PREPARATION OF HUMM2 ASSAY REAGENTSAND STANDARDS FOR qPCR ANALYSIS

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1. INTRODUCTION

1.1. The HumM2 assay targets a single copy chromosomal gene in *Bacteroides*-like microorganisms for the detection of human fecal contamination using quantitative polymerase chain reaction (qPCR). A gBlock® fragment containing a 333 bp HumM2 qPCR target sequence is used as the standard in the HumM2 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 spike are used as positive controls for the HumM2 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 and 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 per SOP PCR-2.0

2.2. Equipment –

Equipment	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
Finish 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 Freeezer	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 Steris Amsco Model 250 LS	S/N 031761851
Or Equivalent	

2.3. Supplies and Consumables –

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

3. REAGENTS AND CHEMICALS

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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)	N/A
TE Buffer	Catalog Number: BP2473-500
Or Equivalent	

4. HUMM2 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 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. See SOP PCR-5.3 for ordering reagents.

4.1.1. **Forward Primer HumM2For**: 5'–CGTCAGGTTTGTTTCGGTATTG–3'

4.1.2. **Reverse Primer HumM2Rev**: 5'–TCATCACGTAACTTATTTATATGCATTAGC–3'

4.1.3. **Taqman® Probe HumM2Probe**: 5'–/56–FAM/TATCGAAAA/ZEN/TCTCACGGATTAACTCTTGTGTACGC/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\COA and Reagent information\Oligo Data Sheet.

4.3. Record 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 HumM2 Forward Primer –

Identity	**HumM2For**
Description	HumM2 Assay Forward Primer
Entered By	Analyst Name
Type	Powder

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Grade	Leave Blank
Purity	Salt-Free
Received	Date received
Expires	730 days (2 years) from received date
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

4.3.3.2. For HumM2 Reverse Primer –

Identity	**HumM2Rev**
Description	HumM2 Assay Reverse Primer
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received	Date received
Expires	730 days (2 years) from received date
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 HumM2 Probe –

Identity	**HumM2Probe**
Description	HumM2 Assay Probe
Entered By	Analyst Name
Type	Powder
Grade	HPLC Grade
Purity	HPLC
Received	Date received
Expires	730 days (2 years) 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

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Storage Temperature	Freezer at <-10 degC
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 Brady Workstationsoftware 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

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

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 purple 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 10 µL volumes into purple 0.5 mL centrifuge tubes.

5.5. When a new batch of primers andprobe 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.

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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.

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 HumM2 Forward Primer –

Identity	**HumM2For**
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-3.8
Description	HumM2 Assay Forward Primer 500 µM concentration
Additional Information	TE Buffer S/N
Solvent Used	AE Bufer
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 HumM2For S/N and weight in µg of forward primer

5.7.3.2. For HumM2 Reverse Primer –

Identity	**HumM2Rev**
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-3.8
Description	HumM2 Assay Reverse 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 HumM2Rev S/N and weight in µg of reverse primer

5.7.3.3. For HumM2 Probe –

Identity	**HumM2Probe**

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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-3.8
Description	HumM2 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 HumM2Probe 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 Brady Workstation software and create labels. Print labels with the reagent name (HumM2For, HumM2Rev, or HumM2Probe), serial number, and expiration date. 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 HumM2 assay. The target sequence for the HumM2 assay is a 333 bp fragment (bold sequences show location of primer binding):

5’ – ATCGCTCACACCGA**CGTCAGGTTTGTTTCGGTATTG**AGTATCGAAAAT
CTCACGGATTAACTCTTGTGTACGCTCTCGAGGACCA**GCTAATGCATATAAA**
TAAGTTACGTGATGAGACCGGCGCACGGGTGAGTAACACGTATCCAACCTG
CCGTCTACTCTTGGCCAGCCTTCTGAAAGGAAGATTAATCCAGGATGGGATC
ATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGA
TGCGTTCCATTAGCTCGAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAAC
GATGGATAGGGGTTCTGAGAGGAAGG – 3’

6.2. This sequence is combined with the target sequence for the CowM2 assay to generate a standard that can be used for both assays. This gBlock fragment is named C2H2 gBlock and is a 449 bp fragment:

5’ –ATCGCTCACACCGAATCGCGGCCAAATACTCCTGATCGTACTCGAGATAG
GCACCTATGTCCTTTACCTCATCAACTACAGACAAAATTATCTCAAGGAACGC
AACAAGCATCGCCTCTAATGGAAAATGCGTCAGGTTTGTTTCGGTATTGAGTA
TCGAAAATCTCACGGATTAACTCTTGTGTACGCTCTCGAGGACCAGCTAATGC
ATATAAATAAGTTACGTGATGAGACCGGCGCACGGGTGAGTAACACGTATCC
AACCTGCCGTCTACTCTTGGCCAGCCTTCTGAAAGGAAGATTAATCCAGGAT
GGGATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGAT
GGGGATGCGTTCCATTAGCTCGAGATAGTAGGCGGGGTAACGGCCCACCTAG
TCAACGATGGATAGGGGTTCTGAGAGGAAGG – 3’

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6.3. No modifications or purifications needed, and 250 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, select **Prepared/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 C2H2 gBlock –

Identity	**C2H2 gBlock**
Description	gBlock standard for CowM2 and HumM2 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 <-10 degC
Additional Information	449 base pairs
Container Size	0.00025
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 250 ng, add 25 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 C2H2 gBlock in TE buffer (see table below) to measure on the Qubit fluorometer. Gently mix each dilution and pulse centrifuge to coalesce droplets.

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Concentration	1:10	1:100
Stock Name	C2H2 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 on 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. Select the checkbox for the data you want to export. If only specific points from a day should be exported, first click the date of the run and then select the specific points. Next select Export→CSV→USB. A check mark will appear on the screen if export is successful. The Qubit data are 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: C2H2_Qubit_YYMMDD. Save the file in \\floridadep\data\LABS\Lab3\Biology\qPCR\Qubit Data.

7.5. Determine the concentration of stock C2H2 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 C2H2 double-stranded gBlock=**2.06 X 10^9 copies of C2 target sequence.**

$$\frac{TSC\ C2H2}{5\ uL} = \left(Stock\ Conc.\ in \frac{ng}{mL}\right)\left(\frac{1\ mL}{1000 uL}\right)\left(\frac{2.06\ X\ 10^9\ TSC}{1\ ng}\right) \times 5$$

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 10.

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7.7. Prepare a 10-fold dilution using the E8 concentration to make 3,000 µL of an E^7 dilution. This will be used as the working standard and extraction control. Use the formula $C_1V_1=C_2V_2$ and refer to table 7.3 as a guide for making dilutions.

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 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.

7.9. 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 C2H2 gBlock DNA –

Identity	**x.xxEx C2H2 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 degC
SOP	PCR-3.8
See Also	Leave Blank
Description	C2H2 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 (25 µL) Click Add→Choose Purchased Material: Enter TE S/N and volume in µL Click Add→Choose Purchased Material: Enter C2H2 S/N and weight in ng (250 ng)

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 take out of the tube. Write this information down every time a new dilution is prepared.

7.9.5. Intermediate C2H2 gBlock DNA Standard –

Identity	**x.xxEx C2H2 (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

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Storage Temperature	Freezer at <-10 degC
SOP	PCR-3.7
See Also	Leave blank
Description	C2H2 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 C2H2 gBlock DNA Standard –

Identity	**x.xxE7 C2H2 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 <-10 degC
SOP	PCR-3.7
See Also	Leave Blank
Description	Resuspended gBlock used for standards for C2H2 assay
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

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

7.9.9. C2H2 Extraction Control –

Identity	**x.xxE7 C2H2 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 degC
SOP	PCR-3.7
See Also	Leave blank
Description	Extraction control for CowM2 and HumM2 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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7.10. Determine the number of tubes to aliquot for C2H2 STD and C2H2 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^7 C2H2 STD or x.xxE^7 C2H2 EC), and expiration date. On the top label include C2H2 STD or C2H2 EC. Print labels and affix to the top and sides of the tubes.

7.12. Aliquot the C2H2 STD in 50 µL volumes and the C2H2 EC in 16 µL volumes in purple 0.5 mL tubes. Store in the same box at -80 °C.

7.12.1. When setting up the reaction plate, the 10^7 standard will be diluted further to 10^6, 10^5, 10^4, 10^3 to make a standard curve for the HumM2 assay. The 10^5 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 C2H2 3rd well SPK –

Identity	**x.xxE5 C2H2 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 <-10 degC
SOP	PCR-3.7
See Also	Leave blank
Description	3rd well SPK for CowM2 and HumM2 assays. C2H2 gBlock diluted from E6 at plate preparation.
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer

8. HUMM2 ASSAY STANDARD CURVES, EXTRACTION CONTROL, AND THIRD WELL SPIKE

8.1. The HumM2 standard curve consists of running concentration series 10^7, 10^6, 10^5, 10^4, and 10^3 TSC/5µL of C2H2 gBlock and should be run on every HumM2 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 C2H2 gBlock.

8.3. HumM2 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

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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.

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

For the CowM2 assay, select the previous C2LFBxxxxxx in the SPK window. In the component window, select CowM2-qPCR. Hit replicate SPK. Enter new ID information. Hit ok. Select the new C2LFB only. Click the Conc column. Enter the new concentration. Click ok. Repeat for all assays:

For CowM2: C2LFBxxxxxx, C2LFBPxxxxxx, C2SPKxxxxxx

For HumM2: H2LFBxxxxxx, H2LFBPxxxxxx, H2SPKxxxxxx

9. QUALITY CONTROL

9.1. Maintain control charts of HumM2 extraction controls. If recoveries show a decreasing trend, DNA standards may be deteriorating and need to be remade.

9.2. Test each new preparation of standards and primer/probe sets. 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. Run a comparison plate of an older primer/probe set alongside a new primer/probe set using the same standard for both curves.

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

10.3. 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*

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

Appendix of Significant Changes

01/05/2021	Updated the gBlock information to the combined C2H2 gBlock.
12/28/2022	Updated new fridge and freezer information in Equipment and Supplies.
11/16/2023	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 C2H2 gBlock. Added formulas and instructions to calculate the concentration of the EC and 3rd well spike and enter this information in LIMS.
01/10/2024	Changed expiration date of Primers/Probe. Updated Qubit and gBlock standard preparation. Added procedure for primers and probes that are purchased as resuspended reagents.

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