CANINE-ASSOCIATED *BACTEROIDALES* DG3 MARKER BY QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) ASSAY

1. SCOPE AND APPLICATION

1.1. This method describes a quantitative Polymerase Chain Reaction (qPCR) procedure that targets a non-ribosomal genetic marker found in members of the phylum *Bacteroidetes*. It is used to detect canine fecal contamination.

1.2. This procedure is used for samples with LIMS test ID of PCR-DG3, PCR-DG3B, PCR-DG3S, and PUR-DG3.

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 detection in a thermocycler system using a primer/probe assay set specific to canine-associated Bacteroidetes. Solid samples are weighed and purified prior to qPCR amplification and detection.

2.2. Interferences: 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 primers/probe set is designed to attach specifically to this sequence.

2.3.3. *Bacteroidetes*: A phylum of anaerobic bacteria that is highly abundant in feces.

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 quantification cycle (C_q) value of each dilution on the y-axis to create a standard curve. This is used to determine the concentration of unknown samples and the performance of the reaction.

3. EQUIPMENT AND SUPPLIES

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3.1. Instrumentation –

Instrument	Serial Number	Manufacturer
QuantStudio3 Thermocycler	272311740	Applied Biosystems
PCR3- Step One Plus Thermocycler	2720010389	Applied Biosystems

3.2. Equipment –

Equipment Name	Notes
Tube Racks	N/A
Micropipettes	N/A
96-Well Plate Holders and Stands	N/A
Autoclave	S/N 031761851
Mettler Toledo MS403TS/00 Balance	S/N B815673069
CBS Optimizer PCR Workstation #1	S/N 007631
CBS Optimizer PCR Workstation #2	S/N 007632
Fisher Mini Vortexers	N/A
Fisher Microcentrifuges	N/A
Thermo Sorval T3 Centrifuge	S/N 309020018
PCR#9, Thermo Refrigerator Isotemp	S/N TR15622511012; Maintained at 4 ± 2°C, Room B246A
PCR#2, Thermo Freezer	S/N 0121174101121218; Maintained at -20 ± 10°C, Room B246A
PCR#4, Thermo Freezer	S/N 0121174201121218; Maintained at -20 ± 10°C, Room B246B
PCR#5, Thermo Fridge/Freezer	S/N 0121174701121218; Maintained at 4 ± 2°C and -20 ± 10°C, Room B246
PCR#7, Thermo Ultra Low Freezer	S/N 128473401090813; Maintained at -80 ± 10°C, Room B246
PCR#10, Thermo TDE Freezer	S/N S/N 1127920701221013; Maintained at -80 ±10°C, Room B246
Or Equivalent	

3.3. Supplies/Consumables –

Supply Name	Notes
Gloves, Latex or Nitriles	N/A
Dispersion Beads, 0.1 mm, Zirconium Silica	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 at 121°C for 15 min (15 psi). Record in LIMS Autoclave Log.
Extraction Tubs, Conical, 2 mL Screw Cap with O-Ring Seal	
Centrifuge Tubes, 0.5, 1.5, and 2.0 mL	N/A
Sterile PCR Grade Filter Tips	N/A
96-Well Reaction Plate, Clear	N/A
Optical Adhesive Seal for Reaction Plates	N/A

4. REAGENTS AND STANDARDS

4.1. Reagents and Standards –

Reagent/Standard Name	Notes
Deionized (DI) Water	N/A
Molecular Biology Grade Water	Corning Molecular Biology Grade Water tested to USP Sterile Water (Catalog Number: MT46000CV)
Bleach Solution, 10% (v/v)	N/A
Ethanol, 80% (v/v)	N/A
Bovine Serum Albumin (BSA), 2 mg/mL	Prepare per SOP PCR-3.0
PerfeCta® qPCR ToughMix™, ROX™	Used with the StepOne Plus Thermocycler
TaqMan™ Environmental Master Mix 2.0	Used with the QuantStudio3 Thermocycler

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Salmon DNA Standards (10^3, 10^2, 10^1)	Prepare per SOP PCR-3.1
Salmon DNA Extraction Buffer, 0.2 µg/mL	
Salmon DNA Forward and Reverse Primers, 500 µM Stock	
Salmon DNA Probe, 100 µM stock	
BRD3 gBlock Standards (10^7, 10^6, 10^5, 10^4, 10^3)	Prepare per SOP PCR-3.6
BRD3 gBlock spikes (10^7, 10^5)	
DG3 primers (Stock concentration: 500 µM each)	**Forward Primer DG3For**: 5'-TGAGCGGGCATGGTCATATT– 3' **Reverse Primer DG3Rev**: 5'-TTTTCAGCCCCGTTGTTTCG– 3' Prepare per SOP PCR-3.6
DG3 Probe 100 µM stock	**DG3Probe**: 5'-[6-FAM]AGTCTACGCGGGCGTACT[MGBE-Q]–3' Prepare per SOP PCR-3.6

5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5.

5.2. Check the calibration of thermometers annually. See SOP LB-035.

5.3. Check the 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 the thermocyclers.

5.5. Refer to SOP BB-035 for calibration, use, and maintenance of the balance.

5.6. Sterilization of the workstation:

5.6.1. UV workstation: After each use , wipe down all surfaces with 10% bleach, then PCR grade water. Turn on UV lamps for at least 3 minutes. Wipe metallic surfaces with 80% ethanol weekly to prevent corrosion. Never mix bleach and ethanol.

5.6.2. Counters: 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 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. Prior to analysis, samples are filtered (per SOP PCR-4.0) and extracted (per SOP PCR-4.1). Inhibited or solid samples are purified according to SOP PCR-4.2.

8. ASSAY PREPARATION

NOTES: Primer/Probe Mix, Master Mix, and Standard preparation for each plate is documented in the PCR-PLATEPREP form. These are saved at \\Floridadep\data\DEAR\Labs\Lab3\Biology\qPCR\RxnPlatePrepWS. The Primer/Probe Mix and Master Mix are prepared in PCR Workstation #1.

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8.1. Primer/Probe Mix Preparation–

8.1.1. DG3 Assay:

Forward Primer, 500 µM	11.2 µL
Reverse Primer, 500 µM	11.2 µL
Probe, 100 µM	4 µL
PCR Grade Water	573.6 µL

In a purple 1.5 µL centrifuge tube, add 11.2 µL of each primer stock and 4 µL of probe to 573.6 µL of PCR grade water to make the primer/probe mix. Mix by tapping then centrifuge briefly.

Label with the date, assay and "PP" and store at 4°C. Use within one week.

8.1.2. SKETA Assay:

Forward Primer, 500 µM	10 µL
Reverse Primer, 500 µM	10 µL
Probe, 100 µM	9 µL
PCR Grade Water	571 µL

In a pink 1.5 mL centrifuge tube, add 10 µL of each primer and 9 µL of probe to 571 µL of PCR grade water to make the primer/probe mix. Mix by tapping then centrifuge briefly.

Label with the date, assay and "PP" and store at 4°C. Use within one week.

8.1.3. Primer/Probe Summary –

	DG3 Assay	SKETA Assay
Forward Primer (500µM stock)	11.2 µL	10 µL
Reverse Primer (500µM stock)	11.2 µL	10 µL
Probe (100µM stock)	4 µL	9 µL
Sterile H_2O	573.6 µL	571 µL

8.2. qPCR Master Mix Preparation Preparation –

8.2.1. Count the number of wells to determine the total volume of reagents needed for the assay. Add an additional 3-4 wells to ensure enough Master Mix is available, as some can be lost in mixing and pipetting. Enter the number of wells in the '#Rxns' column in the Master Mix Preparation section of the reaction plate worksheet.

8.2.2. For each assay, add 10 µL X total wells of PerfeCta® qPCR ToughMix® with ROX™ for the Step One Plus or TaqMan™ Environmental Master Mix 2.0 for the QuantStudio3, 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 for the SKETA Master Mix and a purple 1.5 mL centrifuge tube for the DG3 Master Mix.Label the tubes 'MM"

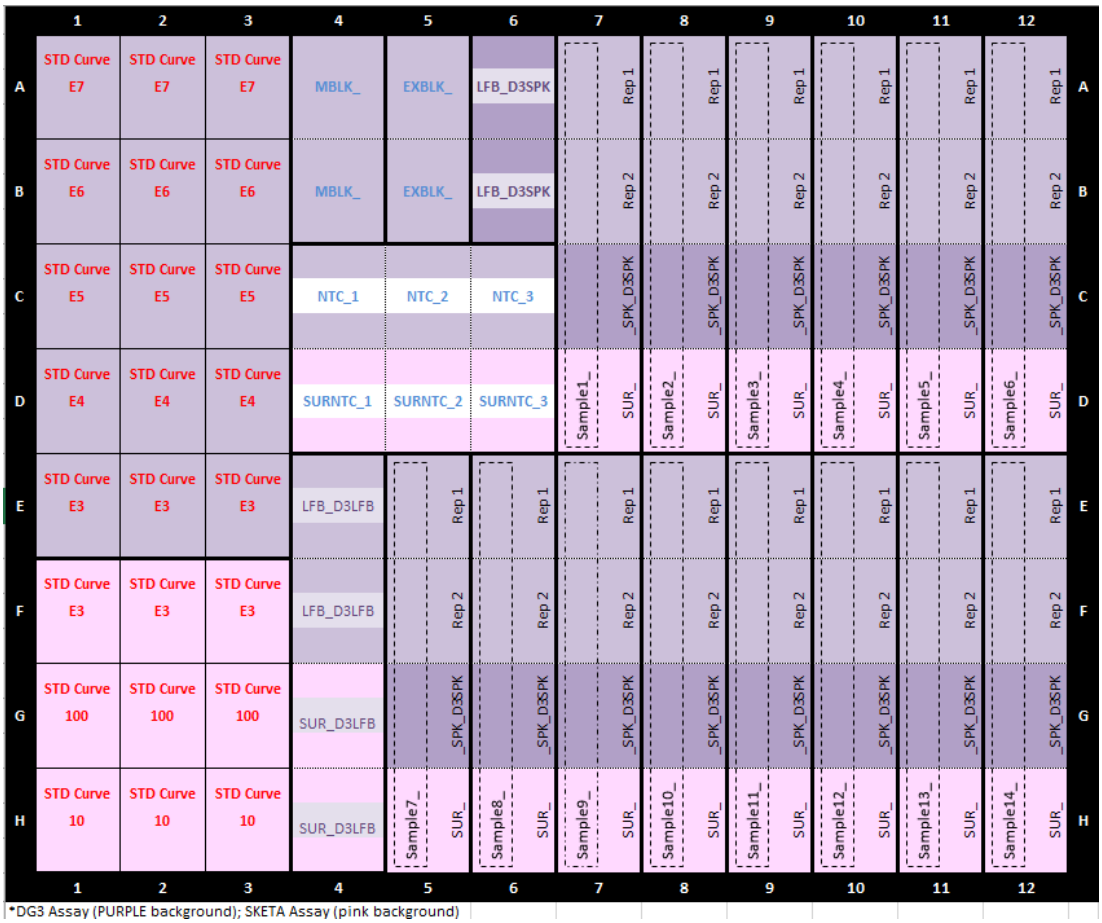
8.2.3. Master Mix Summary –

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Reagent	DG3 Assay		SKETA Assay	
	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 MM	10	720	10	310
Primer/Probe Mix	3	216	3	93

9. REACTION PLATE PREPARATION

9.1. Plate Layout Map –



*DG3 Assay (PURPLE background); SKETA Assay (pink background)

9.2. Collect a 96 well plate and holder. In PCR workstation #1, add 15 µL of the appropriate qPCR Master Mix to each well of a reaction plate using the electronic Xplorer Plus pipette. Use the figure in Section 9.1. as a guide. Add the DG3 Master Mix to the purple wells and add the SKETA Master Mix to all the pink wells. Place the Master Mix, BSA, and any extra labeled PP mix back in PCR#9 - refrigerator. Carefully move the plate to PCR workstation #2.

9.2.1. Make sure to re-mix the Master Mix occasionally by tapping the tube, followed by a pulse spin in the minicentrifuge. This ensures that the Master Mix is evenly distributed throughout the plate.

9.2.2. 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.

9.3. In PCR workstation #2, add 5 µL of DNA sample extract or control to each well using a 10 µL pipette. Use the figure in Section 9.1. 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 hood thoroughly cleaned. Use the following order for addition of the template to wells:

9.3.1. Add the DG3 Standard Curve controls (12.1.) to wells A1-E3.

9.3.2. Add the SKETA Standard Curve controls (12.2.) to wells F1-H3.

9.3.3. Add the method blank (MBLK) control (12.3.2.) to wells A4-B4.

9.3.4. Add the extraction blank (EXBLK) control (12.3.3.) to wells A5-B5.

9.3.5. Add PCR grade water for the No Template Controls (NTC, SURNTC; 12.3.1.) and 3rd well spike control (LFB_D3SPK; 12.4.3.) to wells A6, B6, and C4-D6.

9.3.6. Add the extraction control (LFB_D3LFB, SUR_D3LFB; 12.4.1.) to wells E4-H4.

9.3.7. Add 1 µL of the DG3 spike standard (LFB_D3SPK, and _SPK_D3SPK; 12.4.3.) to the spike control and 3rd samples in wells A6-B6, C7-C12, and G5-G12.

9.3.8. Add the sample extracts to appropriate wells (4 wells per sample). Add the sample to the three DG3 wells first, change tips, then add the sample to the SKETA well.

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

9.5. Centrifuge plate at 1300 g for 2 minutes (Program 1 on the MegafugeST4F Plus 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 thermocycler and start run. 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 below).

		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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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. DG3 Assay Standard Curves

12.1.1. Analyze a DG3 Assay standard curve per plate. Run five serially diluted standards in triplicate at the following concentrations: 10^7, 10^6, 10^5, 10^4, and 10^3 target sequence copies (TSC)/5µL. Dilute standards fresh from the 10^7 standard each time in TE buffer. Refer to SOP PCR-3.6 for preparing standard dilutions.

12.1.2. Standard curve target efficiency is 85–110% and R^2 should be ≥0.99. Re-analyze samples and discuss with supervisor if efficiency is <70% or R^2 is <0.97.

12.2. SKETA Assay Standard Curves

12.2.1. Analyze a SKETA Assay standard curve per plate. Run three serially diluted standards in triplicate at the following concentrations: 10^3, 10^2, and 10^1. Refer to SOP PCR-3.1 for preparing standard dilutions.

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. For each Master Mix used on a reaction plate, add 15 µL of Master Mix and 5 µL of sterile water to three wells.

12.3.1.2. The DG3 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 C_q or more than one NTC well amplifies, the reaction plate may have contamination. Re-run if samples are affected and notify a supervisor.

12.3.2. Method Blank (MB) – labeled MBLK_#

12.3.2.1. Analyze a method blank for each batch of samples filtered (max. of 13 samples). Filter 50 mL of sterile PBS at the beginning of each sample batch. Extract and analyze with the same batch of samples. Add 5 µL of the MB extract to duplicate wells containing 15 µL DG3 Master Mix.

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12.3.2.2. If the MB has a result above the MDL, there may be possible sample contamination. Discuss with supervisor or senior analyst. 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 ten times 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 DG3 master mix.

12.3.3.2. If the EB has a result above the MDL, there may be possible contamination at the extraction step. Samples may be affected and should be re-analyzed. Discuss with supervisor or senior analyst.

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 10^7 BRD3 gBlock standard onto the filter and add 595 µL of salmon extraction buffer.

12.4.1.2. The DG3 assay EC is labeled, LFB_D3LFB#. The SKETA assay EC is labeled, SUR_D3LFB#.

12.4.1.3. The extraction control helps determine extraction recovery. Percent recovery of the extraction control for both the DG3 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. Salmon DNA is added to the extraction buffer and each sample is analyzed for both the DG3 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 DG3 assay.

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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 expected recovery, then the sample may be inhibited.

12.4.3. Inhibition control (third well spike)

12.4.3.1. A known amount of DG3 standard is added to each sample as a matrix spike to check for inhibition and DNA recovery.

12.4.3.2. For each sample and replicate, spike the third well of the DG3 assay with 1 µL of E5 BRD3 gBlock spike standard. Also add 1 µL 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, 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 and re-analyzed. Discuss with supervisor or senior analyst.

12.5. Replicate Sample

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

12.5.2. The target replicate Relative Percent Difference (RPD) should be ≤25%. If RPD >25%, the duplicate should be "J" qualified at the job authorization step. 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 DG3-qPCR component" 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.

14. REFERENCES

14.1. PerfeCta® qPCR ToughMix™, Rox™ Protocol

14.2. DEP Laboratory Quality Manual

14.3. Health and Safety Plan

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

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

14.6. DEP SOP BB-035, *Calibration, Use and Maintenance of Mettler Toledo MS403TS/00 Balance*

14.7. DEP SOP PCR-2.3, *Use and calibration of StepOne Plus™ Real-Time PCR System*

14.8. DEP SOP PCR-2.4, *Calibration and Use of Mechanical Pipettes using the Artel® PCS®3 Pipette Calibration System*

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

14.10. DEP SOP PCR-3.1, *Preparation of SKETA assay standards and reagents for qPCR analysis*

14.11. DEP SOP PCR-3.6, *Preparation of DG3 Assay Reagents and Standards for qPCR analysis*

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

14.13. DEP SOP PCR-4.1, *Crude Lysate Extraction Method for qPCR samples*

14.14. DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen DNeasyPowerSoil Pro Kit*

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

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

Appendix of Significant Changes

01/10/2023 Added new refrigerator, freezer in Equipment and Supplies section. Updated link to quality manual 2023. 11/16/2023 Reformatted SOP and added tables to Section 3, 4, 8 and 10.

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