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

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 an added known concentration of *B. dorei* DNA and is added as a third replicate well to 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. Cryodot labels

2.5. 50 Nalgene bottles, 50 mL

2.6. Micropipettes with sterile PCR grade filter tips

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

2.7.1. Calibrate and use per SOP PCR-2.2.

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

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

2.10. Fisher Mini Vortexer

2.11. Fisher Minicentrifuge

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

2.13. Qubit 2.0 Fluorometer (S/N 1301008411)

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2.13.1. Calibrate and use per SOP PCR-2.0

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

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

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

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

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

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. AE Buffer (Prepare per SOP PCR-3.0)

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

3.6. *Bacteroides dorei* (*B. dorei*) DSM 17855 active 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 needs to be ordered with the 5’ 6-FAM dye and 3’ TAMRA quencher. 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’

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

4.1.3. Taqman® Probe BthetP1: 5’-6FAM-CTGAGAGGAAGGTCCCCCACATTGGA-TAMRA

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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\AppliedBiology\Molecular Biology\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 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 Forward Primer HF183: Identity: = HF183F; Description = HF183 Assay Forward Primer; Entered by: = Analyst Name; Type: = Powder; Grade: = blank; Purity: = desalted; 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 (shown on data sheet in µg); 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 = 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 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 (shown on data sheet in µg); Units = mg;

4.3.3.4. Select "Yes" for the "Has Certificate of Analysis" drop down list.

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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. 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**. Place the label on the Oligonucleotide Data Sheet, before scanning it (from Step 6.2).

4.3.7. 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 AE 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 AE buffer into the tube containing the dried primer to make a 500 µM concentration. Dissolve by gentle vortexing (or tapping the tube). Allow the solution to equilibrate for 5 minutes. Mix again and pulse centrifuge to coalesce droplets. Aliquot 12 µL volumes into blue 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 AE buffer into the tube containing the dried probe to make a 100 µM concentration. Dissolve by gentle vortexing (or tapping the tube). Allow the solution to equilibrate for 5 minutes. Mix again and pulse centrifuge to coalesce droplets. Aliquot 12 µL volumes into blue 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**).

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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 exist 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: = 180 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: = AE 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: = 180 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: = AE 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: = 180 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: = AE 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.

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5. *B. dorei* CULTURE

5.1. Using a sterile syringe and needle aspirate 1 ml of *B.dorei* culture suspension and inoculate a 10 mL PRAS PY Glucose w/Tween™ 80 broth tube. Do not introduce air into the tube. Make sure to push out air from the syringe before inserting into the culture tube.

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

5.3. Using a sterile syringe and needle aspirate 2 mL of culture from the PRAS tube for making stock *B. dorei* standards.

5.4. 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 -80°C.

5.5. Start with the purchased active culture. The culture can be transferred to new broth for up to 5 passages. Bring the stored culture to room temperature and mix with gentle vortexing at speed 7 for 1minute before transferring to a new broth tube. After 5 passages, lab strains may develop and new culture should be purchased. Always use freshly grown (24-48 hours) culture to purify for gDNA.

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 AE buffer and resuspend the pellet by vortexing. 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 AE buffer.

6.4. Extract total gDNA from the culture per SOP PCR-4.2, using the MO BIO PowerLyzer PowerSoil DNA extraction kit.

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

6.5.1. Mix 50 µL of *B. dorei* gDNA with 450 µL of AE buffer to make a 1:10 dilution of *B. dorei* gDNA.

6.5.2. Mix 100 µL of the 1:10 *B. dorei* gDNA with 900 µL of AE buffer to make a 1:100 dilution of the *B. dorei* gDNA.

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6.5.3. Mix 100 µL of the 1:100 *B. dorei* gDNA with 900 µL of AE buffer to make a 1:1000 dilution of the *B. dorei* gDNA.

6.5.4. Mix 100 µL of the 1:1000 *B. dorei* gDNA with 900 µL of AE buffer to make a 1:10000 dilution of the *B. dorei* gDNA.

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 continue the serial dilution down to a concentration of 10^1 in 1000 µL volumes.

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, dilution 1:100 is 7.03 X 10^4, and dilution 1:1000 is 7.03 X 10^3. Continue the serial dilution by mixing 100 µL of the 7.03 X 10^3 dilution with 900 µL of AE buffer to make a dilution with a concentration of 7.03 X 10^2. Then make one further dilution by mixing 100 µL of the 7.03 X 10^2 dilution with 900 µL of AE buffer to make a dilution with a concentration of 7.03 X 10^1. This will complete the serial dilution series of the stock *B. dorei* gDNA.

6.9.2. 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, dilution 1:100 is 7.03 X 10^4, and dilution 1:1000 is 7.03 X 10^3. Continue the serial dilution by mixing 100 µL of the 7.03 X 10^3

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dilution with 900 µL of AE buffer to make a dilution with a concentration of 7.03 X 10^2. Then make one further dilution by mixing 100 µL of the 7.03 X 10^2 dilution with 900 µL of AE buffer to make a dilution with a concentration of 7.03 X 10^1. This will complete the serial dilution series of the stock *B. dorei* gDNA.

6.10. Using blue 0.5 mL tubes, dispense the stock *B. dorei* gDNA and any dilutions that are 10^6 or greater in 120 µL aliquots. For dilutions that are less than 10^6, aliquot in 18 µL volumes. Place red cryodots on the tops of all tubes. Label the dots with the concentration (e.g. E4) and the volume.

6.11. Store the stock *B. dorei* gDNA and dilutions that are 10^6 or greater in a cryobox in the -20°C freezer in room B246B.

6.12. The 10^5, 10^4, 10^3, 10^2, and 10^1 are used as standard curves for the HF183 assay. Group the five 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.

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

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

6.13.2. 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.

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

6.13.3.1. <u>For Stock *B. dorei* gDNA</u>: Identity: = B. DOREI DNA; Type: = Stock Standard; Holding Time: = 365 days; Entered by: = Analyst Name; Location: = B246B; 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 = AE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 50.00; Units: = µL;

<u>Note</u>: The stock gDNA will essentially be used up to make working standards. It still needs to be entered into LIMS.

6.13.3.2. <u>For working gDNA Standards</u>: 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: = B246B; 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 =

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AE Buffer S#; Storage Temperature: = Freezer at <-10 degC; Solvent Used: = AE Buffer; Final Volume: = 1000.00 (500 for the 1:10 dilution); Units: = µL;

6.13.4. 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 *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**.

6.13.5. 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.

7. HF183 ASSAY STANDARD CURVES

7.1. A set of 10^5, 10^4, 10^3, 10^2, and 10^1 should be run on every plate that uses the HF183 assay.

7.2. When new standards are needed, use the largest dilution available in the freezer, starting with the 10^6 dilution, to make a dilution series (10^5, 10^4, 10^3, 10^2, and 10^1) as shown in Section 5. If no dilutions are available, make stock HF183 and proceed as in Section 5 to make dilutions for the standard curves.

8. *B. dorei* EXTRACTION CONTROL AND 3RD WELL SPIKE

8.1. The extraction control (EC) has a concentration of 10^5 GEU/5 uL of *B. dorei* gDNA and the third well spike has a concentration of 10^4 GEU/5 uL of *B. dorei* DNA.

8.2. Use the 10^6 *B. dorei* gDNA working standard to make the 10^5 EC dilution by mixing 40 µL of the 10^6 dilution with 360 µL of AE buffer.

8.3. Use the 10^5 EC dilution to make 10^4 third well spike by mixing 80 µL of the 10^5 dilution with 720 µL of AE buffer.

8.4. Aliquot the EC into 0.5 mL tubes in 6 µL volumes and keep frozen at -20°C in B246B. Aliquot the 3rd well spike into 0.5 mL tubes in 18 µL volumes and keep frozen at -20°C in B246B. Place white cryodots on the tops of all tubes. Label the dots with the concentration E6 or E4 and the volume.

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8.5. 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 SPIKE 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 similar 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 http://www.dep.state.fl.us/labs/library/progplan.htm

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

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

10.4. DEP SOP PCR-1.0 *Human-Associated Bacteroidales HF183 Marker in Water by Quantitative Polymerase Chain Reaction (qPCR*)

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-3.0 *Preparation of Reagents for quantitative Polymerase Chain Reaction (qPCR)*

10.9. DEP SOP PCR-4.2 *Purification of Filtered Samples Using the MO BIO PowerLyzer Powersoil DNA Isolation Kit*