Standard Operating Procedure for:

Preparation of *Bacteroides dorei* (*B. dorei*) Assay Reagents and Standards for qPCR analysis

DEPARTMENT OF ENVIRONMENTAL PROTECTION LABORATORIES
BIOLOGY PROGRAM
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1. INTRODUCTION

1.1. A purified and quantified *Bacteroides dorei* DSM 17855 genomic DNA is used as a standard in the *Bacteroidales* quantitative Polymerase Chain Reaction (qPCR) assay. DNA standards are used to generate standard curves for determination of performance characteristics of the qPCR assays.

1.2. Extraction control and third well spike: The extraction control contains an added known concentration of *B. dorei* DNA that is extracted and analyzed in the same manner as environmental samples. The third well spike also contains a known concentration of *B. dorei* DNA which is added to the third replicate well of each environmental sample during reaction plate setup. Both extraction control and third well spikes are used as positive controls for the *Bacteroidales* target sequence and qPCR assay.

2. EQUIPMENT AND SUPPLIES

2.1. Gloves, latex or nitriles

2.2. Tube racks

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

2.4. Micropipettes with sterile PCR grade filter tips

2.4.1. Calibrate and use per SOP PCR-2.4.

2.5. Forceps

2.6. Syringe, 1 ml

2.7. Hypodermic needle, 23 gauge

2.8. Gassing cannula -Nitrogen or Nitrogen/Argon mix gas cylinder

2.9. Bunsen burner

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

2.10.1. Calibrate and use per SOP PCR-2.2.

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

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

2.13. Fisher Mini Vortexer

2.14. Fisher Minicentrifuge

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2.15. Eppendorf Centrifuge Model 5418 (S/N 5418AG715992)

2.16. Qubit 2.0 Fluorometer (S/N 1301008411)

2.16.1. Calibrate and use per SOP PCR-2.0

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

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

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

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

2.21. PCR#6, Thermo UXF Freezer (S/N 0132376801130826), maintained at -80ºC ± 10ºC, Room B246

2.22. Incubating mini shaker (S/N 150747)

3. REAGENTS AND CHEMICALS

3.1. Deionized (DI) water

3.2. PCR grade water – Autoclave ultrapure water for 15 minutes. Record in LIMS Autoclave Log.

3.3. Bleach solution, 10% (v/v)

3.4. TE Buffer

3.5. PRAS (Pre-reduced anaerobic system) PY Glucose w/Tween™ 80 broth tubes

3.6. *Bacteroides dorei* (*B. dorei*) DSM 17855 freeze-dried culture

3.6.1. May also be purchased as genomic DNA(gDNA)

4. HF183 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 is ordered with the 5' 6-FAM dye and 3' Zen-Iowa Black FQ quencher from IDT. When ordered, a confirmation email is sent. Verify that sequences are correct on the confirmation email. Keep stored at -20°C.

4.1.1. Forward Primer HF183: 5'-ATCATGAGTTCACATGTCCG- 3'

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4.1.2. Reverse Primer BthetR1: 5'-CGTAGGAGTTTGGACCGTGT- 3'

4.1.3. Taqman® Probe BthetP1: 5'-56-FAM/ CTG AGA GGA /ZEN/ AGG TCC CCC ACA TTG GA/3IABkFQ/-3'

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

4.3. Record each preparation 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 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 Forward Primer HF183: Identity: = HF183F; Description = HF183 Assay Forward Primer HF183F; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = desalted; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer: = Integrated DNA Technologies (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 Reverse Primer BthetR1: (NOTE: Reverse Primer BthetR1 will be entered as HF183R for consistency with the HF183 qPCR assay). Identity = HF183R; Description = HF183 Assay Reverse Primer BthetR1; Entered by = Analyst Name; Type = Powder; Grade = blank; Purity = desalted; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Integrated DNA Technologies (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 BthetP1Probe: (NOTE: Probe BthetP1 will be entered as HF183P for consistency with the HF183 qPCR assay). Identity = HF183P; Description = HF183 Assay Probe BthetP1; Entered by = Analyst Name; Type = Powder; Grade = HPLC Grade; Purity = HPLC; Received: = Date of receipt; Expires: = 1 year from receive date; Manufacturer = Eurofins Operon; 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 "Yes" for the "Has Certificate of Analysis" drop-down list.

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. Click **Yes** on the "Print label?" pop up window. Choose the label printer in the "Print" pop-up window and click **OK**. Place the label on the Oligonucleotide Data Sheet, before scanning it (from Step 6.2).

4.3.7. The mass of primer/probes will vary with each probe. This is listed in the "Amount of Oligo" 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 "For 100 µM: add X µL". 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. Pulse centrifuge tubes prior to opening. The lyophilized contents may have been dislodged during shipping. Do not over mix the oligonucleotide.

4.5. For each primer, pipette appropriate amount of TE buffer 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 blue 0.5 ml centrifuge tubes. Print labels with serial number, HF183F or HF183R, and expiration date and affix labels to the side of each tube. The top label should include HF183F or HF183R, and the serial number.

4.6. For the probe, pipette appropriate amount of TE buffer 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 8 µL volumes into blue 0.5 ml centrifuge tubes. Print labels with serial#, HF183P, and expiration date and affix labels to the side of each tube. The top label should include HF183P, and the serial number.

4.7. Group in sets of forward primer, reverse primer, and probe in a cryo safe 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**).

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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. For HF183 Forward Primer: Identity: = HF183F; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 360 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183 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 HF183 Reverse Primer: Identity = HF183R; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 360 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183Assay 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 HF183 Probe: Identity = HF183P; Type: = Working Reagent; Entered by: = Analyst Name; Holding Time: = 360 Days; Prepared = Date/Time of preparation and Analyst; SOP: = PCR-3.2; See Also: = leave blank; Location: = B246A; Description = HF183 Probe; 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;

4.8.4. 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 mg and choose "mg" 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. Choose the label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquotted tubes before storage in the freezer.

5. *B. dorei* CULTURE

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5.1. Start with the purchased lyophilized (freeze-dried) culture of *B.dorei* in a double-vial ampule. Before opening the ampule, identify the culture by the label on the inner vial which indicates the strain number and date of preservation. Confirm that the ampoule is under vacuum by checking the color of the desiccant at the bottom of the outer vial.

Note: The freeze-dried pellet of most anaerobic strains available from DSMZ (Germany) is protected against short exposure to oxygen by amorphous ferrous sulfide (FeS) which confers a black color to the pellet.

5.2. Open ampule and rehydrate the freeze-dried culture pellet:

5.2.1. Remove the glass ampoule from the secondary packaging.

5.2.2. While wearing proper protective glasses, heat the tip of the ampoule on a Bunsen burner. Place two or three drops of water onto the hot tip to crack the glass.

5.2.3. Carefully strike off the glass tip with forceps. Remove the insulation material with forceps and gently take out the inner vial containing the freeze-dried pellet.

5.2.4. Lift and remove the cotton plug using forceps. Flame the top of the inner vial and immediately insert a gassing cannula to keep the pellet under oxygen-free conditions. Replace the cotton plug half-way so that it keeps the gassing cannula snugly inside the vial.

5.2.5. Aseptically pull approximately 0.5 ml of the PRAS medium from a PRAS PY Glucose w/Tween™ 80 broth tube using a 1 ml syringe with a hypodermic needle and add to the vial. Resuspend the cell pellet completely. This may take up to 5 minutes. Transfer the cell suspension by using a 1 ml syringe with the hypodermic needle to the PRAS tube.

5.2.6. Invert tube 2-3 times to mix well and incubate in mini shaker at 37.0°C ± 0.5°C for 24-48 hours.

5.2.7. The culture can be transferred to new broth for up to 5 passages. Bring the stored culture to room temperature and mix by vortexing at speed 7 for 1 minute before transferring to a new broth tube. After 5 passages, genetic mutations may arise and new culture should be purchased. Always use freshly grown (24-48 hours) culture to purify for gDNA.

5.2.8. After removing culture for standards, the remaining culture broth can be kept at 4°C for up to one week. For long term (up to 1 year) storage keep frozen at -20°C.

5.3. Record the *B. dorei* culture in LIMS Standard Preparation Tracker module (Laboratory/Technical/Standard Preparation Tracker).

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

5.3.2. Select **Purchased/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.

5.3.3. In the "Purchased Standard" pop up window enter the following information:

5.3.3.1. For *B. dorei* culture: Identity: = B. DOREI CULTURE; Description: = Bacteroides dorei DSM 17855 pure culture; Entered by: = Analyst Name; Prepared = Date of reviving the culture; Received: = Date received; Expires: = 365 days; Type: = pellet; Has certificate of Analysis: = YES; Manufacturer: = DSMZ Germany; Lot #: = (enter from the vial); Storage Location: = B246B; Storage Temperature: = Freezer <10 degC; Additional information: = Use with SOP = PCR-3.2.

6. STOCK *B. dorei* gDNA, AND WORKING STANDARDS

6.1. Using a sterile syringe and needle aspirate 2 mL of culture from the PRAS tube, and add to a 2 mL micro centrifuge tube. Centrifuge at 14,000 rpm for 10 minutes to pellet the *B.dorei* culture. Remove supernatant without disturbing the pellet.

6.2. Add 2 mL of TE buffer and vortex to resuspend the pellet. Centrifuge at 14,000 rpm for 5 minutes to pellet the *B.dorei* culture. Remove supernatant without disturbing the pellet.

6.3. Repeat step 6.2. two more times to finish washing the media from the culture. Resuspend the pellet in 50 µL TE buffer.

6.4. Extract total gDNA from the culture per SOP PCR-4.2, using the Qiagen DNeasy PowerLyzer® PowerSoil® kit (see SOP PCR4.2-1.2).

6.5. Make a serial dilution (1:10, 1:100, 1:1000, 1:10000) of the *B. dorei* gDNA using TE buffer as the diluent. Gently mix each dilution and pulse centrifuge to coalesce droplets. Overmixing can affect DNA amplification.

6.5.1. Mix 10 µL of *B. dorei* gDNA with 90 µL of TE buffer to make a 1:10 dilution of *B. dorei* gDNA.

6.5.2. Mix 10 µL of the 1:10 *B. dorei* gDNA with 90 µL of TE buffer to make a 1:100 dilution of the *B. dorei* gDNA.

6.5.3. Mix 10 µL of the 1:100 *B. dorei* gDNA with 90 µL of TE buffer to make a 1:1000 dilution of the *B. dorei* gDNA.

6.5.4. Mix 10 µL of the 1:1000 *B. dorei* gDNA with 900 µL of TE buffer to make a 1:10000 dilution of the *B. dorei* gDNA.

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6.6. Analyze the 1:100 and 1:1000 dilutions (and 1:10000 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. If the 1:100 and 1:1000 dilutions are not 10X from each other, but the 1:1000 and 1:10000 are 10X from each other, then the 1:100 may be too concentrated and can be ignored in the calculations below. If none of the dilutions are 10X from each other, remake dilutions and re-read on the Qubit fluorometer.

6.7. Determine the concentration of stock *B. dorei* gDNA in ng/mL.

6.7.1. 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.).

6.7.2. Use the average of these as the final measured concentration of the stock *B. dorei* gDNA. The stock should have at least a 10^5 concentration. If not, not enough DNA was recovered from the culture and the stock should be remade.

6.8. Determine the concentration of the stock *B.dorei* gDNA in GEU/5 µL using the following equation with the conversion factor of 1 ng of *B.dorei* double-stranded gDNA = 1.69 X 10^5 copies of *B.dorei* genome.

$$\frac{GEU\ B.dorei}{5\ uL} = (Stock\ Conc.\ in\ ng/mL)\left(\frac{1\ mL}{1000\ uL}\right)\left(\frac{1.69\ \times\ 10^5\ GEU}{1\ ng}\right) \times 5$$

6.9. Determine the concentrations of the dilutions in GEU/5 µL units and make up a 10^5 dilution in 2000 µL volume.

6.9.1. For example, if the concentration of the stock *B. dorei* gDNA is 7.03 X 10^6, the dilution 1:10 is 7.03 X 10^5.

6.9.2. Store the stock *B. dorei* gDNA in a cryobox in the -80°C freezer in room B246. For the 10^5 dilution for standard curve make aliquots of 50 µL, and for the 10^5 dilution spike for extraction control make aliquots of 16 µL using blue 0.5 mL tubes. Print labels and affix them on the top and side of the tubes. Side label for 10^5 dilution for standard curve should include serial#, **X** E5 *B.Dorei* STD, and expiration date. Top label should read *B.Dorei* STD. The 10^5 dilution spike tubes will be labeled similarly but SPK instead STD. Store 10^5 tubes for standard curve and extraction control spike at -80°C in room B246.

6.9.3. When setting up the reaction plate, perform a serial dilution of the 10^5 standards to create a standard curve set and 3rd well spike for the HF183 assay. See Section 7.

6.10. Record the stock *B. dorei* gDNA and dilutions in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**).

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

6.10.2. 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.

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

6.10.3.1. For Stock *B. dorei* gDNA: Identity: = B. DOREI DNA; Type: = Stock Standard; Holding Time: = 365 days; Entered by: = Analyst Name; Location: = B246; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.2; See Also: = leave blank; Description: = *B. dorei* gDNA standard at x.xxEx GEU per 5 uL; Additional Information = TE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 120.00; Units: = µL;

Note: The stock gDNA will be used to make working standards and the original tube will be stored at -80°C.

6.10.3.2. For working gDNA Standard: Identity: = x.xxEx B. DOREI DNA (use the measured concentration of the standard in GEU/5 µl), e.g. 7.03E5); Type: = Working Standard; Holding Time = 365 days; Entered by: = Analyst Name; Location: = B246; Prepared = Date/time of preparation and analyst; SOP: = PCR-3.2; See Also: = leave blank; Description: = *B. dorei* gDNA standard at x.xxEx GEU per 5 uL; Additional Information = TE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 2000.00 ; Units: = µL;

6.10.3.3. 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 *B. dorei* stock gDNA, 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**.

6.10.3.4. Click **OK** in the "Standard Solution" pop up window. Click **Yes** in the "Confirm" pop up window to print labels. Choose the label printer in the "Print" pop-up window and click **OK**. Place labels on all aliquoted tubes before storage in the freezer.

7. HF183 ASSAY STANDARD CURVES AND 3RD WELL SPIKE

7.1. A set of 10^5, 10^4, 10^3, 10^2, and 10^1 GEU/5 uL *B. dorei* genomic DNA should be run on every HF183 plate. The 3rd well spike has a concentration of 10^4 GEU/5 uL.

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7.2. *B. dorei* standards and 3rd well spike will be diluted before each run from 10^5 concentration. To make the 10^4 standard, dilute by mixing 10 µL of the 10^5 dilution with 90 µL of TE buffer. 10^3, 10^2, and 10^1 will be made similarly by serial dilution.

7.3. Record the 3rd well spike standard in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Step 6.9.7.2, except add SPK to the end of the Identity name.

7.4. When new standards are needed, dilute the stock gDNA and measure as described in Section 6. Reorder as needed.

8. *B. dorei* EXTRACTION CONTROL

8.1. The extraction control (EC) has a concentration of 10^5 GEU/5 uL of *B. dorei* gDNA and is prepared following instructions in section 6.9.

8.2. Record the standards in LIMS Standard Preparation Tracker module (**Laboratory/Technical/Standard Preparation Tracker**) as a working standard per Step 7.12.3, except add SPK to the end of the Identity name.

9. QUALITY CONTROL

9.1. Maintain control charts of HF183 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 similarly to older standards. If older standards are not available, compare to the control chart information.

10. REFERENCES

10.1. DEP Laboratory Quality Manual
http://publicfiles.dep.state.fl.us/dear/labs/lab_qualitymanual_18.pdf

10.2. Health and Safety Plan Health and Safety Plan

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

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

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

10.6. DEP SOP PCR-4.2 *Purification of Filtered Samples Using the Qiagen DNeasy PowerLyzer® PowerSoil® Kit*

10.7. *Qiagen DNeasy PowerLyzer® PowerSoil® Kit (Catalog#12855)*

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Appendix of Changes

06/14/2017 – Added table of contents and cover page, made major revision to working stock standards procedure and added new culturing procedure.

06/05/2018 – Updated the primers/probe and label information.

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