**Real Time PCR Protocol for Primer Sets *D.* Cs1-4 #1 and *D. a.* #1**

Perform **ALL STEPS** in a biological safety cabinet. **Always use filter tips**. Take care to not introduce contaminants into the safety cabinet, and **spray ethanol onto any object** you bring in.

Materials

* Bio-Rad iTaq ™ Universal SYBR **®**  Green Supermix
* Primer Set diluted to 10 µM (*D.* Cs1-4 #1 or *D. a*. #1—See Prior to Experimentation step A.)
* DNA template diluted to 4 different concentrations: 10 ng/µL, 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL (*Delftia sp.* Cs1-4 or *Delftia acidovorans* SPH-1—See Prior to Experimentation step B.)
* Ultra Clean Deionized Water
* 5 sets of PCR strip tubes and PCR strip tube caps
* Microcentrifuge tubes
* p1000 filter pipette tips
* p200 filter pipette tips
* p100 filter pipette tips
* p20P (note: do not use p20E tips. This will alter the experiment results.) filter pipette tips
* p10 filter pipette tips
* CFX Connect Real Time System and Software

Prior to Experimentation

1. i. Calculate the amount of liquid to resuspend the dry powder in by using the primer resuspension calculator at this URL: <https://www.idtdna.com/Calc/resuspension/>.

ii. Resuspend both forward and reverse primers to 100 µM in Ultra Clean from dry powder if this has not already been done.

iii. To create a working stock of primer, dilute some of the 100 µM stock to 10 µM in a separate microcentrifuge tube.

1. i. Create a working stock of the DNA template at 10 ng/µL. To determine initial concentration of DNA after isolation, use a Nanodrop as detailed in the Biofilm gDNA Isolation Protocol, step 7.

ii. Create 4 dilutions of the DNA template used in the reactions—10 ng/µL, 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL by tenfold dilutions. For example, to create a dilution of 1 ng/µL, add 1 µL of the 10 ng/µL dilution to 9 µL of Ultra Clean diH20.

1. Use the CFX Connect software to create a protocol to run. Name the protocol and the output files appropriately. Set the following as parameters of the run:

**Polymerase Activation and DNA Denaturation:** 95° C for 5 minutes.

**Denaturation:** 95° C for 30 seconds.

**Annealing, Extension, and Plate Read:** 55° C for 30 seconds.

**Repeat Denaturation and Annealing, Extension, and Plate Read 40x**

**Melt Curve Analysis** 72° C for 1 minute and 65° C for 5 seconds.

Protocol

When possible, leave reagents on ice. Do not bring ice into biological safety cabinet.

1. Turn on CFX Connect Real Time PCR System in order for it to have time to warm up.
2. Label 5 PCR strip tube sets (on the side of the caps, NOT THE TOP) respectively—Negative control, 0.01, 0.1, 1, and 10.
3. Create a Master Mix for 21 reactions:

* 210 µL Bio-Rad iTaq with SYBR Green (10/Rxn)
* 42 µL Forward Primer\* (2/Rxn)
* 42 µL Reverse Primer\* (2/Rxn)
* 105 µL Ultra Clean Deionized Water (5/Rxn)

\*Primers used (only one primer pair at a time): *D. a.* #1 if using SPH-1 DNA and *D*. Cs1-4 #1 if using Cs1-4 gDNA.

1. Add 19 µL of the master mix to the first four tubes in each set of PCR strip tubes. Each set of strip tubes corresponds to 1 concentration of DNA—10 ng/µL, 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, or the negative control.
2. **Negative Control Set--**Add 1 µL of water to each of the four tubes in the set of strip tubes labeled Negative Control. Put on ice.
3. Add 1 µL of 0.01 ng/µL DNA to each of the four tubes in the set of strip tubes labeled 0.01. Put on ice.
4. Add 1 µL of 0.1 ng/µL DNA to each of the four tubes in the set of strip tubes labeled 0.1. Put on ice.
5. Add 1 µL of 1 ng/µL DNA to each of the four tubes in the set of strip tubes labeled 1. Put on ice.
6. Add 1 µL of 10 ng/µL DNA to each of the four tubes in the set of strip tubes labeled 10. Put on ice.
7. Centrifuge all sets of strip tubes for 7 seconds.
8. Arrange the sets of strip tubes in the CFX Connect Machine. Record their order.
9. Run Protocol created in Prior to Experimentation step C.