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 and Gull2 assay as a sample processing control and inhibition control. The salmon DNA is added to the extraction buffer and is therefore added to all samples and controls during extraction.

1.2. Each sample for Test IDs, PCR-HF183 and PCR-GULL2, are also 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. Cryodot 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

2.14. Qubit 2.0 Fluorometer (S/N 1301008411)

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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. PCR grade water – Autoclave ultrapure water for 15 minutes.

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 needs to be ordered with the 5' 6-FAM dye and 3' TAMRA quencher.

4.1.1. Forward Primer (SketaF2): 5'-GGTTTCCGCAGCTGGG- 3'

4.1.2. Reverse Primer (SketaR2): 5'-CCGAGCCGTCCTGGTC- 3'

4.1.3. Taqman® Probe (SketaP2): 5'-6FAM-AGTCGCAGGCGGCCACCGT – TAMRA

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\AppliedBiology\Molecular Biology\Certificates of Analysis\Oligo Data Sheet.

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

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

4.3.2. Select **Purchased/New Reagent/Material** from the menu. If a similar reagent exist then 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; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = Salt-Free; Received Date: = Date of receipt; Manufacturer: = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location: = B246A; Storage Temperature: = Freezer at <-10 degC; Container Size: = weight in mg (shown on data sheet in µg); 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 = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location = B246A; Storage Temperature = Freezer at <-10 degC; Container Size = weight in mg (shown on data sheet in µg); 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 = Eurofins Operon; Lot # = Barcode ID from data sheet; Storage Location = B246A; Storage Temperature = Freezer at <-10 degC; Container Size = weight in mg (shown on data sheet in µg); 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. Click **Yes** on the "Print label?" pop up window. Select the **3/4" x 1-1/4"** label size in the "Select Label Size & Copies" pop up window. Uncheck the **Use Increment Range** and click **OK.** Choose the Large label printer in the "Print" pop-up window and click **OK**.

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Place the label on the Oligonucleotide Data Sheet, before scanning it (from Step 4.2).

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.

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. Do not over mix the oligonucleotide. The primer/probes are made up of very small DNA fragments that are fragile.

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. Aliquot 12 µL volumes into pink 0.5 ml centrifuge tubes. Label clearly, and affix cryodots (green dot – forward primer; pink dot – reverse primer) to the top 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. Aliquot 12 µL volumes into pink 0.5 ml centrifuge tubes. Label clearly, and affix cryodots (yellow dot) to the top of each tube.

4.7. Group in sets of forward primer, reverse primer, and probe in a tube rack 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: = 180 Days;

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Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See Also: = leave blank; Location: = B246A; Description = Salmon SKETA Assay Forward Primer; 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: = 180 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See Also: = leave blank; Location: = B246A; Description = Salmon SKETA Assay Reverse Primer; 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: = 180 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.1; See Also: = leave blank; Location: = B246A; Description = Salmon SKETA Probe; 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. Click **Yes** in the "Confirm" pop up window to print labels. In the "Select Label Size & Copies" window, select the 1/2" x 9/10" label size in the "Label Size" section. Make sure that **Use Increment Range** is checked and the "Increment Range:" reflects the number of aliquots made for the primer/probe. Click **OK**. Choose the small label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquotted tubes before storage in the freezer.

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, vortex at a low speed for 2-4 hours to dissolve. 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 10 µL as the sample volume. 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.

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

5.4.1. For each rep 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. Determine the concentrations of the dilutions and continue the serial dilution down to a concentration of 10^1 in 1000 µL volumes.

5.5.1. For example, if the concentration of the stock Salmon DNA is 2.50×10^6, the dilution 1:10 is 2.50×10^5, dilution 1:100 is 2.50×10^4, and dilution 1:1000 is 2.50×10^3. Continue the serial dilution by mixing 100 µL of the 2.50×10^3 dilution with 900 µL of TE buffer to make a dilution with a concentration of 2.50×10^2. Then make one further dilution by mixing 100 µL of the 2.50×10^2 dilution with 900 µL of TE buffer to make a dilution with a concentration of

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2.50 X 10^1. This will complete the serial dilution series of the stock salmon DNA.

5.6. Using pink 0.5 mL tubes dispense the stock salmon DNA and any dilutions that are 10^4 or greater in 120 µL aliquots. For dilutions that are less than 10^4, aliquot in 18 µL volumes. Place red cryodots on the tops of all tubes.

5.7. Store the stock salmon DNA and dilutions that are 10^4 or greater in a cryobox in the -20°C freezer in room B246B.

5.8. The 10^3, 10^2, and 10^1 are used as standard curves for the SKETA assay. Group the three dilutions as a set in a tube rack and store in the -20°C freezer in room B246B. This will make it easier to retrieve when performing standard curves.

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

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

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

5.9.3. In the "Standard Solution" pop up window enter the following information:

5.9.3.1. <u>For Stock Salmon DNA</u>: Identity: = SKETA 10 MG; Type: = Stock Standard; Holding Time: = 365 days; Entered by: = Analyst Name; Location: = B246B; 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;

5.9.3.2. <u>For working DNA Standards</u>: Identity: = x.xxEx SKETA (use the measured concentration of the standard, e.g. 2.28E5); Type: = Working Standard; Holding Time = 365 days; Entered by: = Analyst Name; Location: = B246B; 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: = 1000.00; Units: = µL;

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5.9.4. Click **Add**. In the "Enter Serial #" pop up window, choose the standard that was used to make the standard being entered from the list, or enter its serial number in the text box. For Stock Salmon DNA, the standard is in the **Purchased Materials** "Select Source:" list. For the other working standards, choose **Existing Standards** for the source list. In the "Enter Amount & Units from..." pop up window, enter the amount of standard added in µL and "Final Conc. Units:" should be entered as "ng/mL". Click **OK**.

5.9.5. Click **OK** in the "Standard Solution" pop up window. Click **Yes** in the "Confirm" pop up window to print labels. In the "Select Label Size & Copies" window, select the 1/2" x 9/10" label size in the "Label Size" section. Make sure that **Use Increment Range** is checked and the "Increment Range:" reflects the number of aliquots made for the standard. Click **OK**. Choose the small label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquotted tubes before storage in the freezer.

6. SKETA ASSAY STANDARD CURVES

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 largest dilution available in the freezer, starting with the 10^4 dilution, to make a dilution series (10^3, 10^2, and 10^1) as shown in Section 5. If no dilutions are available, make stock salmon DNA and proceed as in Section 5 to make dilutions for the standard curves.

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 working standard. Make a 10 mL volume for the extraction buffer working standard. Make a 2.5 mL volume for the MOBIO purification working standard.

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. 10 ml or 2.5 ml)

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7.3. Working standard for extraction buffer: Add the E5 SKETA to a 30 mL centrifuge tube and bring up to 10 mL volume. Gently mix and pulse centrifuge to coalesce droplets. Aliquot into 0.5 mL tubes in 250 µL volumes and keep frozen at -20°C in B246B.

7.4. Working standard for purification: Add the E5 SKETA to a 10 mL centrifuge tube and bring up to 2.5 mL volume. Gently mix and pulse centrifuge to coalesce droplets. Aliquot into 0.5 mL tubes in 50 µL volumes and keep frozen at -20°C in B246B.

7.5. Record the standards in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Step 5.9.3.

8. SALMON DNA EXTRACTION BUFFER

8.1. Determine the amount of extraction buffer needed for the week. Multiply 600 by the total number of samples to be extracted, including 4 controls for each batch (method blank, lab duplicate, extraction blank, extraction control). This will be the amount of buffer needed in µL for the week. Bring up to the next mL to ensure there is enough buffer for accurate dispensing of 600 µL per extraction tube. For example, if the volume of buffer needed is 10500 µL, make 11 mL.

$$Volume\ of\ buffer\ needed\ (\mu L) = [(\#of\ samples) + (\#of\ extraction\ batches \times 4)](600\ \mu L)$$

8.2. Determine the volume of 1E4 SKETA needed to make 0.2 µg/mL extraction buffer. Use the following $C_1V_1 = C_2V_2$ equation.

$$V_1 = \frac{(V_2)(0.2\ \mu g/mL)\left(\frac{1000\ ng}{1\ \mu g}\right)}{1 \times 10^4\ ng/mL}$$

Where: V_1 = volume in µL of 1E4 SKETA to add for extraction buffer

V_2 = volume in µL of extraction buffer to be made (final volume)

8.3. Remove 1E4 SKETA working standard tubes (250 µL per tube) and thaw at room temperature. Do not freeze/thaw tubes for more than 3 cycles. Combine the thawed standard into one tube and vortex for 5 minutes. Pulse centrifuge to coalesce droplets.

8.4. In a 30 mL centrifuge tube, add the correct volume of 1E4 SKETA standard and bring up to final volume with TE buffer. Gently mix and store at 4°C for a maximum of 1 week. Leftover 1E4 SKETA standard may be refrozen up to 3 times for subsequent use. Label the tube with volume leftover.

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

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

8.5.2. Select **Prepared/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.

8.5.3. In the "Standard Solution" pop up window enter the following information:

8.5.3.1. Identity: = SALMON EXT BUFF; Type: = Working Standard; Holding Time = 7 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;

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

8.5.5. Click **OK** in the "Standard Solution" pop up window. Click **Yes** in the "Confirm" pop up window to print labels. In the "Select Label Size & Copies" window, select the **3/4" x 1-1/4"** label size. Uncheck the **Use Increment Range** and click **OK.** Choose the Large label printer in the "Print" pop-up window and click **OK**. Place on the centrifuge tube before storage in the refrigerator.

9. QUALITY CONTROL

9.1. Maintain control charts of SKETA standard curves and 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 similar to older standards. If older standards are not available, compare to the control chart information.

10. REFERENCES

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10.1. FDEP Bureau of Labs Quality Manual
http://www.dep.state.fl.us/labs/library/progplan.htm

10.2. Health and Safety Plan http://depnet/burlabs/safety.htm

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™*

09/13/16 Updated buffer to TE buffer, removed SOPs reference that were not needed.