PREPARATION OF SKETA ASSAY REAGENTS AND STANDARDS FOR qPCR ANALYSIS

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1. INTRODUCTION

1.1. Sperm cells from salmon testes of the species, *Oncorhynchus keta* (*O. keta*), are used for the SKETA assay. The SKETA assay is used alongside the HF183, Gull2, DG3, HumM2, CowM2, GFD, and BacR assays as a sample processing and inhibition control. The salmon DNA is added to the extraction buffer which is added to all samples and controls during extraction.

1.2. Each sample for Test IDs PCR-HF183, PMA-HF183, PUR-HF183, PCR-GULL2, PUR-GULL2, PCR-DG3, PUR-DG3, PCR-CowM2, PUR-CowM2, PCR-HumM2, PUR-HumM2, PCR-GFD, PUR-GFD, PCR-BACR, and PUR-BACR are analyzed with the SKETA assay to measure extraction efficiency and check for possible inhibition. Refer to the individual method SOPs for the use of SKETA assay controls.

2. EQUIPMENT AND SUPPLIES

2.1. Instrumentation –

Instrument Name	Serial Number	Manufacturer	Notes
Qubit 2.0 Fluorometer	1301008411	Invitrogen	See SOP PCR-2.0 for calibration and use

2.2. Equipment –

Equipment	Notes
Tube Racks	N/A
Micropipettes	See SOP PCR-2.4 for calibration and use
Mettler Toledo MS403TS/00 Balance	S/N B815673069; See SOP BB-035 for calibration and use
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
Centrifuge	N/A
Analog Dry Bath Incubator with Thermometer	S/N C1648131158860
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#9, Thermo Refrigerator Isotemp	S/N TR1562211012; Maintained at 4 ± 2°C, Room B246A
PCR#10, Thermo Revco Freezer	S/N 1127920701221013; Maintained at -80 ± 10°C, Room B246
Incubating Mini Shaker	S/N 150747
Or Equivalent	

2.3. Supplies/Consumables –

Supplies/Consumables	Notes
Sterile Gloves, Latex or Nitriles	N/A
Centrifuge Tubes, 0.5 mL, 1.5 mL, and 2.0 mL	N/A
Brady FreezerBondz Laboratory labels	Catalog Number 19-140-586
50 mL Centrifuge Tubes	N/A
Pipette Tips	N/A

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Or Equivalent

3. REAGENTS AND STANDARDS

3.1. Reagents and Standards –

Reagent/Standard	Notes
Deionized (DI) Water	N/A
Molecular Biology Grade Water	Corning Molecular Biology Grade Water tested to USP sterile water. Catalog Number: MT46000CV
Bleach Solution, 10% (v/v)	N/A
Tris-EDTA, 1X Solution, pH 8.0, Molecular Biology	Catalog Number: BP2473500
Lyophilized Salmon Testes DNA	Sigma D1626 or equivalent. Stored at 2–8°C
Or Equivalent	

4. SALMON DNA 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 should be ordered with 5' 6-FAM dye and 3' Zen-Iowa Black FQ quencher from IDT.

4.1.1. **Forward Primer SketaF2**: 5'-GGT TTC CGC AGC TGG G- 3'

4.1.2. **Reverse Primer SketaR2**: 5'-CCG AGC CGT CCT GGT C- 3'

4.1.3. **Taqman® Probe SketaP2**: 5'-56 FAM/AGT CGC AGG /ZEN/ CGG CCA CCG T/3IABkFQ/ -3'

4.2. Each order includes an Oligonucleotide Data Sheet. Record the date received on this sheet and review the sequences for correctness. Scan the data sheet into the following folder: \\FLDEP1\DEAR\Labs\Lab5\Biology\MolecularBiology\Certificates of Analysis\Oligo Data Sheet

4.3. Enter each purchased primer/probe set as soon as received 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 SKETA Forward Primer –

Identity	SKETAF2
Description	Salmon SKETA Assay Forward Primer

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Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received	Date of Receipt
Expires	365 days from received date
Has certificate of analysis	Yes
Manufacturer	IDT
Additional information	Concentration in nmoles
Lot Number	Barcode ID from Data Sheet
Storage Location	B246A
Storage Temperature	Freezer at <-20 deg C
Container Size	Weight in mg
Units	mg

4.3.3.2. For SKETA Reverse Primer –

Identity	**SKETAR2**
Description	Salmon SKETA Assay Reverse Primer
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	Salt-Free
Received Date	Date of Receipt
Expires	365 days from received date
Has certificate of analysis	Yes
Manufacturer	IDT
Additional information	Concentration in nmoles
Lot Number	Barcode ID from Data Sheet
Storage Location	B246A
Storage Temperature	Freezer at <-20 deg C
Container Size	Weight in mg
Units	mg

4.3.3.3. For SKETA Probe –

Identity	**SKETAP2**
Description	Salmon SKETA Assay Probe
Entered By	Analyst Name
Type	Powder
Grade	Leave Blank
Purity	HPLC
Received Date	Date of Receipt
Expires	365 days from received date
Has certificate of analysis	Yes
Manufacturer	IDT
Additional information	Concentration in nmoles
Lot Number	Barcode ID from Data Sheet
Storage Location	B246A
Storage Temperature	Freezer at <-10 deg C
Container Size	Weight in mg

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Units	mg

4.3.4. Click **Add**, and "Identity" name will self-populate in the "Components" table. 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.

4.3.5. Click **OK** to save the entry and receive a serial number. Open the LabelMark 6 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. Place the label on the Oligonucleotide Data Sheet, before scanning (from Section 4.2.).

5. PRIMERS AND PROBE RESUSPENTION

5.1. The concentration of primers and probes 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 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.

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 12 µL volumes into pink 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 12 µL volumes into pink 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 Laboratory Information Management System (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 SKETA Forward Primer –

Identity	**SKETAF2**
Type	Working Reagent
Holding time	365 days from received date
Entered By	Analyst Name
Prepared	Enter Date, Time and Analyst name
SOP	PCR-3.1
See Also	Leave Blank
Location	B246A
Description	Salmon SKETA Assay Forward Primer 500 µM concentration
Storage Temperature	Freezer at -20 deg C
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Enter volume of TE added, Units: µL Click Add: Choose Purchase Materials: Enter TE S/N and volume in µL Click Add: Choose Purchase Materials: Click Add: Enter SKETA Forward S/N and weight in µg

5.7.3.2. For SKETA Reverse Primer –

Identity	**SKETAR2**
Type	Working Reagent
Holding Time	365 Days from received date
Entered By	Analyst Name
Prepared	Enter Date, Time and Analyst name
SOP	PCR-3.1
See Also	Leave Blank
Location	B246A
Description	Salmon SKETA Assay Reverse Primer 500 µM concentration
Storage Temperature	Freezer at <-10 deg C
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Enter volume of TE added, Units: µL Click Add: Choose Purchase Materials: Enter TE S/N and volume in µL Click Add: Choose Purchase Materials:

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	Click Add: Enter SKETA Reverse S/N and weight in µg

5.7.3.3. For SKETA Probe –

Identity	**SKETAP2**
Type	Working Reagent
Holding Time	365 Days from received date
Entered By	Analyst Name
Prepared	Enter Date, Time and Analyst name
SOP	PCR-3.1
See Also	Leave Blank
Location	B246A
Description	Salmon SKETA Probe 100 µM concentration
Storage Temperature	Freezer at <-20 deg C
Additional Information	TE Buffer S/N
Solvent Used	AE Buffer
Components Table	Final Volume: Enter volume of TE added, Units: µL Click Add: Choose Purchase Materials: Enter TE S/N and volume in µL Click Add: Choose Purchase Materials: Click Add: Enter SKETA Forward S/N and weight in µg

5.7.4. 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 (**SKETAFor**, **SKETARev**, or **SKETAProbe**), 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.

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

6. STOCK SALMON DNA AND WORKING STANDARDS

6.1. Enter the lyophilized Salmon testes DNA in Laboratory Information Management System (LIMS) Standard Preparation Tracker module.

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

6.1.2. 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.

6.1.3. In the "Purchased Material" pop-up window, enter the following information:

Identity	**Salmon DNA**
Description	DNA sodium salt from Salmon sperm
Type	Powder
Received	Enter date received

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Expires	Use expiration date in the bottle
Grade	If available in datasheet
Purity	If available in datasheet
Has Certificate of Analysis	Yes
Manufacturer	Fisher or Sigma
Lot #	Enter number on tube
Storage Location	B246A
Storage Temperature	Refrigeration at 4 degC
Additional information	Use as SOP PCR 3.1.
Container size	1
Units	g

6.1.4. Click **OK** to save the entry and print the serial number. Place serial number on the bottle and store in the fridge.

NOTES: Lyophilized Salmon DNA should be stable past the expiration date; however, the standard will need to be recertified. Document steps for validation.

6.2. Weigh 10 mg of lyophilized salmon DNA into a sterile 2.0 mL centrifuge tube. Add 1 mL of TE buffer to make a concentration of 10 mg/mL. Heat the solution to 60°C in the analog dry bath incubator or benchtop shaker for 20 mins, then vortex at a low speed for 2–4 hours to dissolve at room temperature. Leave overnight at 4°C to help completely dissolve DNA. Solution should have an even consistency.

6.3. Make a serial dilution (1:10, 1:100, 1:1000, 1:10000) of the 10 mg/mL stock salmon DNA using TE buffer as the diluent (see table below). Measure on. Gently mix each dilution and pulse centrifuge to coalesce droplets. Overmixing can affect DNA amplification.

Concentration	**1:10**	**1:100**	**1:1000**	**1:10000**
Stock Name	10 mg/mL Stock Salmon DNA	1:10	1:100	1:1000
Amount of Stock	100 µL	100 µL	100 µL	100 µL
Amount of TE	900 µL	900 µL	900 µL	900 µL

6.4. Determine the concentration of each dilution

Measure the 1:100, 1:1,000, and 1:10,000 dilutions in triplicate on the Qubit fluorometer as per SOP PCR-2.0. Use 5 µL as the sample volume for the 1:100 dilution and 20 µL for the 1:1000 and 1:10,000.

6.4.1. Each dilution should be 10X greater than the next higher dilution. The 1:100 dilution may be too viscous and not read accurately. If this occurs and the 1:1,000 and 1:10,000 dilutions are 10X from each other, the 1:100 readings can be ignored in the determination of the concentration of the stock salmon DNA. If none of the dilutions are 10X from each other, remake dilutions and re-read on the Qubit fluorometer.

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6.4.2. 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.

6.4.3. 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 SKETA_Quibit_Template file. Rename the file as: SKETA_Qubit_YYMMDD. Save the file in X:\Labs\Lab3\Biology\qPCR\Qubit Data.

6.5. Determine the concentration of the stock salmon DNA in ng/mL.

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

6.5.2. 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 1:1,000 dilution by ten and the 1:10,000 dilution by 100 to compare the values to the 1:100 dilution. The 1:100 dilution should be 10X greater than the 1:1,000 dilution, which in turn should be 10X greater than the 1:10,000. If this is not the case, use a more concentrated or dilute sample as needed.

6.5.3. The worksheet will calculate the average of all the readings and use this average to calculate the concentration of the stock salmon DNA and all dilutions made.

6.5.3.1. **The stock salmon DNA should have at least a 10^6 concentration. If not, then insufficient DNA dissolved in the solution and the stock should be remade.**

6.6. Aliquot 50 µL volumes of the 10^3 standard into pink 0.5 mL centrifuge tubes and store at -80°C. The 10^3 standard is the high standard of the SKETA assay standard curve. The 10^2 and 10^1 standards used in the standard curve are diluted fresh from the 10^3 standard with each plate.

6.7. Record the stock salmon DNA and each of the dilutions in LIMS Standard Preparation Tracker module.

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 add the following information:

6.7.3.1. For Stock Salmon DNA –

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Identity	**SKETA 10 MG**
Type	Stock Standard
Holding Time	365 days
Entered By	Analyst Name
Location	B246
Prepared	Date/Time of preparation and analyst
SOP	PCR-3.1
See Also	Leave blank
Description	Stock Salmon DNA for use as PCR standard and inhibition control
Additional Information	TE Buffer S/N
Storage Temperature	Freezer at <-10 deg C
Solvent Used	AE Buffer
Final Volume	1000.00
Units	µL
Click Add on the Components Table	Enter S/N for Salmon DNA used from the Purchased Material list, Amount: 10 mg, Final Concentration: ng/mL

6.7.3.2. For working DNA Standards:

Identity	x.xxEx SKETA (use the measured concentration of the standard)
Type	Working Standard
Holding Time	365 days
Entered By	Analyst Name
Location	B246
Prepared	Date/Time of preparation and analyst
SOP	PCR-3.1
See Also	Leave blank
Description	Salmon DNA for use as PCR standard and inhibition control
Additional Information	TE Buffer S/N
Storage Temperature	Freezer at <-10 deg C
Solvent Used	AE Buffer
Final Volume	Final total volume of preparation in µL
Units	µL
Click Add on the Components Table	Add the TE Buffer: Enter S/N for TE Buffer used from the Purchased Material list, Amount: amount in µL, Final Concentration: ng/mL Add the Salmon DNA: Enter S/N for the 1E4 Salmon DNA used from the Prepared Material list, Amount: Amount in µL, Final Concentration: ng/mL

6.7.4. Click OK to save the entry and receive a serial number. Open the Labelmark software and create labels (see SOP PCR-5.0 for details). Print labels with the standard name and concentration (SKETA STD, xxEx) serial number, and expiration date and affix labels to the side of each tube. The top label should include the standard name and concentration. Place labels on all aliquoted tubes before storage at -80°C.

7. SKETA ASSAY STANDARD CURVES

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7.1. A set of 10^3, 10^2, and 10^1 dilutions should be run on every plate that uses the SKETA assay.

7.2. When new standards are needed, use the stock SKETA DNA available in the freezer and recalculate the concentration using a dilution series as described in Section 5. If no stock DNA is available, make a new stock salmon DNA and proceed as in Section 5.

8. SALMON DNA WORKING STANDARD FOR EXTRACTION BUFFER (1E4 SKETA)

8.1. Use the E^5 SKETA working standard to make the 1 X 10^4 ng/mL standard. Make 25 mL for the extraction buffer and 2.5 mL for the Qiagen purification kit.

8.2. To determine the volume of E^5 SKETA working standard needed, use the following $C_1V_1 = C_2V_2$ equation.

$$V_1 = \frac{(1 \times 10^4\ ng/mL)(V_2)\left(\frac{1000\ \mu L}{1\ mL}\right)}{[E5\ SKETA]}$$

Where: V_1 = Amount of E5 SKETA in µL needed
[E5 SKETA] = Concentration of the E^5 SKETA standard in ng/mL
V_2 = Final volume of stock standard in mL (i.e. 25 ml or 2.5 ml)

8.3. Working standard for extraction buffer: Add the corresponding amount of E5 SKETA to a 50 mL centrifuge tube and bring up to 25 mL volume with TE buffer. Gently mix and pulse centrifuge to coalesce droplets. Aliquot 1 mL into pink 1.5 mL tubes and store at -80°C.

8.4. Working standard for purification: Add the corresponding amount of E^5 SKETA to a 15 mL centrifuge tube and bring up to 2.5 mL volume with TE buffer. Gently mix and pulse centrifuge to coalesce droplets. Aliquot 50 µL into pink 0.5 mL tubes and store at -80°C.

8.5. Record the standards in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Section 5.6.3.2.

9. SALMON DNA EXTRACTION BUFFER

9.1. Remove 1.xxE^4 SKETA working standard (1 mL per tube) and thaw at room temperature. Vortex and pulse centrifuge to coalesce droplets.

9.2. In a 50 mL centrifuge tube, add 1 mL of 1.xx E^4 SKETA standard and 49 mL of TE buffer to make a final concentration of 0.2 µg/mL. Gently mix and store at 4°C for a maximum of 3 months. Make additional extraction buffer as needed in increments of 50 mL.

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9.3. Record the extraction buffer in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

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

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

9.3.3. In the "Standard Solution" pop up window add the following information:

Identity	SALMON EXT BUFF
Type	Working Standard
Holding Time	90 Days
Entered By	Analyst Name
Location	B246B
Prepared	Date/Time of preparation and analyst
SOP	PCR-3.1
See Also	Leave blank
Description	0.2 µg/mL Salmon DNA/TE extraction buffer
Additional Information	TE Buffer S/N
Storage Temperature	Refrigeration at 4 deg C
Solvent Used	AE Buffer
Final Volume	Enter correct final volume in mL
Units	mL

9.3.4. Click **Add**. In the "Enter Serial #" pop up window, choose the standard that was used to make the buffer or enter its serial number in the text box. Choose **Existing Standards** for the source list. In the "Enter Amount & Units for.." pop up window, enter the amount of standard added in µL and "Final Conc. Units:" as "ng/mL". Click **OK**.

9.3.5. Click **OK** to save the entry and receive a serial number. Open the Labelmark software and create labels (see SOP PCR-5.0 for details). Print labels with the standard name and concentration (SKETA STD, 1.xxE4), serial number, and expiration date and affix labels to the side of each tube. The top label should include the standard name and concentration. Place labels on all aliquoted tubes before storage at -80°C.

10. QUALITY CONTROL

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

10.2. 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.

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11. REFERENCES

11.1. DEP Laboratory Quality Assurance Manual

11.2. Laboratory Health and Safety Plan

11.3. Sigma Catalog # D1626 Product Information Sheet and FAQ

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

11.5. DEP SOP BB-36, *Calibration, Use, and Maintenance of Mettler Toledo MS303S Balance*

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

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

Appendix of Significant Changes

09/13/2016 Updated buffer to TE buffer, removed SOPs reference that were not needed.

10/18/2017 Updated probe quencher and manufacturer, storage of standards, and extraction buffer prep.

09/23/2019 Updated standard preparation procedures. Add instructions to print labels and the SOP-5.0 reference. Add test code HumM2 to the list of available tests.

08/13/2021 Add test code GFD TaqMan to the list of available tests.

08/01/2023 Formatted SOP and added SOP tables. Added additional guidance in Section 6 for Qubit calculations.

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