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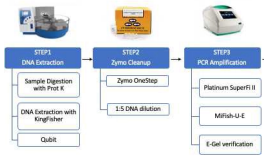
12S PCR Metabarcoding Protocol for Fish Detection in Estuarine Samples



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We use this protocol and it's working

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Abstract

This methodology has been developed for the use of MiFish-U and MiFish-E primers multiplexed with Platinum SuperFi II (2X) and Zymo clean up under a touchdown PCR program, aimed at the specific amplification of fish species from estuarine samples with inhibitors. The primers, originally developed by Miya et al., 2015, and Kawato et al., 2021, target the hypervariable region of the mitochondrial DNA 12S rRNA gene in fish species including Elasmobranchii. Platinum SuperFi II (2X) is a high fidelity polymerase and is well suited for multiplexing as it can work using primers with different melting temperatures at 60°C.

Guidelines

Before preparing the PCR mix and adding DNA, thoroughly clean all work surfaces with a 5%-10% bleach solution, followed by distilled water (dH2O). Ensure that surfaces are cleaned both before and after use. Always wear lab coats, gloves, and change them when moving between different work areas to prevent cross contamination. Clean pipettes regularly using a 5%-10% bleach solution, followed by a rinse with dH2O. Dispose of all hazardous waste in designated disposal areas to maintain a safe and orderly laboratory environment.

Materials

1. PCR Inhibition Removal

- Zymo clean up kit (for sites with known PCR inhibition) **D6035**.

2. Primers

	A	B	C
	MiFish-MIX-Forward Primers	**Sequence 5'-3'**	**Concentration**
	MiFish-U-F	GTCGGTAAAACTCGTGCCAGC-	5 µM
	MiFish-E-F	GTTGGTAAATCTCGTGCCAGC-	5 µM
	MiFish-MIX-Reverse Primers		
	MiFish-U-R	CATAGTGGGGTATCTAATCCCAGTTTG	5 µM
	MiFish-E-R	CATAGTGGGGTATCTAATCCTAGTTTG	5 µM

3. PCR Reagents

- PCR grade water (Thermofisher, CAT: AM9932)
- MiFish-U and MiFish-E Primers
- Platinum SUPERFI II Master Mix (2X) (CAT: **12368010**)
- DNA cleaned with Zymo and diluted 1:5

4. PCR Setup Equipment and Consumables

- Microcentrifuge tubes
- 96 well PCR plates
- PCR plate foil/caps
- Pipettes and pipette tips
- Thermal cycler for PCR
- Microseal 'B' PCR Plate Sealing Film (Biorad, CAT: MSB1001)

5. Post-PCR Analysis

- 2% agarose gel or E-Gel 2% for PCR product verification
- 100 bp DNA Ladder (diluted 1:10)
- Gel documentation system or UV light source for visualizing DNA bands

Safety warnings

This protocol has been optimized on a Biorad T-100 Thermocycler and was not tested on machines from other manufacturers. For the preparation of PCR mix, always utilize PCR grade water. Ensure that spaces for pre-PCR and post-PCR procedures are distinctly separated. Additionally, maintain a separate area for mixing PCR reagents apart from where DNA is added. Strictly follow cleaning protocols for the working surfaces.

DNA clean up using Zymo

1 Before setting up the PCR mix, purify the DNA using the Zymo OneStep PCR Inhibitor Removal Kit as indicated by the manufacturer (CAT: **D6030** or **D6035**). After the clean up, dilute the DNA at a 1:5 ratio. Follow the the **Guidelines & Warnings** for lab space preparation and clean up. See **Description** above for a general overview of this protocol.

Prepare the primer mix for multiplexing

2 Prepare an equimolar mix of **MiFish-U-F** and **MiFish-E-F** (with TruSeq or Nextera illumina adapters) of the Forward primers and an equimolar mix of the Reverse primers (final concentration for each primer in the PCR mix is 5 µM). See the **Materials** section above for details.

Prepare the Forward primer mix with equimolar concentration (final 5 µM)

- **Prepare a combined Forward primer mix:**

- 90 µL of PCR grade water
- Add 5 µL of MiFish-U-F stock solution (100 µM)
- Add 5 µL of MiFish-E-F stock solution (100 µM)

This results in a mix containing both Forward primers at a final concentration of 5 µM each.

- **Prepare a combined Reverse primer mix:**

- 90 µL of PCR-grade water
- Add 5 µL of MiFish-U-R stock solution (100 µM)
- Add 5 µL of MiFish-E-R stock solution (100 µM)

This results in a mix containing both Reverse primers at a final concentration of 5 µM each.

Prepare PCR reagents

3 IMPORTANT: The PCR is run in triplicate for each sample and the products are pooled together before running the E-Gel and preparing the Library.

Set up the PCR reaction for a total reaction volume of 20 µl by mixing the following components (amounts are per sample):

PCR grade H2O: 2 µl
Platinum SUPERFI II Master Mix (2X): 10 µl
Forward primer mix (5 µM): 2 µl
Reverse primer mix (5 µM): 2 µl

Aliquot 16 µL of the mixture into each well to be used in the 96 well PCR plate

Add 4 µL of DNA (Zymo cleaned and diluted at a 1:5 ratio)
Total Volume: 20 µl

Set up Touchdown program on thermocycler

4 We use a touchdown protocol by setting up an initial annealing temperature of 69.5°C to increase the specificity for the target.

	Temperature	Duration	Description
	95°C	3 minutes	Initial denaturation
	94°C	30 seconds	Denaturation
	69.5°C	30 seconds	Annealing (-1.5°C each cycle, touchdown)
	72°C	90 seconds	Extension
	----------	----------	Repeat steps 2-4 for 13 cycles
	94°C	30 seconds	Denaturation
	60°C	30 seconds	Annealing
	72°C	45 seconds	Extension
	----------	----------	Repeat steps 6-8 for 25 cycles
	72°C	10 minutes	Final extension
	4°C	Hold	Storage temperature

PCR check

5 After amplification, check the success of the PCR reaction by loading the sample onto a 2% agarose gel using a 1:3 dilution of the products and a 100 bp ladder (diluted 1:10).
NOTE: In the same lane, the gel should show one main band slightly below 300 bp. In some cases another band will appear (mainly off-target) at slightly below 400 bp.

Protocol references

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J.Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H. and Kondoh, M., 2015. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society open science*, *2*(7), p.150088.

Kawato, M., Yoshida, T., Miya, M., Tsuchida, S., Nagano, Y., Nomura, M., Yabuki, A., Fujiwara, Y. and Fujikura, K., 2021. Optimization of environmental DNA extraction and amplification methods for metabarcoding of deep-sea fish. *MethodsX*, *8*, p.101238.

ZYMO D6035 OneStep-96 PCR Inhibitor Removal Kit
https://files.zymoresearch.com/sds/_d6035_onestep-96_pcr_inhibitor_removal_kit.pdf