PREPARATION OF HF183 ASSAY REAGENTS AND STANDARDS FOR qPCR ANALYSIS

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1. INTRODUCTION

1.1. The HF183 assay targets the 16S rRNA gene in Bacteroidales and detects fecal contamination from the gastrointestinal tract of humans using quantitative polymerase chain reaction (qPCR). This procedure describes the preparation of HF183 reagents and standards. A gBlock® fragment containing a 171 bp HF183 qPCR target sequence is used as a standard in the HF183 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 HF183 Bacteroidales 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 of each environmental sample during reaction plate setup.

2. EQUIPMENT AND SUPPLIES

2.1. Instrumentation –

Instrument Name	Serial Number	Manufacturer	Notes
Qubit 4.0 Fluorometer	2322624050130	Invitrogen	Calibrate and use per SOP PCR-2.0

2.2. Equipment –

Equipment	Notes
Tube Racks	N/A
Micropipettes	Calibrate and use per SOP BB-039
CBS Optimizer PCR Workstation #1	S/N 007631
CBS Optimizer PCR Workstation #2	S/N 007632
Fisher Mini Vortexer	N/A
Fisher Microcentrifuge	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, Thermo TDE -80 Freezer	S/N 1127920701221013; Maintained at -80 ± 10°C, Room B246
Incubating Mini Shaker	S/N 150747
Autoclave	S/N 0317618851
Or Equivalent	

2.3. Supplies and Consumables –

Supply/Consumable	Notes
Gloves, Latex or Nitriles	N/A
Centrifuge Tubes, 0.5mL, 1.5mL, and 2.0mL	N/A
Brady FreezerBondz II Laboratory Labels	Fisher Catalog Number: 19-140-586
Brady™ B30 Series Heavy-Duty Printer Ribbon B30-R6400	Fisher Catalog number: 19-140-589
Sterile PCR Grade Filter Tips	N/A
Or Equivalent	

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3. REAGENTS AND CHEMICALS

3.1. Reagents and Chemicals –

Reagent/Chemical	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)	General lab supply is prepared by Taxonomy staff per SOP IZ-09
TE Buffer	Catalog Number: BP2473-500
Or Equivalent	

4. HF183 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 ordered with the 5' 6-FAM dye and 3' MGBEQ quencher from Eurofins or IDT. Store at -20°C.

4.1.1. **Forward Primer HF183F**: 5'–ATCATGAGTTCACATGTCCG–3'

4.1.2. **Reverse Primer BacR287**: 5'–CTTCCTCTCAGAACCCCTATCC–3'

4.1.3. **Probe BacP234MGB**: [6–FAM]–5'–CTAATGGAACGCATCCC–[MGBEQ]–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\COA and Reagent information\Oligo Data Sheet

4.3. Record each preparation in 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 HF183 –

Identity	**HF183F**
Description	HF183 Assay Forward Primer HF183F
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank

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Purity	Salt-Free
Received	Date Received
Expires	730 days (2 years) from received date
Manufacturer	Integrated DNA Technologies (IDT)
Lot Number	Barcode ID from Data Sheet
Storage Location	B246A
Storage Temperature	Freezer at <-10 degC
Container Size	Weight in mg
Units	mg

4.3.3.2. For Reverse Primer BacR287 –

Identity	**HF183R**
Description	HF183 Assay Reverse Primer BacR287
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received	Date received
Expires	730 days (2 years) from received
Manufacturer	Integrated DNA Technologies (IDT)
Has Certificate of Analysis	Yes
Lot Number	Barcode ID from Data Sheet
Storage Location	B246A
Storage Temperature	Freezer at <-10 degC
Container Size	Weight in mg
Units	mg

4.3.3.3. For BacP234MGB Probe –

Identity	**HF183P**
Description	HF183 Assay Probe BacP234MGB
Entered By	Analyst Name
Type	Powder
Grade	HPLC Grade
Purity	HPLC
Received	Date received
Expires	730 (2 years) days from received date
Has Certificate of Analysis	Yes
Manufacturer	Integrated DNA Technologies (IDT)
Lot Number	Barcode ID from Data Sheet
Additional Information	Concentration in nmoles
Storage Location	B246A
Storage Temperature	Freezer at <-10 degC
Container Size	Weight in mg
Units	mg

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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 Brady Workstation 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 Step 4.2.).

5. PRIMERS AND PROBE RESUSPENSION AND ALIQUOTS

Note: New batches should be tested in a timely manner after receipt from the vendor to avoid delays if there are quality issues.

5.1 **Resuspended primers and probes:** If primers and probes are received resuspended, they can be frozen until ready to test and aliquot. If they are frozen, thaw, then gently mix and pulse spin each tube. Run a comparison plate of the new and old batches of primers and probes using the same lot of standard. The standard curves should perform similarly. Determine the number of aliquots needed based on the volume of the probe since it has a lower yield than the primers. Aliquot 10 µL of primers into 0.5 ml tubes, and aliquot 8 ul of probe into 0.5 ml tubes only in PCR Workstation #1. Skip to section 5.8.5 to print labels.

5.2. **Dry primers and probes**: The concentrations of primers and probe will vary with each order. This is listed in the "Amount of Oligo" 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 "For 100 µM: add X µL" column. Use this amount for a 100 µM concentration. For a 500 µM concentration, divide this number by 5.

5.3. 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.

5.4. **For the primers:** Add the appropriate amount of TE buffer into the tube containing the dried primer to make a 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 10 µL volumes into blue 0.5 mL centrifuge tubes.

5.5. **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.

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Allow the solution to equilibrate for 5 minutes. Mix again and pulse centrifuge to coalesce droplets. Aliquot 8 µL volumes into blue 0.5 mL centrifuge tubes.

5.6. 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.

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

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

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

5.8.2. 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.

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

5.8.3.1. For HF183 Forward Primer –

Identity	**HF183F**
Type	Working Reagent
Holding Time	2 years from received date
Entered By	Analyst Name
Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 degC
SOP	PCR-3.2
Description	HF183 Assay Forward Primer 500 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	TE 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 HF183F S/N and weight in µg of forward primer

5.8.3.2. For HF183 Reverse Primer –

Identity	**HF183R**
Type	Working Reagent
Holding Time	2 years from received date
Entered By	Analyst name
Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 degC
SOP	PCR-3.2
Description	HF183 Assay Reverse Primer 500 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	TE Buffer

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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 HF183R S/N and weight in µg of forward primer

5.8.3.3. For HF183 Probe –

Identity	**HF183P**
Type	Working Reagent
Holding Time	730 days (2 years) from received date
Entered By	Analyst Name
Prepared	Enter Date, Time, and Analyst name
Location	B246A
Storage Temperature	Freezer at <-10 degC
SOP	PCR-3.2
Description	HF183 Probe 100 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	TE 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 HF183P S/N and weight in µg of probe

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

5.8.5. Open the LabelMark6 software and create labels. Print labels with the reagent name (HF183F, HF183R, or HF183P), serial number, and expiration date. The top label should include the reagent name and serial number.

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

6. GBLOCK STANDARD

6.1. A gBlock® gene fragment is ordered from IDT and is used as a standard for the HF183 assay. The target sequence for the HF183 assay is a 171 bp fragment (bold sequences show location of primer binding):

5’–CCGGCGCACGGGTGCGTAAC**ATCATGAGTTCACATGTCCG**CATGATTAA
AGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTAG
GCGGGGTAACGGCCCACCTAGTCAACGAT**GGATAGGGGTTCTGAGAGGAA**
GGAATACCGCGAGGTGGAGC–3’

6.2. This sequence is combined with the target sequence for the HF183 assay to generate a standard that can be used for both assays. This gBlock fragment is named HFG2GD gBlock and is a 725 bp fragment:

5’–CAAAACTTTTAACTGATGCTATCATGAGTTCACATGTCCGCATGATTAAAG
GTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTAGGC
GGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGTGC
ATCGACCTAAAGTTTTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTG
CCCATCAGAGGGGGACAACACTTGGAAACAGGTGCTAATACCGCATAATACA

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GAGAACCGCATGGTTCTTTGTTGAAAGGCGCTTCTGGTGTCGCTGATGGATGG
ACCCGCGGTGCATTAGCTAGACGGTGAGGTAACGGCTCACCGTGGCAATGAT
GCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCC
AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCT
GACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGT
TGTTAGAGAAGAACAGGAGCGATAGTAACTGCTCGCTCTTTGACGGTATCTA
ACCAGAAAGCCATCGGCTGAGCACTCTAGGGAGACTGCCTTCGTAAGGAGGA
GGAAGGTGAGGACGACGTCAAGTCATCATGGCCCTTACGCCTAGGGCTACAC
ACGTGCTACAATGGGATGTACAAAGAGACGCAATACCGCGAGGTGGAGC–3’

6.3. No modifications or purifications needed, and 500 ng of the gBlock arrives dried 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.

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 HF183 gBlock –

Identity	**HFG2GD gBlock**
Description	gBlock standard for HF183, Gull2, and GFD assays
Entered By	Analyst Name
Type	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 <-80 degC
Additional Information	725 base pairs
Container Size	0.0005
Units	mg

6.4. Click **OK** to save the entry and print the label in LIMS. Place LIMS label with LIMS serial # on the COA document (specification sheet) that came with gBlock.

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 TE. Vortex briefly, then incubate at 50°C for 20 minutes in benchtop incubator. 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 HFG2GD gBlock in TE buffer (see table below) to measure on Qubit fluorometer. Gently mix each dilution and pulse centrifuge to coalesce droplets.

Concentration	**1:10**	**1:100**
Stock Name	HFG2GD gBlock	1:10
Amount of Stock	10 µL	50 µL
Amount of TE Buffer	90 µL	450 µL

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 4.0 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.

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: HFG2GD_Qubit_YYYYMMDD. Save the file in the appropriate yearly folder in \\floridadep\data\LABS\Lab3\Biology\qPCR\Qubit Data\.

7.5. Determine the concentration of stock HFG2GD 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 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 or redo dilutions 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.

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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 HFG2GD double-stranded gBlock=**1.28 X 10^9 copies of HF target sequence.**

$$\frac{TSC\ HFG2GD}{5\ uL} = (Stock\ Conc.\ in\ ng/mL)\left(\frac{1\ mL}{1000\ uL}\right)\left(\frac{1.28\ \times\ 10^9\ TSC}{1\ ng}\right) \times 5$$

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

7.6.2.2. To determine the concentration of the 1:100 dilution, divide the 1:10 concentration by ten.

7.7. Once the concentration has been calculated, continue to serially dilute to make 3,000 µL of an E6 solution. This will be used as a working standard and extraction control.

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

7.8. Prepare a comparison plate to analyze alongside an older standard. Serially dilute the newly prepared E6 to make a standard curve from 10^6-10^2. New standards should perform similarly to older standards. Check efficiency and R^2 of E7-E2. If results are acceptable, proceed to Section 7.9., if not, remake the E6 standards.

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

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

7.9.2. 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.

7.9.3. For Stock HFG2GD gBlock DNA –

Identity	**x.xxEx HFG2GD 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 <-80 degC
SOP	PCR-3.2
See Also	Leave Blank
Description	HFG2GD 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 HFG2GD S/N and weight in ng (500 ng)

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7.9.4. 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.

7.9.5. Intermediate HFG2GD gBlock DNA Standard –

Identity	**x.xxEx HFG2GD (use the measured concentration in TSC/5µL)**
Type	Intermediate Standard
Holding Time	365 days from received date
Entered By	Analyst Name
Prepared By	Entered By, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-10 degC
SOP	PCR-3.2
See Also	Leave blank
Description	HFG2GD gBlock diluted from EX (with X changing from 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.

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

7.9.7. Working HFG2GD gBlock DNA Standard –

Identity	**x.xxE6 HFG2GD gBlock (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 <-80 degC
SOP	PCR-3.2
See Also	Leave Blank
Description	Resuspended gBlock used for standards for HFG2GD
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

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

7.9.9. HFG2GD Extraction Control –

Identity	**x.xxE6 HFG2GD EC (use measured concentration in TSC/5µL)**
Type	Working Standard
Holding Time	365 days from received date
Entered By	Analyst Name

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Prepared By	Enter Date, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-80 degC
SOP	PCR-3.2
See Also	Leave blank
Description	Extraction control for HF183, Gull2, and GFD 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

7.10. Determine the number of tubes to aliquot for HFG2GD STD and HFG2GD EC (50 µL volumes for the STD and 16 µL volumes for the EC).

7.11. Open the Brady Workstation software and create labels. On the side label, include the serial number, name, concentration, (x.xxE^6 HFG2GD STD or x.xxE^6 HFG2GD EC), and expiration date. On the top label include HFG2GD STD or HFG2GD EC. Print labels and affix to the top and sides of the tubes.

7.12. Aliquot the HFG2GD STD in 80 µL volumes and the HFG2GD EC in 20 µL volumes in blue 0.5 mL tubes. Store in the same box at -80 °C.

7.12.1. When setting up the reaction plate, the 10^6 standard will be diluted further to 10^5, 10^4, 10^3, 10^2 to make a standard curve for the HFG2GD assay. The 10^4 dilution will also serve as a third well spike.

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

7.13.1. HFG2GD 3rd well SPK –

Identity	**x.xxE4 HFG2GD 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 at <-10 degC
SOP	PCR-3.2
See Also	Leave blank
Description	3rd well SPK for HF183, Gull2, and GFD assays. HFG2GD gBlock diluted from E5 at plate preparation.
Additional Information	TE Buffer S/N
Solvent Used	TE Buffer

8. HF183 ASSAY STANDARD CURVES, EXTRACTION CONTROL, AND THIRD WELL SPIKE

8.1. The HF183 standard curve consists of running concentration series 10^6, 10^5, 10^4, 10^3, and 10^2 TSC/5 µL HFG2GD gBlock and should be run on every HF183 plate. The third well spike is run at a concentration of 10^4 TSC/5 µL.

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8.2. The extraction control (EC) has a concentration of 10^6 TSC/5 µL of HFG2GD gBlock.

8.3. HFG2GD standards and third well spike will be made by serial dilution diluted before each run from 10^6 concentration according to the table below.

Concentration	**10^5**	**10^4**	**10^3**	**10^2**
Stock Name	10^6	10^5	10^4	10^3
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 at \\floridadep\data\DEAR\Labs\Lab5\Biology\MolecularBiology\Validation and calibration\calculations.

8.4.1. $Crude\ Concentration = \frac{x.xxE6*(\frac{5\mu L}{600\mu L})}{5\mu L}$

8.4.2. $Pure\ Concentration = \frac{x.xxE6*(\frac{5\mu L}{100\mu L})}{5\mu L}$

8.4.3. $3rd\ Well\ SPK\ Concentration = \frac{x.xxE4*(\frac{1\mu L}{5\mu L})}{5\mu L}$

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 Table→SPK. Carefully enter the new LFB, LFBP, and SPK as in the example below:

For the HF183 assay, select the previous BDLFBxxxxxx in the SPK window. In the component window, select HF183-qPCR. Hit replicate SPK. Enter new ID information. Hit ok. Select the new BDLFB only. Click the Conc column. Enter the new concentration. Click ok. Repeat for all assays:

For HF183: BDLFBxxxxxx, BDLFBPxxxxxx, BDSPKxxxxxx

For Gull2: G2LFBxxxxxx, G2LFBPxxxxxx, G2SPKxxxxxx

For GFD: GDLFBxxxxxx, GDLFBPxxxxxx, GDSPKxxxxxx

Record the third 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 SPK to the end of the Identity name.

9. QUALITY CONTROL

9.1. Maintain control charts of HF183 extraction controls. If recoveries show a decreasing trend, DNA standards may be deteriorating or binding to the tubes and need to 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.

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

10. REFERENCES

10.1. DEP Quality Manual

10.2. Health and Safety Plan

10.4. DEP SOP BB-039, *Calibration and Use of Mechanical Pipettes Using the Sartorius® Cubis II Balance*

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

10.6. SOP PCR2.0 Calibration, Use, and Maintenance of the Qubit 4.0 Fluorometer

Appendix of Significant Changes

06/14/2017 Added table of contents and cover page, made major revision to working stock standards procedure and added new culturing procedure.

06/05/2018 Updated the primers/probe and label information.

05/30/2019 Updated link and printer information.

02/03/2022 Added changes from B. dorei gDNA to HFG2GD gBlock®.

01/17/2023 Updated primers/probe sequences. Added new fridge and freezer information. Removed old fridge and freezer S/Ns. Added Ethanol 80% as cleaning reagent.

11/20/2023 Removed instructions to prepare E7 gBlock concentration.

12/22/2025 Increased the aliquot volumes of the E6 standard and the EC for storage. Replaced references to Artel with reference for Sartorius SOP for calibration and use of pipettes. Added SOP reference for Qubit fluorometer.

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