reviewed date>
<Last reviewed date>

5.

<Owner> <RPN>