PREPARATION OF GULL2 ASSAY REAGENTS AND STANDARDS FOR qPCR ANALYSIS

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1. INTRODUCTION

1.1. The GULL2 assay targets a seabird-associated 16S rRNA gene in *Catellicoccus marimammalium* and detects fecal contamination from gulls and other costal birds using quantitative Polymerase Chain Reaction (qPCR). A gBlock® fragment containing a 452 bp Gull2 qPCR target sequence is used as a standard in the Gull2 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 Gull2 *C. marimammalium* 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 Minicentrifuge	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 Freezer	S/N 1127920701221013; Maintained at -80 ± 10°C, Room B246
Incubating Mini Shaker	S/N 150747
Autoclave	S/N 031761851
Or Equivalent	

2.3. Supplies/Consumables –

Supplies/Consumables	Notes
Gloves, Latex or Nitriles	N/A
Centrifuge tubes, 0.5mL, 1.5 mL, and 2.0 mL	N/A
Brady FreezerBondz Laboratory Labels	Catalog Number: 19-140-586

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PCR Grade Filter Tips	N/A
Or Equivalent	

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 SOPB BG-05
TE Buffer	Catalog Number: BP2473-500
Ethanol 80% (v/v)	N/A
Or Equivalent	

4. GULL2 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 Taqman® probe should be ordered with the 5' 6-FAM dye and 3' Zen-Iowa Black FQ quencher from Integrated DNA Technologies (IDT). Store at -20°C.

4.1.1. **Forward Primer Gull2For**: 5'–TGCATCGACCTAAAGTTTTGAG–3'

4.1.2. **Reverse Primer Gull2Rev**: 5'–GTCAAAGAGCGAGCAGTTACTA–3'

4.1.3. **Taqman® Probe Gull2Probe**: 5'–/6FAM/CTGAGAGGG/ZEN/TGA TCGGCCACATTGGGACT/3IABkFQ/–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 Gull2For –

Identity	**Gull2For**

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Description	Gull2 Assay Forward Primer
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received Date	Date received
Expires	365 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 deg C
Container Size	Weight in mg
Units	mg

4.3.3.2. For Reverse Primer Gull2Rev –

Identity	**Gull2Rev**
Description	Gull2 Assay Reverse Primer
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received Date	Date received
Expires	365 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 deg C
Container Size	Weight in mg
Units	mg

4.3.3.3. For Gull2 Probe –

Identity	**Gull2Probe**
Description	Gull2 Assay Probe
Entered By	Analyst Name
Type	Powder
Grade	HPLC grade
Purity	HPLC
Received Date	Date received
Expires	365 days from received date
Has Certificate of Analysis	Yes
Manufacturer	IDT DNA
Lot Number	Barcode ID from Data Sheet
Additional Information	Concentration in nmoles
Storage Location	B246A
Storage Temperature	Freezer at <-10 deg C

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Container Size	Weight in mg
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 label 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 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 yellow 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 14 µL volumes into yellow 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.

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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.

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 Gull2 Forward Primer –

Identity	**Gull2For 500**
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-3.3
Description	Gull2 Assay Forward 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 Gull2For S/N and weight in µg of forward primer

5.7.3.2. For Gull2 Reverse Primer –

Identity	**Gull2Rev 500**
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-3.3
Description	Gull2 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 Gull2Rev S/N and weight in µg of reverse primer

5.7.3.3. For Gull2 Probe –

Identity	**Gull2Probe 100**
Type	Working Reagent

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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-3.3
Description	Gull2 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 Gull2Probe 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 (see SOP PCR-5.0 for details). Print labels with the reagent name (**Gull2For, Gull2Rev, or Gull2Probe**), serial number, and expiration date. Affix labels to the side of each tube. The top label should include the reagent 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 IDT and is used as a standard for the Gull2 assay. The target sequence for the Gull2 assay is a 452 bp fragment (bold red sequences show location of primer binding):

5’ – CAAAACTTTTAACTGATGCT**TGCATCGACCTAAAGTTTTGAG**TGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAGGGGGACAACACTTGGA
AACAGGTGCTAATACCGCATAATACAGAGAACCGCATGGTTCTTTGTTGAAA
GGCGCTTCTGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGACGGT
GAGGTAACGGCTCACCGTGGCAATGATGCATAGCCGACCTGAGAGGGTGATC
GGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGA
AGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAGGAGCGATA
GTAACTGCTCGCTCTTTGACGGTATCTAACCAGAAAGCCA – 3’

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

5’ – CAAAACTTTTAACTGATGCT**ATCATGAGTTCACATGTCCG**CATGATTAA
AGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGCTCGAGATAGTAG
GCGGGGTAACGGCCCACCTAGTCAACGAT**GGATAGGGGTTCTGAGAGGAA
GTGCATCGACCTAAAGTTTTGAG**TGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCCATCAGAGGGGGACAACACTTGGAAACAGGTGCTAATACCGCAT
AATACAGAGAACCGCATGGTTCTTTGTTGAAAGGCGCTTCTGGTGTCGCTGAT
GGATGGACCCGCGGTGCATTAGCTAGACGGTGAGGTAACGGCTCACCGTGGC
AATGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACA

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CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACG
AAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAA
ACTCTGTTGTTAGAGAAGAACAGGAGCGA**TAGTAACTGCTCGCTCTTTGA**C
GGTATCTAACCAGAAAGCCA**TCGGCTGAGCACTCTAGGG**AGACTGCCTTCG
TAAGGAGGAGGAAGGTGAGGACGACGTCAAGTCATCATGGCCCTTACGCCTA
GGGCTACACACGTGCTA**CAATGGGATGTACAAAGAGACGC**AATACCGCGA
GGTGGAGC —3’

6.3. 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. 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 HFG2GD 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 -80 degC
Additional Information	725 base pairs
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.

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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 the 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 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 Quibit_Template file. Rename the file as: HFG2GD_Qubit_YYMMDD. Save the file in \\floridadep\data\DEAR\Labs\Lab3\Biology\qPCR\QubitData.

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 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 HFG2GD double-stranded gBlock=**1.28 X 10^9 copies of Gull2 target sequence.**

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

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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 10.

7.7. Prepare 1,000 µL of an E7 dilution using the 1:100 dilution and TE buffer. This is an intermediate dilution used to prepare E6.

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

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 E7 dilution.

7.8.2. Export the data as in Section 7.5.

7.8.3. Determine the concentration of the E7 in Excel. This is the concentration of the standard and will be used for quantification of samples in qPCR analysis.

7.9. Once the E7 solution is prepared, serially dilute to make 2,000 µL of an E6 solution. This solution should be a 10X dilution of the E7. This will be used as a 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 10^8-10^2 and run the E6 dilution prepared in Section 7.9. as an unknown. New standards should perform similarly to older standards Check efficiency and R^2 of E^8-E^2If results are acceptable, proceed to Section 7.11., if not, remake the E7 and E6 standards.

NOTE: The 10^8 and 10^7 standards are **NOT** used on the standard curve for analytical runs. However, they should be run on the initial comparison plate.

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 HFG2GD gBlock DNA –

Identity	**x.xxEx HFG2GD gBlock (use the measured concentration in TSC/5µL)**
Type	Stock Standard
Holding Time	365 days from received date

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Entered By	Analyst Name
Prepared By	Enter Date, Time, and Analyst name
Location	B246
Storage Temperature	Freezer at <-10 deg C
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	AE 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)

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 HFG2GD gBlock DNA Standards –

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	Enter Date, Time, and Analyst Name
Location	B246
Storage Temperature	Freezer at <-10 deg C
SOP	PCR 3.2
See Also	Leave blank
Description	HFG2GD gBlock diluted from EX (with X changing for each dilution)
Additional Information	xE^x copies/5 µL
Solvent Used	AE 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 HFG2GD gBlock® DNA Standard –

Identity	**x.xx E^6 HFG2GD STD (Use measured concentration in TSC/5 µL)**
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	B246A
Storage Temperature	Freezer at <-10 deg C
SOP	PCR 3.2
See also	Leave blank
Description	HFG2GD gBlock diluted from E8. Standard for HF183, Gull2, and GFD assays
Additional information	TE Buffer S/N
Solvent used	AE buffer
Components	FFinal 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

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

7.11.9. HFG2GD Extraction Control –

Identity	**x.xx E^6 HFG2GD EC (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 deg C
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	AE 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 HFG2GD STD and HFG2GD EC (50 µL volumes for the 10^6 HFG2GD STD and 16 µL volumes for HFG2GD EC).

7.13. Open the LabelMark6 software and create labels. On the side label, include the serial number, name, concentration, (x.xx E^7 HFG2GD STD or x.xx E^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.

7.14. Aliquot the HFG2GD STD in 50 µL volumes and the HFG2GD EC in 16 µL volumes in blue 0.5 mL tubes. Store in the same box at -80C.

7.14.1. When setting up the reaction plate, the 10^6 standard will be diluted further to 10^5, 10^4, 10^3, and 10^2 to make a standard curve for the Gull2 assay. The 10^4 dilution will also serve as a third well spike.

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

7.15.1. HFG2GD 3rd well SPK –

Identity	**x.xx E4 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 -80 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	AE buffer

8. GULL2 ASSAY STANDARD CURVES, EXTRACTION CONTROL, AND THIRD WELL SPIKE

8.1. The Gull2 standard curve consists of running concentration series 10^6, 10^5, 10^4, 10^3, and 10^2 TSC/5µL HFG2GD gBlock and should be run on every Gull2 plate. The third well spike is run at a concentration of 10^4 TSC/5 µL.

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

8.3. Gull2 standards and third well spike will be made by serial dilution before each run from 10^7 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. Calculate the LIMS concentrations for crude, pure, and 3rd well SPK as follows:

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 thermalcycler. In the sample table, click Edit→Limits Tables→SPK. Carefully enter the new LFB, LFBP, and SPK as in the example below:

For the HF183 assay, Select previous BDLFBxxxxxx in the SPK window. In the component window, select HF183-qPCR. Hit replicate SPK. Enter New ID

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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 GFD: GDLFBxxxxxx, GDLFBPxxxxxx, GDSPKxxxxxx

For Gull2: GPLFBxxxxxx, GPLFBPxxxxxx, GPSPKxxxxxx

9. QUALITY CONTROL

9.1. Maintain control charts of Gull2 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.

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 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

11/29/2021 Added changes from Gull2 gBlock to HFG2GD gBlock.

12/06/2022 Added new -80°C freezer and +4°C refrigerator. Removed references to old standard.

11/01/2023 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 HFG2GD 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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