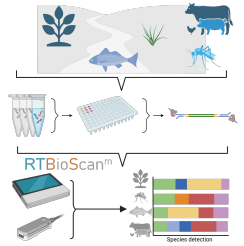


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Universal plant and animal eDNA metabarcoding using Nanopore sequencing

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We use this protocol and it's working

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Abstract

Environmental DNA (eDNA) is a powerful, non-invasive tool for biodiversity monitoring, but traditional approaches face challenges including limited sensitivity, taxonomic restrictions, lengthy processing times, and reliance on specialized lab equipment and computational resources. To address these limitations, we present an integrated eDNA workflow from extraction to real-time analysis. Our approach combines the Tagsteady protocol for multiplexed eDNA metabarcoding with Oxford Nanopore library preparation and sequencing, enabling simultaneous detection of animals (Metazoa) and plants (Viridiplantae) using universal markers (COI and ITS2). By integrating metabarcoding and barcoding in a single protocol, this workflow can assess species presence in environmental samples and barcode individual specimens to expand reference databases, providing a streamlined solution for broad, field-based biodiversity assessment.

Image Attribution

Image partly created with Biorender



Guidelines

It is crucial for the quality of the data obtained to work in the cleanest possible manner. Not only is the targeted DNA present in the sample at a very low concentration, but importantly **due to our degenerate primer design almost any animal or plant DNA can be a source of contamination**. We suggest to **physically separate Pre- and Post-PCR areas and wipe surfaces**, gloves and equipment regularly with either diluted bleach or nucleic acid removal solutions like RNaseAway.

Before start

Clean all surfaces, pipettes, centrifuges, magnets and your gloves with RNaseAway before starting. The DNA extraction protocol can be performed comfortably with 16 samples at a once, using a 16 sample tube magnet. Timings in the DNA extraction part assume 16 samples and the use of a repeater pipette to add buffers etc. to samples.



Before Starting

1m

- 1 Add 1,040 µl Proteinase K Storage Buffer to each Proteinase K (20 mg) tube prior to use. Store at -20°C after mixing.

1m

Sample Preparation

52m

- 2 Add up to 50 mg material to a bead beating tube containing DNA/RNA Shield and mechanically homogenize your sample on a vortex mixer at maximum speed for 3 minutes.
- 3 Add 20 µl Proteinase K and mix well. Incubate at RT for 30 min.
- 4 Centrifuge the sample at 10,000 x g for 1 min to pellet the debris and transfer up to 400 µl of lysate into a new tube.

20m

30m

2m

DNA Purification

1h 58m

- 5 Add 400 µl Quick-DNA MagBinding Buffer to 400 µl digested sample and mix by pipetting or inverting 10 times.
- 6 Add 33 µl of MagBinding Beads to each sample. If using a repeater pipett use 30 µl
- 7 Mix by shaking for 10 min at 1100 – 1500 rpm or by inverting regularly
- 8 Transfer the sample to the magnetic rack, wait until beads have separated from the solution
- 9 Discard the supernatant using a P1000 pipette
- 10 Transfer the sample off the magnetic rack, add 500 µl Quick-DNA MagBinding Buffer.

5m

2m

10m

3m

5m

5m



- | | | |
|----|---|-----|
| 11 | Mix by shaking or inverting regularly for 10 min | 10m |
| 12 | Transfer the sample to the magnetic rack, wait until beads have separated from the solution and discard the supernatant using a P1000 pipette | 5m |
| 13 | Transfer the sample off the magnetic rack, add 500 µl DNA Pre-Wash Buffer and mix the sample by pipetting. | 5m |
| 14 | Transfer the sample to the magnetic rack, wait until beads have separated from the solution and discard the supernatant using a P1000 pipette | 5m |
| 15 | Transfer the sample off the magnetic rack, add 900 µl g-DNA Wash Buffer and mix the sample by pipetting. | 5m |
| 16 | Transfer the sample to the magnetic rack, wait until beads have separated from the solution and discard the supernatant using a P1000 pipette | 5m |
| 17 | Repeat steps 15-16 two more times. | 20m |
| 18 | Air dry for 20 minutes. | 20m |
| 19 | Add 40 µl of DNA Elution Buffer to each sample. | 3m |
| 20 | Mix by shaking at RT for 5 min. | 5m |
| 21 | Transfer the sample to the magnetic rack, wait until beads have separated from the solution and transfer the eluted DNA to a new tube. | 5m |

Multiplex PCR with Qiagen Multiplex PCR Kit

3h 49m

- | | | |
|----|---|----|
| 22 | Set up a no template control reaction (NTC) and if used a positive control containing high quality DNA of animals and/or plants | 2m |
|----|---|----|

**Note**

NTC: 10µl 2x Qiagen Multiplex PCR mix, 6.5 µl H₂O
Positive Control: 10µl 2x Qiagen Multiplex PCR mix, 6.5 µl diluted control DNA (max. 1ng total)

23 Add 19.5 µl eDNA to one well per sample (6.5 per technical triplicate)

5m

24 Add 30µl 2x Qiagen Multiplex PCR mix to each well

1m

25 Mix sample and PCR mastermix with a multichannel pipette and distribute 16.5 µl into a total of 3 wells per sample.

5m

26 Add 3.5 µl of forward reverse barcode mix per well. Or 1.75 µl forward mix and 1.75 µl reverse mix when not using pre-mixed primers. See Notes in "Before you start" section

5m

**Note**

Use **each forward/reverse barcode combination just once** per experiment. This is crucial to be able to demultiplex the data from different wells. **Note down which primer combination was used for each well meticulously**

27 When using a microwell plate, seal very well to avoid cross contamination.

1m

Note

We recommend using thick plastic seals like MicroAmp clear Adhesive Film

28 Incubate as indicated below

3h 25m

	A	B	C	D
	Step	Temperature	Time	Repeat
	Initial denaturation	95 °C	15m	



	A	B	C	D
	denaturation	95 °C	30s	35 cycles
	annealing	55 °C	1m 30s	
	extend	72 °C	1m 30s	
	final extension	72 °C	15m	
	store	12 °C	Inf	

Multiplex PCR cycling conditions

- 29 Choose one normalisation strategy here. **Concentration based** normalisation gives you **more information** about the PCR **amplification success** for each sample, while **Bead based** normalisation is **faster, cheaper and more scalable**.

STEP CASE

Concentration based normalisation using Qubit 59 steps

Here you can choose to either measure each sample's concentration and normalise using this information or adjust concentration using normalisation beads. For recipes of bead normalisation buffer and homemade SPRI beads check "Before you start" section

Bead clean up

38m

- 30 Equilibrate SPRI bead solution to RT and vortex until the solution appears homogeneous. 1m
- 31 Pool 3 replicates of each sample in one tube/well using a multichannel pipette 2m
- 32 Add 1.5x volume beads (with 60 µl of sample, add 90 µl of beads). 5m
- 33 Mix 10 times with a pipette and incubate for 5 min. 5m
- 34 Place the tubes on the magnet and leave it until the supernatant has cleared. 2m



- 35 Discard the supernatant. 1m
- 36 Wash twice in 100 μ l freshly prepared 80% EtOH. 5m
- 37 Air-dry beads for 5 min on the magnet. 5m
- 38 Remove the samples from the magnet and add 30 μ l ddH₂O to the beads. 2m
- 39 Mix 10 times with a multichannel pipette and incubate for 5 min. 7m
- 40 Put back on the magnet until the supernatant has cleared and transfer the DNA to a new tube. 3m

Concentration measurement

30m

- 41 measure 1 μ l of the cleaned up PCR product using a Qubit flourometer and the Qubit HS DNA Kit according to manufacturers instructions. 5m
- 42 In a spreadsheet calculate the amount of DNA to add for each sample. We asume here that the amplicons will have a similar length distribution in all samples. 10m
- 43

Note

Typically we aim for 300 fmol total input
- 44 Based on the amounts calculated pool amplicons equimolarly and add H₂O to 30 μ l if necessary. 15m

End Repair

39m

- 45 For each library make a master mix: 3m



A	B
Reagent	Amount
Klenow fragment exo- (5 U/μl)	1.5 μl
T4 PNK (10 U/μl)	2 μl
dATP (10mM)	0.5 μl
T4 DNA ligase buffer (10x)	4 μl
Reaction booster	2 μl
Total	10 μl

46 Add 10 μl of master mix to each 30 μl amplicon pool, mix by pipetting and spin down.

1m

47 Incubate as indicated below:

35m

A	B
Temperature	Time
37 °C	30m
65 °C	5m
4 °C	inf

Adapter Ligation

46m

48

Note

Here we use the Oxford Nanopore Library Preparation Kit (Ligation Sequencing Kit V14)

49 Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice.

1m



50 Thaw Ligation Buffer (LNB), Elution Buffer (EB) and Short Fragment Buffer (SFB) at RT and place on ice immediately after thawing and mixing.

1m

51 Add 20 µl ddH₂O to the sample.

1m

52 In a 1.5 ml DNA LoBind tube, mix in the following order:

	A	B
	Reagent	Amount
	Endrepaird Amplicon pool	60 µl
	Ligation Buffer (LNB)	25 µl
	NEBNext Quick T4 DNA Ligase	10 µl
	Ligation Adapter (LA)	5 µl
	Total	100 µl

53 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

2m

54 Incubate the reaction for 10 min at RT.

10m

55 Resuspend the AMPure XP Beads (AXP).

1m

56 Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.

1m

57 Incubate for 5 min at RT.

5m

58 Spin down the sample, place it on the magnet and pipette off the supernatant when it is clear.

2m

59 Wash the beads twice with 250 µl Short Fragment Buffer (SFB) and discard the supernatant.

5m

- 60 Spin down, place the tube back on the magnet and pipette off any residual supernatant. 2m
- 61 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB). 1m
- 62 Spin down and incubate for 10 min at RT. 10m
- 63 Pellet the beads on the magnet until the eluate is clear. 1m
- 64 Remove 15 µl of eluate and perform concentration measurement using Qubit HS DNA kit according to manufacturers instructions. 1m
- 65 Calculate molarity using following formula:
average fragment size for a library containing COI and ITS2 is ~500 bp 1m

Molarity in nM= concentration (ng/µl)/660*mean fragment length (bp)*1e6

µl for 50 fmol= (50 fmol*660* mean fragment length (bp) *1e6)/concentration (ng/µl)
- 66 Prepare 50 fmol in 12 µl of Elution Buffer (EB). 1m

Priming and Loading the MinION

21m

- 67 Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at RT and place on ice after thawing. 1m
- 68 To prepare the flow cell priming mix with BSA, combine the following reagents:

A	B
Reagent	Amount
Flow Cell Flush (FCF)	1170 µl
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl



69

Note

For detailed instructions on how to load a MinION flow cell consult Nanopores documentation

70 Open the MinION device lid, slide the flow cell under the clip and press down firmly on the flow cell. 1m

71 Slide the flow cell priming port cover clockwise to open the priming port. 1m

72 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: 1m

72.1 Set a P1000 pipette to 200 μ l. 2m

72.2 Insert the tip into the priming port.

72.3 Turn the wheel until the dial shows 220-230 μ l, to draw back 20-30 μ l or until you can see a small volume of buffer entering the pipette tip.

73 Load 800 μ l of the priming mix into the flow cell via the priming port and wait for 5 min. 6m

74 In a new 1.5 ml DNA LoBind tube, prepare the library for loading as follows: 3m

	A	B
	Reagent	Amount
	Sequencing Buffer (SB)	37.5 μ l
	Library Beads (LB)	25.5 μ l
	DNA library (50 fmol)	12 μ l



75 Complete the flow cell priming:

2m

75.1 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

75.2 Load 200 μ l of the priming mix into the flow cell priming port, avoiding the introduction of air bubbles.

76 Mix the prepared library gently by pipetting up and down just prior to loading.

1m

77 Add 75 μ l of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion.

1m

78 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

1m

79 Place the light shield onto the flow cell.

1m

Sequencing and starting the BioScanRT pipeline

80

Note

Here we assume you have already set up and tested the BioScanRT pipeline following the instructions in github and you have the newest version of MinKnow installed (either on the Minlon device (Mk1c) or on the computer used to control the run (Mk1B or Mk1D))

81 Run the Flow Cell check. This can also be done already before the Priming and Loading section to save time.

82 Start the MinKnow run with standard parameters for the Library Preparation Kit (Ligation Sequencing Kit V14), disable bascalling as this will be done on the Laptop or Macbook, depending on library size (<200bp) select to include small fragments



83 Start the BioScanRT pipeline as outlined in the corresponding github repository.

Protocol references

Carøe, C. & Bohmann, K. Tagsteady: A metabarcoding library preparation protocol to avoid false assignment of sequences to samples. *Molecular Ecology Resources* 20, 1620–163