Standard Operating Procedure for:

Human-associated HumM2 marker by quantitative Polymerase Chain Reaction (qPCR) assay

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BIOLOGY SECTION
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1. SCOPE AND APPLICATION

1.1. This method describes a quantitative Polymerase Chain Reaction (qPCR) procedure that targets a single copy chromosomal gene in *Bacteroidales*-like microorganisms to detect human fecal contamination.

1.2. This procedure is used for samples with LIMS Test ID PCR-HUMM2, PCR-HUMM2B, PCR-HUMM2S, and PUR-HUMM2.

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 specific to human-associated fecal bacteria.

2.2. Interferences

2.2.1. 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

2.3.1. 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. This is used to determine the concentration of unknown samples, as well as the performance of the reaction by using several parameters including the slope and correlation of the graph.

3. EQUIPMENT AND SUPPLIES

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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 15 min (15 psi). Record in LIMS Autoclave Log.

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

3.6. Micropipettes with sterile PCR grade filter tips

3.7. 96-well Reaction Plate, clear

3.8. 96-well plate holders and stands

3.9. Optical adhesive Seal for reaction plates

3.10. Autoclave (S/N 031761851)

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

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

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

3.14. Fisher Mini Vortexers

3.15. Fisher Minicentrifuges

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

3.17. QuantStudio3 Thermocycler (S/N 272311740)

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

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

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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 Step One Plus Thermocycler

4.6. TaqMan™ Environmental Master Mix 2.0 to use with QuantStudio3 Thermocycler

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-3.8.

4.8.1. C2H2 gBlock Standards (10^7, 10^6, 10^5, 10^4, 10^3)

4.8.2. C2H2 gBlock spikes (10^7, 10^5)

4.8.3. HumM2 primers (Stock concentration: 500 µM each)

4.8.3.1. Forward HumM2For: 5’- CGT CAG GTT TGT TTC GGT ATT G

4.8.3.2. Reverse HumM2Rev: 5’- TCA TCA CGT AAC TTA TTT ATA TGC ATT AGC

4.8.4. HumM2 Probe 100 µM stock

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4.8.4.1. Probe HumM2Probe: 5'- /56-FAM/TAT CGA AAA /ZEN/TCT CAC GGA TTA ACT CTT GTG TAC GC/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 BB-36 for calibration and use of the balance.

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 extraction.

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

8. ASSAY PREPARATION

8.1. Primers/Probe Mix – In PCR Workstation #1, prepare primer/probe mix for each assay (HumM2 and SKETA) separately as follows:

8.1.1. HumM2 Assay: Add 8 µL of each 500 µM primer (forward and reverse) stock and 3.2 µL of 100 µM probe to 580.8 µL of PCR grade water to make primer/probe mix. Use a purple 1.5 mL centrifuge tube labeled "PP". Mix by tapping then centrifuge briefly.

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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”. 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 – Create a reaction plate worksheet as per SOP PCR-5.0. In PCR Workstation #1, prepare Master Mix for each assay (SKETA and HumM2) 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 wells to ensure enough Master Mix is available, as some 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 the appropriate Master Mix depending on the qPCR platform (PerfeCta® qPCR ToughMix® with ROX™ for the Step One Plus and 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 labeled “MM” for the SKETA Master Mix. Use a purple 1.5 mL centrifuge tube labeled “MM” for the HumM2 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 the appropriate qPCR Master Mix to each well of a reaction plate using the electronic Xplorer pipette with a 100 µL tip. Use Figure 15.1 as a guide. Add the HumM2 Master Mix to the purple wells. Add the SKETA Master Mix to the pink wells. Place the Master Mix, BSA, and any labeled extra PP mix back in Thermo Refrigerator B246A. Carefully move the plate to PCR workstation #2.

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

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.

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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. To ensure proper mixing, tap to 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 hood thoroughly cleaned. Use the following order for addition of the template to wells:

9.2.1. Add the HumM2 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_H2SPK) to wells A6, B6, and C4-D6. See Section 12.3.1 and 12.4.3.

9.2.4. Add 1 µL of the HumM2 spike standard (LFB_H2SPK, and _SPK_H2SPK) to the spike control andthird 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_H2LFB, SUR_H2LFB) 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 HumM2 wells first, change tips, then add the sample to the 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. 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 StepOnePlus 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. HumM2 Assay Standard Curves

12.1.1. Analyze a HumM2 Assay standard curve per plate. Use 5 serially diluted standards at 10^7, 10^6, 10^5, 10^4, and 10^3 concentrations 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 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 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 HumM2 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 amplify before 35 Cq or more than one NTC well amplifies, the reaction plate may have contamination and should be re-run if samples are affected. Discuss with supervisor or senior analyst.

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. Add 5 µL of the blank extract to duplicate wells containing 15 µL HumM2 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. 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 tube labeled EB. Add 600 µL of salmon buffer and extract along with the samples. Add 5 µL of the blank extract to duplicate wells containing 15 µL HumM2 master mix.

12.3.3.2. If the EB has a result above the MDL, there may be possible contamination at the extraction step. 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 tube labeled EC. Pipette 5 µL of 10^7 C2H2 gBlock standard onto the filter and add 595 µL of salmon buffer.

12.4.1.2. The HumM2 assay EC is labeled, LFB_H2LFB#. The SKETA assay EC is labeled, SUR_H2LFB#.

12.4.1.3. The extraction control helps determine extraction recovery. Percent recovery of extraction control for both the HumM2 and SKETA assays 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 HumM2 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 HumM2 Assay. See Figure 15.1 for the plate layout.

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 HumM2 DNA standard is added to each sample as a matrix spike to check for inhibition. For each sample and replicate, spike the third well of the HumM2 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, 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. Notify supervisor or senior analyst.

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 named SAMPLE ID_DUP_A and SAMPLE ID_DUP_B and 100 mL is filtered for each.

12.5.2. The target replicate RPD (Relative Percent Difference) should be ≤ 25%. If RPD > 25%, the duplicate sample 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 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.

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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 Bureau of Labs 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*

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-3.8, *Preparation of HumM2 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 DNeasy PowerSoil DNA Isolation 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, *Data upload, Verification, and Sample Authorization*

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15. TABLES AND DIAGRAMS

Table 15.1 - Primer/Probe Mix Summary

	HumM2 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

Table 15.2 – Master Mix Summary

	HumM2 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
15 µL of Master Master Mix + 5 µL of DNA template = 20 µL per rxn				

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 E7	STD Curve E7	STD Curve E7	MBLK_	EXBLK_	LFB_H2SPK	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	Rep 1	A
B	STD Curve E6	STD Curve E6	STD Curve E6	MBLK_	EXBLK_	LFB_H2SPK	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	Rep 2	B
C	STD Curve E5	STD Curve E5	STD Curve E5	NTC_1	NTC_2	NTC_3	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	C
D	STD Curve E4	STD Curve E4	STD Curve E4	SURNTC_1	SURNTC_2	SURNTC_3	Sample1_ SUR_	Sample2_ SUR_	Sample3_ SUR_	Sample4_ SUR_	Sample5_ SUR_	Sample6_ SUR_	D
E	STD Curve E3	STD Curve E3	STD Curve E3	LFB_H2LFB	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_H2LFB	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_H2LFB	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	_SPK_H2SPK	G
H	STD Curve 10	STD Curve 10	STD Curve 10	SUR_H2LFB	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	

*HumM2 Assay (PURPLE background); SKETA Assay (pink background)

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