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Mosquito DNA extraction using the Phenol-Chloroform-Isoamyl alcohol method.

Created on: Mar 26, 2019; **Last modified by**: Nov 2, 2022, **Version:** 2.0

DNA extraction is a crucial and essential step for many molecular assays and applications such as genetic testing, polymorphism analysis, genomic studies, and molecular epidemiology. Current DNA extraction methods have several limitations for the high-throughput processing of samples: high-cost, low overall DNA yields and their use of molecular grade and toxic compounds. Phenol–chloroform extractions are a liquid-liquid extraction approach used in molecular biology to separate nucleic acids from proteins and lipids. Aqueous solutions (lysed cells, or homogenised tissue) are mixed with equal volumes of a phenol:chloroform which, after centrifuging, form two distinct phases and upper aqueous phase and a lower organic phase. Phenol is only slightly denser than water which requires it be mixed with chloroform to give it a much higher density. The hydrophobic lipids will segregate to the lower organic phase, the proteins will remain at the interphase while the nucleic acids (as well as other contaminants such as salts, sugars, etc.) remain in the upper aqueous phase. For DNA extractions, the upper aqueous phase is transferred to a new tube without carrying the organic phase or the interface contents. If the mixture is acidic, DNA will precipitate into the organic phase while RNA segregates to the aqueous phase as DNA is readily neutralised (in comparison to RNA). This procedure yields large double stranded DNA that can be used in several downstream applications. The disadvantages of this technique are that it is time-consuming, uses hazardous and toxic reagents and because of the risk of contaminating constituents of phases. Nevertheless, phenol-chloroform extractions have long been regarded as the golden standard for DNA, RNA and Protein extractions but rely on proteolytic enzymes, carcinogens (Phenol). This protocol describes our optimized approach for mosquito DNA extraction with special interest in assessing novel DNA extraction methods as a reference.

Procedure

1) Kill captured mosquitos by placing in BSL2 -80 ºC ultra-low freezer for 15 minutes.

2) Remove and discard mosquito abdomen and place remnants (thorax, head, legs, and wings) in 1.5 mL Eppendorf microcentrifuge tube.

3) Add 25 µL of lysis buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCl and 1% SDS, pH 8.0) and homogenize mosquito using the locally manufactured glass pestle (see below).

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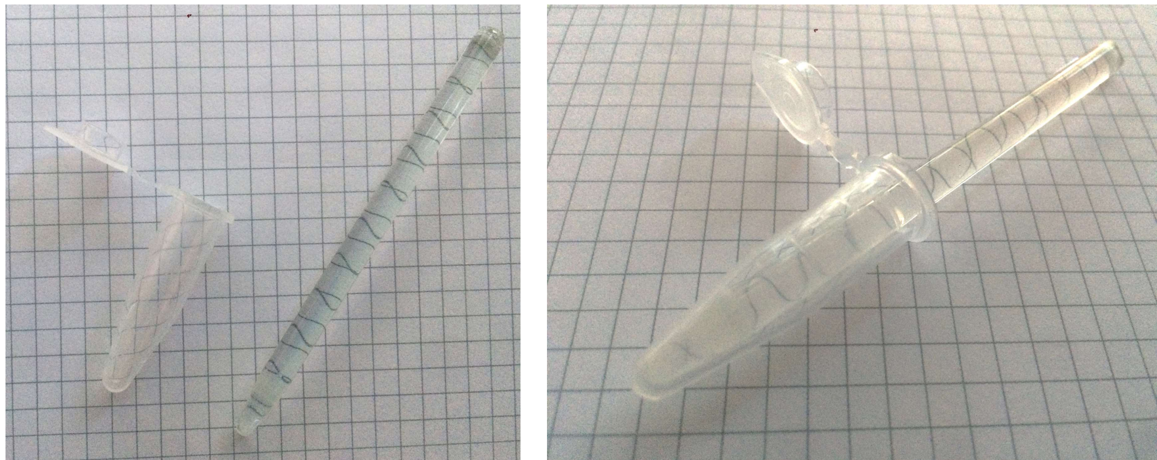

NOTE: this pestle was improvised by our lab to allow for the processing of small samples against the inside conical end of a 1.5 mL Eppendorf tube. In our experience this method has shown to produce less aerosols than the use of tissue homogenizers and as such is amenable for use in procedures involving BSL3 discipline.

4) Add 12.5 µL of proteinase K (10 mg/mL) and vortex briefly, add 437.5 µL of lysis buffer and mix by finger tapping.

5) Spin at 15,000 G for 30 seconds.

6) Incubate in the thermomixer at 55°C and 350 rpm for 4 hours.

7) Add 500 µL of Phenol-chloroform-isoamyl alcohol (PCI) solution and incubate in the thermomixer at 550 rpm and 25 °C for 10 minutes.

8) Centrifuge at 15,000 G for 5 minutes.

9) Transfer the upper aqueous phase to a new 1.5 mL microtube.

10) Repeat the PCI extraction (steps #4 to #5)

11) Transfer the aqueous phase (~350 µL) to a new 1.5 mL microtube.

12) Add 14.5 µL of 5M NaCl (to achieve a final concentration of 0.2 M NaCl).

13) Add 700 µL of 96% ethanol at -20 °C, mix by inversion and incubate at -20 ºC overnight.

14) Centrifuge at 15,000 g for 10 minutes.

15) Discard the supernatant and add 500 µL of 70% ethanol, mix by inversion and incubate at -20 ºC for 30 minutes.

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16) Centrifuge at 15,000 g for 5 minutes.

17) Discard the supernatant and let the DNA pellet dry for about 15 to 30 minutes.

18) Dissolve the DNA pellet in 50 μL of Tris-HCl 10mM, pH 8.0.

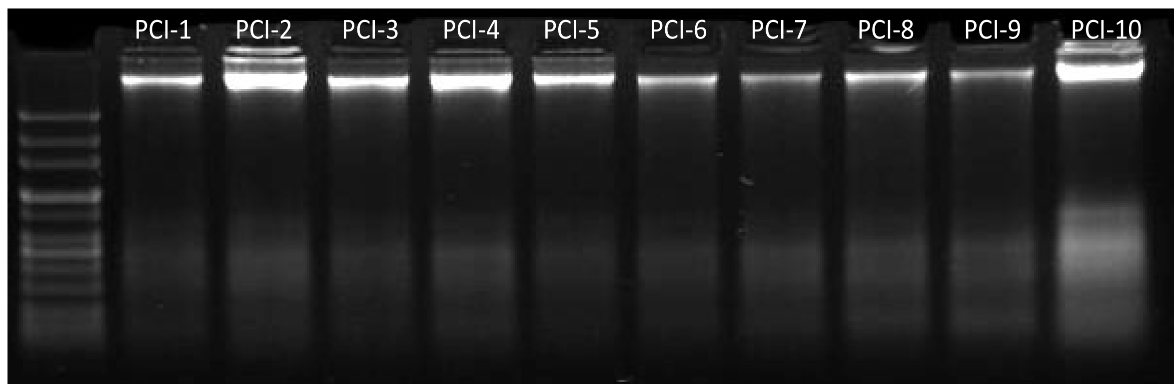
19) Resuspend the DNA in the thermomixer at 40°C and 350 RPM for 30 minutes.

Assessing DNA yield, quality, integrity and downstream application.

20) For DNA quantitation and quality assessment refer to our online multimedia protocol "Spectrophotometric evaluation of DNA using a Nanodrop ND-1000" available through YouTube (https://www.youtube.com/watch?v=k2tZTDDyxaw&feature=youtu.be).

    Note: Common spectrophotometric parameters for DNA extracted using this protocol are 110.84 ± 37.4 ng/μL for a total of 5.5 μg of total DNA extracted with 1.84 ± 0.08 A^{260}/A^{280} ratio and 1.48 ± 0.18 A^{260}/A^{230} ratio.

21) To assess DNA integrity load 18 μL of a mix including 15 μL extracted DNA with 5 μL of 6x orange loading buffer into a 1.5% agarose gel and run at 90 VDC (3.6 V/cm) for 80 minutes. Good integrity DNA should appear as a distinctive smear between 3kb and the gel wells. Fragmented DNA will either not show up on gel or appear as a smear spreading from 200 bp to 3 kb.



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Note: Typical good DNA integrity result for 10 individual mosquito samples (PCI-1 through 10) as seen in 1.5% agarose gel after 80 minutes at 80 volts.

22) Final tests of DNA should involve any downstream procedure, either qPCR or endpoint PCR. At our Laboratory we usually employ mosquito DNA for molecular taxonomy applications and, as such, subject all extracted DNAs to this endpoint PCR technique.

Notes

1) All reagents used for cell lysis buffer, alcohol solutions and protein/DNA precipitation are ACS grade. However, the alcohol used should not be denatured.

2) To prepare 1 litre of cell lysis buffer add 6.057 g of Tris, 1.861 g of EDTA, 5.844 g of NaCl and 100 mL of 10% SDS to a 500 ml flask and top up to 400 mL with distilled water and mix using a magnetic stirrer. Allow all reagents to dissolve completely then transfer to a 1 litre graduated cylinder and top up to 1 litre with distilled water. Dispense 50 mL aliquots and store under refrigeration (do not freeze).

3) To prepare the proteinase K solution 10 mg/mL add 10 mL of water ACD grade to a 100 mg flask of proteinase K (Vivantis Cat. No.: PC0712). Dispense 1 mL aliquots and store al -20ºC.

4) To prepare 1 L of PCI solution, first mix 240 mL of chloroform and 10 mL of isoamyl alcohol. Separately prepare 750 mL of 90% phenol solution by adding 675 g of crystalized phenol into 75 mL of ACS water. Mix the solution with a magnetic stirrer and heating the solution to 60º C. Once the phenol is completely dissolved, add the 250 mL of chloroform-isoamyl alcohol solution and mix with a magnetic stirrer. Transfer the solution to an amber flask and store at room temperature.

References

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Revision history

1.0 Original document.

2.0 Optimized protocol volumes and scalling

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