Preparation of BacR Assay Reagents and Standards for qPCR Analysis

1. INTRODUCTION

1.1. This quantitative Polymerase Chain Reaction (qPCR) assay targets a 16S rRNA gene in members of the phylum *Bacteroidetes*. It is used to detect fecal contamination from cattle, deer, sheep, goats, and other ruminants. A gBlock® fragment containing a 158 bp BacR qPCR target sequence is used as a standard in the BacR assay, which generates a standard curve for determination and performance characteristics of the qPCR assay.

1.2. Extraction control and 3rd well spike: The extraction control contains an added known concentration of gBlock DNA that is extracted and analyzed in the same manner as environmental samples. The third well spike also contains an added known concentration of gBlock DNA and is added as a third replicate well to each environmental sample during reaction plate setup. Both extraction control and 3rd well spikes are used as positive controls for the BacR 16S rRNA target sequence and qPCR assay.

2. EQUIPMENT AND SUPPLIES

2.1. Gloves, latex or nitriles

2.2. Tube racks

2.3. Centrifuge tubes, 0.5ml, 1.5 ml, and 2.0 ml

2.4. Cryodot labels

2.5. Micropipettes with sterile PCR grade filter tips

2.5.1. Calibrate and use per SOP PCR-2.4.

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

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

2.8. Fisher Mini Vortexer

2.9. Fisher Minicentrifuge

2.10. Eppendorf Centrifuge Model 5418 (S/N 5418AG715992)

2.11. Qubit 2.0 Fluorometer (S/N 1301008411)

2.11.1. Calibrate and use per SOP PCR-2.0

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2.12. PCR#3, Thermo Refrigerator (S/N 0121179201121219), maintained at 4ºC ± 2ºC, Room B246B

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

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

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

2.16. Incubating mini shaker (S/N 150747)

3. REAGENTS AND CHEMICALS

3.1. Deionized (DI) water

3.2. PCR grade water – Autoclave ultrapure water for 15 minutes. Record in LIMS Autoclave Log.

3.3. Bleach solution, 10% (v/v)

3.4. TE Buffer

4. BACR PRIMER/ PROBE STOCK

4.1. Always purchase primer/ probes together as a set. Primers should be dry, salt free, and at a 1 µmol scale. The probe should be dry, HPLC purified and at a 1 µmol scale. The probe is an MGB (minor groove binder) Taqman® probe ordered from Eurofins with the 5’ 6-FAM dye and 3’ MGB-Eclipse quencher. Keep stored at -20°C.

4.1.1. Forward Primer BacRFor: 5’- GCGTATCCAACCTTCCCG - 3’

4.1.2. Reverse Primer BacRRev: 5’- CATCCCCATCCGTTACCG - 3’

4.1.3. Probe BacRProbe: 5’- [6-FAM] CTTCCGAAAGGGAGATT [MGBE-Q] – 3’

4.2. Each order includes an Oligonucleotide Data Sheet. Record received date on this sheet and review the sequences on the sheet for correctness. Scan the data sheet into the following folder: \\Fldep1\dear\Labs\Lab5\Biology\MolecularBiology\Certificates of Analysis\Oligo Data Sheet

4.3. Record each preparation in Laboratory Information Management System (LIMS) Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

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4.3.1. In LIMS select **Laboratory/Technical/Standard Preparation Tracker** from the menu. Choose **BIO-PCR** from the "Group" drop down list.

4.3.2. Select **Purchased/New Reagent/Material** from the menu. If a similar reagent exists, select **Purchased/Duplicate Reagent/Material** from the menu and choose the reagent to duplicate from the list.

4.3.3. In the "Purchased Material" pop-up window, enter the following information:

4.3.3.1. For Forward Primer BacRFor: Identity: = BacRFor; Description = BacR Assay Forward Primer; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = Salt-free; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer: = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location: = B246A; Storage Temperature: = Freezer at <-10 degC; Container Size: = weight in mg (shown on data sheet in µg); Units: = mg;

4.3.3.2. For Reverse Primer BacRRev: Identity = BacRRev; Description = BacR Assay Reverse Primer; Entered by = Analyst Name; Type = Powder; Grade = blank; Purity = Salt-free; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location = B246A; Storage Temperature = Freezer at <-10 degC; Container Size = weight in mg (shown on data sheet in µg); Units = mg;

4.3.3.3. For Probe BacRProbe: Identity = BacRProbe; Description = BacR Assay Probe; Entered by = Analyst Name; Type = Powder; Grade = HPLC Grade; Purity = HPLC; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location = B246A; Storage Temperature = Freezer at <-10 degC; Container Size = weight in mg (shown on data sheet in µg); Units = mg;

4.3.3.4. Select "Yes" for the "Has Certificate of Analysis" drop down list.

4.3.4. If a component is not already in the components list, click **Add**, and "Identity" name will self-populate in the "Components" table.

4.3.5. Double click the component and add the concentration of the reagent. Use the concentration in nmol from the data sheet. The **Add** or **Remove** buttons can be used to add or remove components.

4.3.6. Click **OK** to save the entry and receive a serial number. Click **Yes** on the "Print label?" pop up window. Select the **1/2" x 9/10"** label size in the "Select Label Size & Copies" pop up window. Uncheck the **Use Increment Range** and click **OK.** Choose the Small label printer in the "Print" pop-up window and click **OK**. Place the label on the Oligonucleotide Data Sheet, before scanning it (from Step 4.2).

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4.3.7. The concentration of primer/probes will vary with each order. This is listed in the "nmol" column of the data sheet. The data sheet will list how much TE buffer is needed to reconstitute the primer/probe to a 100 µM concentration in the "Add X µL for 100 µM" column. Use this amount for a 100 µM concentration. For a 500 µM concentration, divide this number by 5.

4.4. All primer/ probe mixing and pipetting must be completed in PCR Workstation #1 (DNA free) using sterile, DNA free supplies. Use only aerosol barrier tips for micropipettes. Before opening the dry oligonucleotide tube, pulse centrifuge to make sure all the oligos are at the bottom of the tube and help prevent a spill. Oligos are shipped by the manufacturer as dried content and appear either a translucent film or a white powder in a centrifuge tube. Do not over mix the oligonucleotide.

4.5. For each primer, pipette appropriate amount of TE buffer into the tube containing the dried primer. The forward primer BacRFor is resuspended at 100 µM concentration and the reverse primer BacR is resuspended at 500 µM concentration. Dissolve by tapping the tube. Allow the solution to equilibrate for 5 minutes. Mix again and pulse centrifuge to coalesce droplets. Aliquot 6 µL volumes into orange 0.5 ml centrifuge tubes. Label clearly, and affix cryodots (green dot – forward primer; purple dot – reverse primer) to the top of each tube.

4.6. For the probe, pipette appropriate amount of TE buffer into the tube containing the dried probe to make a 100 µM concentration. Dissolve by tapping the tube. Allow the solution to equilibrate for 5 minutes. Mix again and pulse centrifuge to coalesce droplets. Aliquot 6 µL volumes into orange 0.5 ml centrifuge tubes. Label clearly, and affix cryodots (yellow dot) to the top of each tube.

4.7. Group in sets of forward primer, reverse primer, and probe in a tube rack and store at -20°C.

4.8. Record each primer/probe preparation in Laboratory Information Management System (LIMS) Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

4.8.1. In LIMS select **Laboratory/Technical/Standard Preparation Tracker** from the menu. Choose **BIO-PCR** from the "Group" drop down list.

4.8.2. Select **Prepared/New Reagent** from the menu. If a similar reagent exists, select **Prepared/Duplicate Reagent** from the menu and choose the reagent to duplicate from the list.

4.8.3. In the "Prepared Reagent" pop-up window, enter the following information:

4.8.3.1. <u>For BacR Forward Primer</u>: Identity: = BacRFor; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.5; See Also: = leave blank; Location: = B246A; Description = BacR Assay Forward Primer; Storage Temperature = Freezer at <-10 degC;

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Additional Information: = TE Buffer S#; Solvent Used: = AE Buffer; Final Volume: = final total volume of preparation in µL; Units: = uL;

4.8.3.2. For BacR Reverse Primer: Identity = BacRRev; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.5; See Also: = leave blank; Location: = B246A; Description = BacR Assay Reverse Primer; Storage Temperature = Freezer at <-10 degC; Additional Information: = TE Buffer S#; Solvent Used: = AE Buffer; Final Volume: = final total volume of preparation in µL; Units: = uL;

4.8.3.3. For BacR Probe: Identity = BacRProbe; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.5; See Also: = leave blank; Location: = B246A; Description = BacR Assay Probe; Storage Temperature = Freezer at <-10 degC; Additional Information: = TE Buffer S#; Solvent Used: = AE Buffer; Final Volume: = final total volume of preparation in µL; Units: = uL;

4.8.4. Click **Add**. Choose the **Purchased Materials** radio button in the "Select Source:" section. In the "Enter Serial #" pop up window, choose the primer/probe that was used to make the preparation being entered from the list, or enter its serial number in the text box. In the "Enter Amount & Units for.." pop up window, enter weight of the primer/probe added in µg and choose "ug" from the "Added Units:" drop down list. Click **OK**.

4.8.5. Click **OK** to save the entry and receive a serial number. Click **Yes** in the "Confirm" pop up window to print labels. In the "Select Label Size & Copies" window, select the 1/2" x 9/10" label size in the "Label Size" section. Make sure that **Use Increment Range** is checked and the "Increment Range:" reflects the number of aliquots made for the primer/probe. Click **OK**. Choose the small label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquotted tubes before storage in the freezer.

5. GBLOCK STANDARD

5.1. A gBlock® gene fragment is ordered from Integrated DNA Technologies (IDT) and is used as a standard for the BacR assay. The target sequence for the BacR assay is a 158 bp fragment (bold sequences show location of primer binding):

CCGGCGCACGGGTGCGTAAC**GCGTATCCAACCTTCCCG**TTACTCAGGGATA
GCCTTCCGAAAGGGAGATTAATACCTGATGGTGTTTGAATTCCGCATGTTATT
CAAACTAAAGATTTAT**CGGTAACGGATGGGGATG**CGTGACATTAGGCAGAT
GGC

5.2. This sequence is combined with the target sequences for the DG3 and GFD assays to generate a standard that can be used for all three assays. This gBlock fragment is named BRD3GD gBlock and is a 513 bp fragment:

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CCGGCGCACGGGTGCGTAACGCGTATCCAACCTTCCCGTTACTCAGGGATAG
CCTTCCGAAAGGGAGATTAATACCTGATGGTGTTTGAATTCCGCATGTTATTC
AAACTAAAGATTTATCGGTAACGGATGGGGATGCGTGACATTAGGCAGATGG
CTTTTTTGAGCGGGCATGGTCATATTATTGTTTAGACCGTGGATGTTTGCTCTG
CATTAACCTCCGTCCGCAGGATATTCAGAAAACCATCAAGGAGGTTCGCCCT
ACAGCCATGTGCAGTGTGCCCCGTTTCTGGGAAAAAGTCTACGCGGGCGTAC
TGGAAAAGATTAACGAAACAACGGGGCTGAAAATTTTTGTCCTTAGTTGCTA
ACAGTTCGGCTGAGCACTCTAGGGAGACTGCCTTCGTAAGGAGGAGGAAGGT
GAGGACGACGTCAAGTCATCATGGCCCTTACGCCTAGGGCTACACACGTGCT
ACAATGGGATGTACAAAGAGACGCAATACCGCGAGGTGGAGC

5.3. There are no modifications or purifications needed, and 500 ng of the gBlock arrives dried content and appear either as a translucent film or a white powder in a centrifuge tube. Record the gBlock in LIMS Standard Preparation Tracker module (Laboratory/Technical/Standard Preparation Tracker).

5.3.1. In LIMS select **Laboratory/Technical/Standard Preparation Tracker** from the menu. Choose **BIO-PCR** from the "Group" drop down list.

5.3.2. Select **Purchased/New Standard** from the menu. If a similar standard exists then select **Prepared/Duplicate Standard** from the menu and choose the standard to duplicate from the list.

5.3.3. In the "Purchased Standard" pop up window enter the following information:

5.3.3.1. <u>For BRD3GD gBlock</u>: Identity: = BRD3GD gBlock; Description: = gBlock standard for BacR, DG3, and GFD assays; Entered by: = Analyst Name; Received: = Date received; Expires: = 365 days; Type: = purchased powder; Has certificate of Analysis: = YES; Manufacturer: = Integrated DNA Technologies; Lot #: = (list number on tube); Storage Location: = B246B; Storage Temperature: = Freezer <10 degC; Additional information: = Use with SOP = PCR-3.5.

5.4.To resuspend, centrifuge the tube briefly then add TE to reach a final concentration of 10 ng/µL. For 500 ng, add 50 µL TE. Vortex briefly, then incubate at 50°C for 20 minutes in benchtop incubator. Briefly vortex and centrifuge.

6. STOCK DNA AND WORKING STANDARDS

6.1. Dilute the resuspended gBlock in TE buffer to measure on Qubit. Gently mix each dilution and pulse centrifuge to coalesce droplets.

6.1.1. Mix 10 µL of resuspended BRD3GD gBlock with 90 µL of TE buffer to make a 1:10 dilution.

6.1.2. Mix 50 µL of the 1:10 dilution with 450 µL of TE buffer to make a 1:100 dilution.

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6.2. Analyze the 1:10 and 1:100 dilutions in triplicate on the Qubit fluorometer per SOP PCR-2.0 to determine the concentration. Use 10 µL as the sample volume. The 1:10 dilution should be 10X greater than the 1:100 dilution. If this is not the case, use a more concentrated or dilute sample as needed.

6.3. Determine the concentration of stock BRD3GD gBlock in ng/mL.

6.3.1. For each dilution, multiply the stock concentration (from Qubit data file) measured on the Qubit by the dilution factor. Use the average of these as the concentration of the stock BRD3GD gBlock.

6.3.2. Compare this value to the expected concentration of 10 ng/µL. If calculated value differs greatly, recheck measurements.

6.4. Determine the concentration of the stock DNA in TSC/5 µL using the following equation with the conversion factor of 1 ng of BRD3GD double-stranded gBlock = 1.81 X 10^9 copies of BacR target sequence.

$$\frac{TSC\ BRD3GD}{5\ uL} = (Stock\ Conc.in\ ng/mL)\left(\frac{1\ mL}{1000\ uL}\right)\left(\frac{1.81\ \times\ 10^9\ TSC}{1\ ng}\right) \times 5$$

6.5. Determine the concentrations of the dilutions in TSC/5 µL units and continue the serial dilution down to a concentration of 10^7 in 1000 µL volumes. This will be used as the working standard.

6.6. Using orange 0.5 mL tubes, aliquot the 10^7 dilution in 50 µL volume. Place red cryodots on the tops of all tubes and label the dots with the concentration (E7). Store the 10^7 tubes at -80°C.

6.6.1. The 10^7 standards will be diluted further to 10^6, 10^5, 10^4, and 10^3 when setting up the reaction plate to make a standard curve for the BacR assay and as a 3rd well spike at a concentration of 10^5.

6.7 Record the stock DNA and dilutions in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

6.7.1 In LIMS select **Laboratory/Technical/Standard Preparation Tracker** from the menu. Choose **BIO-PCR** from the "Group" drop down list.

6.7.2 Select **Prepared/New Standard** from the menu. If a similar standard exists then select **Prepared/Duplicate Standard** from the menu and choose the standard to duplicate from the list.

6.7.3 In the "Standard Solution" pop up window enter the following information:

6.7.3.1 <u>For Stock BRD3GD gBlock DNA</u>: Identity: = x.xxEx BacR gBlock (use measured concentration in TSC/5µL); Type: = Stock Standard; Holding Time:

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= 365 days; Entered by: = Analyst Name; Location: = B246B; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.5; See Also: = leave blank; Description: = BacR gBlock reconstituted at 10 ng/uL in TE buffer; Additional Information = TE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 50.00; Units: = µL;

Note: The stock DNA will be used to make working standards and the original tube will be stored at -80°C.

6.7.3.2 For working BRD3GD gBlock Standard: Identity: = x.xxE7 BRD3GD gBlock (use measured concentration in TSC/5µL); Type: = Working Standard; Holding Time = 365 days; Entered by: = Analyst Name; Location: = B246B; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.5; See Also: = leave blank; Description: = resuspended gBlock used for standards for BacR, DG3, and GFD assays; Additional Information = TE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 1000.00 ; Units: = µL;

6.7.3.3 Click **Add**. In the "Enter Serial #" pop up window, choose the standard that was used to make the standard being entered from the list, or enter its serial number in the text box. For BRD3GD gBlock stock DNA, the standard is in the **Purchased Materials** "Select Source:" list. For the other working standards, choose **Existing Standards** for the source list. In the "Enter Amount & Units from.." pop up window, enter the amount of standard added in µL and "Final Conc. Units:" should be entered as "ng/mL". Click **OK**.

6.7.3.4 Click **OK** in the "Standard Solution" pop up window. Click **Yes** in the "Confirm" pop up window to print labels. In the "Select Label Size & Copies" window, select the 1/2" x 9/10" label size in the "Label Size" section. Make sure that **Use Increment Range** is checked and the "Increment Range:" reflects the number of aliquots made for the standard. Click **OK**. Choose the small label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquoted tubes before storage in the freezer.

7. BACR ASSAY STANDARD CURVES AND 3RD WELL SPIKE

7.1. The BacR standard curve will consist of running concentration series 10^7, 10^6, 10^5, 10^4, and 10^3 TSC/5 uL, and the 3rd well spike is run at a concentration of 10^5 TSC/5 uL.

7.2. BacR standards and 3rd well spike will be diluted before each run from 10^7 concentration. To make the 10^6 standard, dilute by mixing 10 µL of the 10^7 dilution with 90 µL of TE buffer. 10^5, 10^4, and 10^3 will be made similarly by serially diluting.

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7.3. Record the 3rd well spike standard in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Step 6.7.3.2, except add SPIKE to the end of the Identity name.

7.4. When new standards are needed, dilute the original gBlock stock and measure as described in Section 6.1. Reorder as needed.

8. BACR EXTRACTION CONTROL

8.1. The extraction control (EC) has a concentration of 10^7 TSC/5 uL of BRD3GD gBlock.

8.2. Use the 10^8 BacR gBlock stock standard to make the 10^7 EC dilution by mixing 40 µL of the 10^8 dilution with 450 µL of TE buffer.

8.3. Aliquot the EC spike into orange 0.5 mL tubes in 6 µL volumes and keep frozen at -20°C in B246B. Place white cryodots on the tops of all tubes. Label the dots with the concentration (E7).

8.4. Record the standards in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Step 6.7.3.2, except add SPIKE to the end of the Identity name.

9. QUALITY CONTROL

9.1. Maintain control charts of BacR standard curves and extraction controls. If recoveries show a decreasing trend, DNA standards may be deteriorating and need to be remade.

9.2. When new standard curve sets are made, analyze alongside an older set for comparison. New standards should perform similarly to older standards. If older standards are not available, compare to the control chart information.

10. REFERENCES

10.1. DEP Laboratory Quality Manual
http://publicfiles.dep.state.fl.us/dear/labs/lab_qualitymanual_18.pdf

10.2. Health and Safety Plan Health and Safety Plan

10.3. EPA Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay (October 2012)

10.4. DEP SOP PCR-2.0 *Calibration, Use and Maintenance of Qubit 2.0 Fluorometer*

10.5. DEP SOP PCR-2.4 *Calibration and Use of Mechanical Pipettes using PCS® Pipette Calibration System and Artel® Pipette Tracker™*

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