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Quantitative multi-target amplicon sequencing workflow

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We use this protocol and it's working

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Abstract

- High-throughput sequencing of marker gene amplicons is a powerful tool for the discovery of biodiversity, at different levels. However, the method has multiple caveats.
- Here, we describe in detail a versatile laboratory protocol for high-throughput and cost-effective characterization of microbiota across large numbers of wild insects. By simultaneously targeting different marker regions, in a quantitative manner, the protocol enables the determination of insect host identity and reconstruction of bacterial diversity, alongside bacterial quantification combined with rigorous contamination control. It can be easily extended to encompass other marker regions and customized depending on specific needs, research questions, or specimen properties.
- The protocols encompasses DNA extraction from individual specimens (specimen homogenization, homogenate purification) and Illumina amplicon library preparation for two target regions: insect mitochondrial cytochrome oxidase I gene and the V4 region of bacterial 16S rRNA gene. It also explores the sequencing options and briefly outlines bioinformatics solutions that are published elsewhere.
- The protocol is labor- and cost-effective and relies on readily available reagents and equipment. It is highly customizable: we suggest alternative solutions, including omission of certain optional elements, or addition of alternative reagents and approaches.
- To date, we have successfully applied the protocol to nearly 20,000 individual insects, obtaining insights into microbial distribution, diversity, and transmission across multiple populations or large multi-species community subsamples of wild insects.

Materials

LIST OF EQUIPMENT

A	B
Item	
Homogenizer Bead Ruptor Elite (Omni International)	Homogenizer
Thermal Block - Digital Dry Baths	Block Heaters
Thermocycler - Labcycler SensoQuest	Thermocycler
Plate Reader - Synergy HTX Reader	Plate reader
Magnetic stand for 96 well plate	
Qubit 2.0 Fluorometer	Fluorometer
Set of adjustable 8-channel pipets (HTL)	HTL
Set of single-channel pipets	
Horizontal Gel Electrophoresis Units	
Vortex Mixers	
GelDoc Go Gel Imaging System	
Agilent 4200 TapeStation	

Reagents:

- Proteinase K (Thermo Scientific, E00491)
- Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Scientific)
- QIAGEN Multiplex PCR Master Mix
- Promega ProNex® Size-Selective Purification System
- Qubit™ 1X dsDNA High Sensitivity (HS) (Thermo Scientific, Q33231) and Broad Range (BR) Assay Kits (Thermo Scientific, Q33266)

Consumables:

- 2ml screw cap tubes for homogenisation and insect storage (Sarstedt, 72.694.006)
- Ceramic beads for homogenisation, sizes 0.5mm and 2.8mm (Omni International)
- 50 ml falcon tubes (Sarstedt, 62.559.001)
- - DNA low binding eppendorf tubes (Eppendorf, 0030108400 - 0.5 ml, 0030108051 - 1.5 ml or Sarstedt, 72.704.700 - 0.5ml, 72.706.700 - 1.5 ml)
- DeepWell Storage plate (e.g. Thermo Scientific, AB0765)
- Black 96 well plate

Buffers preparation

LYSIS BUFFER – VESTERINEN

	500 ml of buffer
NaCl	11,7 g
1M Tris-HCl	5 ml
0,5M EDTA	2 ml
20% SDS	50 ml
ddH ₂ O (sterile)	Up to 500 ml
Proteinase K	Added to each sample separately

Buffer parameters:

0,4M NaCl
 10mM Tris-HCl
 2mM EDTA
 2% SDS
 20ug/ml proteinase K

Chemicals needed:

NaCl (powder)
 1M Tris-HCl (solution)
 0,5M EDTA (solution)
 20% SDS (solution)
 Proteinase K – added to each sample separately

It is good to put sterile magnetic stirrer into a bottle. It helps with mixing all of the chemicals.

SPRI BEADS

Materials:

- Sera-mag SpeedBeads (Merck, GE # 65152105050250)
- PEG-8000
- 0.5 M EDTA, pH 8.0
- 1.0 M Tris, pH 8.0
- Tween 20
- 5 M NaCl

Steps:

1. In a 50 mL conical using sterile stock solutions, prepare TE (10 mM Tris-HCl, 1 mM EDTA = 500 µL 1 M Tris pH8 + 100 µL 0.5 M EDTA, fill conical to 50 mL mark with dH₂O).
2. Mix Sera-mag SpeedBeads and transfer 1 mL to a 1.5 mL microtube.
3. Place SpeedBeads on magnet stand until beads are drawn to magnet.
4. Remove supernatant with P200 or P1000 pipetter.

5. Add 1 mL TE to beads, remove from magnet, mix, return to magnet.
6. Remove supernatant with P200 or P1000 pipetter.
7. Add 1 mL TE to beads, remove from magnet, mix, return to magnet.
8. Remove supernatant with P200 or P1000 pipetter.
9. Add 1 mL TE to beads and remove from magnet. Fully resuspend and set microtube in rack (i.e. not on magnet stand).
10. Add 9 g PEG-8000 to a new 50 mL, sterile conical.
11. Add 10 mL 5 M NaCL (or 2.92 g) to conical.
12. Add 500 µL 1 M Tris-HCL to conical.
13. Add 100 µL 0.5 M EDTA to conical.
14. Fill conical to ~ 49 mL using sterile dH2O. You can do this by eye, just go slowly.
15. Mix conical for about 3-5 minutes until PEG goes into solution (solution, upon sitting, should be clear).
16. Add 27.5 µL Tween 20 to conical and mix gently.
17. Mix 1 mL SpeedBead + TE solution and transfer to 50 mL conical.
18. Fill conical to 50 mL mark with dH2O (if not already there) and gently mix 50 mL conical until brown.
19. Test against AMPure XP using aliquots of ladder. See below - SPRI BEADS Testing.
20. Wrap in tinfoil (or place in dark container) and store at 4°C.
21. Test monthly.

SPRI BEADS Testing

Materials:

- Perfect Ladder 100-1000bp (EURx, E3141-02)
- TE x1
- SPRI BEADS (if stored in a fridge, remember to take them out 1 hour before the test)
- 80% EtOH (freshly prepared)
- Deep well plate
- Magnetic stand

1. Dilute the ladder two times with TE x1.
2. Mixed 20 ul of diluted ladder with SPRI BEADS in ratios: 0.8, 0.9, 1.2, 1.5, 2.0 (16ul, 18ul, 24ul, 30ul, 40ul of beads respectively).
3. Follow the protocol for PCR cleaning using SPRI magnetic beads.
4. After cleaning make a small 2.5% agarose gel and verify the results of cleaning.

Protocol overview

1 Protocol overview, general instructions, and considerations

Quantitative Multi-Target Amplicon Sequencing is an approach originally developed for the characterization of microbiota in large collections of diverse insects. It enables the molecular identification of the host insects and the characterization of the abundance and genotype- and clade-level diversity and distribution of their associated bacteria. This is achieved through simultaneous amplification of insect (COI) and bacterial (16S rRNA) marker genes, the latter in a quantitative manner, and the preparation of Illumina-compatible sequencing libraries in a highly multiplexed manner.

This protocol has been initially developed and optimized for the cost-effective preparation of large numbers of amplicon sequencing libraries for these two target regions. However, it can be easily adapted to incorporate other marker regions for different organisms potentially present in samples, or adapted for other types of biological samples. Parts of the protocol can be executed on their own and combined with other methods. Many of the solutions implemented in the protocol, including quantification of targets or use of "phased" primers, are optional.

The protocol presented here starts with a set of 88 diverse ethanol-preserved insects selected from a Malaise trap. This number, alongside different positive and negative controls, is sufficient to fill a 96-well plate.

It ends with a library pool suitable for sequencing using one of Illumina platforms compatible with at least 500-cycle kits.

Specifically, the protocol comprises the following steps:

A. DNA extraction

- > Sample homogenization
- > Quantification spike-in preparation and addition
- > Homogenate purification
- > DNA quantification

B. Amplicon library preparation

- > The overview of the library preparation process
- > First PCR
- > PCR product validation
- > PCR product purification
- > Second (indexing) PCR

C. Amplicon library pooling, QC, and sequencing

- > Estimating library concentrations

- > Library pooling
- > Library pool QC and preparation for sequencing
- > Sequencing options and considerations

D. An overview of the data analysis workflow

>

A. DNA extraction

5h 25m

2 Specimen selection and preparation

- Typically, we start with a batch of ethanol-preserved insects from a sweep net or a Malaise trap catch
- Generally, we pre-select insects based on morphology, to maximize diversity and avoid sequencing large numbers of specimens representing the same species.
- We have also successfully used insect specimens pre-barcoded following Meyer protocols that include HotSHOT DNA extraction step (<https://link.springer.com/article/10.1186/s12915-021-01141-x>). See Andriienko et al. (2024) for details (<https://doi.org/10.1101%2F2024.04.30.591865>).
- We typically roughly sort insects by size. Very large insects may require a modified treatment, such as using a greater volume of lysis buffer (see section 3).
- We use 88 individual insects per plate (one per well). The remaining wells are used for negative and positive controls.
- We carefully organize and preserve sample metadata
- We recommend rinsing specimens in clean ethanol before processing, especially if they originate from dense multi-species samples, and may have dust or body fragments of other insects attached.
- It is recommended to air-dry at least the larger insects before processing them, to avoid the inhibitory effects of excess ethanol.... but wait until tubes for the DNA extraction (next step) are ready to go

3 Sample homogenization

3h 30m

Material:

- Lysis buffer „Vesterinen” [0.4M NaCl, 10mM Tris-HCl, 2mM EDTA, 2% SDS] – before use, place in a container with warm water to help dissolve any precipitate
- Proteinase K (stored frozen at -20°C)
- Ceramic beads: 2,8mm and 0,5mm
- 2 ml screw-cap tubes
- 50 ml Falcon tubes, tweezers, Petri dish, paper towels, metal spatula, torch

Equipment:

- Homogenizer. We use Bead Ruptor Elite (Omni International), with a block suitable for 24 2ml screw cap tubes. A different homogenizer may require different tubes.

Make sure that the tubes are made for plastic resistant for bead beating!

Homogenization:

1. Prepare 91 tubes and sign them with sample ID on the side (the inscriptions on the lid may wear off). For each plate you will have **88 insects and 3 negative controls - "extraction blanks" (3C, 6C, 9C)**.
2. For standard size insects¹⁾ (size 1-2) in 50 ml falcon tube prepare a mix of a "Vesterinen" lysis buffer and proteinase K for 100 samples. For one sample in that case we use **195 ul of lysis buffer and 5 ul of proteinase K**, so for a 100 – 19 500 ul of buffer and 500 ul proteinase K. However, if you see that the insects are bigger prepare more buffer, because you will use more than 200 ul of buffer mix per sample.
3. Open the tubes and place them on a 96 well rack. Make sure that the caps are placed on aluminium foil to avoid contamination from the bench. To each tube **add 200 ul of prepared buffer mix**. Then into each tube add one small spoon of 0.5mm beads (make sure to sterilize the spoon under the flame first!) and one big bead 2.8mm. You can add big beads by hand, but before change gloves to minimize the risk of contamination.
Note that bigger insects will need more buffer mix and more big beads (2-3 instead of 1).
4. Close the tubes with blanks – 3C, 6C, 9C.

5. Transfer your specimens to the tubes. Sterilize the tweezers at least between the different eppendorfs with insects to homogenization. If each individual is in separate eppendorf, then sterilize the tweezers between every individual. Make sure to put an insect on a paper towel before you put in a tube with buffer, to get rid off external ethanol. Close the tube after adding the insect²⁾.

(Remember to note on the homogenization sheet³⁾ all of the important informations about the sample – trap ID, size of an insect, volume of a buffer, info about the Order/Group/species, sex. Sterilize the tweezers with a flame after each sample.)

6. Place the tubes in the homogenizer and grind two times for 30 s with a 5m/s speed.
7. Place the samples in a thermal block/water bath at 55°C for 2 hours. If possible, vortex samples 1-2 times during incubation.
8. Remove the samples from the thermal block and, after cooling to room temperature, start DNA extraction or store in fridge or freezer.

¹⁾ Insect size categories: [1] up to 2mm, [2] up to 5mm, [3] up to 1cm, [4] up to 2cm, [5] over 2 cm. In case of size category 1, 2 and most of the 3 standard amount (200 ul) of

homogenization mix is enough. If you need to homogenize really big insects, e.g. bees, you need to cut it into smaller pieces. Divide bee into two pieces and put each into a 2 ml screw cap tube. Don't forget to sterilize the tweezers/scalpel after each sample. After incubation pour the content of one tube into another, vortex and store in a fridge or freezer.

2)



Insect samples placed individually in 2ml screw cap tubes filled with Vesterinen lysis Buffer and Proteinase K.

3)



PLATE ID: HGR19			DATE: 23.02.23			PERSON: VA						
	1	2	3	4	5	6	7	8	9	10	11	12
A	ID:HGR1901	HGR1909	17	25	HGR1933	41	49	57	65	73	81	89
	SIZE: 3	SIZE: 4	SIZE: 3	SIZE: 3	SIZE: 4	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 2
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 400	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
	LEPID	OTHER			Diptera							
B	2	HGR1910	18	26	HGR1934	42	50	58	66	74	82	90
	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 1	SIZE: 1	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 3
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
					HYMEN							
C	HGR1903	HGR1911	19	27	35	43	51	59	67	75	83	HGR1911
	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 1	SIZE: 1	SIZE: 2	SIZE: 1	SIZE: 3	SIZE: 3	SIZE: 3
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
	OTHER	Diptera	BLANK		BLANK			BLANK				HYMEN
D	4	12	20	28	36	44	52	60	HGR1908	76	HGR1904	
	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 3	SIZE: 3
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
									MOSQ			Diptera
E	5	13	21	29	37	45	53	61	HGR1909	77	HGR1905	
	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 1	SIZE: 1
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
									Diptera			HYMEN
F	6	14	22	30	38	46	54	62	70	78	86	
	SIZE: 4	SIZE: 2	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 2
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
G	7	15	23	31	39	47	HGR1955	63	71	79	87	
	SIZE: 4	SIZE: 3	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 2
	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
									Diptera			
H	8	16	24	32	40	48	HGR1956	64	72	80	88	
	SIZE: 4	SIZE: 3	SIZE: 3	SIZE: 3	SIZE: 1	SIZE: 2	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 3	SIZE: 2	SIZE: 2
	H.vol: 400	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200	H.vol: 200
									MOSQ			

SIZE: [1] up to 2mm, [2] up to 5mm, [3] up to 1cm, [4] up to 2cm, [5] more than 2 cm
BLANK – lysis buffer (195µl) + proteinase K (5µl) + one big bead + one spoon of small beads, NO INSECT!

Filled homogenisation sheet.

4 Quantification 16S rRNA spike-in plasmid preparation

10m

A. Sequence synthesizing, plasmid production, transformation into *E. coli* and bacteria culture.

Plasmid sequences with artificial 16S rRNA targets are derived from Toulousse et al., 2016.

In our workflow we are using **Ec5502** at the stage of DNA extraction, and **Ec5001** during PCR I.

Use commercial services (i.e. Eurofins) to synthesise spike-in sequences and insert them onto pEX-A128 plasmid harboring ampicillin resistance.

After obtaining the plasmid you need to transfer them into *E. coli* and culture the bacteria. For the description of those steps please look into the protocol by E. Iwaszkiewicz-Eggebrecht et al.

(2023) dx.doi.org/10.17504/protocols.io.14egn33ryl5d/v2

B. Plasmid isolation from *E. coli*

The plasmids were isolated from *E. coli* using a GeneMATRIX PLASMID MINIPREP (EURx) kit following the manufacturer's instructions.

C. Plasmid digestion with restriction enzymes.

To avoid complications during PCR that might have arisen because of circular shape pf plasmids, we decided to linearise them by cutting with a restriction enzyme.

For plasmid Ec5502 we choose EcoRI-HF enzyme (NEB) and for Ec5001 AatII enzyme (NEB). Both are cutting the plasmid effectively and leaving the barcode region intact.

Materials:

- Restriction enzyme EcoRI-HF (for Ec5502) or AatII (For Ec5001) - **we are using New England Biolabs enzymes, for the enzymes from a different manufacture the digestion protocol might be different**
- CutSmart Buffer (provided with enzyme)
- Molecular water (nuclease free)

PREPARE ON ICE!

C.1. Measure the concentration of isolated plasmid DNA on Qubit.

C.2. Remove restriction enzyme and CutSmart buffer from the freezer and put it on ice.

C.3. Calculate how many ul of plasmid you need to add to the reaction in order to have ca. 100 ng of plasmid DNA in each reaction. At first add molecular water to the tube, then plasmid and CutSmart buffer. As a last component add restriction enzyme. Do not vortex the reaction! Just mix it by pipetting. Centrifuge shortly.

C.4. Put the strip tube/plate in a thermal cycler and set up the program. The incubation and inactivation temperatures and time can be found in table below.

A	B	C	D
Plasmid ID	Restriction enzyme	Incubation temp. and time	Inactivation temp. and time
Ec5502	Eco-RI HF (NEB)	37°C, 5-15 mins	65°C, 20 mins
Ec5001	AatII (NEB)	37°C, 5-15 mins	80°C, 20 mins

C.5. After digestion freeze the samples or prepare 220 MLN copies single-use aliquots according to the instruction below.

D. Preparing 220 MLN copies single-use aliquots.

Materials and equipment:

- PolyA 100 (TE with PolyA tails in conc. 100 ng/ul)
- Digested plasmid from previous step
- Qubit fluorometer and Qubit HS assay kit

D.1. Measure digested plasmid DNA concentration using Qubit fluorometer.

D.2. Based on the received information calculate how many copies of a plasmid you have in one μl .

You can do this based on the assumption that 1 ng of 16S plasmid DNA contains 220 MLN copies of the plasmid.

(This assumption is based on a calculation taking into account the size of the DNA molecule)

D.3. Based on performed calculations dilute spike-in's samples with PolyA 100 into the concentration of 220 mln of copies/ μl and prepare single-use aliquots of 10 μl each.

To avoid molecule binding to the surface of the tube, use DNA low-binding eppendorf tube.

Store in a freezer in -20°C.

E. Preparing 10 000 copies dilutions.

In our laboratory we are using, as a standard dose, 20 000 copies per 40 μl of homogenate (20% of the sample/insect).

Materials:

- Ec5502 16S spike-in – 10 μl aliquote from previous step
- sterile water
- DNA low bind eppendorf tubes

Preparation:

- (1) add 210 μl of sterile water into aliquot tube (concentration after dilution 10 mln/ μl)
- (2) to the fresh tube add 180 μl of sterile water and 20 μl of dilution from step 1 (concentration after dilution 1 mln/ μl)
- (3) to the fresh tube add 1980 μl of sterile water and 20 μl of dilution from step 2.

The final concentration after 3 dilutions is 10 000 copies/ μl .

!!! Start preparing plasmids only after you have completed point 1 of DNA Extraction protocol. It is important to minimize plasmid concentration loss due to the adhesion of plasmid molecules to the tube walls.

5 Homogenate purification

1h

Materials:

- SPRI magnetic beads (stored in the fridge) – take out from the fridge and keep in room temperature
- 1 hour before use
- deepwell storage plate
- 80% ethanol (freshly made)
- TE x 1

- magnetic stand
- clean PCR plates with caps

DNA extraction:

1. From the tubes after digestion transfer 40 μ l of homogenate into proper well on deep well plate.

If the tubes after digestion were stored in a fridge or freezer make sure to warm them up on thermoblock before (the homogenate in the fridge becomes thick and difficult to pipet).

2. If you are using spike-in add 2 μ l of freshly prepared 10 000 copies/ μ l dilution into each well.

3. Add 80 μ L (x 2.0) of SPRI magnetic beads, and mix by pipetting (or vortex).

4. Incubate samples at room temperature for 5 minutes.

5. Place the plate on the magnetic stand for 2 min.

6. Remove the supernatant. Make sure to not disturb the magnetic beads.

7. Pour alcohol into the plastic trough (reservoir) and add 200 μ L of 80 % EtOH to each well using multichannel pipett.

8. Make sure that the deep well plate still stays on the magnetic stand. Incubate for 30 seconds.

9. Remove the supernatant.

10. Repeat steps 7 – 9 for two ethanol washings.

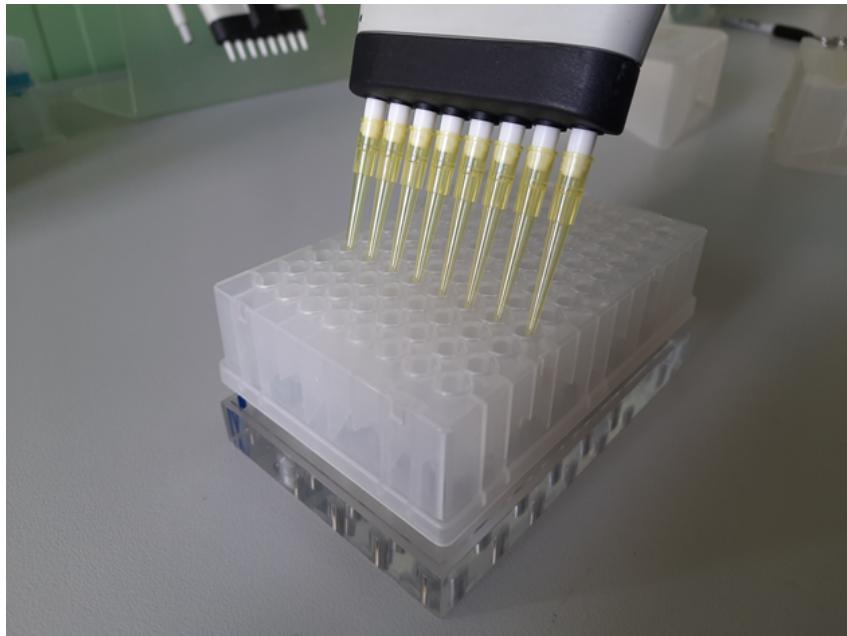
11. Wait 5 min until dry (on magnetic stand).

12. Remove the plate from the magnetic stand, add 20 μ L of TE x 1 into each well and mix by pipetting (or vortex).

13. Incubate the samples at room temperature for 5 minutes to elute the DNA.

14. Place on magnet stand for 2 min.

15. Transfer supernatant (20 μ L), containing DNA, to new 96-well PCR plate. Sign the plate and store in fridge (for few days) or freezer.



Deep well plate on magnetic stand.

6 Measuring DNA concentration

45m

Materials:

- Quant-iT™ PicoGreen™ dsDNA Assay Kit (stored in the fridge) - allow the Quant-iT PicoGreen reagent to warm to room temperature before opening the vial. It might take a while, so before using check if the vial content is liquid(!!!).
- Black, flat bottom 96-well plate

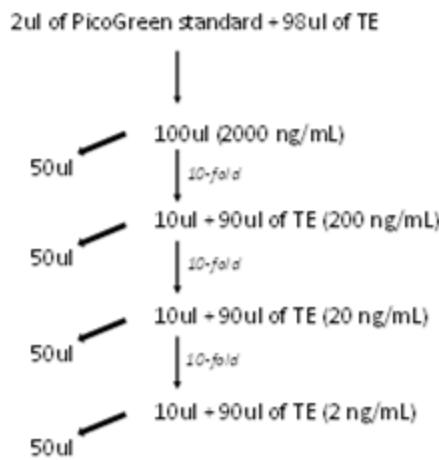
Equipment:

- Synergy HTX reader

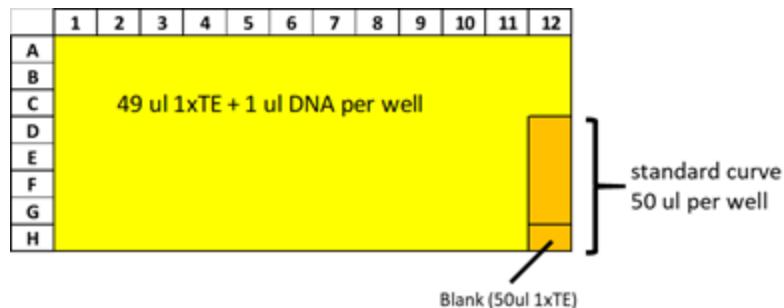
Protocol for whole 96-well plate:

1. Prepare 10 ml of 1xTE working solution (used for diluting the Quant-iT PicoGreen reagent, DNA samples and PicoGreen standard) by mixing 9.5 ml of sterile, distilled water with 0.5 ml of 20xTE concentrate buffer.
2. Mix 4975 ul of 1xTE, that you prepared in point one, with 25 ul of concentrated DMSO to obtain 5 ml of Quant-iT PicoGreen reagent working solution. Cover tube with reagent with aluminum foil, as PicoGreen reagent is susceptible to photodegradation.

3. Prepare PicoGreen five-point standard curve. Add 2ul of PicoGreen standard (100 ug/mL) to 98ul of 1xTE. Take 10ul of this solution and add 90ul of 1xTE to obtain 10-fold dilution. Repeat this step twice to obtain 100 and 1000-fold dilutions. Arrange diluted standard on the plate adding 50ul of each dilution, starting from the highest concentration and ending with blank (50ul of 1xTE).



4. Prepare the 96-well plate as in the picture:



5. Add 50ul of Quant-iT PicoGreen reagent working solution to each well (standard curve and DNA samples). Use plastic reagent tray and multi-channel pipette to speed up the process. Cover plate with aluminum foil, mix well and incubate 2-5 minutes.

6. Samples are ready to measure DNA concentration on Synergy HTX reader [15 min].

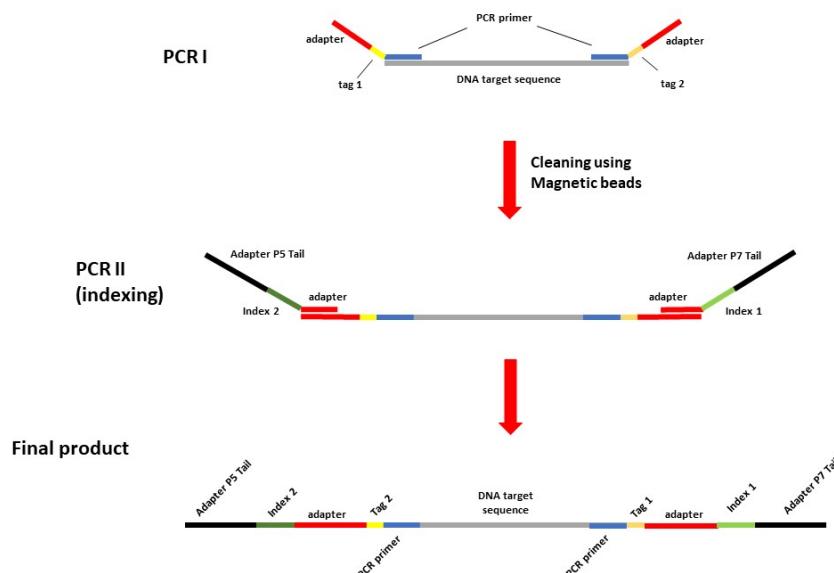
B. Amplicon library preparation

5h 55m

7 Overview

For amplicon library preparation we use a **two-step PCR** approach. During the first step (PCR1) we amplify three target genes: COI, 16S V1-2 region and 16S V4 region, in multiplex reaction.

Next we clean PCR1 products with magnetic beads. Finally, during the second step (PCR2) we add the indexes and illumina adapters to the amplified sequences.



Two-step PCR overview

8 PCR I primers preparation

For each target gene we have 8 forward and 12 reverse primers with unique tags. What gives us in total 96 unique pairs combinations. Informative variable-length inserts (tags) are used to mitigate index switching that occurs during sequencing on Illumina platform.

Primers scheme example:

Primer_name	Sequence	comment
COIBF3_P5	ACACTCTTCCCTACACCGACGCTCTTCCGATCT-C CCHGAYATRGCHTTYCCHCG	
COIBF3_P5_ins1	ACACTCTTCCCTACACGGACGCTCTTCCGATCT- ACCHGAYATRGCHTTYCCHCG	
COIBF3_P5b_ins2	ACACTCTTCCCTACACGGACGCTCTTCCGATCT- GA CCHGAYATRGCHTTYCCHCG	
COIBF3_P5b_ins3	ACACTCTTCCCTACACGGACGCTCTTCCGATCT- TGA CCHGAYATRGCHTTYCCHCG	purple - temple-specific primer; black - partial Illumina adapter sequence; Bold red - variable-length insert

Using stock primers [100uM] we are preparing primer mixes (separately for forward and reverse). Each mix contains three primers in concentration: COI [2.5uM], 16S V4 [10uM] and

16S V1-2 [5uM].

2.5uM	10uM	5uM	
COIBF3 ---- P5	515F ---- P5	27F ---- P5	Mix
COIBF3_P5	515F_P5	27F_P5	MA_P5
COIBF3_P5_ins1	515F_P5_ins1	27F_P5_var1	MB_P5
COIBF3_P5b_ins2	515F_P5_ins2	27F_P5_var2	MC_P5
COIBF3_P5b_ins3	515F_P5_ins3	27F_P5_var3	MD_P5
COIBF3_P5_var1	515F_P5_var1	27F_P5_var4	ME_P5
COIBF3_P5_var2	515F_P5_var2	27F_P5_var5	MF_P5
COIBF3_P5_var3	515F_P5_var3	27F_P5_var6	MG_P5
COIBF3_P5_var4	515F_P5_var4	27F_P5_var7	MH_P5
15 ul +	60 ul +	30 ul +	495 ul TEx1

Forward primer mixes

2.5uM	10uM	5uM	
COIBF3 ---- P7	806R ---- P7	338R ---- P7	Mix
COIBR2_P7	806R_P7	338R_P7	M1_P7
COIBR2_P7_sub1	806R_P7_ins1	338R_P7_var1	M2_P7
COIBR2_P7_ins1	806R_P7_ins2	338R_P7_var2	M3_P7
COIBR2_P7_ins2	806R_P7_ins3	338R_P7_var3	M4_P7
COIBR2_P7_var1	806R_P7_var1	338R_P7_var4	M5_P7
COIBR2_P7_var2	806R_P7_var2	338R_P7_var5	M6_P7
COIBR2_P7_var3	806R_P7_var3	338R_P7_var6	M7_P7
COIBR2_P7_var4	806R_P7_var4	338R_P7_var7	M8_P7
COIBR2_P7_var5	806R_P7_var5	338R_P7_var8	M9_P7
COIBR2_P7_var6	806R_P7_var6	338R_P7_var9	M10_P7
COIBR2_P7_var7	806R_P7_var7	338R_P7_var10	M11_P7
COIBR2_P7_var8	806R_P7_var8	338R_P7_var11	M12_P7
10 ul +	40 ul +	20 ul +	330 ul TEx1

Reverse primer mixes

Finally primer mixes aliquots should be distributed into strip tubes: MA_P5 - MH_P5, M1_P7 - M8_P7 and M9_P7 - M12_P7. This will allow you to use multi-channel pipette during adding primer mixes to the PCR reactions.

9 PCR I

2h 40m

Set up PCR 1 by creating a master mix as described below. We carry out PCR 1 in a total volume of 10 µL. To prepare the master mix for a 96-well plate, multiply the volumes for one reaction by 100. Plate will contain: 91 samples, two negative controls (with and without PCR spike-in), two positive controls and one empty well for PCR 2 negative control.

Component	reactions number [volume]	
	x1 [ul]	x100 [ul]
2x Qiagen Multiplex	5	500
primer mix MA_P5 - MH_P5	1	unique primer
primer mix M1_P5 - M12_P5	1	unique primer
dd H2O	1	100
DNA	2	
total	10	600

1. In sterile tube prepare PCR reaction mix by mixing 2x QIAGEN Multiplex PCR Master Mix and sterile water, according to the table above.
2. If you are using PCR spike-in add 6 ul of PCR mix prepared in point one to well D12 of your PCR plate, it will be first negative control (without PCR spike-in). Otherwise skip point 2 and go directly to point 4.
3. Prepare plasmid spike-in in similar manner as for DNA extraction. You need to use different plasmid than before (e.g. Ec5001) and dilute it to lower concentration, 1000 copies per sample. Add 4 ul of diluted spike-in, containing 99 000 copies of plasmid to PCR mix and vortex it.
4. Distribute 6 ul of PCR mix to each well on the plate, except D12 (if you use plasmids) and H12.
5. Using multi-channel pipette add correct primers mixes to the correct wells, according to the scheme below. Each well should contain unique pairs of primers.



	reverse primer	M1_P7	M2_P7	M3_P7	M4_P7	M5_P7	M6_P7	M7_P7	M8_P7	M9_P7	M10_P7	M11_P7	M12_P7
forward primer		1	2	3	4	5	6	7	8	9	10	11	12
MA_P5	A	MA_P5-M1_P7	MA_P5-M2_P7	MA_P5-M3_P7	MA_P5-M4_P7	MA_P5-M5_P7	MA_P5-M6_P7	MA_P5-M7_P7	MA_P5-M8_P7	MA_P5-M9_P7	MA_P5-M10_P7	MA_P5-M11_P7	MA_P5-M12_P7
MB_P5	B	MB_P5-M1_P7	MB_P5-M2_P7	MB_P5-M3_P7	MB_P5-M4_P7	MB_P5-M5_P7	MB_P5-M6_P7	MB_P5-M7_P7	MB_P5-M8_P7	MB_P5-M9_P7	MB_P5-M10_P7	MB_P5-M11_P7	MB_P5-M12_P7
MC_P5	C	MC_P5-M1_P7	MC_P5-M2_P7	MC_P5-M3_P7	MC_P5-M4_P7	MC_P5-M5_P7	MC_P5-M6_P7	MC_P5-M7_P7	MC_P5-M8_P7	MC_P5-M9_P7	MC_P5-M10_P7	MC_P5-M11_P7	MC_P5-M12_P7
MD_P5	D	MD_P5-M1_P7	MD_P5-M2_P7	MD_P5-M3_P7	MD_P5-M4_P7	MD_P5-M5_P7	MD_P5-M6_P7	MD_P5-M7_P7	MD_P5-M8_P7	MD_P5-M9_P7	MD_P5-M10_P7	MD_P5-M11_P7	MD_P5-M12_P7
ME_P5	E	ME_P5-M1_P7	ME_P5-M2_P7	ME_P5-M3_P7	ME_P5-M4_P7	ME_P5-M5_P7	ME_P5-M6_P7	ME_P5-M7_P7	ME_P5-M8_P7	ME_P5-M9_P7	ME_P5-M10_P7	ME_P5-M11_P7	ME_P5-M12_P7
MF_P5	F	MF_P5-M1_P7	MF_P5-M2_P7	MF_P5-M3_P7	MF_P5-M4_P7	MF_P5-M5_P7	MF_P5-M6_P7	MF_P5-M7_P7	MF_P5-M8_P7	MF_P5-M9_P7	MF_P5-M10_P7	MF_P5-M11_P7	MF_P5-M12_P7
MG_P5	G	MG_P5-M1_P7	MG_P5-M2_P7	MG_P5-M3_P7	MG_P5-M4_P7	MG_P5-M5_P7	MG_P5-M6_P7	MG_P5-M7_P7	MG_P5-M8_P7	MG_P5-M9_P7	MG_P5-M10_P7	MG_P5-M11_P7	MG_P5-M12_P7
MH_P5	H	MH_P5-M1_P7	MH_P5-M2_P7	MH_P5-M3_P7	MH_P5-M4_P7	MH_P5-M5_P7	MH_P5-M6_P7	MH_P5-M7_P7	MH_P5-M8_P7	MH_P5-M9_P7	MH_P5-M10_P7	MH_P5-M11_P7	empty

6. Add 2ul of DNA to the wells A1 - C12. Add 2ul of sterile water to wells D12 and E12. Add to the wells F12 and G12 2ul of DNA samples, that contains all target genes, so it will work as positive controls.

7. Put the lids on the plate, centrifuge the plate and place in the thermocycler.

8. Run PCR 1 using the following conditions [~2h]:

Step	Temp. [°C]	Time [s]	Cycles
Initial denaturation	95	900	1
Denaturation	94	30	25 or 27*
Annealing	50	90	25 or 27*
Extension	72	90	25 or 27*
Final extension	72	600	1
Store	8	∞	

* Number of cycles depends on DNA concentration

10 Electrophoresis I

1h

Run PCR 1 products on a 2,5% agarose gel (2 µL PCR product + 2 µL loading dye). It is enough to run just a subset of samples on the gel to see if the PCR reaction went properly. However remember to add all negative and positive controls on the gel.

11 PCR product cleaning

45m

Clean up PCR 1 products by following the steps below. Make sure you prepare SPRI Beads as explained in the "Materials" section before starting this step.

1. Prepare fresh aliquots of 80% EtOH.
2. Transfer all of PCR1 product to a 96 deep-well plate (8 - 10 µL per sample) and add 20 µL of SPRI Beads, and mix by pipetting (or vortex the entire plate using a plate shaker).
3. Incubate mixture for 5 min at room temperature.
4. Place the plate on a magnetic stand for 2 min.
5. Remove supernatant (use multichannel pipette).
6. Pour alcohol into a 100 mL reagent reservoir and, using a multichannel pipette, transfer 100 µL of 80% EtOH to each well.
7. Incubate on a magnetic stand for 1 min.
8. Remove supernatant.
9. Repeat steps 6 – 8 above.
10. Wait up to 4 min until the magnetic bead pellet gets dry (on magnetic stand).
11. Remove samples from the magnetic stand, add 20 µL of TE buffer and mix by pipetting up and down (or vortex the entire plate using a plate shaker).
12. Incubate mixture for 5 min at room temperature.
13. Place back the plate on the magnetic stand for 2 min.
14. Transfer supernatant (20 µL) to a new 96-well plate.
15. Mark the plate with a unique identifier and store at 4°C (for a few days) or at -20°C (for a longer period).

12 PCR 2 (indexing)

1h 30m

Set up PCR 2 by creating a master mix as described below. We carry out PCR 2 in a total volume of 10 µL. To prepare the master mix for a 96-well plate, multiply the volumes for one reaction by 100. Plate will contain all samples from PCR 1 plus PCR 2 negative control in well H12.

	reactions number [volume]	
Component	x1 [ul]	x100 [ul]
2x Qiagen Multiplex	5	500
10 uM Index i5	1	unique index
10 uM Index i7	1	unique index
dd H2O	2	200
Clean PCR 1 product	1	
total	10	700

1. In sterile tube prepare PCR reaction mix by mixing 2x QIAGEN Multiplex PCR Master Mix and sterile water, according to the table above.
2. Distribute 7 ul of PCR mix to each well on the plate.
3. Using multi-channel pipette add correct indexing primers to the correct wells, that each well will contain unique pair of primers.
4. Add 1ul of clean PCR 1 product to each well. Add 2ul of sterile water to well H12 (negative control of indexing).
7. Put the lids on the plate, centrifuge the plate and place in the thermocycler.
8. Run PCR 1 using the following conditions [~1h]:

Step	Temp. [°C]	Time [s]	Cycles
Initial denaturation	95	900	1
Denaturation	94	30	7-9*
Annealing	50	90	7-9*
Extension	72	90	7-9*
Final extension	72	600	1
Store	8	∞	

* Number of cycles depends on PCR 1 gel bands intensity

13 Electrophoresis II

1h

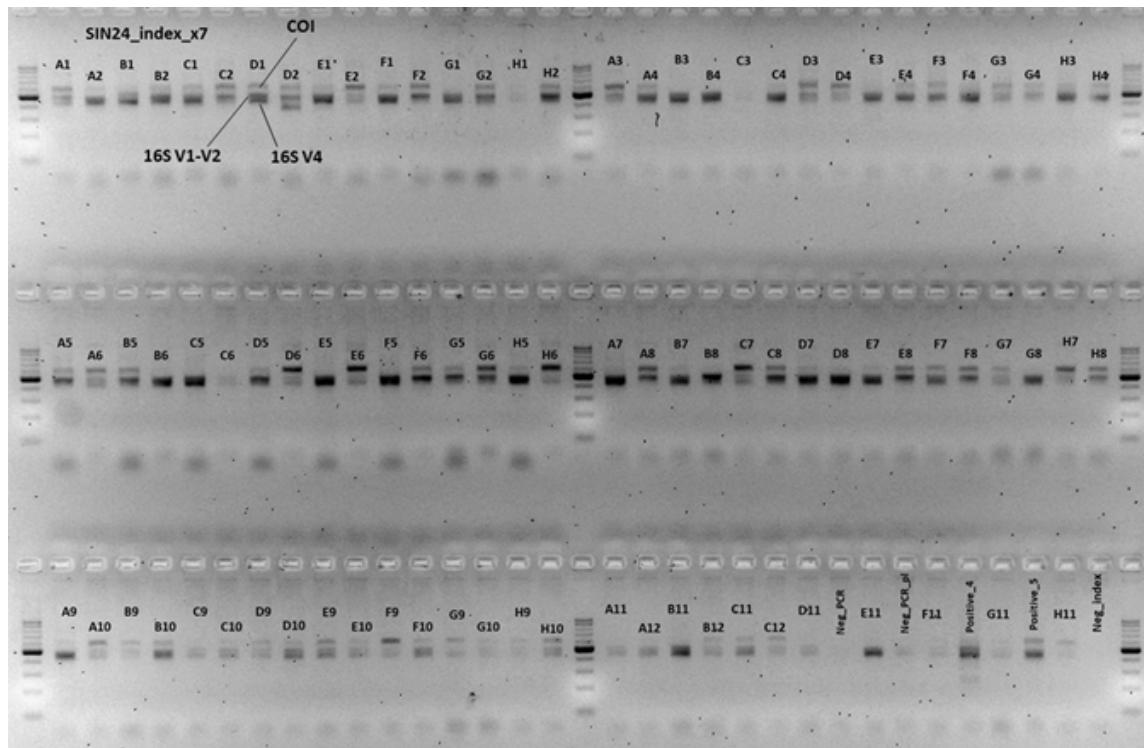
Run PCR 2 products on a 2,5% agarose gel (2 µL PCR product + 2 µL loading dye).

C. Amplicon library pooling, QC, and sequencing

14 Pooling

Create a pool of samples (libraries) from each 96-well plate by mixing PCR 2 products in an 1.5 mL microtube, based on gel band intensity:

- very strong band - 0.5 µL
- strong band - 1 µL
- medium band - 2 µL
- weak band - 4 µL
- no visible band - 8 µL



Example of libraries pooling - gel

SIN 24	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

0.5 ul	0.0 ul	
2 ul		empty
2ul		
4ul		
8ul		

Example of libraries pooling - plate scheme of volumes to mix

15 Pools cleaning

Clean the "plate pools" created above with Promega ProNex® Size-Selective Purification System. Follow the manufacturer's instructions, using 1/1.5 (v/v) pool to magnetic beads ratio. As standard we use 80 µL of pool for cleaning.

16 **Measure DNA concentrations of the "plate pools" on Qubit Fluorometer using dedicated kit.**
Please follow the manufacturer's instructions.

You can use Qubit™ 1X dsDNA High Sensitivity (HS) or Broad Range (BR) Assay Kits.

17 **Master Pool**

Create Master Pool by mixing together the "plate pools" obtained in step 12, based on sample number (not all plates had to be full) and the DNA concentration. If pools that you are mixing together differs in target genes composition (e.g one plate has only COI barcode and other plates all three target genes) then you have to take it into account.

18 **Master Pool cleaning**

Clean Master Pool by repeating point 15, but use 1/1.3 (v/v) pool to magnetic beads ratio. Suspend the final product in TE x 0.1 (or ddH₂O). Volume in which to suspend depends on desired DNA concentration.

19 **Measure DNA concentrations of the Master Pool on Qubit Fluorometer using dedicated kit.**
Please follow the manufacturer's instructions.

You can use Qubit™ 1X dsDNA High Sensitivity (HS) or Broad Range (BR) Assay Kit.

20 **Quality check**

Check quality of Master Pool (e.g. lack of primer-dimmers) by running them on Bioanalyzer or Tapestation before submitting for sequencing. In an unlikely event of detecting primer-dimers, repeat cleaning step as in point 16.

21 **Sequencing options and considerations**

The library pool is compatible with all Illumina platforms that produce at least 2x250 bp reads. The currently available platforms - Illumina MiSeq, NextSeq 2000, and NovaSeq 6000 - differ dramatically in output, cost of a sequencing flow cell, and cost per million read pairs, as listed in **Table X.1** below:

Sequencer	Flow cell	Read pairs/flow cell	Cost of flow cell	Cost of 1M read pairs
MiSeq	nano v2 2x250bp	1M	500 EUR	500 EUR
MiSeq	v2 2x250bp	15M (12M)*	1800 EUR	150 EUR

Sequencer	Flow cell	Read pairs/flow cell	Cost of flow cell	Cost of 1M read pairs
MiSeq	v3 2x300bp	25M (20M)*	2300 EUR	115 EUR
NextSeq 200	P1 2x300bp	100M	3000 EUR	33 EUR
NextSeq 200	P2 2x300bp	300M	5500 EUR	18 EUR
NovaSeq 6000	SPrime 2x250 bp	800M	7000 EUR	8.75 EUR

Table X1. The Illumina sequencing platforms providing at least 2x250bp reads, as of July 2023. The prices quoted are approximate, and based on recent quotes by different European providers - but can vary substantially among facilities. For NextSeq and NovaSeq platforms, the expected numbers of read pairs per flow cell match manufacturer-provided upper limit, and often exceed it; for MiSeq, in our experience, read numbers from amplicon-sequencing lanes are often some 20% below that limit.

Amplicon libraries prepared according to the current protocol can generally be combined with other library types, for example, metagenomic libraries prepared using Illumina TruSeq or NEBNext Ultra DNA kits, **as long as there is no direct overlap in index sequences:** we have never encountered issues.

On the other hand, because relatively few researchers use longer-read flow cells on NextSeq and NovaSeq platforms, sequencing facilities may be unwilling to combine your samples with those submitted by other researchers for sequencing in a single lane or flow cell (as is done more often for 2x150bp sequencing submission), and **you will generally need to pay for the whole sequencing flow cell.**

When selecting the sequencing platform, **we would recommend aiming for not less than 50,000 read pairs per library on average.** To maximize the value per read and per library, you would ideally submit pools comprising many libraries, and use highest-throughput platforms. For example, the cost of sequencing a library to an average coverage of 100,000 read pairs on NovaSeq 6000 platform may be less than 1 EUR when submitting 8000 libraries at once - but the price would increase more than ten-fold when using MiSeq for the sequencing. At the same time, few projects so far aimed to sequence thousands of amplicon libraries --- and even if they did, preparing and pooling that many libraries will likely take a long time, translating to a long wait for the data. Also, the availability of sequencers and the turnaround time may be a consideration - and may be greater for lower-throughput platforms. Then, **despite NovaSeq's superior per-read value, for logistics reasons, NextSeq or MiSeq may be preferred options for your project.** Alternatively, you may consider pooling amplicon libraries from across many projects, and combining amplicons with genomic/metagenomic libraries.

When selecting sequencing platforms, it is also good to be aware of differences in data characteristics. For example, in sequencing data produced by the MiSeq platform, read quality tends to decrease substantially towards the end of the read, and is much lower and drops more quickly for the second read in the pair. The decrease in quality along the read length tends to be

much less in data generated using platforms that rely on the newer patterned flow cell - NextSeq and NovaSeq (**Fig. X1**). On the other hand, platforms using patterned flow cells may be affected more by cross-contamination among samples that happens during sequencing: <https://www.illumina.com/techniques/sequencing/ngs-library-prep/multiplexing/index-hopping.html>

When sequencing libraries using flow cells with more than one lane (specifically, NovaSeq SPrime flow cell has two lanes), you may want to submit a single library Master Pool for sequencing on both lanes --- or alternatively, submitting different Master Pools, even with overlapping index combinations, for sequencing on different lanes. The latter approach will allow you to pack your libraries less densely in index space, likely reducing the extent of cross-contamination among samples.

Protocol references

Tourlousse, D. M., Yoshiike, S., Ohashi, A., Matsukura, S., Noda, N., & Sekiguchi, Y. (2016). Synthetic spike-in standards for high-throughput 16S rRNA gene amplicon sequencing. *Nucleic Acids Research*, gkw984. doi:10.1093/nar/gkw984

Iwaszkiewicz-Eggebrecht, E., Prus-Frankowska, M., Łukasik, P. (2023) Synthetic COI spike-ins for use in metabarcoding-based insect biodiversity surveys V.2 dx.doi.org/10.17504/protocols.io.14egn33ryl5d/v2

SPRI Beads protocol is derived from: Rohland N, Reich D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Research* 22: 939-946.