Jul 03, 2021

Insects and DNA metabarcoding - InsectMobile laboratory protocols

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dx.doi.org/10.17504/protocols.io.bmunk6ve

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DISCLAIMER

There may be some slight differences between the attached protocols and this online protocol - the online protocol is the must updated version.

ABSTRACT

This document is a collection of laboratory protocols for the citizen science and DNA metabarcoding research project 'InsectMobile' at the Natural History Museum of Denmark/University of Copenhagen 2017-2021. The protocol(s) cover all methodological steps from sample pre-processing to library build for next-generation-sequencing on a HiSeq 4000 150 bp PE platform.

Samples are collected with car rooftop-mounted insect nets by citizen scientists in the month of June 2018-2020 and the detachable end of the net containing flying insects is shipped to the laboratories at the Natural History Museum of Denmark/GLOBE Institute in containers containing 96% ethanol.

The insects are then size sorted, the large size fraction is identified by morphology and counted, and the sample dry-weight is obtained after which the bulk samples are processed with a DNA metabarcoding protocol. Morphological identification of the insects is not covered by this protocol.

ATTACHMENTS

ExtractionProtocol_Insektmobilen.docx
Purification_Protocol_QIASymphony_Insektmobilen.docx
PCR protocol_Insektmobilen.docx
Protocol for library build_Insektmobilen.docx

DOI

dx.doi.org/10.17504/protocols.io.bmunk6ve

PROTOCOL CITATION

Cecilie S Svenningsen, Lene Bruhn Pedersen 2021. Insects and DNA metabarcoding - InsectMobile laboratory protocols . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bmunk6ve

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Svenningsen, C.S., Frøslev, T.G., Bladt, J., Pedersen, L.B., Larsen, J.C., Ejrnæs, R., Fløjgaard, C., Hansen, A.J., Heilmann-Clausen, J., Dunn, R.R. and Tøttrup, A.P., 2021. Detecting flying insects using car nets and DNA metabarcoding. *Biology Letters*, *17*(3), p.20200833. https://doi.org/10.1098/rsbl.2020.0833

KEYWORDS

insects, DNA metabarcoding, Next Generation Sequencing, Illumina, QIASymphony, molecular ecology

IMAGE ATTRIBUTION

Lene Bruhn Pedersen

CREATED

Sep 29, 2020

LAST MODIFIED

Jul 03, 2021

PROTOCOL INTEGER ID

42606

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GUIDELINES

The laboratory protocols should be followed in order: 1) DNA extraction, 2) Purification, 3) PCR and 4) Library build.

The extraction buffer used was developed by

Gilbert MT, Moore W, Melchior L, Worobey M (2007). DNA extraction from dry museum beetles without conferring external morphological damage. PloS one.
http://10.1371/journal.pone.0000272

and modified by

Nielsen, Martin; Gilbert, M. Thomas P.; Pape, Thomas; Bohmann, Kristine (2019). A simplified DNA extraction protocol for unsorted bulk arthropod samples that maintains exoskeletal integrity. Environmental DNA.
http://10.1002/edn3.16

The PCR primers used in this study are two universal insect COI primer pairs: fwhF2 + fwhR2n from

Vamos, Ecaterina; Elbrecht, Vasco; Leese, Florian (2017). Short COI markers for freshwater macroinvertebrate metabarcoding. Metabarcoding and Metagenomics.
http://10.3897/mbmg.1.14625

and ZBJ-ArtF1c + ZBJ-ArtR2c from

Zeale MR, Butlin RK, Barker GL, Lees DC, Jones G (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces.. Molecular ecology resources.
https://doi.org/10.1111/j.1755-0998.2010.02920.x

and we also used a universal insect 16S primer pair: Inse0 from

Pierre Taberlet, Aurélie Bonin, Lucie Zinger, and Eric Coissac (2018). Environmental DNA: For Biodiversity Research and Monitoring. Oxford Scholarship Online.
http://10.1093/oso/9780198767220.001.0001

MATERIALS TEXT

PB binding

buffer **Qiagen Catalog #19066** Step 5.17

Buffer

EB **Qiagen Catalog #19086** In 4 steps

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626** In 2 steps

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HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #AC-60005** Step 5.33

Liva 1 l **ABENA Catalog #478904** In 5 steps

Ethanol ≥70% (v/v) TechniSolv® **VWR Chemicals Catalog #83801.360** In 5 steps

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** Step 3

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #Q32853** Step 3

Clelands reagent ≥98% **VWR Chemicals Catalog #441496P** Step 1.13

Proteinase K recombinant PCR Grade **Merck Catalog #3115844001** Step 1.13

Ethanol GPR RECTAPUR® **VWR Chemicals Catalog #20824.365** In 2 steps

TruSeq DNA PCR-Free High Throughput Library Prep Kit (96 samples) **Illumina, Inc. Catalog #20015963** Step 5.14

IDT for Illumina – TruSeq DNA UD Indexes (24 Indexes 96 Samples) **Illumina, Inc. Catalog #20020590** Step 5.14

MinElute PCR Purification Kit **Qiagen Catalog #28004** Step 5.2

QIAsymphony DSP DNA Mini Kit (192) **Qiagen Catalog #937236** In 2 steps

Calcium chloride 1 mol/l in aqueous solution for biotechnology sterile **VWR Chemicals Catalog #E506-500ML** Step 1.13

Sodium dodecyl sulphate (SDS) 20% in aqueous solution for biotechnology **VWR Chemicals Catalog #0837-500ML** Step 1.13

Tris-HCl 1M solution pH 8.0 Molecular Biology Grade Ultrapure **Thermo Scientific Catalog # J22638.AP** Step 1.13

Sodium chloride 5 mol/l (5 N) in aqueous solution for biotechnology sterile **VWR Chemicals Catalog #E529-500ML** Step 1.13

Ultrapure dNTPs as set of 100 mM. Stringent QC at ISO-certificated BULK-Production **GeneON.net Catalog #110-010** Step 4

BSA, molecular biology grade, 20 mg/ml **New England Biolabs Catalog # B9000S** Step 4

AmpliTaq Gold™ DNA Polymerase with Gold Buffer and MgCl2 **Thermo Fisher Catalog #4311820** Step 4

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BLS half mask in silicone, twin filters with bayonet fitting
Respiratory protection
BLS **28-BS-4000S-S-M**

Recyclable ABEK1P3 R multifilter, BLS 4000 series, double filter
Respiratory protection
BLS **28-BS-222**

Micro tube 2ml, PP
Screw Cap Micro Tube, 2 ml, PP, with skirted base, with knurls, with assembled cap, with printed writing space and graduation, sterile, 100 pcs./bag
Sarstedt **72.694.006**

QIAsymphony SP
Automated DNA and RNA purification
QIAGEN **9001297**

Filter-Tips, 200 µl, (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube and the QIAsymphony SP/AS instruments
Qiagen **990332**

Sample Prep Cartridges, 8-well (336)
8-well sample prep cartridges for use with the QIAsymphony SP
Qiagen **997002**

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Filter-Tips, 1500 µl, Qsym SP (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAsymphony SP/AS instruments

Qiagen **997024**

8-Rod Covers (144)
8-Rod Covers for use with the QIAsymphony SP

Qiagen **997004**

Invitrogen™ Qubit™ 3 Fluorometer
Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays

Invitrogen™ Q33216 **Q33216**

SAFETY WARNINGS

Some reagents and chemicals should only be used in either a laminar flow hood or with proper safety equipment.

DISCLAIMER:

> There may be some slight differences between the attached protocols and this online protocol - the online protocol is the must updated version.

BEFORE STARTING

If you do not wish to use all three primer pairs, we recommend to use fwhF2 + fwhR2n since it yields the most reads and the most diversity of all the three primer pairs. We did not use ZBJ-ArtF1c + ZBJ-ArtR2c for our 2019 samples since it did not perform as well as fwhF2 + fwhR2n. In our experience, Inse0 does not perform that well, i.e. does not yield as many reads as the COI primer pairs, however, we have not examined why that is (yet).

Non-destructive DNA extraction

1 The first step prior to DNA extraction is to remove the insects from the sampling net, dry them, obtain biomass and then move on to DNA extraction. The general steps covered in this section are:

1. Prepare labels
2. Weigh empty tubes with labels
3. Remove insects from the sampling net
4. Dry insect samples
5. Weigh the dry insects
6. Sample digestion
7. Extract digest and wash insects for sample preservation

Prepare labels

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1.1 **12 samples** (11 samples + 1 blank) NB. A maximum of 12 samples can be extracted each day (per person).

Make sure to make falcon tube labels for 'ND extract' and 'ND extracted insects', Microtube (Sarstedts 2 mL) labels for robot purification 'QIASymphony aliquot' and 'QIASymphony purified DNA' and four top labels for each sample (label size also fits Eppendorf tubes).

Prepare labels for print. Label example:

Example of a 15/50 falcon tube label max. 5 lines/cells wide + three cells long

Example of a 1.5/2 mL Eppendorf tube label, max. 3 cells wide + 1 cell long

Example of Eppendorf tube top label, max. two lines wide

Weigh empty tubes with labels

1.2 Designate and label tubes and weigh the empty tubes with labels standing on the lid – you will weigh them again after the insects have dried to get the dry weight of the sample

Removal of insects from sample net (day 1)

1.3 Clean the work area and laminar flow hood with1:10 diluted bleach and 70% ethanol. Sort insects in the laminar flow hood.

Material and reagents needed (day 1)

- 1:20 Bleach for cleaning

☒ Liva 1

ABENA Catalog #478904

- 1 glass tub with spout for removal of insects from sampling net (can be substituted with plastic trays)
- 70 & 96% EtOH

☒ Ethanol ≥70% (v/v) TechniSolv® **VWR**

Chemicals Catalog #83801.360

☒ Ethanol GPR RECTAPUR® **VWR**

Chemicals Catalog #20824.365

- Forceps
- Vacuum filtration device
- Filters

☒ Qualitative filter papers standard grades grade 4 and 4V Whatman™ **VWR**

Scientific Catalog #512-0412

- Laminated mm paper for size sorting
- 15/50 mL tubes for drying insects (label them with sample number and "<10" (S = small) or ">10" (L = large) for size)
- Mask for respiratory protection during vacuum filtration

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BLS half mask in silicone, twin filters with bayonet fitting
Respiratory protection
BLS 28-BS-4000S-S-M

Recyclable ABEK1P3 R multifilter, BLS 4000 series, double filter
Respiratory protection
BLS 28-BS-222

1.4 Take a glass tub (with spout) and clean it with diluted bleach and 70% EtOH.

1.5 Pour the 96% EtOH from the sample into the tub and place the tub on the laminated mm paper

1.6 Take out a sample net and turn it inside out in the 96% EtOH in the glass tub. Most of the insects should let go when they come into contact with the ethanol

1.7 Try dipping the net up and down to make as many insects let go as possible. Add 70% ethanol if needed.

1.8 For the remaining insects, take a cleaned forceps (bleach + EtOH) and carefully remove them, releasing them into the 96% EtOH

1.9 When all the insects are free from the net, rinse the net and let it dry.

1.10 Take out any insects that are above 10 mm – use the mm paper for picking them out. Make sure there is no smaller insects sticking to the larger insects. Place the larger insects in a separate falcon tube (**15 mL** or **50 mL** depending on how big they are). Each **50 mL** tube should only contain max. **30 mL** of insects and should be divided into two if they take up too much space.

1.11 Work with mask to avoid fumes from ethanol and bleach - alternatively work under fume extractor.

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BLS half mask in silicone, twin filters with bayonet fitting
Respiratory protection
BLS 28-BS-4000S-S-M

Recyclable ABEK1P3 R multifilter, BLS 4000 series, double filter
Respiratory protection
BLS 28-BS-222

The remaining insects in the ethanol are poured through a cleaned (bleach + EtOH) vacuum filtration device and the insects that remain on the use-once filter.

Qualitative filter papers standard grades grade 4 and 4V Whatman™
filter papers
Whatman 512-0412

(i.e. the small size fraction) is transferred to sample tubes (**15 mL** or **50 mL** depending on amount)

Drying samples (predigestion) (day 1)

1.12 Samples (with no lids) are placed in an oven at **50 °C** to dry. The fan should be off inside oven (if possible)! Lids are placed in a way to avoid contamination (e.g. within sheets of aluminium foil). Include a blank (an empty, open 15 mL tube) for each extraction day.

Check the samples roughly every hour. EtOH leaking out of the insects can be removed with a p1000 to speed up the drying process and enable further processing the same day.

Sample digestion (day 1 or 2)Non-destructive DNA extraction

1.13 When samples are dry (some samples may have ethanol residue almost impossible to get rid of) ad 'magic buffer' based on the original recipe:

Gilbert, M. Thomas P. ; Moore, Wendy; Melchior, Linea; Worobey, Michael. DNA Extraction from Dry Museum Beetles without Conferring External Morphological Damage. PLoS ONE. http://10.1371/journal.pone.0000272

and later modified here:

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Nielsen, Martin; Gilbert, M. Thomas P.; Pape, Thomas; Bohmann, Kristine. A simplified DNA extraction protocol for unsorted bulk arthropod samples that maintains exoskeletal integrity. Environmental DNA.
http://10.1002/edn3.16

and lyse the samples. We use the modified version of the buffer.

Material and reagents needed (day 1 or 2)

- 70% EtOH for cleaning

☒ Ethanol ≥70% (v/v) TechniSolv® **VWR Chemicals Catalog #83801.360**

- 1:20 Bleach for cleaning

☒ Liva 1
l **ABENA Catalog #478904**

- Weight
- Weighing boat
- Magic Buffer stock aliquot (see references for recipe)

☒ Calcium chloride 1 mol/l in aqueous solution for biotechnology sterile **VWR Chemicals Catalog #E506-500ML**

☒ Sodium dodecyl sulphate (SDS) 20% in aqueous solution for biotechnology **VWR Chemicals Catalog #0837-500ML**

☒ Tris-HCl 1M solution pH 8.0 Molecular Biology Grade Ultrapure **Thermo Scientific Catalog # J22638.AP**

☒ Sodium chloride 5 mol/l (5 N) in aqueous solution for biotechnology sterile **VWR Chemicals Catalog #E529-500ML**

- DTT

☒ Clelands reagent ≥98% **VWR Chemicals Catalog #441496P**

- Proteinase K

☒ Proteinase K recombinant PCR Grade **Merck Catalog #3115844001**

- P1000 pipette + tips

1.14 Clean the flow hood + pipette and pipette tip box prior to use

1.15 Note the volume of the dried insects + falcon tube type (mL)

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1.16 Weigh the dry weight of the insects (biomass) in the falcon tube by placing the tube on its head in the center of the weight (mg).

1.17 Calculate how much buffer is needed for all samples:

- The volume of insects in each tube + **5 mL** buffer* (to ease the transfer of the supernatant), e.g. in sample a:
  3 mL of dried insects + **5 mL** buffer = **8 mL** for sample a

*If there is less than **1 mL** of insects in the tube, subtract the amount from the **5 mL** total, e.g. **0.5 mL** insects – **5 mL** buffer = **4.5 mL** buffer should be poured on the sample.

If there is <= **1 mL** of 1-2 individuals of slim large insects (>=1 cm), add volume of insects + **2 mL** buffer, e.g. in sample: **0.5 mL** of 1 Syrphidae, **0.5 mL** + **2 mL** = **2.5 mL**

Add up the total amount of buffer need for all samples, and add 2 mL to the blank and prepare 1-2 extra mL to ensure you have enough buffer aliquot.

1.18 Take out an aliquot based on your calculation from the 500 mL stock in the fridge.

1.19 Calculate how much DTT is needed for the reaction, e.g.: (**40 mL** buffer aliquot · **6.17 mg/ml**) / 1000 = **0.2468 g**

1.20 Calculate how much Proteinase K is needed for the reaction, e.g.: **40 mL** buffer aliquot · **6.17 µl/mL** = **400 µl**

1.21 Add DTT and then Proteinase K and gently mix the reagents before adding it to the samples and make sure the DTT completely dissolved. A good trick is to add DTT first, then buffer, and Proteinase K in the end. This way the DTT dissolves quickly. Add the buffer amount calculated for each sample, e.g. sample a = 8 mL of the buffer aliquot.

1.22 Place the samples in a rotator and put the rotator in an oven at **50 °C** overnight. The rotator speed is the lowest setting (10).

Extracting digest (day 2 or 3)Non-destructive DNA extraction

1.23 The supernatant is ready to be extracted from the insect samples. The insects should be preserved in 96% EtOH as bulk voucher samples.

Material and reagents needed (day 2 or 3)

- 70% EtOH for cleaning

Ethanol ≥70% (v/v) TechniSolv® **VWR Chemicals Catalog #83801.360**

- 1:20 Bleach for cleaning

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Liva 1

ABENA Catalog #478904

- Large centrifuge
- p1000 pipette + p1000 tips
- 5 mL Eppendorf tubes for supernatant
- 2 mL Sarstedt for aliquot of supernatant for purification

Micro tube 2ml, PP
Screw Cap Micro Tube, 2 ml, PP, with skirted base, with knurls, with assembled cap, with printed writing space and graduation, sterile, 100 pcs./bag

Sarstedt **72.694.006**

- 96% EtOH for conserving the insects

Ethanol GPR RECTAPUR® **VWR**

Chemicals Catalog #20824.365

1.24 Spin samples (only small size fraction) in the large centrifuge for about **2000 x g, 00:03:00** . This will separate the insects somewhat from the supernatant to ease removal of the digest. If you have many small insects, then try to centrifuge the sample tube at 4000 g for 3 min **4000 x g, 00:03:00** .

1.25 Extract digest with p1000 pipette (or p200/20 if many small insects get stuck in the tips) into 5 mL tubes (if there is more than 5 mL digest then discard the rest). This is a tedious process, but do make sure that no insects are transferred to the supernatant.

1.26 Fill sample tube with insects with 96% EtOH up to **8 mL** and leave them until you finished extracting digest from all samples.

1.27 Spin the samples (only small size fraction) in the large centrifuge for about **500 x g, 00:01:00** (if needed)

1.28 Remove 96% EtOH from the samples and completely fill the tube with fresh 96% EtOH. If the EtOH looks clear then you can store the sample in the box. The samples that remain blurry should be flushed several times until the EtOH looks clear – this is occasionally the case for the samples with many small insects were it was difficult to remove the supernatant.

1.29 Vortex + spin the supernatant. Centrifuge settings **2000 x g, 00:01:00** .

1.30 Add **230 µl** supernatant to sample tubes (2 mL Sarstedt tubes) for QIASymphony purification and prepare tubes with labels for the eluate.

Purifying digest

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2 Purify the **230 µl** aliquots in the QIASymphony robot. 24 samples can be purified each time and always include one extraction blank aliquot as a robot blank. The robot runs 24 samples in one hour. The protocol draws on the following resources:

·**DNA Handbook**: EN-QIAsymphony-DNA-Handbook.pdf
·**Low content purification protocol**: HB-0977-S09_002_PS_QS_LowHighContent_V7_DSP_DNA_UV_0818_WW.pdf
·**General description** of the QIASymphony SP/AS robot: QIASymphony_SPAS_GeneralDescription.pdf
·**User manual**: QIAsymphony_SPAS_User_Manual_Operating_the_QIAsymphony_SP_1211.pdf

QIAsymphony_SPAS_User_Manual_Operating_the_QIAsymphony_SP_1211.pdf
QIASymphony_SPAS_GeneralDescription.pdf **EN-QIAsymphony-DNA-Handbook.pdf**
HB-0977-S09-002_PS_QS_LowHighContent_V7_DSP_DNA_UV_0818_WW.pdf

General information

- 200 µl tip boxes for QIACube works in QIASymphony (QS), but not the 1000 µl tips. QIASymphony uses 1500 µl tips (Cat No./ID: 997024)
- On QIAGEN's webpage search for instrument for instrument information and kit for kit information
- On the specific kit webpage, one can find handbooks for the kit under the tab 'ressources'
- The document called 'labware test' shows, which protocol and labware are recommended for a specific application/kit. Green = good, Yellow = has not been tested, red = not recommended
- QIAGEN can help with a custom protocol if a custom elution is required
- Every time you open the hood the machine asks you whether you have changed anything
- Tip chute can be cleaned with normal dishwashing detergent if needed
- Drop catchers should be checked occasionally for spillage and cleaned with dishwashing detergent and 70% EtOH if needed
- Tips tend to stack in the waste bag and this can cause malfunction, so kick the bag occasionally to make sure they are evenly distributed
- DO NOT USE EtOH ON PLEXIGLASS & THE SCREEN!
- Change the O-rings every month to keep the pipetting precise
- You can work in the next drawer while the machine checks the previous drawer

QIAsymphony SP
Automated DNA and RNA purification
QIAGEN **9001297**

QIAsymphony DSP DNA Mini Kit
(192) **Qiagen Catalog #937236**

Filter-Tips, 200 µl, (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube and the QIAsymphony SP/AS instruments
Qiagen **990332**

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Sample Prep Cartridges, 8-well (336)
8-well sample prep cartridges for use with the QIAsymphony SP

Qiagen 997002

Filter-Tips, 1500 µl, Qsym SP (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAsymphony SP/AS instruments

Qiagen 997024

8-Rod Covers (144)
8-Rod Covers for use with the QIAsymphony SP

Qiagen 997004

All catalog numbers are according to QIAGENS webpage (26.11.2018).

2.1 When loading the robot you always go from the end (elution) to the beginning (samples) (right to left).

2.2 Choose the tab 'Sample preparation' for running samples

Elution drawer

2.3 An adaptor = a cooling rack and you use EMTR plates for eluation (96 tubes/samples, deep-well (special tube racks required)) OR single tubes (24 samples). Elution slot 1 is the only slot with cooling.

2.4 If you use single tubes, label them and place them in the rack without lids on

2.5 When placing the cooling rack, make sure the marked 'A' on the plate is placed at the 'A' mark on the drawer – THIS IS IMPORTANT for knowing which samples are which and the old racks can be placed both ways. For 96 plates: When the bottom is removed from the rack the lid should be on

2.6 Adaptor holes in the bottom show how it should be placed on slot 1 (=cold, **4 °C**)

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2.7 Press ‘ID’ and register the type of rack you are using - use the document ‘labware test’ because the plate should match the adaptor and to run properly the robot needs to know which type it is

2.8 Press ‘Rack ID’ to give the rack a name

2.9 Press ‘OK’. Now the system will check whether the drawer has been loaded correctly

Waste drawer

2.10 Make sure there are waste buckets in the empty slots and remove the lid from partially filled waste buckets

2.11 Make sure there is room in the bag for used tips. Waste drawer layout:

2.12 Press ‘OK’. The robot will check the ‘Waste’ drawer and the bucket closest to you, which needs to be empty if the machine is turned off. If the bucket is full then place a lid on it and throw it out

Reagents and consumables drawer

2.13 You can run two kits, even different kits, at the same time. The robot will let you know prior to a run, whether you need to load more consumables, based on the number of samples. When restocking consumables, use the whole unit box to minimize the risk of contamination. You can scan specific slots for the drawer, which comes in handy if you only make changes to e.g. tip stocks.

Load tips, cartridges and rod covers, if needed.

Sample Prep Cartridges, 8-well (336)
8-well sample prep cartridges for use with the QIAsymphony SP

Qiagen **997002**

Filter-Tips, 1500 µl, Qsym SP (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAsymphony SP/AS instruments

Qiagen **997024**

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8-Rod Covers (144)
8-Rod Covers for use with the QIAsymphony SP
Qiagen 997004

Filter-Tips, 200 µl, (1024)
Disposable Filter-Tips, racked; (8 x 128). For use with the QIAcube and the QIAsymphony SP/AS instruments
Qiagen 990332

QIAsymphony DSP DNA Mini Kit
(192) **Qiagen Catalog #937236**

2.14 Make sure the orientation of the tip holders is correct. You do not need to put tips in a specific slot for the machine to work.

2.15 Vortex the beads trough for min. 3 minutes to re-suspend the beads. The lid can be greasy when you take it off, and make sure to wipe the barcode of if it is dirty, so the machine can read it

2.16 Follow the protocol in the kit. If the reagents are unused, a piercing lid has to be placed on the reagent cartridge. Be careful – the piercing lid is sharp

2.17 The original lid can be re-used to hold the sealing lids if you are re-using a reagent cartridge

2.18 Mount the enzyme rack on the side of the reagent cartridge. Lids can be placed in the holders below the enzyme rack

2.19 Load the reagent cartridge

2.20 Let the robot check whether you loaded the drawer correctly. This check takes a long time (5-10 min) 10m

Sample drawer

2.21 The robot uses four-channel pipettes, so make sure everything is loaded in four, e.g. if you have vials of different size, then they need to be loaded in fours. The 3B adapter in the sample racks is used for the

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Sarstedt microtubes.

Micro tube 2ml, PP
Screw Cap Micro Tube, 2 ml, PP, with skirted base, with knurls, with assembled cap, with printed writing space and graduation, sterile, 100 pcs./bag

Sarstedt **72.694.006**

2.22 Remove the lids from the sample tube and place them in the sample rack. NOTE THE ORDER OF THE SAMPLES

2.23 Load the sample rack in a lane by supporting the rack below with one hand and slide the rack to the first line and wait until the camera comes out

2.24 When the light blinks, push the rack steadily into the robot and make sure the robot has registered all the tubes (this step take practice and you might need to reload it several times). The robot tells you if you need to reload the samples

2.25 Choose the batch on the screen to assign names to your samples and specify the protocol and vial type

2.26 Select all samples and assign the tube type. We use the vial holder 3B and the tubes are called Sarstedt screw skirted 72.694(.006)

2.27 Unless you use a barcode reader, you will need to manually assign names to each sample in the system. Choose each sample and press ‘sample id’ to assign a name

2.28 Choose your setup and which protocol you use (InsectMobile uses ‘DNA Tissue LC’). Press “Next”

2.29 Press “Queue” and then “Run”

2.30 The robot might tell you that you need to load more tips, rod covers or sample prep cartridges. You can specify which consumables that that should be scanned after you make the changes

2.31 If you use the USB stick, then transfer the run file by pressing “Tools”→”User Management”→”File transfer”→”Save to USB”→”Results file”→”Transfer”

After the last run of the day & general cleaning

Citation: Cecilie S Svenningsen, Lene Bruhn Pedersen (07/03/2021). Insects and DNA metabarcoding - InsectMobile laboratory protocols . https://dx.doi.org/10.17504/protocols.io.bmunk6ve

2.32 See ‘End of working day’ sheet in the yellow plastic pocket next to the machine (you do not need to follow general description maintenance instructions unless there has been a spill).

2.33 Clean QS every Friday (if it has been in use) by removing all plastic in the drawers and turning on the UV-light for half an hour.

2.34 Change the O-rings every month (kit and protocol can be found under the machine)

Diluting DNA for standard input in PCR

3 Quantify the DNA concentration in each sample with QuBit HS (BR if too high).

Invitrogen™ Qubit™ 3 Fluorometer
Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays
Invitrogen™ Q33216 **Q33216**

Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Qubit™ dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #Q32853**

Use the template to calculate how much DNA and molecular grade water should be used for a final volume of **50 µl** with an approximate DNA concentration of **2 ng/µl** **Extraction dilutions_Template.xlsx** Make the dilutions in 8-strip PCR tubes and include a blank (molecular grade water) in every 6th row. PCRs can then be carried out for full libraries of 96 samples in each library.

Materials needed
- Molecular grade water aliquot
- PCR strips
- 20 ul or 200 ul empty tip container for holding strips
- Permanent marker for writing on PCR tubes (IM_numbers)
- 10 or 20 ul tips
- 200 ul tips
- 200 and 20/10 ul pipettes
- Rack for water aliquot

3.1 In the “Dilutions” sheet input the *PCRID* and associated QuBit measurement. Input HS if this worked otherwise input BR.

3.2 If your sample is too low for QuBit to perform a measurement then put the final volume in the column *DNA added*

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3.3 **Generally, we want a final volume of 50 µl .** However, this is not always possible. If you sample measurement is below **2 ng/µl** but it is not too low, then still treat it as a too low sample and add the finale volume in the column *DNA added*

3.4 If your sample has a very high DNA concentration then you need to increase the finale volume → try to get the volume around 1 in the column *DNA added*

tagged PCR

4 When the samples have been extracted, purified, measured with Qubit and diluted to 2 ng/µL, they are ready to be amplified by polymerase chain reaction (PCR). Ideally, PCR setup prior to DNA input should be carried out in a DNA-free lab. We set up the MM and add the tagged primers in the DNA-free prior to adding the 2 ng/µL sample in DNA lab.

Setup your PCR spreadsheet prior to entering the lab and print it. It should contain the tag combination of your primers for each tube, which samples go in each tube and the calculated reaction volume of Master Mix (MM). See attached example of PCR spreadsheets setup and thermocycler settings. Example setup for the fwh primer pair (COI): **07082019_PCR_29_Carnet2018(QS)_IM18_433-521.xlsx** , example setup for the art primer pair (COI): **05082019_PCR_28_Carnet2018(QS)_art.xlsx** , example setup for the ins primer pair (16S): **08082019_PCR_30_Carnet2018(QS)_ins.xlsx**

Remember to add a couple of samples to the sample size, so you make enough MM, e.g. if you have 24 samples add 2 more so you make MM for 26 samples. The tag combination of each sample allows us to identify the origin of the sample when we get our sequencing results, so it is important it is consistent or else we will not be able to identify the sample in the data analysis. Switch tag combination for each library by either jumping 8 tags on the reverse or the forward primer (be consistent), since it makes it easier using the multichannel pipette.

Materials needed

- 70% EtOH

Ethanol ≥70% (v/v) TechniSolv® **VWR Chemicals Catalog #83801.360**

- 1:20 Bleach

Liva 1 l **ABENA Catalog #478904**

- Waste bin
- Pipette holder
- Pipettes of the volume needed (see recipe/spreadsheet)
- Multichannel pipette
- Repeater (electronic pipette)
- Pipette tip boxes that match the pipettes
- Pipette tip for the repeater
- Primers from the freezer
- dNTPs in the correct dilution

Ultrapure dNTPs as set of 100 mM. Stringent QC at ISO-certificated BULK-Production **GeneON.net Catalog #110-010**

- BSA

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☒BSA, molecular biology grade, 20 mg/ml **New England Biolabs Catalog # B9000S**

- $MgCl_2$
- PCR buffer
- Tag Gold polymerase

☒AmpliTaq Gold™ DNA Polymerase with Gold Buffer and MgCl2 **Thermo Fisher Catalog #4311820**

- Molecular grade H_2O aliquot in 50 mL tube
- PCR tubes in strips of 8 with individual lids
- 2 x Eppendorf tubes for MM & H_2O
- Freezing rack for PCR buffer reagents, Master Mix (MM) and H_2O
- Freezing rack for PCR tube strips
- Two rack for primers
- Permanent marker

DNA free laboratory

4.1

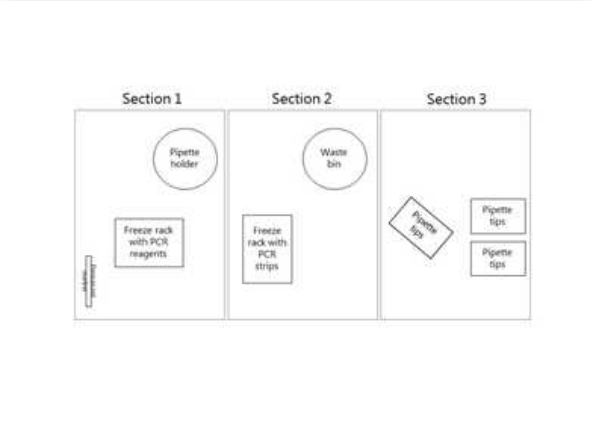
Turn on the flow hood and clean the workbench during the startup of the flow hood

4.2 Clean the pipette holder and the pipettes needed

4.3 Clean the flow hood and place the pipettes, pipette holder and waste bin in the hood

4.4 Clean and place a freeze rack in the hood and place PCR reagents (buffers, dNTPs, BSA) in the freeze rack to thaw

4.5



Flow hood setup

Take 2 x 1.5 mL Eppendorf tubes – 1 for MM and 1 for molecular grade H_2O. Label them.

4.6 Fill the water tube with the water required for the PCR reaction and some extra for PCR negatives

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4.7 Mix the MM. Remember to add TagGold second last and H_2O last
SLOWLY PUM UP AND DOWN WHEN ADDING THE H_2O TO MIX THE BUFFER

4.8 Take out the primers you need and place them in the first rack. The second rack is for the primer strips you are done with

4.9 Take out as many PCR strips as you need, place them in the freeze rack and close the lids

4.10 Label PCR strips. The PCR tubes should be labelled like this:

All tubes should be labelled on the side, but only label the ends and the middle on the lids on the lid opener (if you write directly on the lid it will be erased in the PCR machine because the cover heats up). Remember each 8-strip is a number e.g. 1, 2, 3… and each tube is a letter (A-H)

4.11 Transfer MM to each PCR tube with the repeater. The amount of MM depends on how much DNA is added – check the recipe (e.g. 1 mL pipette tip, 19 µL MM, 32 x)

4.12 Add primers to your PCR tubes. Use the multichannel pipette → place the first one on a tip and "rollover"" to get the rest of the tips. Make sure they all are fastened by rolling the pipette a bit from side to side while pressing down. First, add forward primers to all PCR tubes, then add the reverse primers

4.13 Put the PCR strips and the rest of the H_2O aliquot in a ziplock bag and store them in the fridge while you clean your workspace

4.14 Take the ziplock bag and go to the DNA lab to add sample DNA

DNA laboratory

4.15 The PCR tubes have MM and tagged primers and are ready for DNA input in the DNA lab.

Materials needed

- 70% EtOH

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Ethanol ≥70% (v/v) TechniSolv® **VWR Chemicals Catalog #83801.360**

- 1:20 Bleach

Liva 1 **ABENA Catalog #478904**

- Waste bin
- Multichannel pipette
- 10 µL pipette tips
- PCR tubes with MM and tagged primers
- Freezing rack for PCR tube strips with MM and primers
- 2 ng/µL DNA dilutions in PCR tube strips
- Rack for 2 ng/µL DNA dilutions PCR strips

4.16 Turn on the flow hood and clean the workbench during the startup of the flow hood

4.17 Clean the pipette, tip box and racks

4.18 Clean the flow hood and place everything you need in the hood

4.19 Take out the DNA dilutions and allow them to thaw

4.20 When the DNA has thawed, vortex for ~ **00:00:05** and spin them down for ~ **00:00:08** 13s

4.21 Add DNA using the multichannel pipette and according to the spreadsheet recipe

4.22 Add some of the leftover PCR reaction H_2O to the PCR blank(s)

4.23 Put the PCR strips and water back in the ziplock bag and store it in the fridge while you clean

PCR laboratory 2h

4.24 Now the DNA has been added to your PCR reaction and you are ready to run the samples on the PCR machine. 3h 30m
Place your samples in a PCR machine and adjust the settings according to the recipe on the spreadsheet. The run usually takes **01:30:00** - **02:00:00** .

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4.25 When there is ~ **00:20:00** . left, go to the gel area and prepare gels (2% agarose) corresponding to the number of samples you have 20m

4.26 When the run is complete and your gels have solidified, prepare a sheet of parafilm

4.27 Use the multichannel pipette to transfer **2 µl** gel red rows corresponding to the number of strips/samples you have

4.28 Vortex and spin your samples

4.29 Put the gels in the gel chambers

4.30 Transfer **5 µl** to the designated gel red droplets and mix the liquids by pumping up and down

4.31 SET THE PIPETTE TO **7.3 µl** AND PIPETTE EVERYTHING UP

4.32 The first well and the last well should have ladder, so skip those

4.33 Starting from the second well, pipette the sample slowly into the bottom of the wells
DO NOT stab the gel! Gently feel your way to ease the tips into the wells. You can feel whether you are in the wells by slowly moving the pipette from side to side in the wells. You should feel the sidewalls of the wells if you are in the wells

4.34 When almost all liquid is out → lift the pipette and push almost all the way down → lift up the pipette slowly and suck in a bit before leaving the gel, to suck up any bubbles

4.35 Run the gel with settings: 130 V, 350 A, **00:30:00** . 30m

4.36 Now you can take pictures of the gels. Remember to put a USB stick in the USB slot and make sure the UV light is off.

4.37 Turn on the machine and go to live mode

4.38 Pour ddH_2O on the surface and gently place plastic foil over it to avoid bubbles forming between the

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surface and the plastic foil.

4.39 Remove the gel from the gel chambers and pour off as much TBE buffer as possible. Make sure the gel does not slide

4.40 Gently push the gel onto the plastic foil and avoid bubbles forming under the gel

4.41 Close the door and turn on the UV-light

4.42 Adjust the setting so band appear visible, but no red colour is on the screen (red = too much light). Make sure to keep the settings the same when taking pictures of multiple gels

4.43 Save a picture on the USB stick by pressing “Save” – it takes some time

4.44 Print a picture by pressing “Print”. Make sure the photo machine is on. Label the photo immediately, so you know which picture is which and so you have a reference for the picture on your USB stick

4.45 Turn off the UV-light

4.46 Throw out the plastic foil and gel

4.47 Wipe the surface from water

4.48 Turn off the machine and take your USB stick

4.49 Immediately go to your computer and label your photo files from the USB stick correctly and update your gel results on the spreadsheet, see example **13052019_PCR_20_FwH_results.xlsx**

Your PCR samples should be stored in the freezer for long time storage or in the fridge for short-time storage. DO NOT store samples in the fridge for more than a couple of days.

Library build (Illumina TruSeq PCR-free)

5 PCR products are now ready to be pooled into libraries for sequencing.

- All lab work must be carried out in the flow hood.
- Pool PCR products so your pools are equimolar.
- Make sure the starting DNA concentration > **250 ng** .

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- Spin your buffers down before using them.
- Remember to take End Repair Mix 2 out of the freezer to thaw some time before beginning the library build

Pool samples

5.1 Pool 96 samples with unique tag combinations, e.g.each tag only appears once on the forward and reverse primer - this should add up based on the PCR runs. This is to avoid tag jumps during sequencing. We pool the samples based on gel band strength, but you can use QuBit to check whether it corresponds to DNA concentration for a subset of samples before you begin. See example spreadsheet for pool setup **Pooling_PCRproduct_example.xlsx**

Purify pools 10m

5.2 Purify using MinElute (fragments <70 bp, > **10 µl**). If the sample volume is over ~ **300 µl** , then split the sample between two columns and combine them again after eluation (end volume = **40 µl**).

MinElute PCR Purification
Kit **Qiagen Catalog #28004**

5.3 Remember to make aliquots for all buffers.

5.4 Aliquot the amount of buffer EB in eppendorf – put in a heatblock at **37 °C** .

5.5 Add 5x volumes (rather more than less) PB buffer. Mix by pipetting up and down.

Sample/Pool	Sample/Pool volume (µl)	PB added (µl)

5.6 Spin **600 µl** per time on each column. **You cannot load all sample at once, so repeat the step until all the PB has passed through the column.** **6000 x g, 00:01:00** + empty. If the sample does not pass at 6,000 x g, then increase the setting.

5.7 PE buffer
Add **720 µl** PE buffer to the column **Qiagen Catalog #19065** .
10000 x g, 00:01:00 + empty.

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5.8 Spin empty column for **14000 x g, 00:02:00** to dry.

5.9 Put the filter in a new Eppendorf tube.

10m

5.10 Add **25 µl** EB to the center of the column**. If the sample is split into two columns, then add 20 in each**. Incubate at **37 °C** for **00:10:00** . Spin **14000 x g, 00:01:00** to collect DNA. Turn tube. Repeat the spin.

5.11 Transfer to a new lo-bind tube and mark (combine the two replicates of each sample, if you split the columns).

5.12 Take out **1 µl** of sample for the Qubit and 1 µL sample for the BA dilutions (conc.: **1 ng/µL**). Now the total volume for the sample is **38 µl** .

Run Qubit (HS – high sensitivity) & Bioanalyzer (run later with other samples for the BA) 10m

5.13

Sample	Qubit Conc. (ng/µL)	BA dilution (1:n)
Library Blank		

Illumnia TruSeq DNA PCR-free Library 10m

5.14 *The following is for each library.*

TruSeq DNA PCR-Free High Throughput Library Prep Kit (96 samples) **Illumina, Inc. Catalog #20015963**

IDT for Illumina – TruSeq DNA UD Indexes (24 Indexes 96 Samples) **Illumina, Inc. Catalog #20020590**

truseq_dna_pcrfree_sampleprep_guide_15036187_a.pdf

illumina-adapter-sequences-1000000002694-09.pdf

5.15 Mix 40 µL End Repair Mix 2, 10 µL EB buffer (use the same as before or from the kit) and 50 µL purified PCR product in EB-buffer (adding EB buffer to ensue total reaction volume is 100 µL).

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Buffer

EB **Qiagen Catalog #19086**

Mix sample and EB buffer first, then add the End Repair Mix 2 (remember to spin down first). Pipet up and down to mix. Vortex and spin.

Sample	400/Qubit Conc. from previous table (µl)	EBΔ (60 – value from the middle column)
Library Blank		60

Δ 60-value in previous table column

Put EB buffer back in heat-block.

Thermocycler 10m

30m

5.16 Pre-heat lid to **105 °C**

30 °C for **00:30:00** .

Hold at **4 °C**

2nd MinElute purification 10m

5.17 PB binding

Take new eppendorfs and add 5x volumes PB. buffer **Qiagen Catalog #19066**

5.18 When the samples are done in the thermocycler, add the samples and mix by pipetting up and down.

5.19 Transfer to spin columns.

5.20 Spin **600 µl** per time on column. **6000 x g, 00:01:00** + empty.

5.21 Add **750 µl** PE buffer to column. **10000 x g, 00:01:00** + empty.

5.22 Spin empty column **14000 x g, 00:02:00** to dry.

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5.23 Put the filter in a new Eppendorf tube.

5.24 10m

Buffer

Add **20 µl** EB to center of filter EB **Qiagen Catalog #19086** . Incubate at **37 °C** for **00:10:00** . Spin for **14000 x g, 00:01:00** to collect DNA. Turn tube. Repeat the spin.

5.25 Transfer the libraries to PCR strip tubes.

Freeze the samples in -20 °C if you do not proceed with the protocol at this time.

5.26 **Mix 17.5 µl product from step 5.24 with 12.5 µl A-Tailing mix.**

Thermocycler 10m

40m

5.27 Pre-heat lid to **105 °C**

30 °C for **00:30:00**

70 °C for **00:05:00**

4 °C for **00:05:00**

Hold at **4 °C**

Process next step immediately.

Add index 10m

5.28 **Mix 30 µl product from step 5.27 with**

2.5 µl Resuspension buffer (EB). **NB.** **Room temperature** .

Buffer

EB **Qiagen Catalog #19086**

2.5 µl DNA adapter Index (unique for each library)

2.5 µl Ligation mix 2. **NB. Add as the last thing (enzyme).**

Index primers

Library	Index
Library Blank	

37.5 µL total volume

Spin down.

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Thermocycler 10m

10m

5.29 Pre-heat lid to **105 °C**

30 °C for **00:10:00** .

Hold at **4 °C**

Spin down.

5m

5.30 **Place strips on cooler. Add 5 µl Stop Ligation Buffer and mix.**

Pipet up and down.

Incubate **00:05:00** in cooler.

Samples can be stored in the freezer at -20°C if you do not proceed with the protocol at this time.

Bead purification 10m

5.31 Take out 1 µL of sample for the Qubit and 1 µL sample for the BA dilutions. General protocol:

HighPrep PCR Protocol_MagBioBeads.pdf

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

5.32 **Check results on Qubit, and then Bioanalyzer.**

Sample	Qubit Conc. (ng/µL)	BA dilution (1:n)
Library Blank		

30m

5.33 Take out

HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #AC-60005**

beads from fridge and equilibrate the bead suspension to **Room temperature** . (Take an aliquot, take the beads out from the fridge at least **00:30:00** before you take the aliquot!)

5.34 Make an (or reuse from library build) EB aliquot and place it in a heat block on **55 °C**

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5.35 Prepare aliquots fresh 80% ethanol from molecular grade ethanol (> **400 µl** per sample) and RNase free water (molecular grade).

5.36 Measure the exact volume of PCR products and transfer to 1.5 ml tubes. Adjust volume to **50 µl** with RNase free water.

Sample	Volume	H2O added
Library Blank		

5.37 Vortex the beads thoroughly.

5.38 Add 0.8X bead volume (relative to PCR volume, 50 * 0.8 = 40 µl) to PCR product. Pipet gently but thoroughly at least 10 times until sample is **completely** homogenous.

5.39 Incubate at **Room temperature** for **00:05:00** . 5m

5.40 Place on magnet for **00:03:00** or until solution has cleared. With the plate still on the magnet, remove the supernatant by pipetting. Save the supernatant. Take care not to disturb the beads, some liquid may remain in the tube. 3m

5.41 While the tubes are still on the magnetic stand, add **200 µl** of 80% ethanol using P200 without disturbing the beads and pipette slowly up and down three times.

5.42 Incubate for **00:00:30** and then discard the supernatant without disturbing the beads. 30s

5.43 Repeat step 5.40 and 5.41 **go to step #5.40** . Remove **all** excess ethanol using P20.

5.44 Air-dry the tubes on the magnetic stand for **00:10:00** - **00:15:00** at **Room temperature** (not more!). The beads should appear crackled. Leftover ethanol will interfere with sequencing. 25m

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5.45 Remove the tubes from the magnet. Add **40 µl** of **55 °C** EB buffer to each sample and

Buffer

Mix by pipetting. EB **Qiagen Catalog #19086**

5.46 Resuspend the beads thoroughly by pipetting 10x.

5.47 Incubate at **Room temperature** for at least **00:02:00** . 2m

5.48 Place the tubes back on magnet at **Room temperature** for > **00:01:00** until the beads clear from the solution. 1m

5.49 Transfer the supernatant with the eluted library to new tubes. **If some beads are still in the liquid, then you can use the pipet tip to push the beads up the side so the magnet can fixate them – you may need to do it a couple of times**. Save the beads.

5.50 Measure DNA concentration on **1 µl** library using Qubit.

Invitrogen™ Qubit™ 3 Fluorometer
Accurately measures DNA, RNA, and protein using the highly sensitive fluorescence-based Qubit quantitation assays

Invitrogen™ Q33216 Q33216

5.51 Dilute samples to approx. **1 ng/µL** for the BioAnalyser

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

5.52 Run samples on the BioAnalyser.

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

Sequencing

6 Example of setup for sequencing:

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Lib #	Index	Primer start F	Primer start R	Sample Info
Library 34	UDI0001	fwhF2_001	fwhR2n_001	Carnet (IM18/19)
Library 35	UDI0002	insF2_001	insR2n_001	Carnet (IM18/19)
Library 36	UDI0003	fwhF2_001	fwhR2n_009	Carnet (IM18/19)
Library 37	UDI0004	insF2_001	insR2n_009	Carnet (IM18/19)
Library 38	UDI0005	fwhF2_001	fwhR2n_017	Carnet (IM19)
Library 39	UDI0006	insF2_001	insR2n_017	Carnet (IM19)
Library 40	UDI0007	fwhF2_001	fwhR2n_025	Carnet (IM19)
Library 41	UDI0008	insF2_001	insR2n_025	Carnet (IM19)
LibBlank5	UDI0009			

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