PREPARATION OF BACR ASSAY REAGENTS AND STANDARDS FOR qPCR ANALYSIS

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1. INTRODUCTION

1.1. The BacR assay targets the 16S rRNA gene in members of the phylum *Bacteroidetes* and detects fecal contamination from cattle, deer, sheep, goats, and other ruminants using quantitative Polymerase Chain Reaction (qPCR). A gBlock® fragment containing a 158 bp BacR qPCR target sequence is used as a standard in the BacR assay to generate standard curves to determine the performance characteristics of the qPCR assay.

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

2. EQUIPMENT AND SUPPLIES

2.1. Instrumentation –

Instrument Name	Serial Number	Manufacturer	Notes
Qubit 2.0 Fluorometer	1301008411	Invitrogen	Calibrate and use per SOP PCR-2.0

2.2. Equipment –

Equipment Name	Notes
Tube Racks	N/A
Micropipettes	Calibrate and use per SOP PCR-2.4
CBS Optimizer PCR Workstation #1	S/N 007631
CBS Optimizer PCR Workstation #2	S/N 007632
Fisher Mini Vortexer	N/A
Fisher Mini Centrifuge	N/A
Eppendorf Centrifuge Model 5418	S/N 5418AG715992
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#10, ThermoTDE Freezer	S/N 112701880122060 3; Maintained at -80 ± 10°C, Room B246
Incubating Mini Shaker	S/N 150747
Autoclave	S/N 031761851
Or Equivalent	

2.3. Supplies and Consumables –

Supply Name	Notes
Gloves, Latex or Nitriles	N/A
Centrifuge Tubes, 0.5 mL, 1.5 mL, and 2.0 mL	N/A
Brady FreezerBondz II Laboratory Labels	Catalog Number: 19-140-586
Sterile PCR Grade Filter Tips	N/A
Or Equivalent	

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3. REAGENTS AND CHEMICALS

3.1. Reagents and Chemicals –

Reagent/Chemical 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
TE Buffer	Catalog Number: BP2473
Or Equivalent	

4. BACR PRIMER/PROBE STOCK

4.1. Always purchase primers/probe 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 a MGB (minor groove binder) TaqMan® probe ordered from Eurofins with the 5' 6-FAM dye and 3' MGB-Eclipse quencher. Store 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 as soon as the primers/probe are received and review the sequences on the sheet for correctness. Scan the data sheet into the following folder: \\floridadep\data\DEAR\Labs\Lab5\Biology\MolecularBiology\Certificates of Analysis\Oligo Data Sheet.

4.3. Enter each purchased Primer/Probe set in the Laboratory Information Management System (LIMS) Standard Preparation Tracker module.

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	Leave Blank
Purity	Salt-free
Received	Date received

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Expires	365 days from received date
Has Certificate of Analysis	Yes
Manufacturer	Eurofins Operon
Lot Number	Barcode ID from Data Sheet
Additional Information	Concentration in nmoles
Storage Location	B246A
Storage Temperature	Freezer at <-10 deg C
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	Leave Blank
Purity	Salt-free
Received	Date received
Expires	365 days from received date
Has Certificate of Analysis	Yes
Manufacturer	Eurofins Genomics
Lot Number	Barcode ID from Data Sheet
Additional Information	Concentration in nmoles
Storage Location	B246A
Storage Temperature	Freezer at <-10 deg C
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 received
Expires	365 days from received date
Has Certificate of Analysis	Yes
Manufacturer	Eurofins Operon
Lot Number	Barcode ID from Data Sheet
Additional Information	Concentration in nmoles
Storage Location	B246A
Storage Temperature	Freezer at <-10 deg C
Container Size	Weight in mg (Shown on data sheet in µg)
Units	mg

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.

4.4. Open the LabelMark6 software and create labels (see SOP PCR-5.0 for details). Print labels with the reagent name, serial number, and expiration date and affix labels to the side of each tube. The top label should include the reagent name. Place labels on all aliquoted tubes. Place the label on the Oligonucleotide Data Sheet before scanning it (from Section 4.2.).

5. PRIMERS AND PROBE RESUSPENSION

5.1. The concentration of primers and probe 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.

5.2. Primer/Probe resuspension and aliquoting 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. Oligos are shipped by the manufacturer as dried content and appear as either a translucent film or a white powder in a centrifuge tube.

5.3. **For the primers:** Add the 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 BacRRev 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.

5.4. **For the probe:** Add the 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 volume into orange 0.5 mL centrifuge tubes.

5.5. When a new primers/probe set is resuspended prepare a comparison qPCR plate and analyze alongside an older primers/probe set. The new set should perform similarly to older sets.

5.6. Group in sets of forward primer, reverse primer, and probe in a freezer box and store at -20°C.

5.7. Record each primer/probe preparation in the LIMS Standard Preparation Tracker module .

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

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5.7.2. 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.

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

5.7.3.1. For BacR Forward Primer –

Identity	**BacRFor**
Type	Working Reagent
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
Description	BacR Assay Forward Primer 100 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Final total volume of preparation in µL, Units: µL Click Add→Choose Purchase Materials: Enter TE S/N and volume in µL Click Add→Choose Purchase Materials: Enter BacRFor S/N and weight in µg of forward primer

5.7.3.2. For BacR Reverse Primer –

Identity	**BacRRev**
Type	Working Reagent
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
Description	BacR Assay Reverse Primer 500 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Final total volume of preparation in µL, Units: µL Click Add→Choose Purchase Materials: Enter TE S/N and volume in µL Click Add→Choose Purchase Materials: Enter BacRRev S/N and weight in µg of reverse primer

5.7.3.3. For BacR Probe –

Identity	**BacRProbe**
Type	Working Reagent
Holding Time	365 days from received date
Entered By	Analyst name

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Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
Description	BacR Assay Probe 100 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Final total volume of preparation in µL, Units: µL Click Add→Choose Purchase Materials: Enter TE S/N and volume in µL Click Add→Choose Purchase Materials: Enter BacRProbe S/N and weight in µg of probe.

5.7.4. Click **OK** to save the entry and receive a serial number.

5.7.5. Open the **LabelMark6** software and create labels. Print labels with the reagent name (**BacRFor, BacRRev or BacRP**), serial number, and expiration date. Affix labels to the side of each tube. The top label should include the primer name and serial number.

5.7.6. Place labels on all aliquoted tubes before storage at -20° C.

6. GBLOCK STANDARD

6.1. 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 (sequences in red show location of primer binding):

5'–CCGGCGCACGGGTGCGTAACGCGTATCCAACCTTCCCGTTACTCAGGGAT
AGCCTTCCGAAAGGGAGATTAATACCTGATGGTGTTTGAATTCCGCATGTTAT
TCAAACTAAAGATTTATCGGTAACGGATGGGGATGCGTGACATTAGGCAGAT
GGC–3'

6.2. This sequence is combined with the target sequence for the DG3 assay to generate a standard that can be used for both assays. This gBlock® fragment is named BRD3 gBlock® and is a 372 bp fragment:

5'–CCGGCGCACGGGTGCGTAACGCGTATCCAACCTTCCCGTTACTCAGGGAT
AGCCTTCCGAAAGGGAGATTAATACCTGATGGTGTTTGAATTCCGCATGTTATTCAAACTAAAG
ATTTATCGGTAACGGATGGGGATGCGTGACATTAGGCAGATGGCTTTTTTGAGCGGGCATGGT
CATATTATTGTTTAGACCGTGGATGTTTGCTCTGCATTAACCTCCGTCCGCAGGATATTCAGAAA
ACCATCAAGGAGGTTCGCCCTACAGCCATGTGCAGTGTGCCCCGTTTCTGGGAAAAAGTCTACG
CGGGCGTACTGGAAAAGATTAACGAAACAACGGGGCTGAAAATTTTTGTCCTTAGTTGCTAACA
GT–3'

6.3. No modifications or purifications are needed, and 500 ng of the gBlock arrives as dried content and appears as either a translucent film or a white powder in a centrifuge tube. As soon as the gBlock is received, record in LIMS Standard Preparation Tracker module.

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

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

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

6.3.3.1. For BRD3 gBlock –

Identity	**BRD3 gBlock**
Description	gBlock standard for BacR and DG3 assays
Entered By	Analyst Name
Type	Purchased powder
Received	Enter date received
Expires	365 days from received date
Grade	If available in datasheet
Purity	If available in datasheet
Has Certificate of Analysis	Yes
Manufacturer	Integrated DNA Technologies (IDT)
Lot Number	Enter number on tube
Storage Location	B246
Storage Temperature	Freezer at <-10 deg C
Additional Information	Use with SOP PCR-3.5
Container Size	0.0005
Units	mg

6.4. Click **OK** to save the entry and read the serial number.

7. STOCK DNA AND WORKING STANDARDS

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

7.2. The stock gBlock is used to make intermediate and working standards. The original tube purchased from IDT must be labeled with a LIMS S/N and stored at -80 °C. When new standards are needed, dilute the stock gBlock and measure as described in this section. Reorder as needed.

7.3. Dilute the resuspended BRD3 gBlock in TE buffer (see table below) to measure on the Qubit fluorometer. Gently mix each dilution and pulse centrifuge to coalesce droplets.

Concentration	**1:10**	**1:100**
Stock Name	BRD3 gBlock	1:10
Amount of Stock	10 µL	50 µL
Amount of TE Buffer	90 µL	450 µL

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7.4. Determine the concentration of each dilution

Measure the 1:10 and 1:100 dilutions in triplicate with the Qubit fluorometer as per SOP PCR-2.0 using 10 µL as the sample volume.

7.4.1. Insert the Qubit 2.0 USB drive into the fluorometer USB port, select Data, then the USB icon. The Qubit data are saved in a **.CSV** file and named with a timestamp. The USB icon should display a green dot if it recognizes the USB drive. A red dot is displayed when there is no USB drive inserted or if it is not recognized by the fluorometer.

7.4.2. Save the data on the lab computer's hard drive. Open the Excel file on the computer and copy and paste the data into a new Qubit_Template file. Rename the file as: BRD3_Qubit_YYMMDD. Save the file in \\floridadep\data\DEAR\Labs\Lab3\Biology\qPCR\QubitData.

7.5. Determine the concentration of stock BRD3 gBlock in ng/mL.

7.5.1. In the .xlsx, add the dilution name to the appropriate data rows.

7.5.2. Copy and paste the stock concentration column (in ng/mL) in the designated location in the excel file. The worksheet will multiply the results of the 1:100 dilution by ten to compare the values to the 1:10 dilution. 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.

7.5.3. The worksheet will calculate the average of the six readings. This is used as the concentration of the gBlock in ng/mL.

7.6. Determine the concentration of the dilutions and stock gBlock in TSC/5µL.

7.6.1. The **.xlsx** file should automatically calculate the concentration of the 1:100, 1:10, and stock solution in TSC/5µL.

7.6.2. To determine the concentration of the 1:10 gBlock manually, use the following equation with the conversion factor of 1 ng of BRD3 double-stranded gBlock=**2.49 X 10^9 copies of BacR target sequence.**

$$\frac{TSC\ BRD3}{5\ uL} = (Stock\ Conc.in\ \frac{ng}{mL})\left(\frac{1\ mL}{1000\ uL}\right)\left(\frac{2.49\ \times\ 10^9\ TSC}{1\ ng}\right) \times 5$$

7.6.2.1. To determine the concentration of the stock in TSC/5µL, multiply the 1:10 concentration by ten.

7.6.2.2. To determine the concentration of the 1:100 dilution in TSC/5µL, divide the 1:10 concentration by ten.

7.7. Prepare 1,000 µL of an E8 dilution using the 1:100 dilution and TE buffer. This is an intermediate dilution used to prepare E7.

7.7.1. To determine the volume of 1:100 needed, use the formula $C_1V_1=C_2V_2$. (Example: To create a E^8 dilution from a 1:100 dilution with the concentration $7E^9$ $V_1=(V_2*C_2)/C_1=(7E^8*1000\ \mu L)/7E^9=100\ \mu L$. Add 100 µL of 1:100 to 900 µL of TE buffer.)

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NOTE: The Qubit Fluorometer only reads concentrations ≥ 0.5 ng/mL. This equates to a concentration of approx. 7E7 TSC/5 µL.

7.8. Determine the concentration of the E8 dilution.

7.8.1. Measure the E8 dilution in triplicate with the Qubit fluorometer as in SOP PCR-2.0.

7.8.2. Export the data as in Section 7.5.

7.8.3. Determine the average concentration of the E8 in Excel. Divide the average E8 value by 10. The **E7** value is the concentration of the standard and will be used for quantification of samples in qPCR analysis. Save the E8 dilution for plate analysis.

7.9. Prepare a 10 fold dilution of E8 to make 2,000 µL of an E7 dilution as described in 7.7.1 This will be used as the working standard and extraction control.

7.10. Prepare a comparison plate to analyze alongside an older standard. Serially dilute the newly prepared E7 to make a standard curve from 10^7-10^3. Add the E8 dilution prepared in 7.7 in triplicate to the plate. New standards should perform similarly to older standards. Check efficiency and R^2 of E8-E3. If results are acceptable, proceed to Section 7.11., if not, remake the E7 standards.

7.11. Record the stock, intermediate, and working standards in LIMS Standard Preparation Tracker module.

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

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

7.11.3. For Stock BRD3 gBlock DNA –

Identity	**x.xxEx BRD3 gBlock (use measured concentration in TSC/5µL)**
Type	Stock Standard
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared By	Enter Date, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
See Also	Leave Blank
Description	BRD3 gBlock reconstituted at 10 ng/µL in TE Buffer
Additional Information	TE Buffer S/N
Solvent Used	TE Buffer
Components	Final Volume: Enter volume of TE added, Units: µL (50 µL) Click Add→Choose Purchased Material: Enter TE S/N and volume in µL Click Add→Choose Purchased Material: Enter BRD3 S/N and weight in ng (500 ng)

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7.11.4. Click **OK** to save the entry and read the serial number.

NOTE: Specify in a note on the cryobox containing the gBlock stock the number of aliquots taken out of the tube. Write this information down every time a new dilution is prepared. Update the word file "gBlock inventory" located at \\floridadep\data\DEAR\Labs\Lab5\Biology\MolecularBiology\Forms\Inventory.

7.11.5. Intermediate BRD3 gBlock DNA Standard –

Identity	**x.xxEx BRD3 (use the measured concentration in TSC/5µL)**
Type	Intermediate Standard
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared By	Enter Date, Time, and Analyst Name
Location	B246
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
See Also	Leave blank
Description	BRD3 gBlock diluted from EX (with X changing for each dilution)
Additional Information	xE^x copies/5 µL
Solvent Used	TE Buffer
Components	Final Volume: Enter volume of TE in µL Click Add→Choose Purchased Materials: Enter TE S/N and volume in µL Click Add→Choose Purchased Materials: Select gBlock stock or intermediate S/N and enter volume added in µL. Select Final Conc Units: ng/µL

NOTE: Record every intermediate dilution prepared.

7.11.6. Click **OK** to save the entry and read the serial number.

7.11.7. Working BRD3 gBlock DNA Standard –

Identity	**x.xxE^7 BRD3 STD (use measured concentration in TSC/5µL)**
Type	Working Standard
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared By	Enter Date, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
See Also	Leave blank
Description	Resuspended gBlock used for standards for BacR and DG3 assays
Additional Information	TE Buffer S/N
Solvent Used	TE Buffer
Components	Final Volume: Enter volume in µL of working gblock prepared. Click Add→Choose Purchased Materials: Enter TE S/N and volume in µL Click Add→Choose Prepared Material: Select gblock dilution S/N and enter the volume added in µL. Select Final Conc Units: ng/µL

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7.11.8. Click **OK** to save the entry and read the serial number.

7.11.9. BRD3 Extraction Control –

Identity	**x.xxE^7 BRD3 EC (use measured concentration in TSC/5µL)**
Type	Working Standard
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared By	Enter Date, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-10 deg C
SOP	PCR-3.5
See Also	Leave blank
Description	Extraction control for BacR and DG3 assays
Additional Information	TE Buffer S/N
Solvent Used	TE Buffer
Components	Final Volume: Enter volume in µL of working gblock prepared. Click Add→Choose Purchased Materials: Enter TE S/N and volume in µL Click Add→Choose Prepared Material: Select gblock dilution S/N and enter the volume added in µL. Select Final Conc Units: ng/µL

7.12. Determine the number of tubes to aliquot for BRD3 STD and BRD3 EC (50 µL volumes for the STD and 16 µL volumes for the EC).

7.13. Open the LabelMark6 software and create labels. One the side label, include the serial number, name, concentration (x.xxE^7 BRD3 STD or x.xxE^7 BRD3 EC), and expiration date. On the top label include BRD3 STD or BRD3 EC. Print labels and affix to the top and sides of the tubes.

7.14. Aliquot the BRD3 STD in 50 µL volumes and the BRD3 EC in 16 µL volumes in orange 0.5 mL tubes. Store in the same box at -80 °C.

7.14.1. When setting up the reaction plate, the 10^7 standard will be diluted further to 10^6, 10^5, 10^4, and 10^3 to make a standard curve for the BacR assay. The 10^5 dilution will also serve as a third well spike.

7.15. Record the third well spike standard in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

7.15.1. BRD3 3rd well SPK –

Identity	**x.xx E^5 BRD3 3rd well SPK (Use measured concentration in TSC/5 µL)**
Type	Working standard
Holding time	365 days from received date
Entered By	Analyst Name
Prepared by	Enter Date, Time and analyst name
Location	B246A
Storage Temperature	Freezer -80 degC
SOP	PCR 3.2
See also	Leave blank

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Description	3rd well SPK for BacR and DG3 assays. BRD3 gBlock diluted from E6 at plate preparation.
Additional information	TE Buffer S/N
Solvent used	AE buffer

8. BACR ASSAY STANDARD CURVES, EXTRACTION CONTROL, AND THIRD WELL SPIKE

8.1. The BacR standard curve consists of running concentration series 10^7, 10^6, 10^5, 10^4, and 10^3 TSC/5 µL BRD3 gBlock and should be run on every BacR plate. The third well spike is run at a concentration of 10^5 TSC/5 µL.

8.2. The extraction control (EC) has a concentration of 10^7 TSC/5 µL of BRD3 gBlock.

8.3. BacR standards and third well spike will be made by serial dilution diluted before each run from 10^7 concentration according to the table below.

Concentration	**10^6**	**10^5**	**10^4**	**10^3**
Stock Name	10^7	10^6	10^5	10^4
Amount of Stock	10 µL	10 µL	10 µL	10 µL
Amount of TE Buffer	90 µL	90 µL	90 µL	90 µL

8.4. Convert the LIMS concentrations to TSC/µL for crude, pure, and 3rd well SPK as follows: See Excel file "LIMS Standards Calculations" located \\floridadep\data\DEAR\Labs\Lab5\Biology\MolecularBiology\Validation

8.4.1. $Crude\ Concentration = \frac{x.xxE7*(\frac{5\mu L}{600\mu L})}{5\mu L}$

8.4.2. $Pure\ Concentration = \frac{x.xxE7*(\frac{5\mu L}{100\mu L})}{5\mu L}$

8.4.3. $3rd\ Well\ SPK\ Concentration = \frac{x.xxE5*(\frac{1\mu L}{5\mu L})}{5\mu L}$

8.5. Enter values calculated in 8.4. into the LIMS QC manager (QCM) module for each thermocycler. In the sample table, click Edit→Limits Tables→SPK. Carefully enter the new LFB, LFBP, and SPK as in the example below:

For the BacR assay, select the previous BRLFBxxxxxx in the SPK window. In the component window, select BacR-qPCR. Hit replicate SPK. Enter new ID information. Hit ok. Select the new BRLFB only. Click the Conc column. Enter the new concentration. Click ok. Repeat for all assays:

For BacR: BRLFBxxxxxx, BRLFBPxxxxxx, BRSPKxxxxxx

For DG3: DGLFBxxxxxx, DGLFBPxxxxxx, DGSPKxxxxxx

9. QUALITY CONTROL

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

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

9.3 All calculations and QCM standard value entries must be verified by another analyst or supervisor.

10. REFERENCES

10.1. DEP Laboratory Quality Manual

10.2. Health and Safety Plan

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

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

Appendix of Significant Changes

11/18/2021 Added changes from BRD3GD gBlock® to BRD3 gBlock®.

11/09/2022 Update new fridge and freezer information with S/N. Removed old fridge and Freezer S/Ns. Added Ethanol 80% as cleaning reagent to use on surfaces after plate preparation.

11/13/2023 Reformatted SOP and added tables to Sections 2, 3, 4, 5, 6, 7, and 8. Added in depth instructions to Section 7 for using the Qubit Fluorometer to measure the BRD3 gBlock. Added formulas and instructions to calculate the concentration of the EC and 3rd well spike and enter this information in LIMS.

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