**MiFish-U and -E primers were pooled to an equimolar working stock**

**PCR 1 Setup:**

* Volume of master mix: 14.5 µL total, made up of:
* 12.5 µL NEBNext Ultra II Q5 Master Mix
* 1 µL pooled forward primers
* 1 µL pooled reverse primers
* Sample volume added: 10.5 µL
* Thermocycler used: BioRad BW
* PCR 1 thermal cycling conditions:
* Initial denaturation: 98°C for 3 minutes
* 20 cycles of:
* 98°C for 20 seconds
* 65°C for 15 seconds
* 72°C for 15 seconds
* Final extension: 72°C for 5 minutes

**PCR 2 Setup:**

* To each PCR 2 reaction, added:
* 12.5 µL NEBNext Ultra Q5 Master Mix
* 5 µL index
* 7.5 µL amplified, cleaned DNA from PCR 1
* PCR 2 thermal cycling conditions:
* Initial denaturation: 98°C for 3 minutes
* 18 cycles of:
* 98°C for 30 seconds
* 55°C for 30 seconds
* 72°C for 30 seconds
* Final extension: 72°C for 5 minutes

**Post-PCR Processing: PCR 2 product stored at 4°C until purification**

**Cleaning**

* Magnetic bead purification using:
* 0.8× ratio of magnetic beads to 20 µL PCR product

**Library Quality Check:**

* 2% agarose gel
* D1000 Tapestation
* Buffer:library ratio of 3:1

**Sequencing:**

* Performed on an Illumina NovaSeq X (1.5MB) sequencer
* Sequencing kit: NovaSeq X Series reagent kit (300-cycles) from Illumina Inc.