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| **Title of resource** |
| --- |
| GN_08: Non-destructive DNA extraction from dipteran specimens |
| **Authored by** |
| When using this protocol, the following should be referenced:<br><br>Harrup, L.E (2018). The Pirbright Institute DNA Barcoding Protocols Version 3. |
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| **Description** |
| This method details the protocol and guidance notes for the preparation of genomic DNA (gDNA) extractions from Dipteran specimens where a morphological voucher specimen is required. This protocol includes ethanol precipitation to remove PCR inhibitors from the DNA extractions, in addition linear acrylamide or glycogen is included as a co-precipitant to improve DNA yield and facilitate pellet visualisation during precipitation steps. Protocol from the Gnatwork Bangladesh workshop, September 3-6$^{th}$ 2018. |
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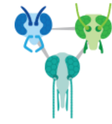
| **Resource history** |
| Updated version of: Harrup, L.E. (2014). The Pirbright Institute *Culicoides* DNA Barcoding Protocols, Version 2, available at www.ibvnet.com |



# GN_08: Non-destructive DNA extraction from dipteran specimens

Citation: Harrup, L.E (2018). The Pirbright Institute DNA Barcoding Protocols Version 3

## A. Introduction

This method details the protocol and guidance notes for the preparation of genomic DNA (gDNA) extractions from Dipteran specimens where a morphological voucher specimen is required. This protocol includes ethanol precipitation to remove PCR inhibitors from the DNA extractions, in addition linear acrylamide or glycogen is included as a co-precipitant to improve DNA yield and facilitate pellet visualisation during precipitation steps.

## B. Materials

### Equipment

- Pipettes (0.1-2 µl, 2-20 µl, 20-200 µl, 100-1000 µl)
- Vortex
- Thermal cycler with 200 µl reaction tube block
- Incubator, thermal cycler or heat block (or equivalent) capable of heating samples to 37°C for 16 hours
- Centrifuge with rotor capable of holding 1.5ml centrifuge tubes at up to 18,500 x g
- 1.5 ml microcentrifuge tube rack
- 50 ml centrifuge rack
- 200 µl reaction tube rack
- Watchmaker number 3 fine point stainless steel forceps (or equivalent)
- Bench top propane burner and lighter (or single use disposable needles (e.g. 1" 25 x G) or equivalent)
- Sharps-disposal bin (if needles used to transfer specimens)
- Timer
- Fridge (+4°C)
- Freezer (-20°C to -80°C)
- Nitrile gloves
- Disposable pipette tips containing hydrophobic filters (0.1-2.0 µl, 1-20 µl, 20-200 µl, 100-1000 µl)
- 50 ml centrifuge tubes
- Plastic or glass measuring cylinder (or large volume serological pipette and pipette filler)
- 250-500 ml plastic or glass bottle
- 200 µl thin-wall reaction tubes as individual tubes, 8 or 12 tube strips or as PCR Plates
- 1.5 ml microcentrifuge tubes (e.g. Eppendorf® Safe Lock Tubes #0030 123.328)
- Label Printer (optional)
- Microcentrifuge tube labels
- Reagent reservoir
- Solvent resistant cryopen

### Reagents

- Sodium dodecyl sulphate (SDS) (e.g. Sigma-Aldrich, #L3771-100G)
- 1 M Tris-HCL pH 8.0 (e.g. ThermoFisher Scientific, #15568025)
- Nuclease (RNase and DNase) free water (e.g. Invitrogen ™ Life Technologies, ThermoFisher Scientific, #10977-035)
- 0.5 M EDTA pH8.0 (e.g. ThermoFisher Scientific, #15575-038)
- 5 M Sodium Chloride Solution (e.g. ThermoFisher Scientific, #24740-011)
- Proteinase K (600 mAU/ml (20 mg/ml) solution) (e.g. ThermoFisher Scientific: # EO0491)

- One of the following nucleic acid co-precipitants:

  Glycogen from Mytilus edulis (Blue mussel) aqueous solution 20 mg/ml (e.g. Roche: 10901393001; Sigma: G1767-1VL)

  or

  Linear acrylamide (5 mg/mL) (e.g. Ambion® ThermoFisher Scientific, AM9520)

- 3 M sodium acetate (NaOAc) pH 5.0 (e.g. Sigma: S7899)
- Invitrogen DNAZap™ Solutions (ThermoFisher Scientific, AM9890) (or equivalent)
- Analytical/molecular grade 100% ethanol

## C. Method

C.1 Put on nitrile gloves.

C.2 Use DNAZap™ (or equivalent) to clean the working any equipment to be used e.g. pipettes where relevant, to reduce/remove any potential DNA contamination.

C.3 Discard used gloves and put on a new pair of nitrile gloves and wear throughout the remainder of the procedure.

C.4 Make a stock solution of 70% ethanol (add 47.75 ml nuclease free water to 100 ml analytical grade 100% ethanol), store at room temp. Use the Gay-Lussac table for alcohol dilution.

C.5 Aliquot a working stock of analytical grade 100% ethanol in to a glass or plastic container and place at -20°C for at least two hours before step C.18 is started.

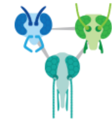
C.6 If a Bunsen burner is to be used to transfer specimens after lysis, aliquot a small volume of analytical grade 100% ethanol into a 50 ml centrifuge tube and a small volume of nuclease free water into a second 50 ml centrifuge tube for use when flame sterilizing forceps. If not proceed directly to step C.7.

C.7 Prepare a stock solution of 10% (*w/v*) SDS in nuclease free water (e.g. for 100 ml weigh out 10 g SDS in a centrifuge tube/bottle then add nuclease free water until a total volume of 100 ml is reached, mix well). Label the stock solution using solvent resistant maker or printed labels with your initials, the name and concentration of the solution and the date the solution was made.

C.8 Following the recipe in Table 1 make sufficient stock solution (200 µl per specimen +10%) of tissue digest solution in a centrifuge tube or bottle, mix well. Label the solution using a solvent resistant maker or printed labels with your initials, the name of solution and the date the solution was made. [Store at +4°C when not in use. Prior to use bring to room temperature, shake tube to mix; ensuring any precipitate formed during storage at +4°C has dissolved].

**Table 1** Components and concentrations of tissue digest solution

| Reagent | Quantity for 10 ml | Final Concentration |
|---|---|---|
| Tris-HCL 1 M pH 8.0 | 1.0 ml | 100 mM |
| NaCl 5 M | 0.4 ml | 200 mM |
| SDS 10% (*w/v*) in Nuclease Free Water | 0.2 ml | 0.2% (*w/v*) |
| EDTA 0.5 M | 100 µl | 5 mM |
| Proteinase K solution (20 mg/ml) | 100 µl | 200 µg/mL |
| Nuclease Free Water | 8.2 ml | |



C.9 Appropriately label 200 µl individual reaction tubes, tube strips or PCR plates so that tubes can be linked to each specimen's unique identifier code.

C.10 Aliquot, from the working solution, 200 µl of tissue digest solution per 200 µl tube.

C.11 Store remaining stock solution of tissue digest solution at +4°C.

C.12 Individually transfer specimens to the reaction tube containing the tissue digest solution from the corresponding reaction tube containing 70% ethanol (retain original tubes containing 70% ethanol for use in step C.14 resealing tubes/plates containing ethanol to prevent evaporation).

C.13 Incubate the reaction tubes containing specimens and tissue digest solution overnight (approximately 16 hours) at 37°C.

C.14 Immediately following the overnight (~16 hour) incubation, using forceps or a single-use disposable needle, individually transfer the specimens from the reaction tubes containing tissue digest solution to their corresponding reaction tube containing ethanol (retained from step C.12), store tubes at 4°C until required for subsequent morphological examination. Flame sterilize forceps between samples to avoid cross-contamination i.e. by dipping forceps in 100% ethanol and igniting on a propane burner for 1-2 seconds, then dipping in the nuclease free water to cool the forceps before handling the next specimen. If a Bunsen burner is not available or is not permitted to be used in the work area use a single-use disposable needle or equivalent to transfer specimens.

C.15 Pre-cool centrifuge to +4°C.

C.16 Double-label sufficient 1.5 ml microcentrifuge tubes and arrange in a microcentrifuge tube rack.

C.17 Bring sodium acetate (NaOAc) solution to room temperature.

C.18 Thaw and vortex chosen nucleic acid co-precipitant (linear acrylamide or glycogen).

C.19 Add 20 µl NaOAc, 600 µl ice-cold 100% ethanol and 1 µl nucleic acid co-precipitant (linear acrylamide or glycogen) to each microcentrifuge tube.

C.20 Transfer each 200 µl of the tissue digest solution (from which the specimen has been removed) from the reaction tube to their corresponding appropriately labelled microcentrifuge tube. Use a new tip per tube to prevent cross-contamination, discard used tips.

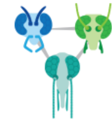
C.21 Close all microcentrifuge tubes and briefly gently vortex tubes (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.22 Store microcentrifuge tubes at -20°C for at least 1 hour.

C.23 Centrifuge microcentrifuge tubes at 18,500 x g (~ 13, 0000 rpm) at 4°C for 30 minutes (position all tubes with the hinge facing outwards in the rotor, the pellet will form on the hinge side of the tube and be easier to identify).

C.24 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.25 Using a pipette carefully remove supernatant without disturbing the pellet. Use a new tip per tube to prevent cross-contamination, discard used tips.



C.26 Add 1 ml 70% ethanol to each microcentrifuge tube. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.27 Close all microcentrifuge tubes and centrifuge all tubes at 18,500 x g (~13,000 rpm) at 4°C for 10 minutes (position all tubes with the hinge facing outwards in the rotor, the pellet will form on the hinge side of the tube and be easier to identify).

C.28 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.29 Using a pipette carefully remove supernatant without disturbing the pellet. Use a new tip per tube to prevent cross contamination, discard used tips.

C.30 Leave the tubes unsealed to air dry until all traces of ethanol have evaporated (approximately 10-30 minutes) (caution over drying DNA pellets may decrease the efficiency with which the pellet can be resuspended).

C.31 Prepare a working stock of 1x TE (10 mM Tris HCL pH 8.0, 0.5 mM EDTA) (for 10 ml add 100 µl 1 M Tris-HCL pH 8.0 and 10 µl 0.5M EDTA to 9.89 ml nuclease free water).

C.32 Add 30-100 µl 1 x TE to each dry microcentrifuge tube to resuspend pellets (volume used for elution is dependent upon the expected yield and the required concentration of the resultant extraction). Use a new tip per well to prevent cross-contamination, discard used tips.

C.33 Close all tubes and store at 4˚C if to be used within 24 hours otherwise store at -20˚C (or for archive storage store at -80°C).

C.34 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential residual DNA contamination.

C.35 Remove and discard gloves.

## D. Results

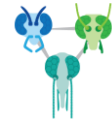
D.1 Results and sample details should be recorded in the appropriate laboratory notebook.

D.2 DNA yield of samples produced from this procedure may be checked using a NanoDrop spectrophotometer or using a Qubit® Fluorometer with the Qubit® High Sensitivity dsDNA Assay Kit (ThermoFisher Scientific).

## E. Tips and troubleshooting

- The tissue digest solution utilised in this method replaced the formally commercially available Qiagen DXT tissue digest reagent with 1% Proteinase K solution previously validated and taught in workshops associated with the following protocols guidebook Harrup (2014) The Pirbright Institute *Culicoides* DNA Barcoding Initiative Protocols v2 DOI: 10.13140/RG.2.1.5026.2642. Qiagen no longer produce DXT tissue digest reagent. The recipe for tissue digest solution included in this protocol has been validated to produce equivalent results and provides greater opportunity for end user modification if adjustments to ingredients/concentrations are required for downstream applications.

- Optimal DNA recovery and specimen preservation is achieved when specimens are stored in 70 to 100% ethanol in the dark at 4°C, cooler temperatures can be used for storage -20°C or -80°C but are not required unless there will be a significant delay in extracting DNA from specimens.



Ideally DNA should be extracted as soon as possible after specimens are collected, with a general rule of thumb for consistent high-quality DNA extraction and PCR amplification of the DNA Barcode region of the mtDNA COI gene region being to extract within one year of collection. However, high-quality DNA Barcodes and slide mounts have been achieved using this protocol in conjunction with protocol GN_06 with specimens which have been stored in the dark at 4°C in 70% ethanol for up to five years post collection.

- A vacuum aspirator with disposable tips may also be used to aid removal of supernatant in steps C.25 and C.29.
- If the DNA yield is too low or high, adjust the elution volume as appropriate.
- When not in use, once cooled, the mobile propane burner together with any spare cartridges should be stored in a flammables cabinet or as per local rules.