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 qPCR procedure that targets a specific gene 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, PCR-GULL2B, and PCR-GULL2S.

2. SUMMARY OF METHOD

2.1 A water sample is filtered through a membrane filter. The filter containing the bacteria is agitated in a centrifuge tube containing zirconium silica beads using a bead beater 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 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. 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 series is developed 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 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.

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

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

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

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

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

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

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

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

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4.5 PerfeCta® qPCR ToughMix® with ROX™

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

4.6.3 Salmon DNA Forward and Reverse Primers, 500 µM Stock

4.6.4 Salmon DNA Probe, 100 µM stock

4.7 Prepare the following per SOP PCR-3.3.

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

4.7.2 Gull2 gBlock Standard spikes (10^6, 10^4)

4.7.3 Gull2 primers 500 µM stock

4.7.3.1 Forward Primer Gull2For: 5'TGCATCGACCTAAAGTTTTGAG

4.7.3.2 Reverse Primer Gull2Rev: 5'GTCAAAGAGCGAGCAGTTACTA

4.7.4 Gull2 Probe 100 µM stock

4.7.4.1 Gull2Probe: 5'-/56-FAM/CTG AGA GGG /ZEN/TGA TCG GCC ACA TTG GGA CT/3IABkFQ/ -3'

5. CALIBRATION AND STANDARDIZATION

5.1 See DEP Laboratory Quality Manual, Section 5.5.

5.2 Check temperatures of refrigerators and freezers twice daily at least 4 hours apart. Use the Checkpoint system to create monthly PDF files of temperature check.

5.3 Check thermometers annually against a NIST certified thermometer. Record in LIMS THERMO_CAL_LOG logbook.

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

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

5.6 See 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.

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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 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:

8.1.1 GULL2 Assay: Add 7 µL of 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 and analyst initials. 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. Add 3-4 extra wells to ensure enough Master Mix is available (some can be lost in mixing and pipetting) to determine a total number of wells per assay.

8.2.2 For each assay add 10 µL X total wells of PerfeCta® qPCR ToughMix® with ROX™, 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 In PCR workstation #1, add 15 µL of appropriate qPCR Master Mix assay to each well of a reaction plate using the 100µL electronic Xplorer pipette. Use Figure 15.4 as a guide. Add the GULL2 Master Mix to the yellow wells shown in Figure 15.4. Add the SKETA Master Mix to all the orange wells in Figure 15.4. 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.

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. Tap to mix, then briefly centrifuge each tube immediately before dispensing liquid. Do not overmix, as this can degrade the DNA and decrease amplification. 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 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 3rd well spike control (LFB_GPSPK) to wells A6, B6, and C4-D6. See Section 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 3rd well samples in wells A6-B6, C7-C12, and G5-G12. Use Figure 15.4 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 15.4 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 all of the reaction mix is at the bottom of each well and no bubbles are present. If not, centrifuge for another minute.

10. SAMPLE ANALYSIS

10.1 Place plate in thermocycler and analyze. Refer to SOP PCR-2.5 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-2.5 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 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 17.4 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 17.4 as a guide

12.2.2 Standard curve efficiency should be between 75-120% 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 bring up to final reaction volume with 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.

12.3.1.3 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.

12.3.2 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 each sample batch. Extract and analyze with the same batch of samples.

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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 tube that is labeled EB. Add 600 µL of salmon DNA extraction buffer and extract along with the samples in the batch. Add 5 µL of the blank extract to duplicate wells containing 15 µL GULL2 master mix.

12.3.3.2 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-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 tube that is labeled EC. Pipette 5 µL of 10^6 GULL2 gBlock standard into the filter and add 595 µL of salmon DNA extraction buffer.

12.4.1.2 The GULL2 assay EC is labeled LFB_GPLFB#. The SKETA assay EC is labeled SUR_GPLFB#.

12.4.1.3 The extraction control helps determine extraction recovery. Percent recovery of 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 used to extract each sample and controls. Each sample is analyzed for the GULL2 Assay and the SKETA assay. The SKETA assay measures the amount of recovery of Salmon DNA and therefore the efficiency of the extraction process. If there is low recovery, then the extraction process was not efficient in bringing the DNA into solution or the sample contained inhibitors that yielded low recovery of DNA. 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 17.4 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, or has >3 C_t difference, then the sample may be inhibited. If the extraction control recovery is <50% of the expected yield, the extraction yield may be low for the batch.

12.4.3 Inhibition control (3rd well spike)

12.4.3.1 A known amount of GULL2 standard is added to each sample as a matrix spike to check for inhibition and DNA amplification.

12.4.3.2 For each sample and replicate, spike the 3rd 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.

12.4.3.3 Compare spike control recovery to sample matrix recovery. If sample recovery is <50% of the control recovery or >3 C_t difference, then the sample matrix may have inhibitory effects.

12.4.4 If both the SPC and 3rd well spike show inhibition, purify the sample and run under the PUR-GULL2 test code. The purification kit may remove inhibitors.

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 is analyzed and within the same batch. The replicates are labeled “DUP_A” and “DUP_B”.

12.5.2 Replicate relative percentage difference (RPD) should be <=25%. If RPD > 25%, it indicates a precision failure.

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 PowerLyzer PowerSoil DNA Isolation 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 15.4 – Plate Layout Map



	1	2	3	4	5	6	7	8	9	10	11	12	
A	STD Curve 1E5	STD Curve 1E5	STD Curve 1E5	MBLK_	EXBLK_	LFB_G2SPK	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	A
B	STD Curve 1E4	STD Curve 1E4	STD Curve 1E4	MBLK_	EXBLK_	LFB_G2SPK	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	B
C	STD Curve 1E3	STD Curve 1E3	STD Curve 1E3	NTC_1	NTC_2	NTC_3	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	C
D	STD Curve 100	STD Curve 100	STD Curve 100	SURNTC_1	SURNTC_2	SURNTC_3	Sample1_ SUR_	Sample2_ SUR_	Sample3_ SUR_	Sample4_ SUR_	Sample5_ SUR_	Sample6_ SUR_	D
E	STD Curve 10	STD Curve 10	STD Curve 10	LFB_G2LFB	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	E
F	STD Curve 1E3	STD Curve 1E3	STD Curve 1E3	LFB_G2LFB	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_G2LFB	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	_SPK_G2SPK	G
H	STD Curve 10	STD Curve 10	STD Curve 10	SUR_G2LFB	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 (orange 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.

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