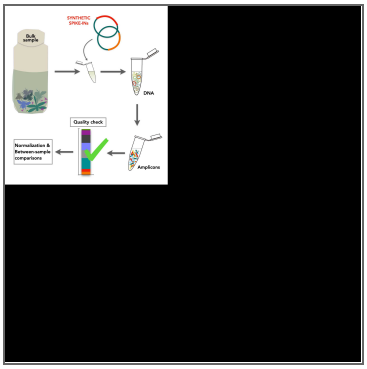

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Protocol status: Working
We used this protocol with ~900 homogenised Malaise trap samples from the Insect Biome Atlas project (insectbiomeatlas.org) and it's working.

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Synthetic COI spike-ins for use in metabarcoding-based insect biodiversity surveys

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ABSTRACT

We have designed two synthetic spike-ins, called *Callio-synth* and *tp53-synth*, for use in metabarcoding arthropod surveys. As the starting point for designing these spike-ins, we used cytochrome *c* oxidase I (COI) barcode region of a blue bottle fly (*Calliphora vomitoria*). We preserved the primer binding sites that match primers BF3-BR2 and fwhF2-fwhR2n as well as a flanking region around each primer site (+/- 6bp). The remainder of the sequence was replaced by i) a random DNA sequence that does not resemble any known sequence deposited in GeneBank, while keeping a GC content similar to the original *C. vomitoria* sequence (*Callio-synth* spike-in); ii) short fragments of the tp53 gene (*tp53-synth*). What follows in this protocol are instructions on how produce and use those spike-ins in arthropod metabarcoding studies. In short, both spike-in sequences (471-bp in total) need to be synthesised and inserted into standard commercially available plasmids. They are then to be transformed into monoclonal bacteria, copied in bacteria and extracted by standard extraction kits. Same numbers of spike-in copies should then be added to every arthropod bulk sample right before the DNA extraction. Subsequently they will be co-extracted, co-amplified and sequenced together with the DNA of the sample. After sequencing, spike-in read counts can be retrieved by matching the known spike-in sequence to the ASV or OTU lists.

MATERIALS

Antibiotics preparation

Ampicillin

	50 mg/ml	100 mg/ml
Ampicillin powder	**1g**	**2g**
ddH2O	**20 ml**	**20 ml**

1. To a sterile 50 ml centrifuge tube (falcon) weight appropriate amount of ampicillin.
2. Add 20 ml of sterile destiled water (e.g. Molecular Biology Grade Water from EURx).
3. Filter stelize solution using a syringes with a filter.
4. Dispense aliquots of 1ml into 1.5 ml eppendorfs. Label the eppendorfs, primarilly with the concentration of the stock.
5. Store in –20˚C freezer.

Plasmid Isolation – Resuspension buffer

Buffer parameters:
50 mM Tris-HCl, pH 8.0
10mM EDTA
20ug RNase A

	15 ml of buffer
1M Tris-HCl	0,75 ml
0,5M EDTA	0,3 ml
RNase A	0,3 ml
ddH20 (sterile)	13,65 ml

Chemicals needed:
1M Tris-HCl (solution)
0,5M EDTA (solution)
RNase A – 10mg/ml

Plasmid Isolation – Lysis Buffer

Buffer parameters:
200 mM NaOH
1% SDS

	15 ml of buffer
1N NaOH	3 ml
20% SDS	0,75 ml
ddH20 (sterile)	11,25 ml

Chemicals needed:
1N (1M) NaOH (solution)
20% SDS (solution)

SAFETY WARNINGS

As a key caveat, we note that cloned plasmids tend to form a long-term contamination of laboratory environments, and therefore recommend the outsourcing or use of separate laboratories for these procedures.

BEFORE START INSTRUCTIONS

We have designed two synthetic spike-ins, called *Callio-synth* and *tp53-synth*, for use in metabarcoding arthropod surveys.

As the starting point for designing these spike-ins, we used cytochrome *c* oxidase I (COI) barcode region of a blue bottle fly (*Calliphora vomitoria*). We preserved the primer binding sites that match primers BF3-BR2 (Elbrecht et al., 2019) and fwhF2-fwhR2n (Vamos et al., 2017) as well as a flanking region around each primer site (+/- 6bp). The remainder of the sequence was replaced by i) a random DNA sequence that does not resemble any known sequence deposited in GeneBank, while keeping a GC content similar to the original *C. vomitoria* sequence (*Callio-synth* spike-in); ii) short fragments of the tp53 gene (*tp53-synth*).

Alignment of the the two synthetic spike-in sequences to the Calliophora vomitoria reference. Green bar on the top shown base-pars that are identical between all sequences. Green boxes indicate primer binding sites.

What follows in this protocol are instructions on how produce and use those spike-ins in arthropod metabarcoding studies.
In short, both spike-in sequences (471-bp in total) need to be synthesised and inserted into standard commercially available plasmids. They are then to be transformed into monoclonal bacteria, copied in bacteria and extracted by standard extraction kits. Same numbers of spike-in copies should then be added to every arthropod bulk sample right before the DNA extraction. Subsequently they will be co-extracted, co-amplified and sequenced together with the DNA of the sample.
After sequencing, spike-in read counts can be retrieved by matching the known spike-in sequence to the ASV or OTU lists.

The proposed spike-ins are designed to work in experiments targeting two different primer pairs (or combination of those primers). Of these, BF3-BR2 produces a 418-bp long fragment of the Folmer region and is commonly used in Illumina-based studies, whereas fwhF2-fwhR2n allows for the amplification of a shorter fragment, only 205-bp long, which is particularly useful when dealing with degraded DNA.

Our proposed protocol was developed for homogenized Malaise trap samples (coming from the Insect Biome Atlas project; insectbiomeatlas.org) and can only be recommended for such types of samples.

Sequence synthesizing, plasmid production and transformati...

1 Download FASTA file with the spike-in sequences from GitHub: https://github.com/ela-iwaszkiewicz/Synthetic_COI_spike-ins

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

3 **Transformation into *E. coli***

3.1 Upon receiving plasmids from your commercial supplier, perform transformation into a monoclonal bacterial strain.
In our experiment, the plasmid was cloned into JO-FI competent cells derived from the E. coli DH5α strain (A&A Biotechnology) using CloneJET PCR Clonning kit (Thermo Fisher Scientific).

3.2 A single successfully transformed colony was transferred into LB medium (A&A Biotechnology) mixed with Ampicilin Sodium (A&A Biotechnology) at the final concentration 100 ug/ml and grown overnight at 37°C.

Bacterial culture

4 **PREPARE LB AGAR PLATES with amplicilin anibioticum**

Tryptone	**5 g**
Yeast Extract	**2,5 g**
NaCl	**5 g**
Agar	**7,5 g**
Water	**Up to 500 ml**
Ampicillin	**0,5ml [100mg/ml] or 1ml [50mg/ml]**

You can also use ready **LB-Agar mix from A&A Biotechnology** (cat. no 2021-250). The proportion of the ingredients is the same. Use **4g of the LB-Agar mix per 100 ml of destilled/sterile water.**

4.1 Mix all of the ingredients (except ampicillin), add water and autoclave it.

4.2 After that cool the molten agar to the temperature of 55˚C.

4.3 Add the proper amount of the antibiotic stock [to obtain final concentration in media 100 ug/ml] and mix it gently.

4.4 Place the bootle under the hood and pour the agar into the sterile, freshly open Petri dishes (plates).

4.5 Leave the plates on the bench/under the laminar to solidify – about 24 h/or overnight.

4.6 Use or store the plates in 4˚C not longer than for a week.

5 **Select bacteria by culturing them on agar with amplicillin**

[All sub-steps in this part of the protocol are copied from : https://www.addgene.org/protocols/inoculate-bacterial-culture/]

Use LB agar plates with amplicillin prepared according to the protocol in the previous step (#3).

5.1 Label the bottom of the plate with the plasmid name, the date and the name of antibiotic (in our case AMP).

5.2 Sterilize and turn on the hood.

5.3 Place fresh plates and you bacteria stock/bacteria plate under the hood.

5.4 Using a sterile pipette tip or toothpick touch the bacteria growing within the punctured area of the stab culture or the top of the glycerol stock.

5.5 Gently spread the bacteria over a section of the plate, as shown in the diagram below, to create streak #1.

Source: https://www.addgene.org/protocols/streak-plate/

5.6 Using a fresh, sterile toothpick, or freshly sterilized loop, drag through streak #1 and spread the bacteria over a second section of the plate, to create streak #2.

5.7 Using a third sterile pipette tip, toothpick, or sterilized loop, drag through streak #2 and spread the bacteria over the last section of the plate, to create streak #3.

5.8 Incubate plate with newly plated bacteria overnight (12-18 hours) at 37 °C.

5.9 It is good to incubate also a blank sample (fresh plate without any bacteria on the surface).

5.10 Prepare liguid LB with antibiotic.

5.11 Prepare fresh, sterile 15 or 50 ml centriguge tubes.

5.12 Sterilize and turn on the hood.

5.13 Transfer ca. 10ml of LB+antibiotic to 15 ml tubes (and ca. 20 ml to 50 ml tubes). Do not forget to label each tube with the date, name of the strain/plasmid.

5.14 Using a sterile pipette tip or toothpick, select a single colony from LB agar plate. You can leave the toothpick or pipette tip inside the LB.

5.15 Loosely cover the culture with sterile aluminum foil or a cap that is not air tight.

5.16 Incubate bacterial culture at 37°C for 12-18 hr in a shaking incubator.

5.17 It is good to incubate also a blank sample (fresh LB without any bacteria in it).

5.18 After incubation you can prepare glycerol long-term stock (section 5) and/or proceed to isolating plasmid DNA (section 6).

Long-term storage of bacteria carrying plasmids

6 **GLYCEROL STOCK**

For long-term storage of plamids it is adviced to create a baterial glycerol stock.
Such stock is stable for years, as long as it kept in –80*C.

To create glycerol stock you need:

- Overnight bacteria culture (in LB medium)
- 50 % glycerol (e.g. 25 g of 100% glycerol + 25ml of dH_2O)

[Source: https://www.addgene.org/protocols/create-glycerol-stock/]

6.1 In a 2ml screw cap tube for freezing put 500ul of overnight colony and 500ul of 50% glycerol. Mix it gently.

6.2 Freeze the tube in –80*C.

6.3 To recover the stock scrap a bit of the frozen bacteria from the top into a fresh agar plate (with Ampicilin).

6.4 Otherwise take a sample of LB medium with bacterial culture for further plasmid isolation.

Plasmid isolation from *E. coli*

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

Plasmid linealization

8 **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.
We chose an AatII enzyme (NEB) as it was cutting the plasmid effectively and leaving the barcode region intact.

(You can always check the parameters of the protocol on the NEB cloner website http://nebcloner.neb.com/#!/)

Materials:

- Restriction enzyme AatII – **we are using New England Biolabs enzymes, for the enzymes from a different manufacture the digestion protocl might be different**
- CutSmart Buffer (provided with enzyme)
- Molecular water (nuclease free)

PREPARE ON ICE!

8.1 Measure the concentration of isolated plasmid DNA on Qubit.

8.2 Remove restriction enzyme and CutSmart buffer from the frezer and put it on ice.

8.3 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.

8.4 Put the strip tube/plate in a termocycler and set up the program. The incubation and inactivation temepratures and time can be found in table below.

	A	B	C
	Restriction enzyme	**Incubation temp. and time**	**Inactivation temp. and time**

	A	B	C
	AatII (NEB)	37˚C, 5-15 mins	80˚C, 20 mins

8.5 After digestion freeze the samples or prepare 300 mln copies single-use aliquots according to the instruction below.

Quantification and dilutions

9 **Preparing 300 mln copies single-use aliquots**

Materials and equipment:

- Qubit fluorometer and Qubit HS assay kit
- TE x1
- Digested plasmid

9.1 Measure digested plasmid DNA concentration using Qubit flurometer.

9.2 Based on the received information calculate how many copies of a plasmid you have in one ul. You can do this based on the assumption that 1 ng of COI plasmid DNA (as we've designed) contains 300 mln copies of the plasmid.
(This assumption is based on a calculation taking into account the size of the DNA molecule)

9.3 Based on performed calculations dilute spike-in's samples with 1xTE into the concentration of 300 mln of copies/ul and prepare **single-use aliquots** of 8ul each. Store in a frezer in 20˚C.

9.4 **Remember that for your experiment you will need to prepare additional dilutions of a spike-ins. You can do it with molecular water (nuclease free).**

Adding spike-ins to samples

10 We added xx ul of the plasmid DNA stock (~1M copies) of each spike-in to the sample right before DNA extraction.