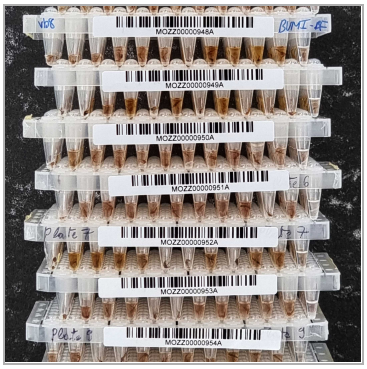

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We use this protocol and it's working

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SOP - Lysis C plate based DNA extraction

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ABSTRACT

This custom minimally morphologically destructive DNA extraction protocol was developed for malaria transmitting *Anopheles* mosquitoes as a cheap and fast alternative to kit-based approaches. DNA extracted using this method can be used unpurified but diluted for singleplex or multiplex PCR. It can also go unpurified straight into Covaris shearing followed by Illumina library preparation or, if quantification or quality is uncertain, it can be purified and used for whole genome sequencing. It is important to note that the times, temperatures, and volumes suggested here were primarily adapted for use with *Anopheles* mosquitoes, but these are quite flexible and can be adapted for different Diptera species (such as a 5-fold increase in volume for larger species like *Glossina*) and probably beyond.

Our SOP is derived from two publications in which lysis buffer C was singled out as the best buffer for both present-day plate-based mosquito DNA extractions (least salt carryover into PCR) and historic museum single-tube DNA extractions (minimally morphologically destructive).

For more information please refer to the original publications:

- Makunin, A., Korlević, P., Park, N., Goodwin, S., Waterhouse, R. M., von Wyschetzki, K., Jacob, C. G., Davies, R., Kwiatkowski, D., St Laurent, B., Ayala, D., & Lawniczak, M. K. N. (2022). A targeted amplicon sequencing panel to simultaneously identify mosquito species and Plasmodium presence across the entire Anopheles genus. Molecular Ecology Resources, 22(1), 28–44. 10.1111/1755-0998.13436
- Korlević, P., McAlister, E., Mayho, M., Makunin, A., Flicek, P., & Lawniczak, M. K. N. (2021). A Minimally Morphologically Destructive Approach for DNA Retrieval and Whole-Genome Shotgun Sequencing of Pinned Historic Dipteran Vector Species. Genome Biology and Evolution, 13(10). 10.1093/gbe/evab226

IMAGE ATTRIBUTION

Stack of 96-well plates containing Anopheles mosquitoes extracted in one go over two days using Lysis C

Figure 1: A plate of *Anopheles* mosquitoes in lysis buffer C after overnight incubation.

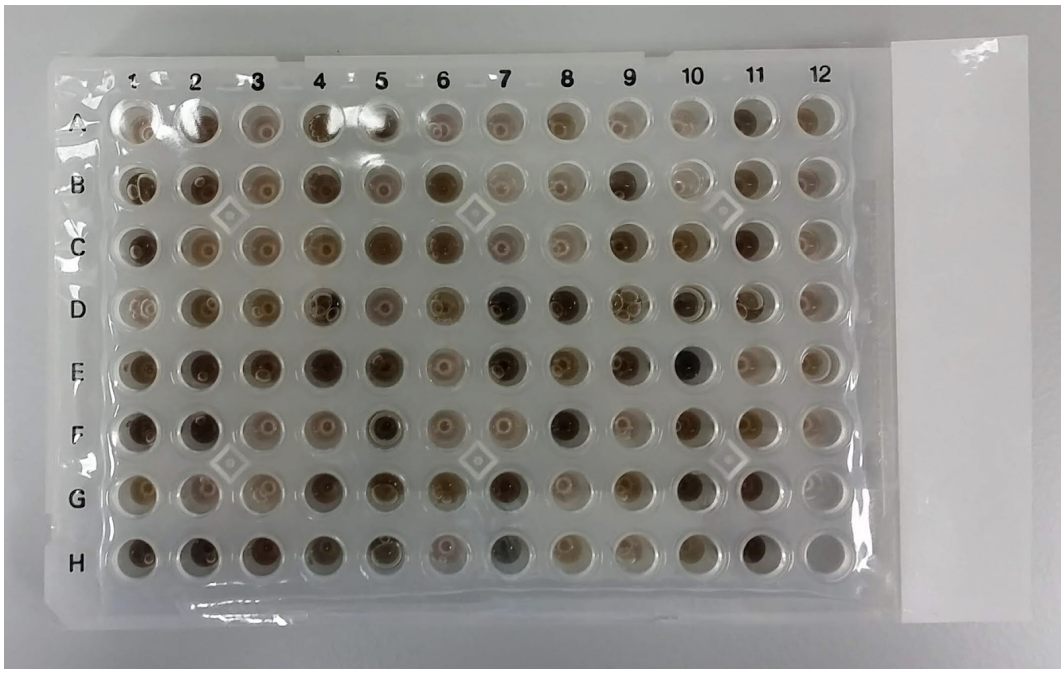


Figure 2: Depending on additional factors, the lysis buffer C color post-extraction can vary substantially (such as mosquitoes with blood meals showing up as dark brown, almost black lysates).

Figure 3: A styrofoam box and paper towel rehydration chamber for pinned dry historic Diptera specimens.

Figure 4: Different insect specimens will require different lysis buffer volumes and extraction containers; pictured here are *Glossina* samples in 1 mL of lysis buffer C in a tissue culturing plate.

MATERIALS

Reagents and consumables:

For the lysis buffer:

- Distilled water (Invitrogen UltraPure DNase/RNase-Free Distilled Water 10977035)
- 1 M Tris-HCl, pH 8.0 (AppliChem A4577)
- 0.5 M EDTA, pH 8.0 (AppliChem A4892)
- 100% Tween-20 (Sigma-Aldrich P5927)
- 20 mg/mL Proteinase K (Qiagen 19131)

For housing extracted mosquitoes at scale:

- ≥99.8% ethanol absolute (AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical 20821.321)
- Domed 8-Cap Strips (Fisherbrand 14-230-231)
- Adhesive PCR Plate Seals (Thermo Scientific AB0558)

For measuring, aliquoting, containing, and sterile technique:

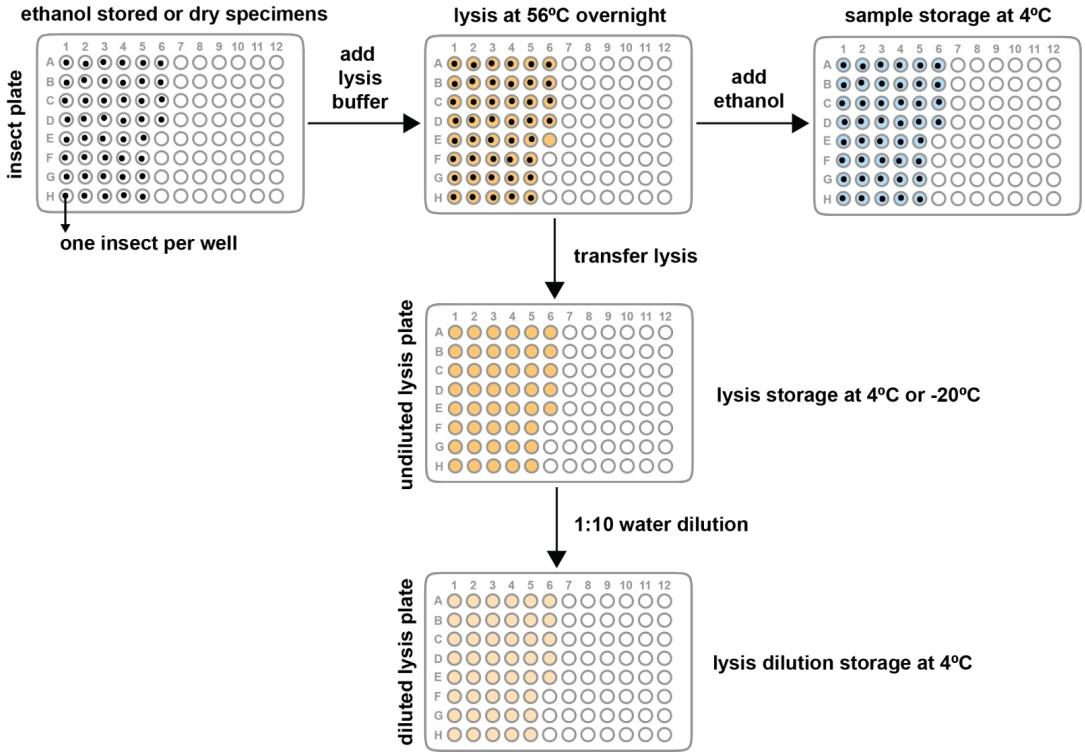
- 15 mL falcon tubes (Corning High Clarity PP Centrifuge Tube Conical Bottom with Dome Seal Screw Cap Sterile 352097)
- 50 mL falcon tubes (Corning High Clarity PP Centrifuge Tube Conical Bottom Sterile 352098)
- 96-well plates (Thermo Scientific SuperPlate PCR Plate low profile skirted AB2800)
- 5 mL Stripette (Corning Costar serological pipettes CLS4487)
- 10 mL Stripette (Corning Costar serological pipettes CLS44818)
- 25 mL Stripette (Corning Costar serological pipettes CLS4489)
- 50 mL Stripette (Corning Costar serological pipettes CLS4490)
- 1,000 µL tips (Graduated TipOne Filter Tip Natural Racks sterile S1126-7810)
- 200 µL tips (Graduated TipOne Filter Tip Natural Racks sterile S1120-8810)
- 20 µL tips (Graduated TipOne Filter Tip Natural Racks sterile S1123-1810)
- 50 mL reservoirs (Corning Costar Sterile Disposable Reagent Reservoirs 10320551)
- Azo wipes (Azo 70% IPA Disinfectant Wipes 81103)
- Kimtech wipes (Kimtech Science Delicate Task Wipes 7558)
- Gloves (material and size as needed)
- Surface cleaning paper wipes
- Any bin/container to dispose of used plasticware

Equipment:

- Single channel pipette (100-1,000 µL, 20-200 µL, 2-20 µL; e.g. Eppendorf Research plus 3-pack 3123000918)
- Multichannel pipette (10-100 µL; e.g. Eppendorf Research plus 3125000036)
- Pipette Controller (e.g. PIPETBOY)
- Oven (should be able to run a stable 56ºC overnight)
- Falcon tube racks
- Vortexer

- Tabletop minifuge
- Plate centrifuge
- PCR strip cap tool (e.g. BioRad #ECT2000)
- Spray bottle (70-80% ethanol for cleaning surfaces)

Schematic:



SAFETY WARNINGS

Do not leave ethanol stored insects close to a flame source.

BEFORE START INSTRUCTIONS

Prepare the appropriate amount of lysis C buffer and add Tween-20 and Proteinase K just before use.

Day 1 (buffer preparation and sample incubation)

1 Prepare an adequate volume of lysis buffer C in a 15 mL or 50 mL falcon tube using the following table (make sure to adjust the initial concentration of stock components if they differ):

Component	Stock or LOT No.	Initial conc.	Final conc.	Final volume (single 100 µL reaction)	Final volume(multiply with No. of samples)
Water				72.95 µL	
Tris pH8		1 M	200 mM	20 µL	
EDTA pH8		0.5 M	25 mM	5 µL	
Tween-20		100%	0.05%	0.05 µL	
Proteinase K		20 mg/ml	0.4 mg/ml	2 µL	

2 Pour the prepared and well mixed lysis buffer C into a 50 mL reservoir for multichannel pipette dispensing or keep in the falcon tube for single pipette dispensing.

NOTE 1: *Add Proteinase K just before use for maximum enzymatic activity.*
NOTE 2: *A larger volume of lysis buffer C without Proteinase K can be stored at room temperature for a prolonged time; just take an adequate aliquot and add Proteinase K to it once you are ready to perform the overnight extraction.*
NOTE 3: *Out of precaution we advise to keep track of stock and LOT numbers of all components in case you notice any contamination or efficiency issues down the line.*
NOTE 4: *For Anopheles mosquitoes we found that* 60 µL *to* 100 µL *per well is an ideal volume in order to submerge the whole specimen and where the DNA concentration is still optimal in a 1:10 dilution for subsequent PCR reactions.*
NOTE 5: *Always include at least one extraction negative control per plate (well with only buffer).*

3 If insects are stored in ethanol:

3.1 Remove as much ethanol from the plate using pipettes while avoiding causing any damage to the insects.

3.2 **OPTIONAL:** Leave the open plate in the incubator at 37 °C until all ethanol evaporates 15m

(typically takes about 00:15:00 if there is < 10 µL of liquid remaining in each well).

3.3 Add an appropriate volume of lysis buffer C (this depends on the size of insect) to each well once ethanol has fully evaporated, making sure the samples are submerged.

4 If insects are dried (e.g. pinned or from silica gel tubes):

4.1 Dispense an appropriate volume of lysis buffer C into each well of a plate. Keep any columns or plates containing aliquoted lysis buffer C loosely closed with strip caps to avoid bits of insects falling in as you are plating them.

4.2 Using tweezers take the individual insects and dip in lysis buffer C (leave any appendages behind in the original tube), clean tweezers thoroughly in between with ethanol and Kimtech wipes or Azo wipes. 5h

NOTE 6: *Dry insects tend to be extremely statically charged, and due to all our plasticware and consumables being equally charged, body parts often detach and fall onto the benchtop or even into plate wells. One thing we saw helped with this issue is to rehydrate the desiccated tissues before submerging in lysis buffer. This can be done by placing the dried samples into a styrofoam box filled with wet paper towels, and incubating in an oven at* 37 °C *for* 02:00:00 *to* 03:00:00 *prior to handling.*

5 Overnight incubation:

5.1 Once all insects are submerged in appropriate lysis buffer volumes, close the plate with strip caps making sure no caps are loose as that might cause evaporation.

5.2 If there are substantial droplets visible on the walls of the wells, do a short spin down in a plate centrifuge.

5.3 Incubate the plate Overnight in an oven at 56 °C. Shorter periods of extraction also work well and can cause less morphological damage, so the exact volumes and times for this protocol could be modified depending on the insect species and size of material being extracted. 15m

Day 2 (DNA extract and specimen plate storage)

6 After incubation is complete, transfer the lysis buffer C (now referred to as “DNA extract”) to a fresh 96-well plate. For long term storage keep this plate sealed with PCR grade foil to avoid evaporation, typically at 4 °C but freezing is also possible depending on the desired downstream application.

7 For downstream amplifications using PCR (e.g. ANOSPP), prepare a 1:10 dilution of the DNA extract with PCR grade water. This can be used both as a DNA template in PCR reactions and for PicoGreen quantification, as the unpurified lysate is too dirty (dyes, salts) to be QC’d accurately without this dilution. In contrast, for whole genome sequencing using Illumina kits, you can start with the unpurified lysate as a template for Covaris shearing without prior purification.

8 For the now extracted insect specimens, add 100 µL – 150 µL of 70-100% ethanol, seal the plates with strip caps and store in the fridge.

NOTE 7: *The volume will depend on the size of the insect, while the concentration will depend on what the sample will be used for in the future (70% for better morphological feature preservation, 100% for leftover DNA preservation)*