**16S PCR for collected samples**

**Purpose:** To amplify 16S RNA in collected samples to prepare samples for deep sequencing

**Methods:**

1. Prepare 24 single PCR tubes
2. Choose samples to amplify and label tubes

We chose:

* + 9 duplicate samples (18 reactions) that were identified as positive for *Delftia acidovorans* in our SYBR green qPCR
	+ 2 duplicate samples (4 reactions) that were identified as negative for *D. acidovorans* in our SYBR green qPCR
	+ 1 duplicate (2 reactions) of *D. acidovorans* SPH-1 genomic DNA as a positive control.
1. Prepare master mix according to:

|  |  |  |  |
| --- | --- | --- | --- |
| Mix for 1 reaction |  | Mix for 25 reactions |  |
| Q5 High-Fidelity 2x Master Mix | 25μL | Q5 High-Fidelity 2x Master Mix | 625μL |
| 10 μM Forward Primer | 2.5μL | 10 μM Forward Primer | 62.5μL |
| 10μM Reverse Primer | 2.5μL | 10 μM Reverse Primer | 62.5μL |
| PCR Grade H2O | 19μL | PCR Grade H2O | 475μL |
| DNA  | 1 μL | DNA per tube | 1μL |

\*total of 50μL per reaction

1. Run in thermal cycler according to:

|  |  |  |
| --- | --- | --- |
| Initial Denaturation | 98 oC | 30 sec |
| Denaturation | 98 oC | 10 sec |
| Annealing | 55 oC | 30 sec |
| Extension | 72 oC | 30 sec |
| Final Extension | 72 oC | 2 min |

\*Steps 2-4 repeated 25x

1. Store samples at -20 oC

***Next Step See Below***

**Barcode/Index PCR of Amplified 16S Samples**

**Purpose:** To add unique identifiers, or “barcodes”, to each sample so they are distinguishable from one another for sequencing

**Methods:**

1. Clean amplified 16S PCR product

\*We used Axygen AxyPrep Mag PCR Clean-up Kit

1. Prepare 24 new single PCR tubes, and fill accordingly

|  |  |
| --- | --- |
| Mix for 1 reaction |  |
| NEB Next Hi-Fi 2x Master Mix | 25μL |
| Barcode 1 | 5μL |
| Barcode 2 | 5μL |
| Amplified 16S Sample | 5μL |
| PCR Grade H2O | 10μL |

\*Total of 50μL per reaction

1. Run in Thermal Cycler according to:

|  |  |  |
| --- | --- | --- |
| Initial Denaturation | 98 oC | 30 sec |
| Denaturation | 98 oC | 10 sec |
| Annealing | 65 oC | 30 sec |
| Extension | 72 oC | 30 sec |
| Final Extension | 72 oC | 5 min |

\*Steps 2-4 repeated 8x

1. Clean Barcoded PCR Product
2. Store at -20 oC