Standard Operating Procedure for:

Bird specific *Helicobacter* GFD marker using a quantitative polymerase chain reaction (qPCR) assay

DEPARTMENT OF ENVIRONMENTAL PROTECTION
BIOLOGY SECTION
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1. SCOPE AND APPLICATION

1.1. This quantitative PCR assay targets a 16S rRNA gene of *Helicobacter* spp. This marker is avian specific and targets gulls, geese, chickens, ducks, and other bird species.

1.2. This procedure is used for samples with LIMS Test ID PCR-GFD.

2. SUMMARY OF METHOD

2.1. A water sample is filtered through a membrane filter. To extract DNA from the bacterial cells, the filter is agitated in a centrifuge tube containing zirconium silica beads using a bead beater and further processed using a purification kit. The extract is analyzed with qPCR amplification and detected in a thermocycler system using a specific primer set.

2.2. Interferences

2.2.1. Colloidal or suspended particulate materials in water can clog the filter and prevent filtration of a sufficient amount of sample. Some substances, including humic and tannic acids, can act as inhibitors and reduce efficiency by either binding to the DNA polymerase or interfering with the annealing and extension cycles. Samples analyzed with the GFD assay are purified, which can help alleviate the effects of inhibitors.

2.3. Definitions

2.3.1. qPCR: Quantitative Polymerase Chain Reaction is a procedure in which the amount of DNA amplified in each PCR cycle is measured using fluorescent dyes to determine the original amount of DNA in the sample.

2.3.2. Target sequence: The DNA sequence in the Gene of Interest (GOI) that is amplified by the primers in each PCR cycle. The primers are developed to attach specifically to this sequence.

2.3.3. *Helicobacter*: A genus of Gram-negative helical-shaped bacteria. Some species are found in the gastrointestinal tract of humans and animals. An uncultured avian specific species contains the target sequence for this method.

2.3.4. Standard Curve: A dilution series of standards with known DNA concentrations is analyzed and plotted on a graph with the log of the known concentrations on the x-axis and the threshold cycle (C_t) value of each dilution on the y-axis to create a standard curve. The standard curve is used to determine the concentrations of unknown samples. It is also used to determine the performance of the reaction using several parameters including the slope and correlation of the graph.

3. EQUIPMENT AND SUPPLIES

3.1. Gloves, latex or nitriles

3.2. Tube racks

3.3. Dispersion beads, 0.1 mm, zirconium silica

3.4. Qiagen DNeasy PowerLyzer PowerSoil Kit

3.5. Centrifuge tubes, 0.5, 1.5, and 2.0 mL

3.6. Micropipettes with sterile PCR grade filter tips

3.7. 96-well Reaction Plate, Clear

3.8. 96-well plate holders and stands

3.9. Optical adhesive Seal for reaction plates

3.10. Autoclave (S/N 031761851)

3.11. Mettler Precision Balance Model XA105DU (S/N B314195659)

3.12. CBS Optimizer PCR Workstation #1 (S/N 007631)

3.13. CBS Optimizer PCR Workstation #2 (S/N 007632)

3.14. Fisher Mini Vortexers

3.15. Fisher Minicentrifuges

3.16. Thermo Sorval T3 Centrifuge (S/N 309020018)

3.17. PCR1 - Step One Plus Thermocycler (S/N 272006852)

3.18. PCR3 – Step One Plus Thermocycler (S/N 2720010389)

3.19. PCR#1, Thermo Refrigerator (S/N 0121179101121219), maintained at 4ºC ± 2ºC, Room B246A

3.20. PCR#2, Thermo Freezer (S/N 0121174101121218), maintained at -20ºC ± 10ºC, Room B246A

3.21. PCR#4, Thermo Freezer (S/N 0121174201121218), maintained at -20ºC ± 10ºC, Room B246B

3.22. PCR#5, Thermo Fridge/Freezer (S/N 0121174701121218), maintained at 4ºC ± 2ºC and -20ºC ± 10ºC, Room B246

3.23. PCR#6, Thermo UXF Freezer (S/N 0132376801130826), maintained at -80ºC ± 10ºC, Room B246

3.24. PCR#7, Thermo Ultra Low Freezer (S/N 128473401090813), maintained at -80ºC ± 10ºC, Room B246

4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. PCR grade water –Autoclave ultrapure water for 15 min. Record in LIMS Autoclave Log.

4.3. Bleach solution, 10% (v/v)

4.4. PerfeCta® SYBR Green SuperMix ROX (Quanta Bio)

4.5. Prepare the following per SOP PCR-3.4.

4.5.1. GFD gBlock Standards (10^7, 10^6, 10^5, 10^4, 10^3)

4.5.2. GFD DNA spikes (10^7, 10^5)

4.5.3. GFD primers 500 µM stock

4.5.3.1. Forward Primer GFDFOR: 5'- TCGGCTGAGCACTCTAGGG- 3'

4.5.3.2. Reverse Primer GFDREV: 5'- GCGTCTCTTTGTACATCCCA- 3'

5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5.

5.2. Check thermometers annually. See SOP LB-035.

5.3. Check calibration of micropipettes quarterly for accuracy and precision. See SOP PCR-2.4.

5.4. Refer to SOP PCR-2.3 for use and maintenance of thermocycler.

5.5. Refer to SOP PCR-2.2 for calibration and use of the balance.

5.6. Refer to SOP PCR-2.5 for calibration and maintenance of support equipment.

5.7. After each use of the UV workstation, wipe down all surfaces with 10% bleach then rinse with PCR grade water. Turn on UV lamps for at least 15 minutes. The UV workstations have timers for the use of the UV lamps.

5.8. Clean work area counters after use. Spray surface of work area with 10% bleach solution and wipe down after 15 minutes. Spray and wipe down with sterile DI water.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1. See DEP Field SOP FS 2000 for sample collection and preservation.

6.2. See SOP PCR-5.0 for details on sample custody and printing sample labels.

7. SAMPLE PREPARATION

7.1. Refer to SOP PCR-4.0 for sample filtration.

7.2. Refer to SOP PCR-4.2 for purification of samples

8. ASSAY PREPARATION

8.1. Primer Mix – In PCR Workstation #1, prepare primer mix for GFD assay as follows:

8.1.1. Add 2.4 µL of 500 µM forward primer stock and 2.4 µL of 500 µM reverse primer stock to 595.2 µL of PCR grade water to make primer mix. Use a green 1.5 µL centrifuge tube labeled “P” for the primer mix. Mix by tapping then centrifuge briefly.

8.1.2. Label tube with the date and analyst initials. Store at 4°C and use within one week.

8.1.3. See Table 15.1 for a summary.

8.1.4. Create a reaction plate worksheet as per SOP PCR-5.0.

8.2. qPCR Master Mix assay – In PCR Workstation #1, prepare Master Mix for GFD assay as follows:

8.2.1. Count the number of wells needed and enter in the reaction plate worksheet under the Master Mix Preparation section under ‘#Rxns’ cell. The calculator adds an additional 3 wells to ensure enough Master Mix is available (some can be lost in mixing and pipetting) to determine total volume of reagents needed for the assay.

8.2.2. Add PerfeCta® SYBR Green SuperMix ROX, primer mix, and sterile water based on the calculated volume in a green 1.5 µL centrifuge tube labeled "MM" for the GFD Master Mix.

8.2.3. See Table 15.2 for a summary.

8.2.4. Fill in the reaction plate worksheet as per SOP PCR-5.0.

9. REACTION PLATE PREPARATION

9.1. In PCR workstation #1, add 15 µL of Master Mix to each well of a reaction plate using the electronic Xplorer pipette with a 100 µL tip. Use Figure 15.4 as a guide. Carefully move the plate to PCR workstation #2.

9.1.1. Make sure to re-mix the Master Mix periodically by tapping the tube, followed by a pulse spin in the minicentrifuge. This ensures that the Master Mix is evenly distributed into all the wells.

9.1.2. Master Mix is more viscous than water and should be pipetted slowly to ensure accuracy. Bubbles can easily form in the pipette tip if pipetted too quickly.

9.2. In PCR workstation #2, add 5 µL of DNA sample extract or control to each well using a 10 µL pipette. Use Figure 15.4 as a guide. To ensure proper mixing, tap mix then centrifuge briefly. Switch tips for each sample or control. If any liquid falls outside of the well or sprays, the plate should be discarded and the procedure should be repeated with a new plate. Use the following order for addition of the template to wells.

9.2.1. Add the GFD Standard Curve controls (see Section 12.1) to wells A1-E3.

9.2.2. Add PCR grade water for the No Template Controls (NTC) and 3rd well spike control (LFB_GDSPK) to wells F3, G3, and H4-H6. See Section 12.2.1 and 12.3.2.

9.2.3. Add 1 µL of the GFD spike standard (LFB_GDSPK and _SPK_GDSPK) to the spike control and 3rd well samples in wells F3, G3, A-G6, A-H9, and A-H12. Use Figure 15.4 as a guide. See Section 12.3.2.

9.2.4. Add the method blank (MBLK) control (see Section 12.2.2) to wells F1 and G1.

9.2.5. Add the extraction blank (EXBLK) control (see Section 12.2.3) to wells F2 and G2.

9.2.6. Add the extraction control (LFB_GDLFBP) to wells H2-H3. See Section 12.3.1.

9.2.7. Add the sample extracts to appropriate wells (3 wells per sample) using Figure 15.4 as a guide.

9.3. Seal plate with optical adhesive. Vortex slowly, on Speed 1, for 30 seconds to mix.

9.4. Centrifuge plate at 2500 rpm for 2 minutes 30 seconds (Program 4 on Thermo Sorval T3 centrifuge). Check to make sure all the reaction mix is at the bottom of each well and the wells are bubble-free. If not, centrifuge for another minute.

10. SAMPLE ANALYSIS

10.1. Place plate in the thermocycler and analyze. Refer to SOP PCR-2.3 for setup and use of the thermocycler. Use the default thermocycler cycling conditions for SYBR Green reagents with a standard ramp speed (see Table 15.3).

11. DATA ARCHIVAL

11.1. Refer to SOP PCR-2.3 for instrument file setup.

11.2. Refer to SOP PCR-5.0 for worksheet instructions.

11.3. Refer to SOP PCR-5.1 for data upload instructions.

12. QUALITY CONTROL

12.1. GFD Assay Standard Curves

12.1.1. Analyze a GFD Assay standard curve per plate using 5 serially diluted standards at 10^7, 10^6, 10^5, 10^4, and 10^3 concentrations. Analyze each concentration in triplicate. These are added to A1-E3 wells on the plate. Use Figure 15.4 as a guide.

12.1.1.1. Standard curve efficiency should be between 90-115% and R^2 should be $\geq$ 0.99. If efficiency is < 70% or R^2 is < 0.97, samples should be re-analyzed. Notify supervisor.

12.2. Negative Controls

12.2.1. No Template Control (NTC)

12.2.1.1. Include on each plate as a negative control. Add 15 µL of Master Mix and 5 µL of sterile water to three wells on the reaction plate.

12.2.1.2. The GFD NTCs are labeled NTC_1, NTC_2, and NTC_3.

12.2.1.3. If any of the NTCs have a result of less than 35 Ct, the reaction plate may have contamination and should be re-run if samples are affected. Notify supervisor.

12.2.2. Method Blank (MB) – labeled MBLK_#

12.2.2.1. Analyze a method blank for each batch of samples (maximum of 20 samples per extraction batch). Filter 50 mL of sterile PBS at the beginning of each sample batch. Extract and analyze with the samples. Add 5 µL of the blank extract to duplicate wells containing 15 µL GFD Master Mix.

12.2.2.2. If the MB has a result above the MDL, there may be possible sample contamination. All sample results in the batch that are less than ten times the amount of contamination should be "V" qualified and the following comment should be added: "Result is V-qualified due to a control blank failure". For samples that are greater than 10X the blank, add the following comment: "Control blank failure. Result is >=10 times blank result".

12.2.3. Extraction Blank (EB) – labeled EXBLK_#

12.2.3.1. Analyze an extraction blank for each batch of samples extracted. For the analytical batch (extracted samples), 20 samples can be analyzed together. Place a sterile filter in an extraction tube that is labeled EB and extract with the purification kit along with the samples. Add 5 µL of the blank extract to duplicate wells containing 15 µL GFD Master Mix.

12.2.3.2. If the EB has a result that is less than 35 Ct, there may be possible contamination at the extraction step. Samples may be affected and should be re-extracted and analyzed. Notify supervisor.

12.3. Positive Controls

12.3.1. Extraction Control (EC)

12.3.1.1. Analyze an extraction control for each batch of samples. Place a sterile filter in an extraction tube that is labeled EC. Pipette 5 µL of 10^7 GFD gBlock standard into the filter and extract with the purification kit along with the samples.

12.3.1.2. The GFD assay EC is labeled, LFB_GDLFBP#.

12.3.1.3. The extraction control helps determine extraction recovery. Percent recovery of extraction control should ideally be 75-150% but is often much lower due to loss of material during purification. Typical yields for GFD EC are 15-30%.

12.3.2. Inhibition control (3^{rd} well spike)

12.3.2.1. A known amount of GFD gBlock standard is added to each sample as a matrix spike to check for inhibition and DNA recovery.

12.3.2.2. For each sample and replicate, spike the 3rd well of the GFD assay with 1 µL of E5 GFD gBlock spike standard. Also add 1 µL of the standard to the spike control wells.

12.3.2.3. Compare spike control recovery to sample matrix recovery. If sample recovery is <50% of the control recovery then the sample matrix may have inhibitory effects.

12.4. Replicate Sample

12.4.1. Analyze at least one replicate per batch. Filter and analyze replicate in the same manner as original sample is analyzed and within the same batch. Replicate samples are numbered SAMPLE ID_DUP_A_PUR and SAMPLE ID_DUP_B_PUR and 100 mL is filtered for each.

12.4.2. Replicate RPD should be ≤25%. If RPD > 25%, a comment regarding precision is automatically added to the report. The duplicate sample should also be "J" qualified.

12.5. Refer to SOP PCR-5.1 for further details on uploading quality control data into LIMS, including calculations and control limits.

13. SAFETY/HAZARDOUS WASTE MANAGEMENT

13.1. All media/bacteria/plates must be autoclaved for 45 minutes before disposal.

13.2. Refer to the Health and Safety Plan. Read MSDS information for reagent handling and disposal.

14. REFERENCES

14.1. EPA Method B (June 2010)

14.2. 2009 TNI Standard

14.3. EPA 815-B-04-001, Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples (October 2004)

14.4. PerfeCta® SYBR Green SuperMix™, Rox™ Protocol

14.5. DEP Bureau of Labs Quality Manual
http://publicfiles.dep.state.fl.us/dear/labs/lab_qualitymanual_19.pdf

14.6. Health and Safety Plan Health and Safety Plan

14.7. DEP SOP FS 2000, *General Water Sampling*

14.8. DEP SOP LB-035, *Calibration of Lab Thermometers using a Constant Temperature Calibration Bath*

14.9. DEP SOP PCR-2.2, *Calibration, Use and Maintenance of Mettler Toledo XA105DU Balance*

14.10. DEP SOP PCR-2.3, *Calibration, Use, and Maintenance of StepOne Plus Thermocycler*

14.11. DEP SOP PCR-2.4, *Calibration and Use of Mechanical Pipettes using PCS® Pipette Calibration System*

14.12. DEP SOP PCR-2.5, *Calibration, Use, and Maintenance of Support Equipment*

14.13. DEP SOP PCR-3.0, *Preparation of Reagents for quantitative Polymerase Chain Reaction (qPCR)*

14.14. DEP SOP PCR-3.4, *Preparation of GFD assay reagents and standards for qPCR analysis*

14.15. DEP SOP PCR-4.0, *Preparation of samples for qPCR*

14.16. DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen PowerLyzer PowerSoil DNA Isolation Kit*

14.17. DEP SOP PCR-5.0, *Sample Custody, Labels, and Worksheet Instructions for Molecular Biology samples*

14.18. DEP SOP PCR-5.1, *Data upload, Verification, and Sample Authorization*

15. TABLES AND DIAGRAMS

Table 15.1 – Primer Mix Summary	GFD Assay
Forward Primer (500µM stock)	2.4 µL
Reverse Primer (500µM stock)	2.4 µL
Sterile H_2O	595.2 µL

Table 15.2 – Master Mix Summary	GFD Assay
Reagent	Volume per reaction (µL)
Primer mix	2
SYBR Green MM	10
Water	3

Table 15.3 – Cycling Conditions			
		PCR	
Step	Enzyme Activation	Cycle (40 cycles)	
	Hold	Denature	Anneal/Extend
Temperature (°C)	95	95	60
Time	10 min.	15 sec.	1 min

*Include melt curve for each run

Figure 15.4 - Plate Layout Map