Standard Operating Procedure for:

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

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1. SCOPE AND APPLICATION

1.1. This quantitative PCR assay targets a sequence in the 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, PUR-GFD, PCR-GFDB, and PCR-GFDS.

2. SUMMARY OF METHOD

2.1. Bacteria present in a water sample are collected by filtration. The filtered sample is processed by a bead beater in the presence of zirconium silica beads to extract the DNA from bacterial cells into a buffer solution. The clarified extract may also be purified using a purification kit. The extract is analyzed with qPCR amplification and detected in a thermocycler system using a primer/probe set specific to bird associated *Helicobacter spp*.

2.2. Interferences

2.2.1. Colloidal or suspended particulate materials in water can clog the filter and prevent filtration of enough sample to detect the target sequence. 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. Sample purification or dilution 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 primer/probe set is designed 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 quantitation cycle (Cq) value of each dilution on the y-axis to create a standard curve. The standard curve is used to determine the

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concentrations of unknown samples and 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. Extraction tubes, conical, 2 mL screw cap with o-ring seal

3.4.1 Weigh out 0.3 ± 0.01 g of dispersion beads into each extraction tube. Seal tube tightly, making sure the O-ring is seated properly. Autoclave @ 121°C for 15min (15 psi). Record in LIMS Autoclave Log.

3.5. Qiagen DNeasy PowerLyzer PowerSoil Kit for DNA purification

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

3.7. Micropipettes with sterile PCR grade filter tips

3.8. 96-well Reaction Plate, Clear

3.9. 96-well plate holders and stands

3.10. Optical adhesive Seal for reaction plates

3.11. Autoclave (S/N 0317618851)

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

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

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

3.15. Fisher Mini Vortexers

3.16. Fisher Minicentrifuges

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

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

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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. Molecular Biology grade water. Corning Molecular Biology Grade Water tested to USP Sterile Water (Cat.# MT46000CV)

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

4.4. Bovine Serum Albumin (BSA), 2mg/mL – Prepare per SOP PCR-3.0.

4.5. PerfeCta® qPCR ToughMix™, ROX™ to use with StepOne Plus Thermocycler.

4.6. Prepare the following per SOP PCR-3.1.

4.6.1. Salmon DNA Standards ($10^3, 10^2, 10^1$)

4.6.2 Salmon DNA Extraction Buffer (0.2 $\mu g/mL$)

4.7. Prepare the following per SOP PCR-3.4.

4.7.1. GFD gBlock Standards (10^6, 10^5, 10^4, 10^3, 10^2)

4.7.2. GFD DNA spikes (10^6, 10^4)

4.7.3. GFD primers 250 µM stock

4.6.3.1 Forward Primer GFDFor: 5'- TCGGCTGAGCACTCTAGGG- 3'

4.6.3.2 Reverse Primer GFDRevMOD: 5'- GCGTCTCTTTGTACATCCCATTG- 3'

4.6.4 *Helicobacter* Probe 100 µM stock

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4.6.4.1 GFDProbe:

5'- /56-FAM/ACGTCAAGT/ZEN/CATCATGGCCCTTACGC/3IABkFQ/ -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-2.5 for calibration and maintenance of support equipment.

5.6 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.

5.7 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-4.1 for sample crude extraction.

7.3 Refer to SOP PCR-4.2 for purification of inhibited or solid samples.

8 ASSAY PREPARATION

8.1 Primer/Probe Mix – In PCR Workstation #1, prepare primer/probe mix for each assay (SKETA and GFD) separately as follows. Record details in Reaction Plate Preparation Worksheet. See SOP PCR-5.0 Section 7 for details.

8.1.1 GFD Assay: Add 3.2 µL of 250 µM forward primer stock and 3.2 µL of 250 µM reverse primer stock, and 3.2 µL of 100 µM probe to 590.4 µL of PCR grade

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water to make primer/probe mix. Use a green 1.5 mL centrifuge tube labeled "PP" for the primer/probe mix. Mix by tapping then centrifuge briefly.

8.1.2 SKETA Assay: Add 10 µL of each 500 µM primer (forward and reverse) stock and 9 µL of 100 µM probe to 571 µL of PCR grade water to make primer/probe mix. Use a pink 1.5 mL centrifuge tube labeled "PP" for the primer/probe mix. Mix by tapping then centrifuge briefly.

8.1.3 Label tubes with the date. Store at 4°C and use within one week.

8.1.4 See Table 15.1 for a summary.

8.2 qPCR Master Mix assay – Record details in Reaction Plate Worksheet as per SOP PCR-5.0 Section 7. In PCR Workstation #1, prepare Master Mix for each assay (SKETA and GFD) separately 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 volume can be lost in mixing and pipetting).

8.2.2 For each assay add 2 µL X total wells of BSA, 10 µL X total wells of PerfeCta® qPCR ToughMix™ Rox™, and 3 µL X total wells of primer/probe mix (PP). Use a pink 1.5 mL centrifuge tube labeled "MM" for the SKETA Master Mix. Use a green 1.5 mL centrifuge tube labeled "MM" for the GFD Master Mix.

8.2.3 See Table 15.2 for a summary.

9 REACTION PLATE PREPARATION

9.1 Collect a 96 well plate and holder. In PCR workstation #1, add 15 µL of Master Mix of appropriate qPCR Master Mix to each well of a reaction plate using the electronic Xplorer pipette with a 100 µL tip. Using Figure 15.1 as a guide, add the GFD Master Mix to the green wells and SKETA Master Mix to all the pink wells. Place the Master Mix, BSA, and any extra labeled PP mix back in the Thermo refrigerator. 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, prepare the serial dilutions 10^5, 10^4, 10^3, and 10^2 from the GFD gBlock Standard 10^6 (see Section 12.1.1) and dilutions 10^2, 10^1 from the

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Salmon DNA Standard 10^3 (see Section 12.2.1). To ensure proper mixing of samples, tap mix then centrifuge briefly. Add 5 µL of DNA sample extract or control to each well using a 10 µL pipette. Use Figure 15.1 as a guide. Switch tips after each sample or control. If any liquid falls outside of the well or sprays, the plate should be discarded, and all contaminated surfaces cleaned (see Section 5.6). 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 the SKETA Standard Curve controls (see Section 12.2) to wells F1-H3.

9.2.3 Add the method blank (MBLK) control (see Section 12.3.2) to wells A4-B4.

9.2.4 Add the extraction blank (EXBLK) control (see Section 12.3.3) to wells A5-B5.

9.2.5 Add PCR grade water for the No Template Controls (NTC, SURNTC) to wells C4-D6. See Section 12.3.1.

9.2.6 Add the extraction control (LFB_GDLFB, SUR_GPLFB) to wells E4-H4. See Section 12.4.1.

9.2.7 Add 1 µL of the GFD spike standard (LFB_GDSPK and _SPK_GDSPK) to the spike control wells A6-B6 and 3rd well samples, C7-C12, and G5-G12. Add 5 µL of PCR grade water to the spike control wells A6-B6 onto the 1 µL spike standard. Use Figure 15.1 as a guide. See Section 12.4.3.

9.2.8 Add the sample extracts to appropriate wells (4 wells per sample) using Figure 15.1 as a guide. Add the sample to the three GFD wells first, change tips, and then add the sample to the final SKETA well.

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 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.

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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 10^6, 10^5, 10^4, 10^3, and 10^2 target sequence copies (TSC)/5µL concentrations. Dilute standards fresh from the 10^6 standard each time in TE buffer. Refer to SOP PCR-3.4 for preparing standard dilutions. Analyze each concentration in triplicate. These are added to A1-E3 wells on the plate. Use Figure 15.1 as a guide.

12.1.2. Standard curve efficiency should be between 85-110% and R^2 should be ≥ 0.99. If efficiency is < 70% or R^2 is < 0.97, samples should be re-analyzed. Discuss with supervisor or senior analyst.

12.2 SKETA Assay Standard Curves

12.2.1. Analyze a SKETA Assay standard curve per plate. Use 3 serially diluted standards at 10^3, 10^2, and 10^1 concentrations. Dilute standards fresh from the 10^3 standard each time in TE buffer. Analyze each concentration in triplicate. These are added to F1-H3 wells on the plate. Use Figure 15.1 as a guide.

12.2.2. Standard curve efficiency should be between 80-120% and R^2 should be ≥ 0.99. Sample results for the GFD marker are not affected if the efficiency or R^2 for SKETA are outside limits.

12.3 Negative Controls

12.3.1 No Template Control (NTC)

12.3.1.1. Include in triplicate on each plate for each target analyzed as a negative control. For each Master Mix assay used on a reaction plate, add 15 µL of Master Mix and 5 µL of sterile water to three wells on the reaction plate.

12.3.1.2. The GFD NTCs are labeled NTC_1, NTC_2, and NTC_3. The SKETA NTCs are labeled SURNTC_1, SURNTC_2, and SURNTC_3.

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

12.3.2 Method Blank (MB) – labeled MBLK_#

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12.3.2.1 Analyze a method blank for each batch of samples (maximum of 13 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.

12.3.2.2. 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".

12.3.3 Extraction Blank (EB) – labeled EXBLK_#

12.3.3.1. Analyze an extraction blank for each batch of samples extracted (maximum of 13 per batch). Place a sterile filter in an extraction bead tube labeled EB. Add 600 µL of salmon DNA extraction buffer and extract along with the samples. Add 5 µL of the EB to duplicate wells containing 15 µL GFD master mix.

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

12.4. Positive Controls

12.4.1. Extraction Control (EC)

12.4.1.1. Analyze an extraction control for each batch of samples. Place a sterile filter in an extraction tube labeled EC. Pipette 5 µL of 10^6 GFD gBlock standard to the filter and add 595 µL of DNA extraction buffer.

12.4.1.2. The GFD assay EC is labeled, LFB_GDLFB#. The SKETA assay EC is labeled SUR_GDLFB#. Use Figure 15.1 as a guide.

12.4.1.3. The extraction control helps determine extraction recovery. Percent recovery of the extraction control should ideally be 75-150% . If recovery is low, extraction efficiency may be low.

12.4.2. Sample Processing Control (SPC) (SKETA Assay)

12.4.2.1 The SKETA assay serves as a sample processing and inhibition control. It helps measure extraction efficiency for each sample matrix. Salmon DNA is added to the extraction buffer and each sample is analyzed for

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both the GFD and SKETA assays. The SKETA assay measures the amount of recovery of Salmon DNA and therefore the efficiency of the extraction process. Low recovery indicates an inefficient extraction process or the presence of inhibition. This control is reported as a surrogate.

12.4.2.2 Analyze each sample, replicate, and extraction control with the SKETA assay alongside the GFD assay. See Figure 15.1 for plate layout details.

12.4.2.3 Compare extraction control Salmon DNA recovery to the Salmon DNA recovery in each of the samples. If sample recovery is < 50% of the extraction control recovery, then the sample may be inhibited.

12.4.3. Inhibition control (3rd well spike)

12.4.3.1. A known amount of GFD gBlock standard is added to each sample as a matrix spike to check for inhibition and DNA amplification. For each sample and replicate, spike the 3rd well of the GFD assay with 1 µL of E4 GFD gBlock spike standard. Also add 1 µL of the standard to the spike control wells.

12.4.3.2. 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.

12.4.4. SPC and 3rd well spike inhibition

12.4.4.1 If both the SPC and 3rd well spike show inhibition, re-run the sample from a diluted or purified extract. The purification kit may remove inhibitors. See SOP PCR-4.2.

12.4.4.2. If only one of the two controls show possible inhibition, the sample may need to be purified or diluted and re-analyzed. Notify supervisor.

12.5. Replicate Sample

12.5.1. Analyze one replicate per filtration batch (maximum of 13 per batch). Filter and analyze replicate in the same manner as original sample and within the same batch. Replicate samples are numbered SAMPLE ID_DUP_A and SAMPLE ID_DUP_B and 100 mL is filtered for each.

12.5.2. The target replicate relative percent different (RPD) is ≤ 25%. If RPD > 25% the duplicate should be “J” qualified. A comment regarding precision is automatically added to the report.

12.5.3. If a replicate sample is not run, all samples in the batch should have the comment “Precision data unavailable for Analysis Batch#_____” added to the results.

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12.6. 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. Green HC, Dick LK, Gilpin B, Samadpour M, Field KG. Genetic markers for rapid PCR-based identification of gull, Canada goose, duck, and chicken fecal contamination in water. Appl Environ Microbiol. 2012 Jan;78(2):503-10.

14.2. Weller, Belias, Green, Roof and Wiedmann. 2020. Landscape, water quality and weather factors associated with an increased likelihood of foodborne pathogen contamination of New York streams used to source water for produce production. Front Sustain Food Syst. 2020 Feb;3:124

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

14.4. Quanta BioSciences PerfeCta® qPCR ToughMix® with ROX™ Protocol

14.5. DEP Laboratory Quality Manual

14.6. 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 BB-36, *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*

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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 DNeasy PowerSoil Pro 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*

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15. TABLES AND DIAGRAMS

Table 15.1– Primer/Probe Mix Summary

	GFD Assay	SKETA Assay
Forward Primer (250µM stock)	3.2 µL	10 µL
Reverse Primer (250µM stock)	3.2 µL	10 µL
Probe (100µM stock)	3.2 µL	9 µL
Sterile H_2O	590.4 µL	571 µL

Table 15.2 – Master Mix Summary

	GFD Assay		SKETA Assay	
Reagent	Volume per reaction (µL)	Volume (µL) per full plate (68 + 4 extra wells)	Volume per reaction (µL)	Volume (µL) per full plate (28 + 3 extra wells)
BSA 2 mg/mL	2	144	2	62
ToughMix®	10	720	10	310
Primer/Probe Mix	3	216	3	93

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

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Figure 15.1 - Plate Layout Map

	1	2	3	4	5	6	7	8	9	10	11	12	
A	STD Curve E6	STD Curve E6	STD Curve E6	MBLK_	EXBLK_	LFB_GDSPK	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	A
B	STD Curve E5	STD Curve E5	STD Curve E5	MBLK_	EXBLK_	LFB_GDSPK	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	B
C	STD Curve E4	STD Curve E4	STD Curve E4	NTC_1	NTC_2	NTC_3	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	C
D	STD Curve E3	STD Curve E3	STD Curve E3	SURNTC_1	SURNTC_2	SURNTC_3	Sample1_ SUR_	Sample2_ SUR_	Sample3_ SUR_	Sample4_ SUR_	Sample5_ SUR_	Sample6_ SUR_	D
E	STD Curve E2	STD Curve E2	STD Curve E2	LFB_GDLFB	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	E
F	STD Curve E3	STD Curve E3	STD Curve E3	LFB_GDLFB	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	F
G	STD Curve E2	STD Curve E2	STD Curve E2	SUR_GDLFB	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	_SPK_GDSPK	G
H	STD Curve E1	STD Curve E1	STD Curve E1	SUR_GDLFB	Sample7_ SUR_	Sample8_ SUR_	Sample9_ SUR_	Sample10_ SUR_	Sample11_ SUR_	Sample12_ SUR_	Sample13_ SUR_	Sample14_ SUR_	H
	1	2	3	4	5	6	7	8	9	10	11	12	

*GFD Assay (green background); SKETA Assay (pink background)

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Appendix of Changes

11/30/2020 Added changes from SYBR qPCR assay to TaqMan qPCR methodology.

10/15/2021 Added Salmon DNA and Extraction Buffer to Reagents and Standards.

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