Feb 05, 2020 Version 1

Environmental DNA (eDNA) metabarcoding protocol for amphibians species V.1

DOI
https://dx.doi.org/10.17504/protocols.io.973h9qn

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.973h9qn

Protocol Citation: Alexander Eiler, Mats Töpel, Tomas Larsson, Johan Andersson 2020. Environmental DNA (eDNA) metabarcoding protocol for amphibians species. **protocols.io** https://dx.doi.org/10.17504/protocols.io.973h9qn

Protocol status: Working
We use this protocol and it's working

Created: December 06, 2019

Last Modified: February 05, 2020

Protocol Integer ID: 30683

Keywords: environmental dna metabarcoding universal primer, environmental dna metabarcoding, protocol for amphibians species, environmental dna, rrna gene, amphibians species, metabarcoding protocol, metabarcoding, universal primer

Abstract

Environmental DNA metabarcoding universal primers targeting the hypervariable region of the 12S rRNA gene

Attachments

PDF

Valentini_et_al-2016...

803KB

Guidelines

Serial dilutions of mock community was prepared as a positive control

Materials

MATERIALS

- Agencourt Ampure XP **Beckman Coulter** **Catalog #**A63AA0
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific** **Catalog #**10977015
- 10 mM dNTPs **Life Technologies** **Catalog #**10297-018
- Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs** **Catalog #**M0491L

Safety warnings

The 1st part of the protocol is performed in the pre-PCR room.
The 2nd part in the post-PCR room.
Never bring back PCR products to the pre-PCR room.
Always add a negative control samples in each PCR run

Before start

Laboratory work space and equipment were sterilized by UV-light and DNase solution and 70% ethanol. Filter pipet tips were used in all steps of the laboratory work.

1 For DNA extraction use Qiagen DNeasy power water sterivex kit.
The quality of the extracted DNA can be estimated using Nanodrop.

Qiagen DNeasy power water sterivex kit:
https://www.qiagen.com/se/resources/resourcedetail?id=c5fe7d5f-070a-4ebe-ac04-4bbf05a13e91&lang=en

2

Perform the first PCR (triplicates/duplicates of each sample) using Illumina adaptor attached primers that target the gene of your choice.
For Amphibians
Group specific mitochondrial 12S primers and human blocking primers (https://www.ncbi.nlm.nih.gov/pubmed/26479867) were used
batra_F: 5′-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN ACA CCG CCC GTC ACC CT-3′ and
batra_R: 5′-AGA CGT GTG CTC TTC CGA TCT NNN NNN GTA YAC TTA CCA TGT TAC GAC TT-3′.
Human blocking primer: batra_blk: TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I which was used to bind to human DNA and prevent its amplification.

Amplification strategy

3 Paired end sequencing on the Miseq platfrom required two steps of PCR

First PCR step

3.1

	Components	Working conc.	Final conc.	1 reaction (µl)
	5x Q5 Reaction Buffer	5X	1X	5
	batra_F	10 µM	0,2 µM	0,5
	batra_R	10 µM	0,2 µM	0,5

	dNTPs	2 mM	0,2 mM	2,5
	batra_blk	50uM	4uM	2
	Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0,25
	Template DNA			5
	Nuclease-Free water			9,25
	Σ			25

For environmental samples add 5 µl of template DNA and for mock community samples add 1 µl DNA

	STEP	TEMP.	TIME
	Initial Denaturation	98 C	30 sec
		98 C	20 sec
	35 cycles	57 C	30 sec
		72 C	1 min
	Final Extension	72 C	7 min
	Hold	6 C	∞

3.2 Check PCR products with Agarose gel electrophoresis (1%).

3.3 Pool PCR triplicated or duplicate samples together and perform purification with magnetic beads (Agencourt AMPure) https://research.fhcrc.org/content/dam/stripe/hahn/methods/mol_biol/Agencourt%20AMPure%20XP.pdf

Second PCR

4 A second PCR is conducted for attaching standard illumina handles and index primers
Multiplex_fwd
AATGATACGGCGACCACCGAGA{TCTACAC}-[i5 index] ACACTCTTTCCCTACACGACG
Multiplex_rev
CAAGCAGAAGACGGCATACGAGAT-[i7 index]-
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
We have in total 20 different forward index/barcode primers and 20 different reverse index/barcode primers.
By combining both primers (20X20), it is possible to generate 400 tags in one final pool for sequencing.

4.1

	Components	Working conc.	Final conc.	1 reaction (20 µl)
	5xQ5 Reaction Buffer	5X	1X	4
	Forward index (i5, illu-N501-N508)	5µM	0.25 µM	1
	Reverse index (i7, illu-N701-N712)	5µM	0.25 µM	1

	dNTPs	2mM	200 µM	2
	Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0.2
	Template from 1st PCR			2
	Nuclease-Free water			9.8
	Σ			20

	STEP	TEMP.	TIME
	Initial Denaturation	98 C	30 sec
		98 C	10 sec
	15 cycles	66 C	30 sec
		72 C	30 sec

	Final Extension	72 C	2 min
	Hold	6 C	∞

4.2 Check second PCR products with Agarose gel electrophoresis (1%)

4.3 Perform purification with magnetic beads (Agencourt AMPure).

5 Use a PicoGreen assay to quantify the concentration of the second PCR product before pooling.

6 Pool the PCR samples in equal DNA amount (ng) or for unequal length amplicons, in equal molecule amount (mol). This results in one tube including a mix of all the samples.

Calculate the volume of each sample to be pooled (DNA amount mixing) as follows:

- Use the lowest concentration sample to define the minimum amount of DNA (ng) that you have available from a single sample:

- DNA concentration (ng/µL) of the lowest concentration sample multiplied with its volume (µL). This will be your target DNA amount for each sample.

- Calculate how many µLs of each sample you need to achieve the target DNA amount: divide the target DNA amount with the concentration of each sample.

- Pipette into one tube the calculated volume of each sample.

- Aim to use the same pipette for all samples (dilute or pipette multiple times) to avoid pipette calibration errors.

7 Check the pooled samples by agarose gel electrophoresis (1%) to make sure only one band is displayed in the gel.

Gel purify the pool and re-quantify with PicoGreen before submitting to sequencing facility.

Sequencing

8

Software

NAME	SOURCE LINK
Illumina MiDeq Next Generation sequencer	https://www.illumina.com/

Sequencing was performed on the Illumina MiSeq platform that generated paired end sequence that where 150 bp in length.

Expected result

Analysis was carried out by DADA2 pipeline https://benjjneb.github.io/dada2/tutorial.html .