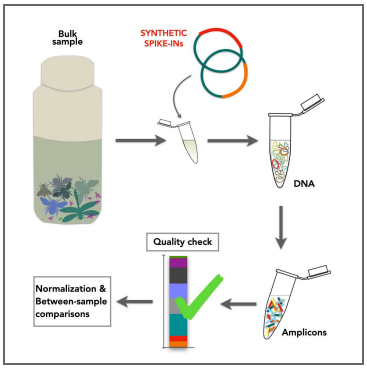

Synthetic COI spike-ins for use in metabarcoding-based insect biodiversity surveys V.2

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VERSION 2

NOV 16, 2023

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.14egn33ryl5d/v2

Protocol Citation: Ela Iwaszkiewicz-Eggebrecht, Monika Prus-Frankowska, Piotr Łukasik 2023. Synthetic COI spike-ins for use in metabarcoding-based insect biodiversity surveys . **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn33ryl5d/v2Version created by Ela Iwaszkiewicz-Eggebrecht

IBA

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ABSTRACT

In the context of insect monitoring, metabarcoding has emerged as a pivotal method, revolutionizing the precision and scope of surveillance efforts. This technique enables rapid and accurate identification of insect species from environmental samples, providing crucial insights into biodiversity, population dynamics, and ecological interactions. While metabarcoding stands out as a powerful tool, numerous challenges persist within the field. The considerable variation in methods, procedures, and protocols across studies introduces complexity and hinders seamless comparisons between datasets. The lack of standardization poses a significant obstacle, emphasizing the need for concerted efforts to establish uniform practices. To address this gap, a viable strategy involves the design and implementation of universal internal standards, such as synthetic spike-ins. Spike-ins serve as sample-specific internal positive controls as well as facilitate standardisation of the results between samples and experiments. Here, we propose two synthetic COI spike-ins designed to be used in metabarcodin-based insect biodiversity surveys. The protocol provides spike-in sequences and a detailed step-by-step description of the procedures involved in production, usage and long-term storage of those spike-ins.

MATERIALS

Antibiotics preparation

Ampicillin

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

Created: Nov 09, 2023

Last Modified: Nov 16, 2023

PROTOCOL integer ID: 90703

Keywords: Spike-in, COI metabarcoding, synthetic DNA, normalisation, calibration

Funders Acknowledgement:
Knut and Alice Wallenberg Foundation
Grant ID: KAW 2017.088
Polish National Agency for Academic Exchange
Grant ID: PPN/PPO/2018/1/00015
Polish National Science Centre
Grant ID: 2018/31/B/NZ8/01158

	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.

The proposed spike-ins are designed to work in experiments targeting only two different primer pairs (or combination of those primers): BF3-BR2 and fwhF2-fwhR2n.

The proposed dilution and numbers of spike-in copies added to samples were optimised for use with homogenized Malaise trap samples (originating from the Insect Biome Atlas project; insectbiomeatlas.org) and can only be recommended for such types of samples.

BEFORE START INSTRUCTIONS

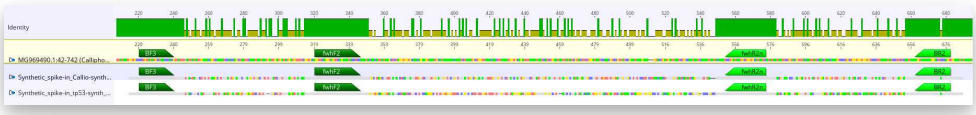
This protocol describes the use of two synthetic spike-ins, called *Calli-synth* and *tp53-synth*, designed for application in metabarcoding-based arthropod surveys.

Spike-ins provide sample-specific internal positive control. They also facilitate between-samples normalisation procedures. By adding the same number of spike-in copies to each sample, sequencing results can be standardized, revealing meaningful differences between samples.

Short description of the spike-in design:
The barcode region of the cytochrome *c* oxidase I (COI) of a blue bottle fly (*Calliphora vomitoria*) was selected as the foundational template. We preserved the sequence of the primer binding sites, corresponding to primers BF3-BR2 (Elbrecht et al., 2019) and fwhF2-fwhR2n (Vamos et al., 2017), along with the inclusion of a flanking region around each primer site (+/- 6bp). The remaining sequence - in between the preserved sites - was modified in two distinct ways:

i) Introduction of a random DNA sequence devoid of any resemblance to known sequences deposited in GenBank. This novel sequence was carefully crafted to maintain a GC content similar to the original *C. vomitoria* sequence. This formed the *Calli-synth* spike-in sequence.

ii) Integration of short, randomly chosen, fragments from the tp53 gene, shaping the *tp53-synth* spike-in sequence.



Alignment of the the two synthetic spike-in sequences to the *Calliophora vomitoria* reference. Green bar on the top of the alignment shows base-pairs that are identical in all sequences (preserved sites). Green boxes above each of the three sequences indicate primer binding sites.

Contents of the protocol:
In this protocol we outline detailed instructions for the synthesis and application of these spike-ins in arthropod metabarcoding studies.

In brief, the two spike-in sequences, totaling 471 base pairs each, are first synthesized and inserted into standard commercially available plasmids (commercial service). Subsequently, the plasmids are transformation into monoclonal bacteria, followed by amplification in bacterial culture. The amplified plasmids are then selectively grown on an antibiotic medium and extracted using standard plasmid extraction kits.
Post-extraction, the plasmids can be linearized and quantified. Subsequently, appropriate spike-in dilutions are prepared based on the DNA concentration. The primary objective is to add an equal number of spike-in copies to each arthropod bulk sample immediately before DNA extraction.
These spike-ins are then co-extracted, co-amplified, and co-sequenced alongside the sample's DNA, ensuring a unified and consistent approach throughout the process.

Sequence synthesizing, plasmid production and transformati...

1 Download FASTA file with 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 µg/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 µg/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. 10 ml 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 500 µl of overnight colony and 500 µl 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 protocol 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 thermal cycler 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**
	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 µl.
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/µl and prepare **single-use aliquots** of 8 µl each. Store in a frezer in 20˚C.

Adding spike-ins to samples

10 **We aim to add 5 mln of EACH plasmid copies to each sample right before DNA extraction. In order to achieve this we prepare fresh dilutions of the plasmid stock prepared in the previous section.**

NOTE: Please bear in mind that 5 mln of copies of each plasmid is what we found and appropriate amount for homogenate bulk insect samples (originating from the Insect Biome Atlas project - insectbiomeatlas.org), where we expected relatively high DNA yields.
*****You must consider adjusting the numbers of copies of spike-ins for your project needs.*****

10.1 Thaw one single-use aliquot of the spike-in (prepared in step 9.3). Measure DNA concentration with qubit to ensure accurate calculations later on (we expect ~1 ng/µl, however we know that small DNA particles stick to walls of plastic and are being lost in crevices).

10.2 Based on the measurement transfer 4 ng of the plasmid DNA (~ 4µl of the aliquot) to a new 1.5ml Eppendorf tube. Fill it up to 500 µl with water or TE buffer. This is your working stock.

The working stock of the spike-in should have a DNA concentration of 0.008 ng/ml.

10.3 Add 2 µl of this working stock to each homogenate sample right before the DNA extraction step. This is ~ 5 mln copies of the plasmid containing spieke-in DNA.

10.4 Throw away what is not used up within a few hours.
Prepare new working stock (repeat steps 10.1-10.4) when needed.