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Title of resource
GN_09: DNA extraction from Dipteran specimens, whole blood and cultured cells using Qiagen DNeasy spin columns
Authored by
When using this protocol, the following should be referenced: 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 no, or only a partial, morphological voucher specimen is required. In addition, to preparation of gDNA extractions from whole blood and cultured cells. Protocol from the Gnatwork Bangladesh workshop, September 3-6th 2018.
Intended use
Scientific research use and training purposes.
Restrictions on use
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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_09: DNA extraction from Dipteran specimens, whole blood and cultured cells using Qiagen DNeasy spin columns

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 no, or only a partial, morphological voucher specimen is required. In addition, to preparation of gDNA extractions from whole blood and cultured cells.

B. Materials

Equipment

- Pipettes (2-20 µl, 20-200 µl, 100-1000 µl)
- Vortex
- Centrifuge with rotor capable of holding 1.5 ml centrifuge tubes and spin columns at up to 20,000 x g
- Dry heat block (or equivalent) (capable of holding 1.5 ml centrifuge tubes at 56˚C)
- Timer
- Fridge (+4°C)
- Freezer (-20°C)
- Disposable pipette tips containing hydrophobic filters (2-20 µl, 20-200 µl, 100-1000 µl)
- 1.5 ml microcentrifuge tubes
- Label Printer (optional)
- Microcentrifuge tube labels
- Solvent resistant cryopen
- Nitrile gloves
- Disposable pellet pestles for 1.5 ml microcentrifuge tubes (e.g. Z359947-100EA (Sigma-Aldrich, UK) (only if manually homogenising Dipteran specimens or tissue samples)
- Qiagen TissueLyser (only if using the TissueLyser to homogenise Dipteran specimens or tissue samples).
- 2 x 24 microcentrifuge tube adaptor plates for Qiagen TissueLyser (only if using the TissueLyser to homogenise Dipteran specimens or tissue samples)
- 3 mm stainless steel balls (optional and only if using the TissueLyser to homogenise Dipteran specimens or tissue samples)

Reagents

- Phosphate buffered saline (PBS) pH 7.2 (50 mM potassium phosphate; 150 mM NaCL) (e.g. ThermoFisher Scientific: 20012019)
- Analytical grade 100% ethanol
- DNAZap™ Solutions (ThermoFisher Scientific: AM9890) (or equivalent)
- RNase Cocktail™ Enzyme Mix (500 U/ml RNase A and 20,000 U/ml RNase T1(ThermoFisher Scientific: AM2286) (or equivalent) (optional only required for RNA-free gDNA extractions)
- Qiagen DNeasy® Blood and Tissue Kit (Qiagen 50 columns: 69504; 250 columns: 69506), includes:
    - Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0)
    - Buffer AL
    - Buffer ATL (not used in this protocol)
    - Buffer AW1
    - Buffer AW2
    - Proteinase K

- DNeasy® mini spin column
- DNeasy® collection tubes

Method

C.1 Put on nitrile gloves.

C.2 Use DNAZap™ (or equivalent) to clean all work areas and equipment to be used e.g. pipettes where relevant, to reduce/remove any potential residual 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 Add 100% ethanol to Buffers AW1 and AW2 from Qiagen DNeasy® Blood and Tissue Kit (see bottle labels for volumes required). Label the bottles with the date and your initials. Store at room temperature.

C.5 Fit an appropriate rotor into the centrifuge capable of holding 1.5 ml microcentrifuge tubes and pre-cool the centrifuge to 21°C.

C.6 Pre-heat the heat block (or equivalent) to 56°C

C.7 If extracting DNA from Dipteran specimens prepare homogenised Dipteran specimens or tissue samples using one of the methods described in C.8, if extracting DNA from whole blood proceed to C.9, or if extracting DNA from cultured cells proceed to C.10.

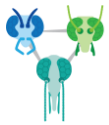
C.8 Dipteran Samples:

Manual homogenisation:

i. Aliquot 200 µl phosphate buffered saline (PBS) into appropriately labelled 1.5 ml microcentrifuge tubes.
ii. Transfer selected Dipteran or tissue samples (~25 mg per sample) individually into the microcentrifuge tubes containing PBS.
iii. Homogenise the selected Dipteran specimens or tissue sample individually using an autoclaved disposable pellet pestle, by rotating the pellet pestle in each microcentrifuge tube for approximately 30 seconds. Use a new pestle per tube to prevent cross-contamination, discard used pellet pestles.
iv. Proceed to step C.11.

Mechanical homogenisation using a TissueLyser:

i. Aliquot 200 µl of PBS into appropriately labelled 1.5 ml microcentrifuge tubes. Optionally add one 3 mm stainless steel ball to each tube/well to improve homogenisation.
ii. Transfer selected Diptera individually into the microcentrifuge tubes containing PBS.
iii. Fit the microcentrifuge into the appropriate TissueLyser adaptor cassette and secure the cassette into the TissueLyser. Ensuring that the tubes are equally distributed and balanced between the two cassettes.
iv. Homogenise at 25 Hz for between 1 to 4 minutes (time dependent on type and robustness of sample being homogenised, 1 minute is sufficient for most Dipteran specimens). Remove the adaptor cassettes from the TissueLyser and remove the microcentrifuge tubes.
v. Pulse spin the microcentrifuge tubes to collect the sample in the base of the tube and reduce the potential for cross-contamination when opening the tubes.
vi. Proceed to step C.11.



C.9 **Whole Blood**: Add either 100 µl of whole anti-coagulated (e.g. defibrinated or K2-EDTA treated) non-nucleated (e.g. mammalian) blood to 1.5 ml microcentrifuge tube containing 100 µl of PBS or add 10 µl of whole anti-coagulated nucleated (e.g. avian or piscine) blood to 1.5 ml microcentrifuge tubes containing 190 µl PBS, then proceed to step C.11.

C.10 **Cultured Cells**: Following a method appropriate to the cell line harvest the required number of cells (max approximately 5x10^6 depending on cell size). Then pellet the harvested cells by centrifugation for 5 minutes at 300 x g. Discard the supernatant and resuspend the cell pellet in 200 µl PBS, transfer the resuspended cells to an appropriately labelled 1.5 ml microcentrifuge tube, then proceed to step C.11.

C.11 Add 20 µl proteinase K to each microcentrifuge tube containing homogenised Dipteran/tissue sample/whole blood/cultured cells. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.12 If RNA-free DNA not required proceed directly to step C.13. However, if RNA-free genomic DNA is required, add 5 µl RNase Cocktail™ Enzyme Mix to each microcentrifuge tube, to digest the RNA, the proceed to step C.13. Use a new tip per tube to prevent cross-contamination, discard used tips, the proceed to step C.13. RNase Cocktail™ Enzyme Mix is a highly concentrated protein solution, if a precipitate is visible in the tube, vortex thoroughly and then microcentrifuge briefly before use.

C.13 Add 200 µl Buffer AL to each microcentrifuge tube. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.14 Immediately seal tubes and briefly vortex gently (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.15 Transfer tubes to dry block heater and incubate tubes at 56°C for 10 minutes.

C.16 Remove tubes from the dry block heater and transfer to a microcentrifuge tube rack.

C.17 Add 200 µl 100% ethanol to each tube. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.18 Immediately seal tubes and briefly vortex gently (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.19 Arrange labelled DNeasy® spin columns in their collection tubes in a microcentrifuge tube rack.

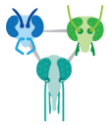
C.20 Pipette the mixture including any precipitate from each tube into the relevant DNeasy® spin column. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.21 Centrifuge at ≥6000 x g (~8000rpm) at 21°C for 1 minute.

C.22 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.23 Transfer DNeasy® spin columns to new collection tubes. Discard flow-through and used collection tubes.

C.24 Add 500 µl Buffer AW1 to each spin column. Use a new tip per tube to prevent cross-contamination, discard used tips.



C.25 Centrifuge tubes at ≥6000 x g (~8,000rpm) at 21°C for 1 minute.

C.26 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.27 Transfer DNeasy® spin columns to new collection tubes. Discard flow-through and used collection tubes.

C.28 Add 500 µl Buffer AW2 to each spin column. Use a new tip per tube to prevent cross-contamination, discard used tips.

C.29 Centrifuge tubes at 20,000 x g (~14,000rpm) at 21°C for 3 minutes.

C.30 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.31 Transfer DNeasy® spin columns to new appropriately labelled flip-top 1.5ml microcentrifuge tubes. Discard used collection tubes containing flow-through.

C.32 Pipette between 50 µl to 100 µl of Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) directly on to the DNeasy® membrane (volume used for elution is dependent upon the expected yield and the required concentration of the resultant extraction). Use a new tip per tube to prevent cross-contamination, discard used tips.

C.33 Incubate at room temperature (approximately 21˚C) for 1 minute.

C.34 Centrifuge tubes at ≥6,000 x g (~8000rpm) at 21°C for 1 minute to elute DNA.

C.35 Transfer tubes from the centrifuge to a microcentrifuge tube rack.

C.36 If a second elution is not required proceed directly to step C.40 (number of elutions and volume per elution is dependent upon the expected yield and the required concentration of the resultant extraction). If a second elution is required repeat steps C.32 to C.35.

C.37 Discard DNeasy® spin column (from which the DNA has been eluted), seal microcentrifuge tubes containing the eluted DNA and store at 4°C if to be used within 24 hours, otherwise store at -20°C (or for archive storage at -80°C).

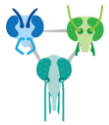
C.38 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential DNA contamination.

C.39 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 Qubit® High Sensitivity dsDNA Assay Kit (ThermoFisher Scientific). Typically, DNA isolated using this protocol has an OD260/280 of >1.8 when samples are diluted in Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0). An OD260/280 of >1.8 indicates that the DNA is reasonably clean of proteins and other UV chromophores that could either interfere with downstream applications or negatively affect



the stability of the stored DNA. Isolated DNA that is free of RNA and proteins should have a OD260/280 ratio > 1.7.

E. Tips and troubleshooting

- Homogenates from up to 25 mg of Dipteran specimen may be applied to the DNeasy® spin column. For example, extraction from 1 individual to pools of 20 *Culicoides* have been tested with this protocol without problem.
- A maximum of 25 mg of animal tissue should be used per spin column. A 2 mm cube of most animal tissues weighs approximately 10-15 mg.
- A maximum of 5x10^6 cultured cells should be used per spin column.
- A maximum of 100 µl non-nucleated in PBS or 10 µl nucleated blood in PBS (total volume 200 µl) should be used per spin column.
- If using frozen blood, equilibrate the sample to room temperature and gently vortex before use to ensure the sample is well mixed (serum and red blood cells may separate during thawing).
- Avoid repeated freeze-thawing samples and minimise the speed/duration samples are vortexed as this can increase the shearing of DNA fragments reducing their size and the quality of the resulting DNA extraction.
- A NanoDrop spectrophotometer or Qubit® Flurometer with Qubit® High Sensitivity dsDNA Assay Kit (ThermoFisher Scientific) may be used to confirm the DNA yield of the DNA extractions produced using DNeasy® spin columns.
- If the DNA yield is too low for downstream applications, for future samples try either a single elution of 50 µl or two elutions of 50 µl (total elution volume should not exceed 200 µl).
- If there is significant risk of cross-contamination of samples when transferring specimens to PBS e.g. specimens have been dissected, the forceps should be flame sterilised between specimens using ethanol and a Bunsen burner. In areas where Bunsen burners are not available or may not be used, single-use disposable needles or equivalent should be used transfer specimens.
- Proteinase K is a broad-range endolytic protease widely used for digestion of proteins in nucleic acid preparations. The proteinase K supplied with the Qiagen DNeasy® Blood & Tissue kit is at 600 mAU/ml solution (~20 mg/ml). Proteinase K is stable for at least one year after delivery when stored at room temperature (15–25°C). However if the proteinase K is to be store for more than one year or if ambient temperature often exceeds 25°C, store proteinase K at 2–8°C.
- RNase Cocktail™ Enzyme Mix contains 500 U/mL RNase A and 20,000 U/mL RNase T1 and can be used to replace RNase A in applications where it is desirable to degrade RNA. RNase A cleaves RNA after C and U residues and generally leaves fragments of RNA large enough to be visible on agarose gels and precipitable in ethanol. RNase T1 cleaves after G residues. Consequently, the use of both enzymes together results in a reduction in RNA fragment size over the use of either enzyme alone. Other RNase A or RNase cocktail solutions may be utilised, however volumes and concentrations may require optimisation dependent upon the amount of RNA required to be removed and/or sensitivity of downstream applications.
- See the Qiagen DNeasy® Blood & Tissue handbook for further information on the kit used in this protocol.

F. References

Qiagen DNeasy® Blood & Tissue handbook