Standard Operating Procedure for:

Preparation of HF183 Assay Reagents and Standards for qPCR analysis

DEPARTMENT OF ENVIRONMENTAL PROTECTION LABORATORIES
BIOLOGY PROGRAM
TALLAHASSEE FLORIDA

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1. INTRODUCTION

1.1. 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 for determination of 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 an added 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. Gloves, latex or nitriles

2.2. Tube racks

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

2.4. Brady FreezerBondz II Laboratory Labels (Cat#19-140-586)

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

2.12. PCR#9, Thermo Refrigerator Isotemp (S/N TR1562211012), maintained at 4ºC ± 2ºC, Room B246A

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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#8, Thermo Revco Freezer (S/N 1127018801220603) maintained at -80ºC ±10ºC, Room B246

2.16. Incubating mini shaker (S/N 150747)

2.17. Autoclave (S/N 031761851)

3. REAGENTS AND CHEMICALS

3.1. Deionized (DI) water

3.2. Molecular Biology grade water. Corning Molecular Biology Grade Water tested to USP Sterile Water (Cat.#MT46000CV)

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

3.4. Ethanol 80% (v/v)

3.5. TE Buffer

4. HF183 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 ordered with the 5' 6-FAM dye and 3' MGBEQ quencher from Eurofins. 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 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 HF183: Identity: = HF183F; Description = HF183 Assay Forward Primer HF183F; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = desalted; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer: = Integrated DNA Technologies (IDT); Lot # = 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 BthetR1: (NOTE: Reverse Primer BthetR1 will be entered as HF183R for consistency with the HF183 qPCR assay). Identity = HF183R; Description = HF183 Assay Reverse Primer BthetR1; Entered by = Analyst Name; Type = Powder; Grade = blank; Purity = desalted; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Integrated DNA Technologies (IDT); Lot # = 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 BthetP1Probe: (NOTE: Probe BthetP1 will be entered as HF183P for consistency with the HF183 qPCR assay). Identity = HF183P; Description = HF183 Assay Probe BthetP1; Entered by = Analyst Name; Type = Powder; Grade = HPLC Grade; Purity = HPLC; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Integrated DNA Technologies (IDT); Lot # = Barcode ID from data sheet; Storage Location = B246A; Storage Temperature = Freezer at <-10 degC; Container Size = weight in mg; 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. 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

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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).

4.3.7. The amount of primer/probes will vary with each probe. 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". 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 as either a translucent film or a white powder in a centrifuge tube.

4.5. For each primer, pipette 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. Print labels with the serial number, HF183F or HF183R, and expiration date and affix labels to the side of each tube. The top label should include HF183F or HF183R and the serial number.

4.6. For the probe, pipette appropriate amount of TE buffer into the tube containing the dried primer 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 8 µL volumes into blue 0.5 mL centrifuge tubes. Print labels with the serial number, HF183P, and expiration date and affix labels to the side of each tube. The top label should include HF183P and the serial number.

4.7. Group in sets of forward primer, reverse primer, and probe in a freezer box 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:

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4.8.3.1. For HF183 Forward Primer: Identity: = HF183F; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183 Assay Forward Primer 500 µM concentration; 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: = µL;

4.8.3.2. For HF183 Reverse Primer: Identity = HF183R; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183Assay Reverse Primer 500 µM concentration; 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: = µL;

4.8.3.3. For HF183 Probe: Identity = HF183P; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183 Probe 100 µM concentration; 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: = µL;

4.8.4. Click **Add**. Choose the **Purchased Materials** - 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 mg and choose "mg" from the "Added Units:" drop down list. Click **OK**.

4.8.5. Click **OK** to save the entry and receive a serial number. Open the LabelMark6 software and create labels (see SOP PCR-5.0 for details). Print labels with the reagent name (HF183F, HF183R, or HF183P), serial number, and expiration date and affix labels to the side of each tube. The top label should include the reagent name and serial number. Place labels on all aliquoted tubes before storage at -20°C.

5. GBLOCK® STANDARD

5.1. A gBlock® gene fragment is ordered from Integrated DNA Technologies (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' -

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CCGGCGCACGGGTGCGTAAC**ATCATGAGTTCACATGTCCG**CATGATTAA
AGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTA
GGCGGGGTAACGGCCCACCTAGTCAACGAT**GGATAGGGGTTCTGAGAGG**
AAGGAATACCGCGAGGTGGAGC – 3

5.2. 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' –
CAAAACTTTTAACTGATGCTATCATGAGTTCACATGTCCGCATGATTAAAGG
TATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTAGGCG
GGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAGTGC
ATCGACCTAAAGTTTTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCCATCAGAGGGGGACAACACTTGGAAACAGGTGCTAATACCGCATAATA
CAGAGAACCGCATGGTTCTTTGTTGAAAGGCGCTTCTGGTGTCGCTGATGGA
TGGACCCGCGGTGCATTAGCTAGACGGTGAGGTAACGGCTCACCGTGGCAA
TGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACAC
GGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGA
AAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAA
ACTCTGTTGTTAGAGAAGAACAGGAGCGATAGTAACTGCTCGCTCTTTGAC
GGTATCTAACCAGAAAGCCATCGGCTGAGCACTCTAGGGAGACTGCCTTCG
TAAGGAGGAGGAAGGTGAGGACGACGTCAAGTCATCATGGCCCTTACGCCT
AGGGCTACACACGTGCTACAATGGGATGTACAAAGAGACGCAATACCGCGA
GGTGGAGC – 3'

5.3. There are no modifications or purifications 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. 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 **Purchased/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 HF183 gBlock</u>: Identity: = HF183 gBlock; Description: = gBlock standard for HF183, Gull2, 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: = B246A; Storage Temperature: = Freezer <10 degC; Additional information: = Use with SOP = PCR-3.2.

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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 HFG2GD 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 HFG2GD 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.

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 HFG2GD 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 HFG2GD 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 HFG2GD double-stranded gBlock® = 1.28 X 10^9 copies of HF183 target sequence.

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

6.5. Determine the concentrations of the dilutions in TSC/5 µL units and continue the serial dilution down to a concentration of 10^6 in 1000 µL volumes. This will be used as the working standard.

6.6. Using blue 0.5 mL tubes, aliquot the 10^6 dilution in 50 µL volumes. Place labeled dots with the concentration (E6) on the tops of all tubes. Store the 10^6 tubes at -80°C.

6.6.1. The 10^6 standards will be diluted further to 10^5, 10^4, 10^3, and 10^2 when setting up the reaction plate to make a standard curve for the HF183 assay and as a third well spike at a concentration of 10^4.

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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, 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. For Stock HFG2GD gBlock® DNA: Identity: = x.xxEX HFG2GD (use measured concentration in TSC/5µL); Type: = Stock Standard; Holding Time: = 365 days; Entered by: = Analyst Name; Location: = B246A; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.2; See Also: = leave blank; Description: = HFG2GD gBlock reconstituted at 10 ng/µL 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 gDNA Standard: Identity: = x.xxEX HFG2GD (use the measured concentration of the standard in TSC/5 µL); Type: = Working Standard; Holding Time = 365 days; Entered by: = Analyst Name; Location: = B246A; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.2; See Also: = leave blank; Description: = resuspended gBlock used for standards for HF183, Gull2, and GFD assays; Additional Information = TE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 2000.00 ; 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 HFG2GD 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 to save the entry and receive a serial number. Open the LabelMark6 software and create labels. On the side label, include the serial number, name, concentration, (_.__ E6 HFG2GD STD or _.__ E6 HFG2GD SPK), and expiration date.

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On the top label, include E6 HFG2GD STD or E6 HFG2GD SPK. Print labels and affix to the top and sides of the tubes. Store at -80°C.

7. HF183 ASSAY STANDARD CURVES AND THIRD WELL SPIKE

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

7.2. HF183 standards and third well spike will be diluted before each run from 10^6 concentration. To make the 10^5 standard, dilute by mixing 10 µL of the 10^4 dilution with 90 µL of TE buffer. 10^3, and 10^2 will be made similarly by serial dilution.

7.3. 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.

7.4. When new standards are needed, dilute the stock gBlock® and measure as described in Section 6. Reorder as needed.

8. HF183 EXTRACTION CONTROL

8.1. The extraction control (EC) has a concentration of 10^{-6} TSC/5 uL of HFG2GD gBlock®.

8.2. Use the 10^7 HFG2GD gBlock® stock standard to make the 10^6 EC dilution by mixing 50 µL of the 10^7 dilution with 450 µL of TE buffer.

8.3. Aliquot the EC spike into blue 0.5 mL tubes in 16 µL volumes and keep frozen at -80°C in B246. Place labeled dots with the concentration (E6) on the tops of all tubes.

8.4. Record the extraction control 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 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 Quality Manual 2023

10.2. Health and Safety Plan

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

10.4. DEP SOP BB-36, *Calibration, Use and Maintenance of Mettler Toledo XA105DU Balance*

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

10.6. DEP SOP PCR-4.2 *Purification of Filtered Samples Using the Qiagen DNeasy PowerSoil® Pro Kit*

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

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.

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