# Standard Operating Procedure for:

## GULL-ASSOCIATED GULL2 MARKER BY QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) ASSAY

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## 1. SCOPE AND APPLICATION

1.1 This method describes a quantitative polymerase chain reaction (qPCR) procedure that targets a sequence in the 16S rRNA gene found in the bacterial species *Catellicoccus marimammalium* commonly associated with seagull feces. *C. marimammalium* is also found in the feces of pelicans, coastal pigeons, and other coastal birds.

1.2 This procedure is used for samples with LIMS test ID of PCR-GULL2, PUR-GULL2, PCR-GULL2B, and PCR-GULL2S.

## 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 the 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 detection in a thermocycler system using a primer/probe assay specific to gull-associated *C. marimammalium*.

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 or probes 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/probe in each PCR cycle. The primers/probe set is designed to attach specifically to this sequence.

2.3.3 *Catellicoccus marimammalium*: A species of facultative anaerobic, Gram-positive, non-spore forming, cocci bacteria that are typically found in the gastrointestinal tract of seagulls. *C. marimammalium* has also been found in the feces of pelicans and other coastal birds.

2.3.4 Standard Curve: A dilution series of standards with known DNA concentrations is analyzed and plotted on a graph with the known concentrations on the x-axis and the quantitation cycle ($C_q$) 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.


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## 3. EQUIPMENT AND SUPPLIES

3.1 Gloves, latex or nitriles

3.2 Tube racks

3.3 Centrifuge tubes, 0.5, 1.5, and 2.0 mL

3.4 Micropipettes with sterile PCR grade filter tips

3.5 96-well Reaction Plate, Clear

3.6 96-well plate holders and stands

3.7 Optical adhesive Seal for reaction plates

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

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

3.10 Fisher Mini Vortexers

3.11 Fisher Minicentrifuges

3.12 Thermo Sorval T3 Centrifuge (S/N 309020018)

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

3.14 PCR#9, Thermo Refrigerator Isotemp (S/N TR15622511012), maintained at 4ºC ± 2ºC, Room B246A

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

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

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

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

3.19 PCR#8, Thermo Revco Freezer (S/N 11270188012206030) maintained at -80ºC ±10ºC, Room B246

3.20 Autoclave (S/N 031761851)

## 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. Number: MT46000CV).

4.3 Bleach solution, 10% (v/v)

4.4. Ethanol 80% (v/v)


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

4.6 PerfeCta® qPCR ToughMix® with ROX™

4.7 Prepare the following per SOP PCR-3.1.

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

4.7.2 Salmon DNA/Extraction Buffer

4.7.3 Salmon DNA Forward and Reverse Primers, 500 µM Stock

4.7.4 Salmon DNA Probe, 100 µM stock

4.8 Prepare the following per SOP PCR-3.3.

4.8.1 Gull2 gBlock Standards ($10^6$, $10^5$, $10^4$, $10^3$, $10^2$)

4.8.2 Gull2 gBlock Standard spikes ($10^6$, $10^4$)

4.8.3 Gull2 primers 500 µM stock

4.8.3.1 Forward Primer Gull2For: 5'TGCATCGACCTAAAGTTTTGAG

4.8.3.2 Reverse Primer Gull2Rev: 5'GTCAAAGAGCGAGCAGTTACTA

4.8.4 Gull2 Probe 100 µM stock

4.8.4.1 Gull2Probe: /56-FAM/CT GAG AGG G/ZEN/T GAT CGG CCA CAT TGG GAC T/3IABkFQ/

## 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 See 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 Ethanol 80%. 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.


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## 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 GULL2) separately as follows. Record details in Reaction Plate Preparation Worksheet. See SOP PCR-5.0 Section 7 for details.

8.1.1 GULL2 Assay: Add 7 µL of each 500 µM primer (forward and reverse) stock and 12 µL of 100 µM probe to 574 µL of PCR grade water to make primer/probe mix. Use a yellow 1.5 mL centrifuge tube labeled "PP" for the primer/probe mix.

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.

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 – In PCR Workstation #1, prepare Master Mix for each assay (SKETA and GULL2) separately as follows:

8.2.1 Count the number of wells needed for each assay. Enter the number of wells in the reaction plate worksheet under the Master Mix Preparation section under '#Rxns' cell. The calculator adds an additional 3-4 extra wells to ensure enough Master Mix is available as some can be lost in mixing and pipetting.

8.2.2 For each assay add 10 µL X total wells of the ToughMix®, 2 µL X total wells of BSA, 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 yellow 1.5 mL centrifuge tube labeled "MM" for the GULL2 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 appropriate qPCR Master Mix to each well of a reaction plate using the 100 µL electronic Xplorer Plus pipette. Use Figure 15.1 as a guide. Add the GULL2 Master Mix to the yellow wells shown in Figure 15.1. Add the SKETA Master Mix to all the pink wells in Figure 15.1. Place the Master Mix, BSA, and any extra labeled PP mix back in Thermo Refrigerator PCR-B246A. Carefully move the plate to PCR workstation #2.


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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. If many bubbles form in the tube when pipetting, tap the tube to remix followed by a pulse spin.

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 15.1 as a guide. Tap to mix, then briefly centrifuge each tube immediately before dispensing liquid. Switch tips for each sample or control. If any liquid falls outside of the well or sprays the plate must be discarded and the hood thoroughly cleaned. Use the following order for addition of the template to wells:

9.2.1 Add the GULL2 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 PCR grade water for the No Template Controls (NTC, SURNTC) and third well spike control (LFB_GPSPK) to wells A6, B6, and C4-D6. See Sections 12.3.1 and 12.4.3.

9.2.4 Add 1 µL of the GULL2 gBlock spike standard (LFB_GPSPK, and _SPK_GPSPK) to the spike control and third well samples in wells A6-B6, C7-C12, and G5-G12. Use Figure 15.1 as a guide. See Section 12.4.3.

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

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

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

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 GULL2 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 the reaction mix is at the bottom of each well. If not, centrifuge for another minute.

## 10. SAMPLE ANALYSIS

10.1 Place plate in 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).


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## 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 GULL2 Assay Standard Curves

12.1.1 Analyze a GULL2 Assay standard curve per plate. Use 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. 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 85-115% and $R^2$ should be ≥ 0.99. If efficiency is < 70% or $R^2$ is < 0.97, samples should be re-analyzed. Notify supervisor.

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. Samples are not affected if the efficiency or $R^2$ 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 GULL2 NTCs are labeled NTC_1, NTC_2, and NTC_3. The SKETA NTCs are labeled SURNTC_1, SURNTC_2, and SURNTC_3. Use Figure 15.1 as a guide.

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_#

12.3.2.1 Analyze a method blank for each batch of samples filtered (maximum of 13 samples per batch). Filter 50 mL of sterile PBS at the beginning of


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each sample batch. Extract and analyze with the same batch of samples. Add 5 µL of the blank extract to duplicate wells containing 15 µL GULL2 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 GULL2 master mix.

12.3.3.2 If the EB has a result that is less than 35 Cq, there may be possible contamination at the extraction step. Samples may be affected and should be re-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 extracted (maximum of 13 per batch). Place a sterile filter in an extraction bead tube labeled EC. Pipette 5 µL of $10^6$ GULL2 gBlock standard onto the filter and add 595 µL of salmon buffer.

12.4.1.2 The GULL2 assay EC is labeled LFB_GPLFB#. The SKETA assay EC is labeled SUR_GPLFB#. Use Figure 15.1 as a guide.

12.4.1.3 The extraction control helps determine extraction recovery. Percent recovery of the extraction control for both the GULL2 and SKETA assay should 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 both the GULL2 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.


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12.4.2.2 Analyze each sample, replicate, and extraction control with the SKETA assay alongside the GULL2 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 (third well spike)

12.4.3.1 A known amount of GULL2 DNA standard is added to each sample as a matrix spike to check for inhibition and DNA amplification. For each sample and replicate, spike the third well of the GULL2 assay with 1 µL of E4 GULL2 gBlock spike standard. Also, add 1µL 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, the sample matrix may have inhibitory effects.

12.4.4 If both the SPC and third 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.

12.4.4.1 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.

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.


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## 14. REFERENCES

14.1 Lu, J., Santo Domingo, J.W., Lamendella, R., Edge, T., Hill, S., 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Applied and Environmental Microbiology 74 (13), 3969-3976.

14.2 Sinigalliano, C.D., Fisher, J.M., Gidley, M.L., et.al, 2010. Traditional and molecular analyses for fecal indicator bacteria in non-point source subtropical recreational marine waters. Water Research 44, 3763-3772.

14.3 SIPP SOP for qPCR Gull-2 assay, received from NOAA-AOML.

14.4 2009 TNI Standard

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

14.6 Quanta BioSciences PerfeCta® qPCR ToughMix® with ROX™ Protocol

14.7 DEP Laboratory Quality Manual

14.8 Health and Safety Plan

14.9 DEP SOP FS 2000, *General Water Sampling*

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 and Artel® Pipette Tracker™*

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-3.3, *Preparation of GULL2 Assay Standards and Reagents for qPCR analysis*

14.15 DEP SOP PCR-4.0, *Preparation of Samples for qPCR Analysis*

14.16 DEP SOP PCR-4.1, *Crude Lysate Extraction Method for qPCR Analysis*

14.17 DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen DNeasy PowerSoil Pro Kit*

14.18 DEP SOP PCR-5.0, *Sample Custody, Preparation Labels, and Worksheet Instructions for Molecular Biology*

14.19 DEP SOP PCR-5.1, *Data upload, Verification, and Sample Authorization for Molecular Biology*


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## 15. TABLES AND DIAGRAMS

**Table 15.1**– Primer/Probe Mix Summary

| | GULL2 Assay | SKETA Assay |
|---|---|---|
| Forward Primer (500µM stock) | 7 µL | 10 µL |
| Reverse Primer (500µM stock) | 7 µL | 10 µL |
| Probe (100µM stock) | 12 µL | 9 µL |
| Sterile $H_2O$ | 574 µL | 571 µL |

**Table 15.2** – Master Mix Summary

| | GULL2 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 | |
|---|---|---|---|
| | Enzyme Activation | Cycle (40 cycles) | |
| Step | 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_GPSPK | 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_GPSPK | 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_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | 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 100 | STD Curve 100 | STD Curve 100 | LFB_GPLFB | 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_GPLFB | Rep 2 | Rep 2 | Rep 2 | Rep 2 | Rep 2 | Rep 2 | Rep 2 | Rep 2 | F |
| G | STD Curve 100 | STD Curve 100 | STD Curve 100 | SUR_GPLFB | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | _SPK_GPSPK | G |
| H | STD Curve 10 | STD Curve 10 | STD Curve 10 | SUR_GPLFB | 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 | |

* GULL2 Assay (yellow background); SKETA Assay (pink background)


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## Appendix of Changes

10/12/2016 Updated S/N for new thermocycler. Added new gblock and probe info.

11/17/2016 Updated links.

11/15/2018 Updated links.

10/13/2021 Updated links.


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