Standard Operating Procedure for:

Ruminant-associated *Bacteroidetes* BacR marker by Quantitative Polymerase Chain Reaction (qPCR) Assay

DEPARTMENT OF ENVIRONMENTAL PROTECTION
BIOLOGY SECTION
TALLAHASSEE FLORIDA

TABLE OF CONTENTS

1. SCOPE AND APPLICATION

1.1. This method describes a qPCR procedure that targets a 16S rRNA genetic marker in members of the phylum *Bacteroidetes*. It is used to detect fecal contamination from cattle, deer, sheep, goats, and other ruminants.

1.2. This procedure is used for samples with LIMS test ID of PCR-BACR and PUR-BACR.

2. SUMMARY OF METHOD

2.1. A water sample is filtered through a membrane filter. 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 kit. The extract is analyzed with real-time PCR amplification and detection in a thermocycler system using a primer/probe assay specific to ruminant-associated *Bacteroidetes*. Solid samples are weighed and purified prior to PCR amplification and detection.

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 necessary 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 series is developed 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 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.

3. EQUIPMENT AND SUPPLIES

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

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

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. 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.

4.5. PerfeCta® qPCR ToughMix™, 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-3.5.

4.7.1. BRD3GD gBlock Standards (10^7, 10^6, 10^5, 10^4, 10^3)

4.7.2. BRD3GD gBlock spikes (10^7, 10^5)

4.7.3. BacR primers (Stock concentration: 100 µM - BacRFor, 500 µM - BacRRev)

4.7.3.1. Forward Primer BacRFor: 5’- GCGTATCCAACCTTCCCG

4.7.3.2. Reverse Primer BacRRev: 5'- CATCCCCATCCGTTACCG

4.7.4. BacR Probe 100 µM stock

4.7.4.1. Probe BacRProbe: 5'- [6-FAM] CTTCCGAAAGGGAGATT [MGBE-Q]

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 PCR-2.2 for calibration and use of the balance.

5.6. Refer to 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 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. Primer/Probe Mix – In PCR Workstation #1, prepare primer/probe mix for each assay (Salmon DNA and BacR) separately as follows:

8.1.1. BacR Assay: Add 4 µL of 100 µM forward primer stock, 4 µL of 500 µM reverse primer stock, and 4 µL of 100 µM probe to 588 µL of PCR grade water to make primer/probe mix. Use an orange 1.5 µL centrifuge tube labeled "PP" for the primer/probe mix. Mix by tapping then centrifuge briefly.

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 µL centrifuge tube labeled "PP" for the primer/probe mix. Mix by tapping then centrifuge briefly.

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 – Create a reaction plate worksheet as per SOP PCR-5.0. In PCR Workstation #1, prepare Master Mix for each assay (SKETA and BacR) separately as follows:

8.2.1. Count the number of wells needed and enter in the reaction plate worksheet under the Master Mix Preparation section under '#Rxns' cell. The calculator adds additional 3 wells in calculations to ensure enough Master Mix is available (some can be lost in mixing and pipetting) to determine total volume of reagents needed for the assay.

8.2.2. Add PerfeCta® qPCR ToughMix™, Rox™, BSA, and primer/probe mix (PP) based on the calculated volume from the reaction plate worksheet in a pink 1.5 µL centrifuge tube labeled "MM" for the SKETA Master Mix. Use an orange 1.5 µL centrifuge tube labeled "MM" for the BacR 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 the appropriate qPCR Master Mix to each well of a reaction plate using the electronic Xplorer pipette with a 100 µL tip. Use Figure 15.4 as a guide. Add the BacR Master Mix to the orange wells shown in Figure 15.4. Add the SKETA Master Mix to all the pink wells in Figure 15.4. Carefully move the plate to PCR workstation #2.

9.1.1. Make sure to re-mix the Master Mix after every 12 wells 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. To ensure proper mixing, tap mix then centrifuge briefly. 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 BacR 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_BRSPK) to wells A6-B6, and C4-D6. See Section 12.3.1 and 12.4.3.

9.2.4. Add 1 µL of the BacR spike standard (LFB_BRSPK, and _SPK_BRSPK) 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_BRLFB, SUR_BRLFB) 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 BacR wells first, change tips, 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 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 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).

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. BacR Assay Standard Curves

12.1.1. Analyze a BacR Assay standard curve per plate. Use 5 serially diluted standards at 10^7, 10^6, 10^5, 10^4, and 10^3 concentrations. Analyze each concentration in triplicate. These are added to A1-E3 wells on the plate. Use Figure 15.4 as a guide.

12.1.2. Standard curve efficiency should be between 90-115% 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. Analyze each concentration in triplicate. These are added to F1-H3 wells on the plate. Use Figure 15.4 as a guide

12.2.1.1. Standard curve efficiency should be between 80-120% and R^2 should be $\geq$ 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 BacR 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. Add 5 µL of the blank extract to duplicate wells containing 15 µL BacR 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 AE buffer, and extract along with the samples. Add 5 µL of the blank extract to duplicate wells containing 15 µL BacR master mix.

12.3.3.2. If the EB has a result of 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 10^7 BRD3GD gBlock standard onto the filter and add 595 µL of salmon DNA extraction buffer.

12.4.1.2. The BacR assay EC is labeled, LFB_BRLFB#. The SKETA assay EC is labeled, SUR_BRLFB#

12.4.1.3. The extraction control helps determine extraction recovery. Percent recovery of extraction control for both the BacR 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 BacR 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 BacR Assay. See Figure 15.4 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 expected recovery, then the sample may be inhibited.

12.4.3. Inhibition control (3rd well spike)

12.4.3.1. A known amount of BacR 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 3rd well of the BacR assay with 1 µL of E5 BRD3GD 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 3rd well spike show inhibition, re-run the sample from a diluted or purified extract. 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 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 samples are analyzed 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. Replicate relative percentage difference RPD should be <=25%. If RPD > 25%, a comment regarding precision is automatically added to the report.

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. EPA Method B (June 2010)

14.2. 2009 TNI Standard

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

14.4. PerfeCta® qPCR ToughMix™, Rox™ Protocol

14.5. DEP Laboratory Quality Manual

14.6. Health and Safety Plan Health and Safety Plan

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

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

14.9. DEP SOP PCR-2.2, *Calibration, Use and Maintenance of Mettler Toledo XA105DU Balance*

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-3.1, *Preparation of SKETA assay standards and reagents for qPCR analysis*

14.15. DEP SOP PCR-3.5, *Preparation of BacR Assay Reagents and Standards for qPCR analysis*

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

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

14.18. DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen PowerLyzer PowerSoil DNA Isolation Kit*

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

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

15. TABLES AND DIAGRAMS

Table 15.1 - Primer/Probe Mix Summary

	BacR Assay	SKETA Assay
Forward Primer (100µM stock-BacR, 500µM stock- SKETA)	4 µL	10 µL
Reverse Primer (500µM stock)	4 µL	10 µL
Probe (100µM stock)	4 µL	9 µL
Sterile H_2O	588 µL	571 µL

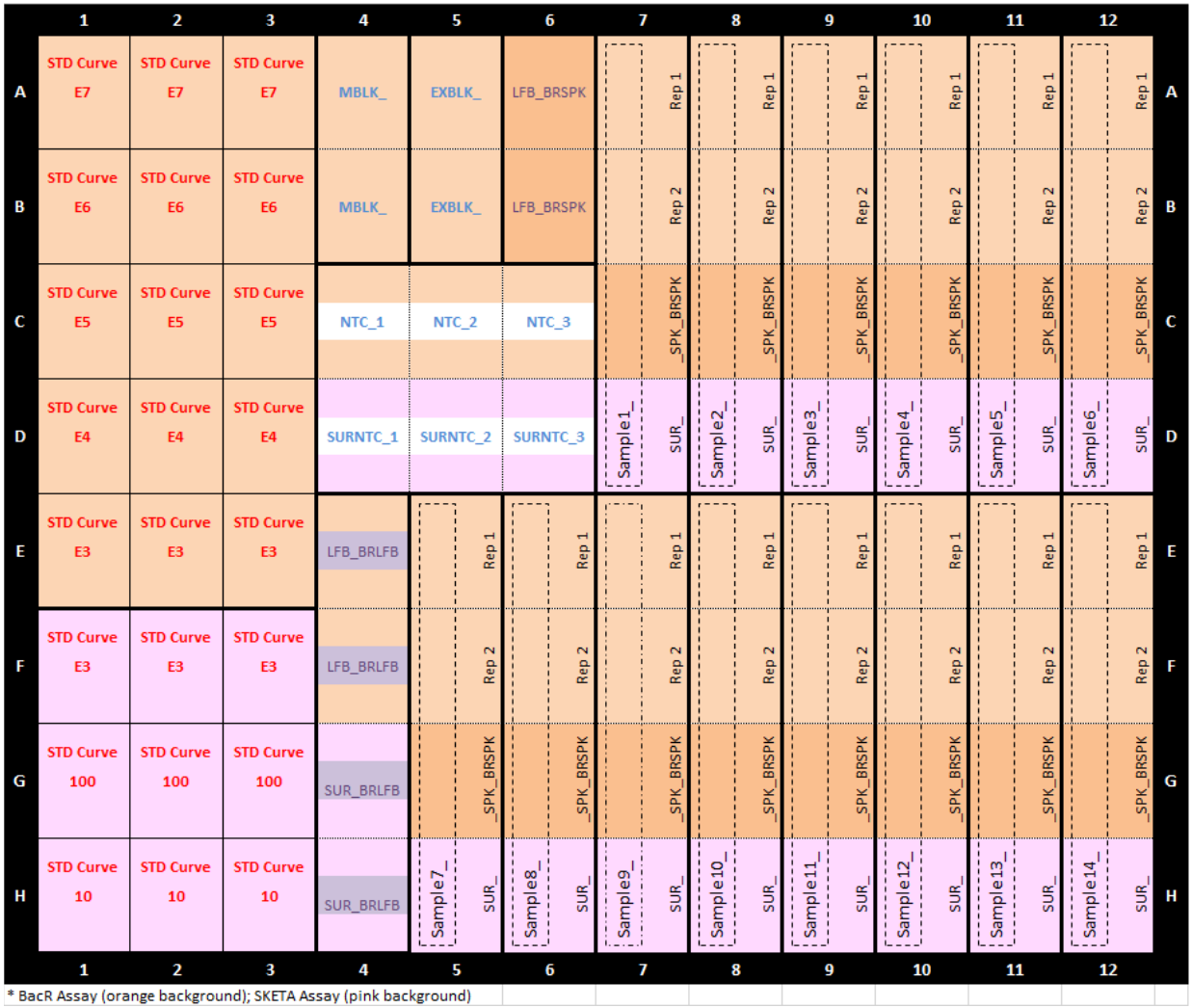
Table 15.2 – Master Mix Summary

	BacR 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

Figure 15.4 – 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_BRSPK	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_BRSPK	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_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	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_BRLFB	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_BRLFB	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_BRLFB	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	_SPK_BRSPK	G
H	STD Curve 10	STD Curve 10	STD Curve 10	SUR_BRLFB	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	

* BacR Assay (orange background); SKETA Assay (pink background)

Appendix of Significant Changes