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# Title: Development and Testing of Species-specific Quantitative PCR Assays for Environmental DNA Applications

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# Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, one site and 2 – 3 buildings**

# Introduction

## 1. Introductory Interview Statements

Videographer NOTE: Interviews were filmed outside, there should be at least one take for each interview without traffic noise.

**REQUIRED:**

1.1. **Katy Klymus:** Well optimized assays are vital for proper interpretation of eDNA data. This protocol describes the necessary design and testing steps to develop such an assay.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Cathy Richter:** In general, environmental DNA is a non-invasive method for detecting a particular species or suite of species. It may reduce costs and be more sensitive compared to conventional survey methods.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

**OPTIONAL:**

1.3. **Katy Klymus:** This method can aid conservation and wildlife management through the detection of DNA from species of interest, allowing one to potentially infer that species' presence in the sampled area.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Cathy Richter:** Because of the great sensitivity of quantitative PCR, contamination can happen easily, and negative controls are needed to identify steps at which contamination was introduced.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.5. **Trudi Frost:** eDNA is a field littered with jargon and shorthand terms. For those not practiced in molecular biology or qPCR, visual demonstrations can aide the planning of an experimental design from conceptualization to data generation.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

**Introduction of Demonstrator on Camera**

1.6. **Cathy Richter:** Demonstrating the procedure will be Dannise Ruiz Ramos, a biologist from our laboratory, and Trudi Frost, a student laboratory aide.

1.6.1. INTERVIEW: Author saying the above.

1.6.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

# Protocol

## 2. Generation of a Sequence Database of Mitochondrial DNA Sequences from Target and Non-target Species of Interest

2.1. To begin, search and download sequences from multiple gene regions for species of interest using NCBI's Nucleotide Database **[1]**.

2.1.1. SCREEN: 61825_screenshot_1(1). 0:00 – 0:16.

2.2. Select all sequences that match the specifications and select **Send to.** Choose **Complete record**, **File**, download format as either **GenBank** or **FASTA**, then **Create File**. These sequences are now saved to the computer **[1]**.

2.2.1. SCREEN: 61825_screenshot_1(1). 0:17 – 0:56.

2.3. Repeat these steps for all the species of interest. Keep sequences for each gene region in a separate file as they will be analyzed separately **[1]**.

2.3.1. SCREEN: 61825_screenshot_2(1). 0:00 – 0:27.

## 3. Assay Design

3.1. To align the sequences from each gene region separately, import the downloaded sequence files into a sequence alignment program, such as Geneious Prime software **[1-TXT]**.

3.1.1. SCREEN: 61825_screenshot_3. 0:02 – 0:39. **TEXT: https://www.geneious.com**

3.2. Create separate folders for each gene region **[1]**. Then, select all the sequences within a folder that contains sequences from one gene region. Use the **Multiple alignment** tool to create a nucleotide alignment such as **Geneious** or **MUSCLE** of the selected sequences **[2]**.

3.2.1. SCREEN: 61825_screenshot_4(1). 0:00 – 0:31.

3.2.2. SCREEN: 61825_screenshot_5. 0:02 – 0:52. *Video Editor: Speed up the alignment running.*

3.3. Choose promising regions for assay design through the visualization of aligned sequence data **[1]**. Select a region that has a lot of sequence data available for the species of interest, is highly divergent among species, and shows low within-species variation **[2]**.

3.3.1. SCREEN: 61825_screenshot_6. 0:02 – 0:06.

3.3.2. SCREEN: 61825_screenshot_6. 0:45 – 1:00.

3.4. Next, design the assay primers and probe. Use qPCR assay design software to design 5 sets of qPCR assays. Paste the selected sequence into the Sequence entry box. If the alignment created spaces, delete those from the sequence **[1-TXT]**.

3.4.1. SCREEN: 61825_screenshot_7. 0:02 – 1:01. **TEXT: IDT's PrimerQuest Tool: https://www.idtdna.com/**

3.5. Select **qPCR 2 Primers + Probe** in the **Choose Your Design** option, then download the recommended assays **[1]**.

3.5.1. SCREEN: 61825_screenshot_7. 1:02 – 1:06.

3.6. Copy the sequences from the forward primer of the first assay, and search for this primer sequence in the previously created alignment. If using Geneious Prime, use the **Annotate and Predict** tool to add the primer region to the alignment. Do this for all the primer and probe combinations **[1]**.

3.6.1. SCREEN: 61825_screenshot_8. 0:01 – 0:30.

3.7. Inspect these regions of the alignment for variation within the target species as well as within the co-occurring species **[1]**.

3.7.1. SCREEN: 61825_screenshot_8. 1:37 – 1:41.

## 4. Assay Screening and Optimization

4.1. Test primers in silico through NCBI's Primer-Blast. Paste primers to the **Use my own primer** box under Primer parameters. In the Primer Pair Specificity Checking Parameters options, select **nr** as the Database and type the taxonomic order or family of the organism of interest in the Organism box. Ensure that the selected primers are not likely to amplify non-target species **[1]**.

4.1.1. SCREEN: 61825_screenshot_9(1). 0:00 – 0:45, search results at 1:18 – 1:34.

4.2. Test assay efficiency in vitro by creating a standard curve and determine the curve's efficiency and linear range. Test at least six 10-fold dilutions of a synthetic DNA standard containing the target sequence, at approximately 1 to a million copies per reaction **[1]**.

4.2.1. SCREEN: 61825_screenshot_10(1).

4.3. Use the qPCR software to plot the Cq value of each standard on the y-axis and the log base 10 of the initial standard concentration in copies per reaction on the x-axis **[1]**. The qPCR software should automatically run a linear regression. Calculate the efficiency from the slope of the regression **[added 4.4.1.2]**.

4.3.1. SCREEN: 61825_screenshot_11. 0:02 – 0:47.

4.4. Visually inspect the standard curve for bias or for poor performance as measured by efficiency and r-squared values. Use an internal positive control to test for PCR

inhibition of actual field samples, which can lead to a decrease in sensitivity and false negatives **[1]**.

4.4.1. SCREEN: 61825_screenshot_11. 0:48 – 0:55.

4.4.1.2 Added Shot: (Technician is inspecting the standard curve on the qPCR machine's software and points to the efficiency values of the curve). NOTE: Use this in 4.3

4.5. Determine the limits of detection and quantification for each assay. Test assays with non-target species to verify specificity and make sure that the assay performs well with an IPC multiplexed. Assays with good sensitivity, specificity and efficiency can move on to the next steps **[1]**.

4.5.1. SCREEN: 61825_screenshot_11. 0:55 – 1:13.

4.6. To perform in situ testing of the assay, obtain multiple water **[1]** samples and process them for qPCR **[added 2]**. *Videographer: This step is important!*

4.6.1. Talent collecting water samples from a tank. Author NOTE: This tank contains the target species, a freshwater mussel, so we should expect to detect DNA from this sample using our designed qPCR assay.

4.6.2. Added shot: Talent taking water samples from a pond that also contains the target species.

4.7. Run the quantitative PCR assay and compare eDNA concentration **[added 4.7.1.4]** and detection frequency with known site differences in occurrence and abundance. Confirm all detections by sequencing **[added 4.7.1.5.1]**. *Videographer: This step is important!*

4.7.1. Talent programming the PCR run. NOTE: Authors added many shots but did not change VO narration, please just show 4.7.1.4 and 4.7.1.5.1

4.7.1.1. Added shot: Making the standard curve. (Here the technician makes a six, 10-fold dilutions of the synthetic DNA standard)

4.7.1.2. Added shot:Adding master mix which also contains the IPC (Internal Positive Control) to plate. (Here the technician adds mastermix to all the wells on the plate).

4.7.1.3.1. Added shot: Adding standard dilutions in triplicate. (Next the technician adds the standard dilutions to the plate in triplicate, so each dilution gets added to three different wells.)

4.7.1.3.2. Added shot: Adding the no template control in triplicate. (Here the technician adds the no template control in triplicate).

~~4.7.1.3.3.~~ Added shot: ~~Adding positive control in triplicate~~.(Remove this step)

4.7.1.4. Added shot: Adding samples to plate.(Next the technician adds the samples to the plate, also in triplicate. The plate is then sealed.)

4.7.1.5. Added shot: Running the samples on the qPCR machine. (Finally the plate and samples are ready to be run on the qPCR thermocycler.)

4.7.1.5.1. Added shot: The technician puts plate in machine and starts the qPCR machine.

# Results

**5. Results: *A. ligamentina* qPCR Assay**

5.1. Available sequences of all Unionidae species in the Clinch river were downloaded and aligned. After designing multiple assays, 5 sets of primers and probe were added to the alignment for visual assessment **[1]**.

5.1.1. LAB MEDIA: Figure 2.

5.2. The primer and probe sets were tested in silico and in vitro. In the lab, all assays were tested using DNA extractions of 27 available species to verify specificity. One assay successfully amplified only the target species **[1]**.

5.2.1. LAB MEDIA: Table 1.

5.3. A successful assay with good efficiency and r-squared values is shown here. The LOD and LOQ for the selected assay were both 5 copies per reaction **[1]**. In contrast, assays that produced a standard curve with poor efficiency were discarded **[2]**.

5.3.1. LAB MEDIA: Figure 3 A and B.

5.3.2. LAB MEDIA: Figure 3 C and D.

5.4. In a quality qPCR, the standard dilutions amplify at evenly spaced cycle quantification values of approximately every 3.3 cycles for each 10-fold difference in concentration **[1]**. In a poor qPCR, standards may exhibit uneven variation in cycle quantification values between dilutions **[2]**.

5.4.1. LAB MEDIA: Figure 3 B and D. *Video Editor: Emphasize B.*

5.4.2. LAB MEDIA: Figure 3 B and D. *Video Editor: Emphasize D.*

5.5. IPC amplification for unknown samples should be compared to the results of the negative template control IPC. If no inhibitors are present in the samples, all IPC amplification should have a tight grouping in the plot with cycle quantification values nearly the same as the no template control **[1]**.

5.5.1. LAB MEDIA: Figure 6.

5.6. For in situ testing of the assay, location sites included the bottom of the mussel bed in stream, bottom of the mussel bed near shore **[1]**, 100 meters downstream of the bed in stream **[2]**, 500 meters downstream of the bed in stream, and 500 meters downstream of the bed near shore **[3]**.

5.6.1. LAB MEDIA: Figure 7. *Video Editor: Emphasize the bottom of bed area.*

5.6.2. LAB MEDIA: Figure 7. *Video Editor: Emphasize the 100 M D.S. area.*

5.6.3. LAB MEDIA: Figure 7. *Video Editor: Emphasize the 500 M D.S. area.*

# Conclusion

**6. Conclusion Interview Statements**

6.1. **Katy Klymus:** Quantitative PCR assays for environmental DNA can aid in the monitoring of both invasive and endangered species as well as improve our understanding of spatial and temporal changes in species detection without having to physically capture the individual organisms.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.