Preparation of SKETA Assay Reagents and Standards for qPCR analysis

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 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-BACR, and PUR-BACR are analyzed with the SKETA assay to measure extraction efficiency and check for possible inhibition. Refer to method SOPS for the use of SKETA assay controls.

2. EQUIPMENT AND SUPPLIES

2.1. Sterile gloves, latex or nitriles

2.2. Tube rack

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

2.4. Brady FreezerBondz Laboratory labels

2.5. 50 mL Nalgene bottles

2.6. Pipette tips

2.7. Micropipettes

2.7.1. See SOP PCR-2.4 for calibration and use.

2.8. Mettler Precision Balance Model XA105DU (S/N B314195659)

2.8.1. See SOP PCR-2.2 for calibration and use.

2.9. CBS Optimizer PCR Workstation #1 (S/N 007631)

2.10. CBS Optimizer PCR Workstation #2 (S/N 007632)

2.11. Fisher Mini Vortexer

2.12. Fisher Minicentrifuge

2.13. Centrifuge

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2.14. Qubit 2.0 Fluorometer (S/N 1301008411)

2.14.1. See SOP PCR-2.0 for calibration and use.

2.15. Analog dry bath incubator with thermometer (S/N C1648131158860)

2.16. PCR#2, Thermo Freezer (S/N 0121174101121218), maintained at -20ºC ± 10ºC, Room B246A

2.17. PCR#3 Thermo Refrigerator (S/N 0121179201121219), maintained at 4ºC ± 2ºC, Room B246B

2.18. PCR#4, Thermo Freezer (S/N 0121174201121218), maintained at -20ºC ± 10ºC, Room B246B

3. REAGENTS AND STANDARDS

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. TE Buffer

3.5. Lyophilized Salmon testes DNA (e.g., Sigma D1626 or equivalent) (stored at 2-8°C)

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 received date on this sheet and review sequences on sheet for correctness. Scan the data sheet into the following folder: \\Fldep1\dear\Labs\Lab5\Biology\MolecularBiology\Certificates of Analysis\Oligo Data Sheet

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4.3. Record each purchased primer/probe set in Laboratory Information Management System (LIMS) Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

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 add the following information:

4.3.3.1. For SKETA Forward Primer: Identity: = SKETAF2; Description: = Salmon SKETA Assay Forward Primer; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = Salt-Free; Received Date: = Date of receipt; Manufacturer: = 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 SKETA Reverse Primer: Identity = SKETAR2; Description = Salmon SKETA Assay Reverse Primer; Entered by = Analyst Name; Type = Powder; Grade = blank; Purity = Salt-Free; Received Date = Date of receipt; Manufacturer = 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 SKETA Probe: Identity = SKETAP2; Description = Salmon SKETA Assay Probe; Entered by = Analyst Name; Type = Powder; Grade = blank; Purity = HPLC; Received Date = Date of receipt; Manufacturer = 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 the "Certificate of Analysis Available" checkmark box.

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 Step 4.2).

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4.3.6. The concentration of primer/probes will vary with each probe. 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. The SKETA probe is reconstituted at 100 µM, and SKETA primers are reconstituted at 500 µM concentration.

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 powder is at the bottom of the tube and help prevent a spill.

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 gentle vortexing (or tapping the tube). Pulse centrifuge to coalesce droplets. Let sit for 5 minutes at room temperature to allow resuspension. Aliquot 12 µL volumes into pink 0.5 mL centrifuge tubes. Label clearly (as in 4.8.5), and affix labels to the top and side of each tube.

4.6. For the probe, pipette appropriate amount of TE buffer into the tube containing the dried probe to make a 100 µM concentration. Dissolve by gentle vortexing (or tapping the tube). Pulse centrifuge to coalesce droplets. Let sit for 5 mins at room temperature to allow resuspension. Aliquot 12 µL volumes into pink 0.5 mL centrifuge tubes. Label clearly (as in 4.8.5), and affix labels to the top and side of each tube.

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

4.8.3.1. <u>For SKETA Forward Primer</u>: Identity: = SKETAF2; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See

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Also: = leave blank; Location: = B246A; Description = Salmon SKETA 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: = uL;

4.8.3.2. For SKETA Reverse Primer: Identity = SKETAR2; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See Also: = leave blank; Location: = B246A; Description = Salmon SKETA Assay 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: = uL;

4.8.3.3. For SKETA Probe: Identity = SKETAP2; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 365 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See Also: = leave blank; Location: = B246A; Description = Salmon SKETA Probe 100 µM concentration; Storage Temperature = Freezer at <-10 degC; Additional Information: = T E Buffer S#; Solvent Used: = AE Buffer; Final Volume: = final total volume of preparation in µL; Units: = uL;

4.8.4. Click **Add**. Choose the **Purchased Materials** radio 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 µg and choose "ug" 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 Labelmark 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. Place labels on all aliquoted tubes before storage at -20°C.

5. STOCK SALMON DNA AND WORKING STANDARDS

5.1. 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 solution to 60°C in the analog dry bath incubator or benchtop shaker for 20 mins, 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.

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5.2. 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. Gently mix each dilution and pulse centrifuge to coalesce droplets. Overmixing can affect DNA amplification.

5.2.1. Mix 100 µL of the 10 mg/mL stock salmon DNA with 900 µL of TE buffer to make a 1:10 dilution of stock salmon DNA.

5.2.2. Mix 100 µL of the 1:10 stock salmon DNA with 900 µL of TE buffer to make a 1:100 dilution of the stock salmon DNA.

5.2.3. Mix 100 µL of the 1:100 stock salmon DNA with 900 µL of TE buffer to make a 1:1000 dilution of the stock salmon DNA.

5.2.4. Mix 100 µL of the 1:1000 stock salmon DNA with 900 µL of TE buffer to make a 1:10000 dilution of the stock salmon DNA.

5.3. Analyze the 1:100 and 1:1,000 dilutions (and 1:10,000 if needed) in triplicate on the Qubit fluorometer, per SOP PCR-2.0, to determine the actual concentration of each dilution. Use 5 µL as the sample volume for the 1:100 dilution and 20 µL for the 1:1000. 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 other two dilutions are 10X from each other, then the 1:100 readings can be ignored in the following calculations to determine 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. Save the data on the lab computer's hard drive. Open the Excel file on the computer and rename the file as: Salmon DNA Qubit year-month-date. Save file in X:\Labs\Lab3\Biology\qPCR\Qubit Data.

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

5.4.1. For each replicate for each dilution, multiply the stock concentration (from Qubit data file) measured on the Qubit by the dilution factor (100X for 1:100, 1000X for 1:1000, etc.).

5.4.2. Use the average of these as the final measured concentration of the stock salmon DNA. The stock salmon DNA should have at least a 10^6 concentration. If not, then not enough DNA dissolved into the solution and the stock should be remade.

5.5. Aliquot the 10^3 standard in 50 µL aliquots and store in the -80°C freezer. The 10^3, 10^2, and 10^1 are used to make the standard curve for the SKETA assay. The 10^2 and 10^1 standards are diluted fresh from the 10^3 standard with each plate.

5.6. Record the stock salmon DNA and each of the dilutions in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

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

5.6.2. 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.

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

5.6.3.1. For Stock Salmon DNA: 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# (add S# of buffer used); Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 1000.00; Units: = µL; Components table: Click **Add**. In the "Enter Serial #" pop up window, choose the TE buffer that was used from the Purchased Material list, Amount:1mL, Final Concentration: ng/mL; Click **Add** and enter S# for Salmon DNA used from the Purchased Material, Amount: 10 mg, Final Concentration ng/mL;

5.6.3.2. For working DNA Standards: Identity: = x.xxEx SKETA (use the measured concentration of the standard, e.g. 2.28E3); 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 an inhibition control; Additional Information = TE Buffer S# (add S# of buffer used); Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = final total volume of preparation in µL; Units: = µL; Components table: Click **Add**. In the "Enter Serial #" pop up window, choose the TE buffer that was used from the Purchased Material list, Amount: amount in µL, Final Concentration: ng/mL; Click **Add** and enter S# for the 1E4 Salmon DNA used from the Prepared Material list (as prepared in section 7), Amount: amount in µL, Final Concentration "ng/mL". Click **OK.**

5.6.4. 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, xxE3) 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.

6. SKETA ASSAY STANDARD CURVES

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

6.2. When new standards are needed, use the stock SKETA DNA available in the freezer and re-determine 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.

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

7.1. Use the E5 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.

7.2. To determine the volume of E5 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] = Actual concentration of the E5 SKETA standard in ng/mL

V_2 = Final volume of stock standard in mL (i.e. 25 ml or 2.5 ml)

7.3. 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. Gently mix and pulse centrifuge to coalesce droplets. Aliquot 1 mL into pink 1.5 mL tubes and store at -80°C.

7.4. Working standard for purification: Add the corresponding amount of E5 SKETA to a 15 mL centrifuge tube and bring up to 2.5 mL volume. Gently mix and pulse centrifuge to coalesce droplets. Aliquot 50 µL into pink 0.5 mL tubes and store at -80°C.

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

8. SALMON DNA EXTRACTION BUFFER

8.1. Remove 1E4 SKETA working standard (1 mL per tube) and thaw at room temperature. Vortex and pulse centrifuge to coalesce droplets.

8.2. In a 50 mL centrifuge tube, add 1 mL of 1E4 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

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maximum of 3 months. Make additional extraction buffer as needed in increments of 50 mL.

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

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

8.3.2. 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.

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

8.3.3.1. 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# (add S# of buffer used); Storage Temperature: = Refrigeration at 4 deg C; Solvent Used: = AE Buffer; Final Volume: = enter correct final volume in mL; Units: = mL;

8.3.4. 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**.

8.3.5. 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, 1E4), 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.

9. QUALITY CONTROL

9.1. Maintain control charts of SKETA 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. FDEP Bureau of Labs Quality Manual https://floridadep.gov/dear/florida-dep-laboratory/content/dep-laboratory-quality-assurance-manual-and-sops

10.2. Laboratory Health and Safety Plan

10.3. Sigma Catalog # D1626 Product Information Sheet and FAQ

10.4. EPA Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay (October 2012)

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

10.6. DEP SOP PCR-2.2, *Calibration, Use and Maintenance of Mettler Toledo XA105DU Balance*

10.7. DEP SOP PCR-2.4 *Calibration and Use of Mechanical Pipettes using PCS® Pipette Calibration System and Artel® Pipette Tracker™*

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

Appendix of 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.

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