Feb 05, 2020

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

Alexander Eiler[1], Mats Töpel[2], Tomas Larsson[1], Johan Andersson[3]

[1]eDNA solutions AB, [2]eDNA solutions AB, [3]Water Circle AB

1 Works for me dx.doi.org/10.17504/protocols.io.973h9qn

eDNAsolutions
Tech. support phone: **+0700264843** email: **omneya@ednasolutions.se**

Omneya Ahmed Osman

ABSTRACT

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

ATTACHMENTS

Valentini_et_al-2016-Molecular_Ecology.pdf

GUIDELINES

Serial dilutions of mock community was prepared as a positive control

MATERIALS

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

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 STARTING

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
∑			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

Illumina MiDeq Next Generation sequencer

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

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