# Environmental DNA (eDNA) 12S metabarcoding Illumina MiSeq NGS PCR Protocol

**Collin Closek,Anni Djurhuus,Katie Pitz,Ryan Kelly,Reiko Michisaki,Kristine Walz,Hilary Starks,Francisco Chavez,Alexandria Boehm,Mya Breitbart**

## Abstract

The 12S protocol is aimed at amplifying the hypervariable region of the mitochondrial DNA 12S rRNA gene in eukaryotes. The primers (MiFish-U-F & MiFish-U-R) used in this protocol were developed by Miya et al., 2015 for metabarcoding environmental DNA (eDNA) from fishes.

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Citation

Miya M et a .2015 MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R.Soc.opensci. 2: 150088. http://dx.doi.org/10.1098/rsos.150088

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## Guidelines

Local containment involves the employment of biological *safety* cabinets for initial specimen disinfection and *DNA* extraction. Such cabinets should not be used for *PCR* setup, as this *procedure* should be conducted apart from the aforementioned disinfection and extraction to minimize contamination.

1. Do not use any tube or plate that is not appropriate for the PCR machine you are using.
2. Make sure tubes and especially plates are well sealed before you begin run.
3. Clean up any spilled solutions and dispose of in appropriate biohazard boxes.
4. Be careful with PCR machine lids. These can be damaged if you slam or drop lids.

5. Make sure PCR heater block is clean before you start a run. Check each tube receptacle before you start.
6. Distribute tubes evenly across block so lid will seat flat against top of tubes for even heating and sealing.
7. Turn PCR machine off when you are done using.

## Before start

Disinfect work surfaces with 10% bleach, followed by 70% ethanol, then RNase Away and pipets with RNase Away. UV pipets, molecular grade water, and tube racks for 20 minutes prior to starting protocol.

## Protocol

PCR

**Step 1.**

PCR reactions for 12S were performed with a two-step amplification protocol for each sample using the MiFish_U primers (Miya et al., 2015).

PCR

**Step 2.**

The primary PCR amplifications were carried out in triplicate 20 µl reactions using:

- 2ul gDNA extract template (1:10 dilution)
- 10 µl Hotstar MasterMix (2X) (Qiagen, USA)
- 0.8 µl of each untagged forward and reverse primer (10 µM)
- 7.2 µl molecular biology grade water

PCR

**Step 3.**

PCR reactions were performed in 8-well strip tubes with a no template control (NTC) for each group of environmental sample triplicates and artificial community as a positive control per amplification round using the 12S primary PCR primers below (all primers listed in 5’ to 3’ direction).

12S MiFish_U (forward):

GTC GGT AAA ACT CGT GCC AGC

12S MiFish_U (reverse):

CAT AGT GGG GTA TCT AAT CCC AGT TTG

PCR

**Step 4.**

Primary 12S cycling parameters:

95 °C for 5 minutes

40 cycles of 95 °C for 15 seconds

55 °C for 30 seconds

72 °C for 30 seconds

Held at 4 °C

PCR

**Step 5.**

Secondary PCR amplifications are conducted in triplicate 20 µl reactions using same reaction master mix as primary PCR, but with 0.8 µl of each tagged 12S forward and reverse primer (10 µM) listed below (forward & reverse with matching unique 6-base tag, indicated by Xs).

12S (forward): NNN XXX XXX GTC GGT AAA ACT CGT GCC AGC

12S (reverse): NNN XXX XXX CAT AGT GGG GTA TCT AAT CCC AGT TTG

PCR

**Step 6.**

The reaction is carried through with 2 µl of each purified environmental as well as positive and NTC samples.

PCR

**Step 7.**

Secondary 12S tagged PCR cycling parameters:

- 95 °C for 5 minutes
- Twenty cycles of 95 °C for 15 seconds

- 57 °C for 30 seconds
- 72 °C for 30 seconds
- Held at 4 °C

Quality control, PCR clean-up

**Step 8.**

After PCR amplification of the marker region, the pooled PCR products were run through an agarose gel to confirm the presence of target bands and absense of non-specific amplification across environmental samples as well as the absence of amplification in no-template controls (NTCs).

Quality control, PCR clean-up

**Step 9.**

PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter, USA).

Quality control, PCR clean-up

**Step 10.**

A second agarose gel was run to confirm primer removal and retention of target amplicons after purification.

Quality control, PCR clean-up

**Step 11.**

Purified products were then quantified using Quant-It Picogreen dsDNA Assay (Life Technologies) on an fmax Molecular Devices Fluorometer with a Qubit dsDNA HS kit.

Quality control, PCR clean-up

**Step 12.**

Equimolar pools were constructed and quantified with Qubit dsDNA HS kit to confirm pool concentration prior to library preparation.

Quality control, PCR clean-up

**Step 13.**

One library was constructed for each location sampled in using the KAPA HyperPrep and Library Quantification kits following manufacturer’s protocol.

Sequencing parameters

**Step 14.**

12S rRNA gene was sequenced at Stanford Functional Genomics Facility.

Sequencing parameters

**Step 15.**

The pooled product for each genetic locus was loaded on a standard MiSeq v2 flow cell and

sequenced in a 2x250bp paired end format using a v2 500-cycle MiSeq reagent cartridge.

Sequencing parameters
**Step 16.**

The MiSeq run was performed and 20% PhiX was added.

Sequencing parameters
**Step 17.**

Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.0.

Sequencing parameters
**Step 18.**

Custom sequencing primers were added to appropriate wells of the reagent cartridge.

*12S rRNA*

FK NEXTflex Library barcode 6: CTTGTA

MB NEXTflex Library barcode 4: GCCAAT

## Warnings

Always observe proper laboratory safety warning and precautions. Wear lab coat, gloves, safety goggles and use UV-proof face shield when visualising gels with UV transilluminator. House the transilluminator in self-contained 'dark room'. All chemicals used as reagents in PCR reaction have Control of Substances Hazardous to Health Regulations (COSHH) storage form available along with procedure COSHH forms for PCR.

DNA visualization within the agarose gels requires the use of potentially hazardous ultraviolet light and ethidium bromide DNA intercollating dye. Personnel exposure to ultraviolet light will be minimized with the use of complete face shields designed to block UV ray transmission, as well as the use of long sleeved lab coats, gloves and the built in shield on the UV light box.