COW-ASSOCIATED COWM2 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 gene in an unknown group of bacteria. It is used to detect bovine (cattle) fecal contamination.

1.2. Tests covered by this SOP are PCR-COWM2 and PUR-COWM2.

2. SUMMARY OF THE METHOD

1.1. 2.1. Bacteria present in a water sample are collected by filtration. The filtered sample is processed using 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 set specific to cow-associated fecal bacteria.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 needed 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:

2.31. qPCR: Quantitative Polymerase Chain Reaction is a procedure in which the amount of DNA amplified in each PCR cycle is measured using fluorophores 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 qPCR cycle. The primers/probe set is designed to attach specifically to this sequence.

2.3.3. 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. The standard curve is used to determine the 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

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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 0317618851
Mettler Precision Balance Model MS403TS/00	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 Minicentrifuges	N/A
Thermo Sorval T3 Centrifuge	S/N 309020018
PCR#9, Thermo Refrigerator Isotemp	S/N TR1562211012; 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 1127920701221013; Maintained at -80 ± 10°C, Room B246
Or Equivalent	

3.3. Supplies and Consumables –

Supply Name	Notes
Gloves, Latex or Nitrile	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 Tubes, 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 Pates	N/A
Or Equivalent	

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)	Prepare per SOP BG-05
Ethanol, 80% (v/v)	N/A

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Bovine Serum Albumin (BSA), 2 mg/mL	Prepare per SOP PCR-3.0
PerfeCta® qPCR ToughMix™, ROX™	Use with the StepOne Plus Thermocycler
TaqMan™ Environmental Master Mix 2.0	Use with the QuantStudio3 Thermocycler
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	
C2H2 gBlock Standards (10^7, 10^6, 10^5, 10^4, 10^3)	Prepare per SOP PCR-3.7
C2H2 gBlock Spikes (10^7, 10^5)	
CowM2 Primers, 500 µM Stock	**CowM2For:** 5'–CGGCCAAATACTCCTGATCGT–3' **CowM2Rev:** 5'–GCTTGTTGCGTTCCTTGAGATAAT–3' Prepare per SOP PCR-3.7
CowM2 Probe, 100 µM Stock	**CowM2Probe:** 5'–/6-FAM/AGGCACCTA/ZEN/TGTCCTTTA CCTCATCAACTACAGACA/3IABkFQ/–3' Prepare per SOP PCR-3.7

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 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 BB-036 for calibration and use 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 with 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 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. 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.

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8. ASSAY PREPARATION

NOTE: 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.

8.1. Primer/Probe Mix Preparation –

8.1.1. CowM2 Assay –

Forward Primer, 500 µM	8 µL
Reverse Primer, 500 µM	8 µL
Probe, 100 µM	3.2 µL
PCR Grade Water	580.8 µL

In a green 1.5 mL centrifuge tube, add 8 µL of each primer stock and 3.2 µL of probe to 580.8 µL of PCR grade water to make primer/probe mix. Mix by tapping then centrifuge briefly.

Label with the date, assay name, 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 stock and 9 µL of probe to 571 µL of PCR grade water to make primer/probe mix. Mix by tapping then centrifuge briefly.

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

8.1.3. Primer/Probe Mix Summary –

	CowM2 Assay	SKETA Assay
Forward Primer (500µM stock)	8 µL	10 µL
Reverse Primer (500µM stock)	8 µL	10 µL
Probe (100µM stock)	3.2 µL	9 µL
Sterile H_2O	580.8 µL	571 µL

8.2. qPCR Master Mix Preparation –

8.2.1. Count the number of wells needed to determine the total volume of reagents needed for the assay. Add an additional 3-4 wells to this total 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.

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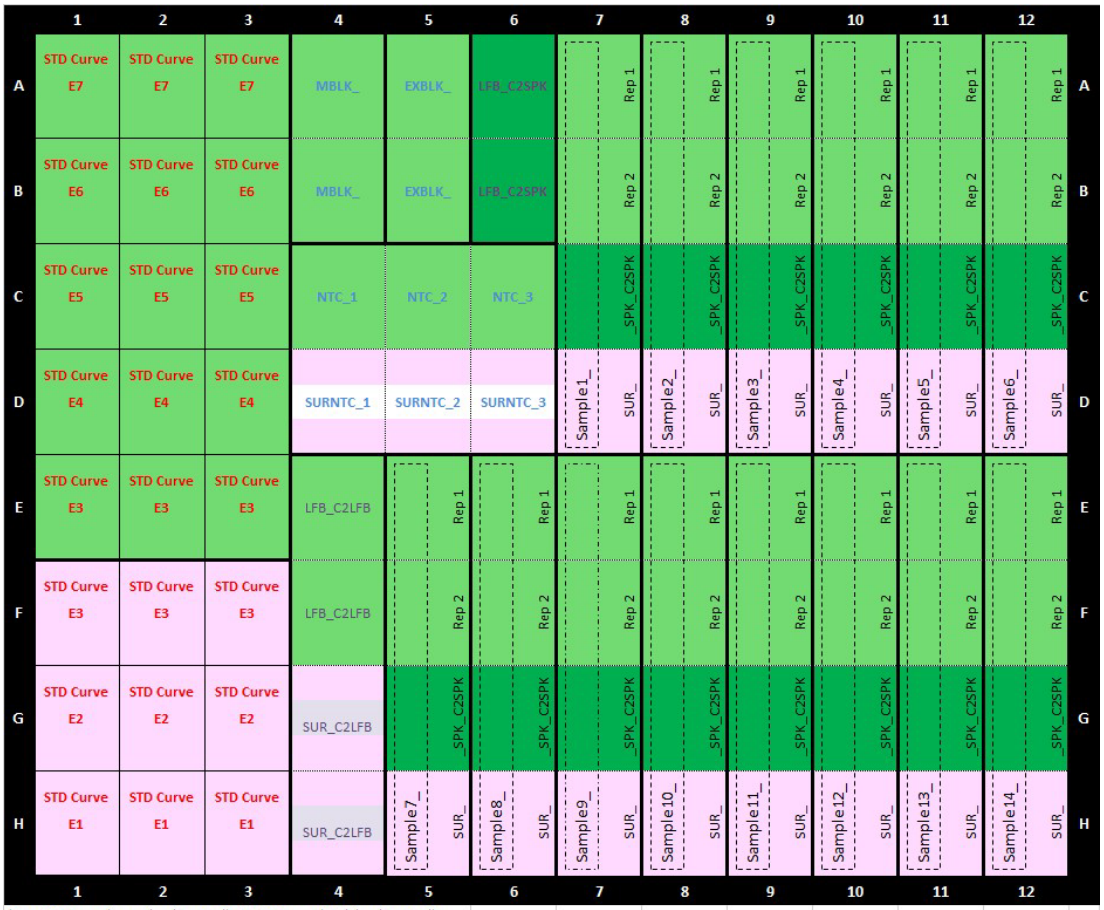
8.2.2. For each assay add 2 µL X total wells of BSA, 10 µL X total wells of PerfeCta® qPCR ToughMix® with ROX™ for the Step One Plus or TaqMan™ Environmental Master Mix 2.0 for the QuantStudio3, and 3 µL X total wells of primer/probe mix (PP) . Use a pink 1.5 mL centrifuge tube for the SKETA Master Mix and a green 1.5 mL centrifuge tube for the CowM2 Master Mix. Label the tubes "MM".

8.2.3. Master Mix Summary –

	CowM2 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 MM	10	720	10	310
Primer/Probe Mix	3	216	3	93
Total of 15 µL of Master Master Mix + 5 µL of DNA template =Total volume of 20 µL per rxn				

9. REACTION PLATE PREPARATION

9.1. Plate Layout Map –



*CowM2 Assay (green background); SKETA Assay (pink background)

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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 CowM2 Master Mix to the green wells and the SKETA Master Mix to the pink wells. Place the Master Mix, BSA, and any extra PP mix back in the PCR#9 refrigerator. Carefully move the plate to PCR workstation #2.

9.2.1. Occasionally re-mix the Master Mix 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.

9.2.2. 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 CowM2 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 control (MBLK; 12.3.2.) to wells A4-B4.

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

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

9.3.6. Add 1 µL of the CowM2 spike standard (LFB_C2SPK and _SPK_C2SPK; 12.4.3.) to the spike control and 3rd well samples, A6-B6, C7-C12, and G5-G12. Then, add 5 µL of PCR grade water to the spike control wells A6-B6.

9.3.7. Add the extraction control (LFB_C2LFB, SUR_C2LFB; 12.4.1.) to wells E4-H4.

9.3.8. Add the sample extracts to appropriate wells (4 wells per sample). Add the sample to the three CowM2 wells first, change tips, and then add the sample to the final 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. If not, centrifuge for another minute.

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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 below).

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

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. CowM2 Assay Standard Curves

12.1.1. Analyze a CowM2 Assay standard curve per plate. Prepare five serially diluted standards at the following concentrations: 10^7, 10^6, 10^5, 10^4, and 10^3 target sequence copies (TSC)/5µL. Analyze each in triplicate.

12.1.2. Standard curve efficiency should be between 85–110% and R^2 should be ≥ 0.99. Reanalyze samples and notify a 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.

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. Label the CowM2 NTCs as NTC_1, NTC_2, and NTC_3. Label the SKETA NTCs as SURNTC_1, SURNTC_2, and SURNTC_3.

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12.3.1.3. If any of the NTCs amplify before 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 (maximum of 13 samples per 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 MB extract to duplicate wells containing 15 µL CowM2 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". No "V" qualifier is needed.

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 samples per batch). Place a sterile filter in an extraction 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 CowM2 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. Reanalyze samples, as they may be affected and notify a 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 samples per batch). Place a sterile filter in an extraction tube labeled EC. Pipette 5 µL of 10^7 CowM2 standard to the filter and add 595 µL of salmon extraction buffer.

12.4.1.2. On the plate, the CowM2 assay EC is labeled LFB_C2LFB#. On the plate, the SKETA assay EC is labeled SUR_C2LFB#.

12.4.1.3. The extraction control helps determine extraction recovery. Percent recovery of extraction control for both the CowM2 and SKETA assays should be 75–150%. If recovery is low, extraction efficiency may be low.

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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 CowM2 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 CowM2 Assay.

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 (3rd well spike)

12.4.3.1. A known amount of CowM2 DNA standard is added to each sample as a matrix spike to check for inhibition and DNA recovery. For each sample and replicate, spike the 3rd well of the CowM2 assay with 1 µL of E5 C2H2 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. 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.

12.4.4.1. If only one of the two controls show possible inhibition, consult a supervisor prior to dilution or reanalysis.

12.5. Replicate Sample

12.5.1. Analyze at least one replicate per filtration batch (maximum of 13 samples per batch). Filter and analyze replicate in the same manner as the original sample and within the same batch. 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 percentage difference (RPD) is ≤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 COWM2-qPCR component” or “Precision data unavailable for COWM2-purified-qPCR component” 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. 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-36, *Calibration, Use, and Maintenance of Mettler Toledo XA105DU Balance*

14.7. DEP SOP PCR-2.3, *Use and calibration of StepOne Plus™ Real-Time PCR System and QuantStudio3™ 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 Reagents and Standards for qPCR Analysis*

14.11. DEP SOP PCR-3.7, *Preparation of CowM2 Assay Reagents and Standards for qPCR Analysis*

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

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

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

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

14.16. DEP SOP PCR-5.1, *LIMS QC Manager Data Upload, Verification and Sample Authorization Instructions for qPCR Samples*

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Appendix of Significant Changes

01/02/2020	Updated autoclave, water, instruments, and master mix
11/29/2021	Updated balance.
12/06/2022	Updated to new fridge and freezer information in Equipment and Supplies.
11/15/2023	Reformatted SOP and added tables to Sections 3, 4, 8, and 10. Removed additional “5” that is not part of the probe sequence.

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