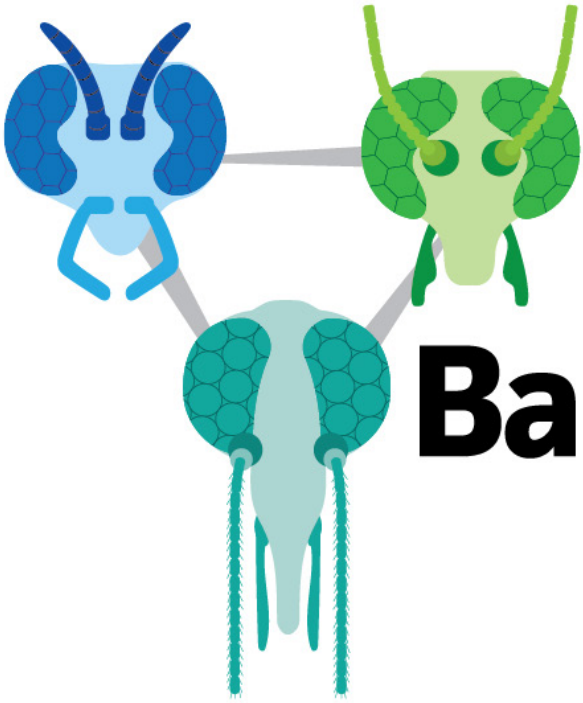

Bangladesh '18

Workshop Booklet
3-6th September 2018

THE Pirbright INSTITUTE

LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

GCRF Global Challenges Research Fund

BBSRC bioscience for the future

Contents

Workshop Agenda

3 September	
Theme: Basic experimental design and introduction to statistics	
Session 1: Basic experimental design and the importance of sample size calculations Prof Mary Cameron, Prof Bob Cheke; Dr Karin Darpel	
Session 2: Statistical methods for entomological data Prof Neal Alexander, Shannon McIntyre, Dr Lara Harrup	
Session 3: Preparing for publication Dr Simon Carpenter; Dr Martin Hall	
10:00-12:00	Session 1
13:00-15:00	Session 2
15:15-17:15	Session 3

4 September	
Theme: Field experiment set-up and trapping of vectors	
Morning discussion on how to collect/trap for each vector type (immature and adult), including the types of trap suitable:	
09:30-10:00	Biting midges Dr Simon Carpenter, Dr Lara Harrup
10:00-10:30	Sandflies Prof Mary Cameron, Shannon McIntyre
10:30-11:00	Blackflies Prof Bob Cheke
Field session	
11:15-15:00 (with break for lunch)	Field experiment set-up. Topics to include: • Field work techniques • Optimal trap locations • Set up of traps
Afternoon session. Towards integrated disease management.	
15:00-15:45	Good lab practice and basic principles of PCR and DNA Barcoding Dr Lara Harrup
16:00-17:00	Workshop session: current issues affecting vector research Audience will be divided into a number of groups randomly and assigned a group leader and note taker from the management board. The following questions will be discussed: • What laboratories are individuals from? • What difficulties do new workers in the field face? • What resources are commonly employed for identification? • What proportion of workers have access to specialist equipment? • Are internet-based resources accessible? What assumptions do workers have on primary vectors in their region?
17:00-17:30	Reporting from workshop session

5 September	
Theme: Vector identification (morphological)	
Trap collection and discussion on basic identification for each vector group:	
08:45-9:30	Sticky trap collection
09:30-10:45	Morphological identification theory. Characteristics of each of the three vectors for basic identification. • Biting midges: Dr Glenn Bellis (09:30-09:55) • Sandflies: Prof Mary Cameron (09:55-10:20) • Blackflies: Prof Bob Cheke (10:20-10:45)
Session 1: Morphological identification practical Prof Mary Cameron, Dr Glenn Bellis, Prof Bob Cheke	
Session 2: DNA extraction for PCR (preparation for DNA Barcoding) Dr Lara Harrup, Dr Karin Darpel	
Session 3: Statistical analysis of entomological data: computer practical Prof Neal Alexander, Prof Mary Cameron	
11:00-13:00	Session 1 – group split in 2
14:00-16:00	Session 2 – group split in 2
16:30-18:30	Session 3 – all together

6 September	
Theme: Vector identification (molecular)	
Session 1: Selection of materials for characterisation and slide mounting practical Dr Martin Hall, Dr Simon Carpenter, Prof Neal Alexander	
Session 2: DNA Barcoding PCR and gel analysis Dr Lara Harrup, Dr Karin Darpel, Dr Glenn Bellis	
Session 3: Putting the training into practice Dr Martin Hall, Dr Simon Carpenter, Prof Bob Cheke	
08:30-10:45	Session 1 (includes bus transfer) – group split in 2
11:15-13:15	Session 2 (includes bus transfer) – group split in 2
14:30-15:30	Session 3 – all together

Organising Committee

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Dr Dinesh Mondal
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Professor Mary Cameron
Network Co-Director
The London School of Hygiene and Tropical Medicine (LSHTM)

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Australian Government, Australia

Dr Karin Darpel
The Pirbright Institute, UK

Dr Lara Harrup
The Pirbright Institute, UK

Dr Martin Hall
Natural History Museum, UK

Professor Neal Alexander
LSHTM, Colombia

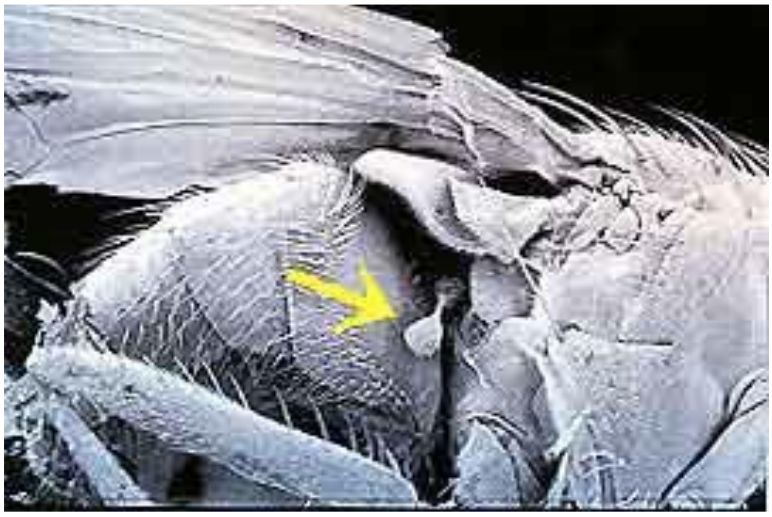
Professor Robert Cheke
University of Greenwich, UK

Shannon McIntyre
LSHTM, UK

GN_01: Key characteristics of vector species

Diptera

All Diptera have only two wings, as their hind wings have been modified into a pair of club-like halteres. This separates Diptera from all other insect Orders.



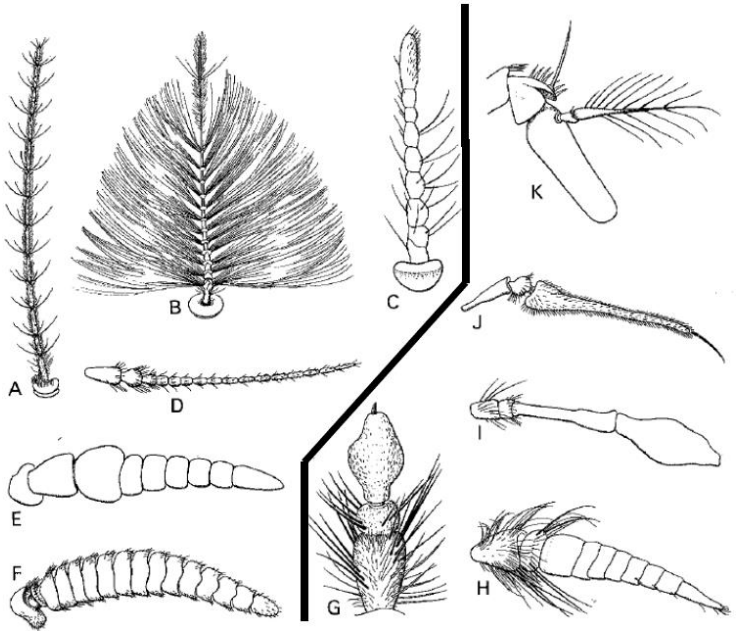
Hamilton *et al.* (2006)

Diptera are separated into 2 major Divisions. These are most easily separated on the shape of the antenna although body shape and wing venation are also useful.

Nematocera are the more primitive group. Most species are slender, delicate flies which have many (usually more than 6) antennal segments of fairly uniform shape. Biting midges, sandflies and blackflies all belong to the Nematocera.

Brachycera are more specialised. Most species are robust and have fewer antennal segments which are usually not of uniform shape. House flies, blow flies and march flies all belong to the Brachycera.

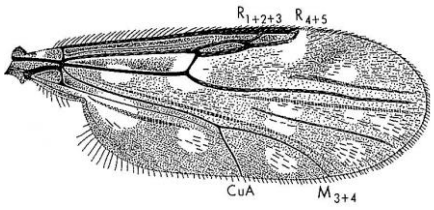
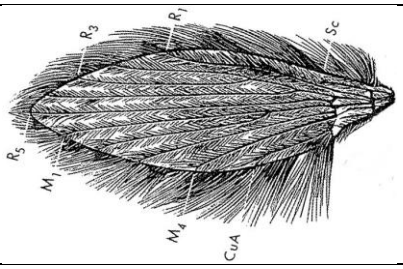
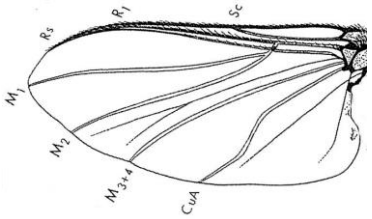
Nematocera Brachycera



CSIRO (1991). Antennae of various Diptera. A: *Aedes alternans*, Culicidae (female); B: *A. alternans* (male); C: *Heptagyia tasmaniae*, Chironomidae (female); D: *Sylvicola dubius*, Anisopodidae (female); E: *Austrosimulium bancrofti*, Simuliidae (female); F: *Keroplatus mastersi*, Mycetophilidae (male); G: *Aplocera asilica*, Apioceridae (female); H: *Scaptia maculiventris*, Tabanidae (female); I *Miltinus viduatus*, Mydidae (male); J: *Rhaphium pudicum*, Dolichopodidae (male); K: *Musca vetustissima*, Muscidae (male).

Nematocera

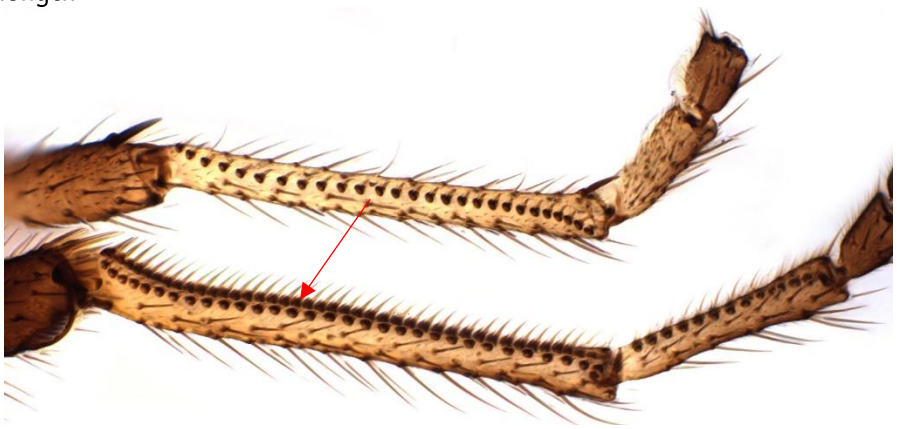
Include the:

Ceratopogonidae (contains *Culicoides* biting midges)	**Psychodidae** (contains phlebotomine sandflies)	**Simuliidae** (contains blackflies)
		
All species have 2 forked veins distally and posteriorly on the wing which separates them from other Nematoceran families	Wings are hairy and veins are numerous, parallel and have no cross-veins in the outer two-thirds of wing	Wings are clear, broad and without hairs or scales. Heavy veins are present near the anterior wing margin, weak veins are present posteriorly

GN_02: Key characteristics of *Culicoides* biting midges

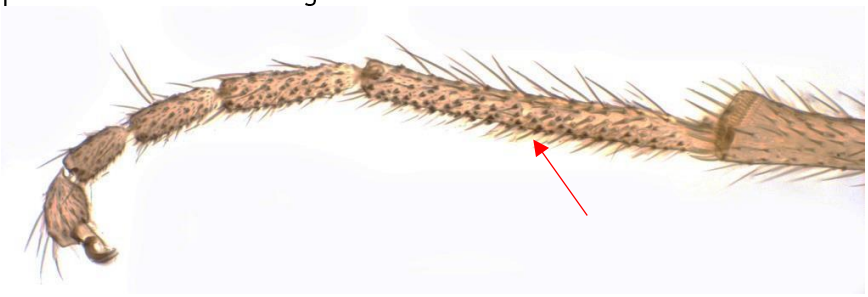
Distinguishing *Culicoides* from other Ceratopogonids

1. *Culicoides* do not have the hind first tarsomere with a row of evenly-spaced palisade setae of even length



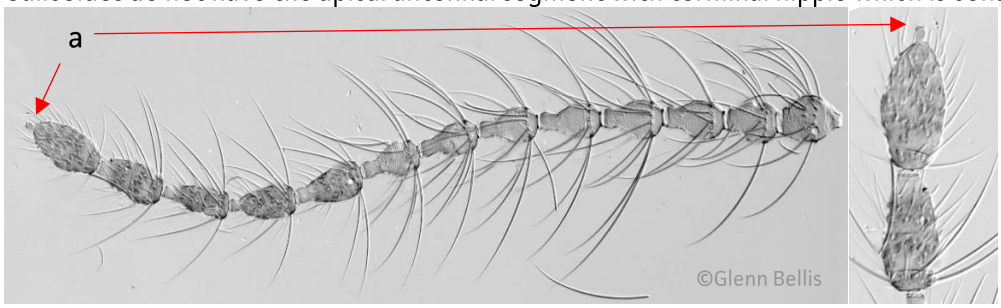
Non-*Culicoides* with evenly-spaced palisade setae of even length
Source: Glenn Bellis

Culicoides do have the hind first tarsomere with scattered setae and without a row of evenly spaced palisade setae of even length



Culicoides with non-evenly-spaced palisade setae
Source: Glenn Bellis

2. *Culicoides* do not have the apical antennal segment with terminal nipple which is constricted basally

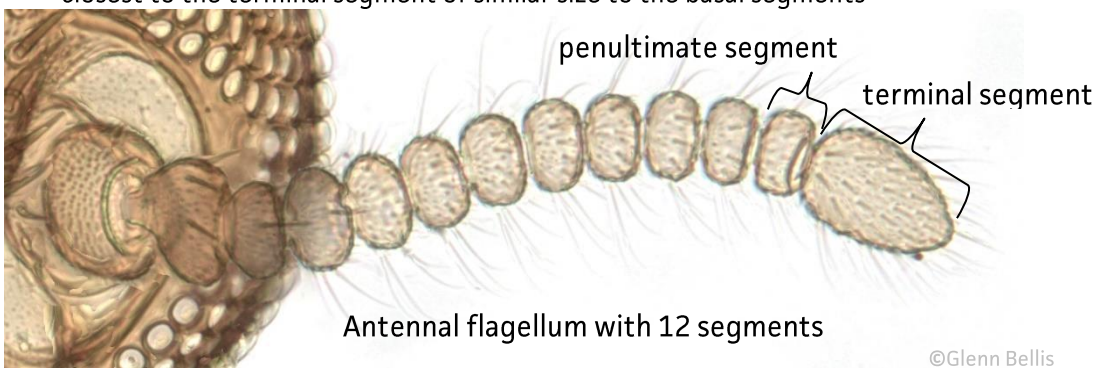


Non-*Culicoides* with terminal nipple
Source: Glenn Bellis

Culicoides do have the apical antennal segment rounded or tapered but without a terminal nipple

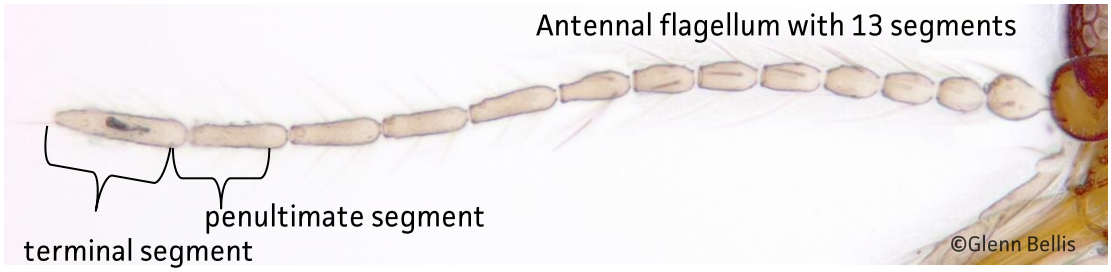
Culicoides without terminal nipple
Source: Glenn Bellis

3. *Culicoides* do not have the female antennal flagella with less than 13 segments, or have the four closest to the terminal segment of similar size to the basal segments



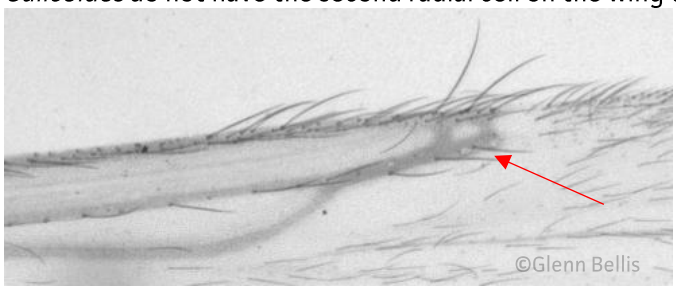
Non-*Culicoides* female antennal flagella
Source: Glenn Bellis

Culicoides do have the female antennal flagella with 13 segments and the four closest to the terminal segment are each longer than the 8 basal segments



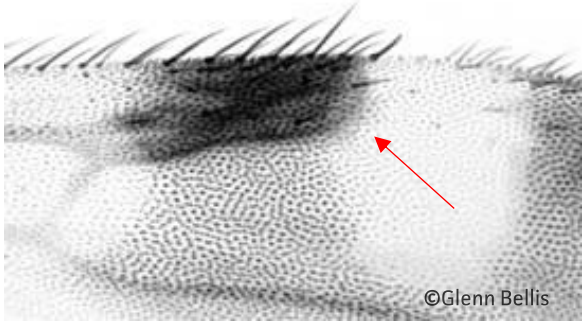
Culicoides female antennal flagella
Source: Glenn Bellis

4. *Culicoides* do not have the second radial cell on the wing truncate apically



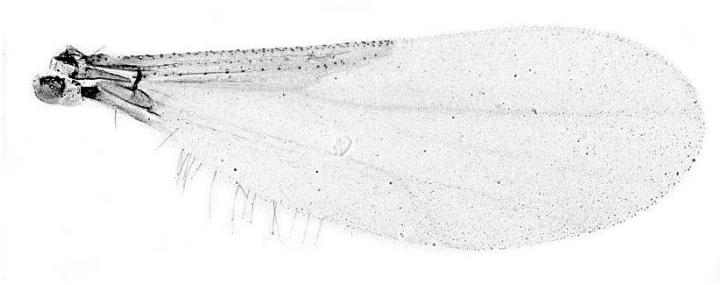
Non-*Culicoides* wing
Source: Glenn Bellis

Culicoides do have the second radial cell on the wing rounded apically



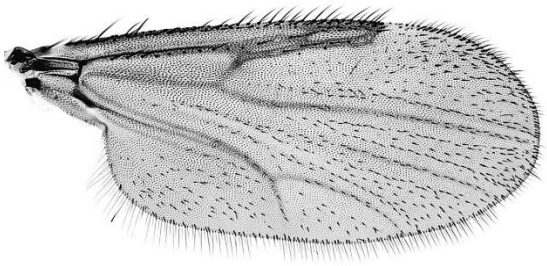
Culicoides wing
Source: Glenn Bellis

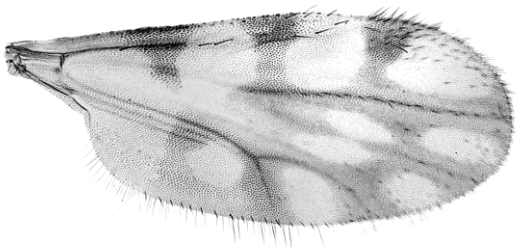
5. *Culicoides* do not have entirely transparent wings



Non-*Culicoides* wing
Source: Glenn Bellis

Culicoides do have grey wings, most species also have pale patches against the grey background



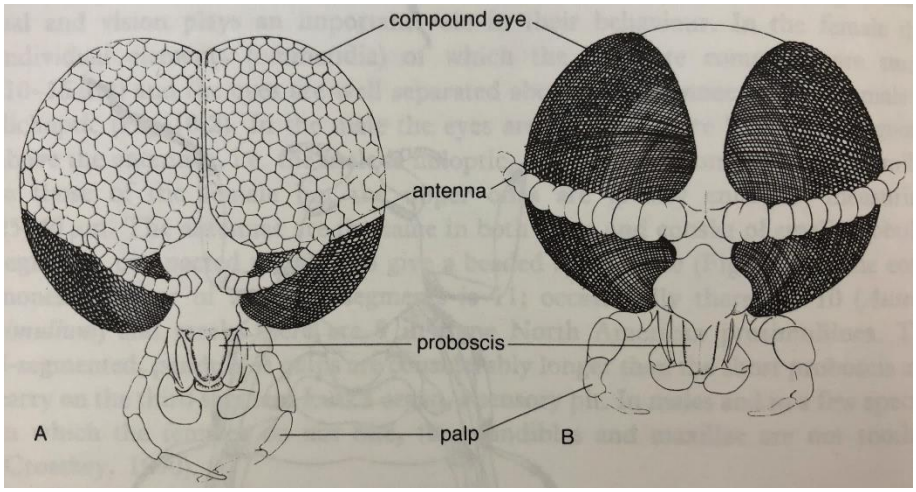


Culicoides wings
Source: Glenn Bellis

GN_03: Key characteristics of blackflies

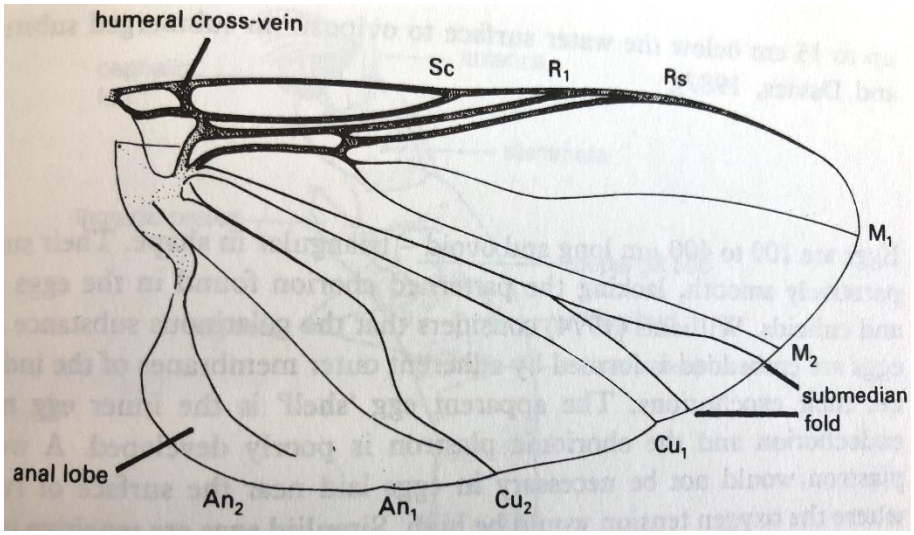
Adult blackfly characteristics

1. Generally small (≤ 4 mm)
2. Stocky
3. Hump-backed
4. Short antennae

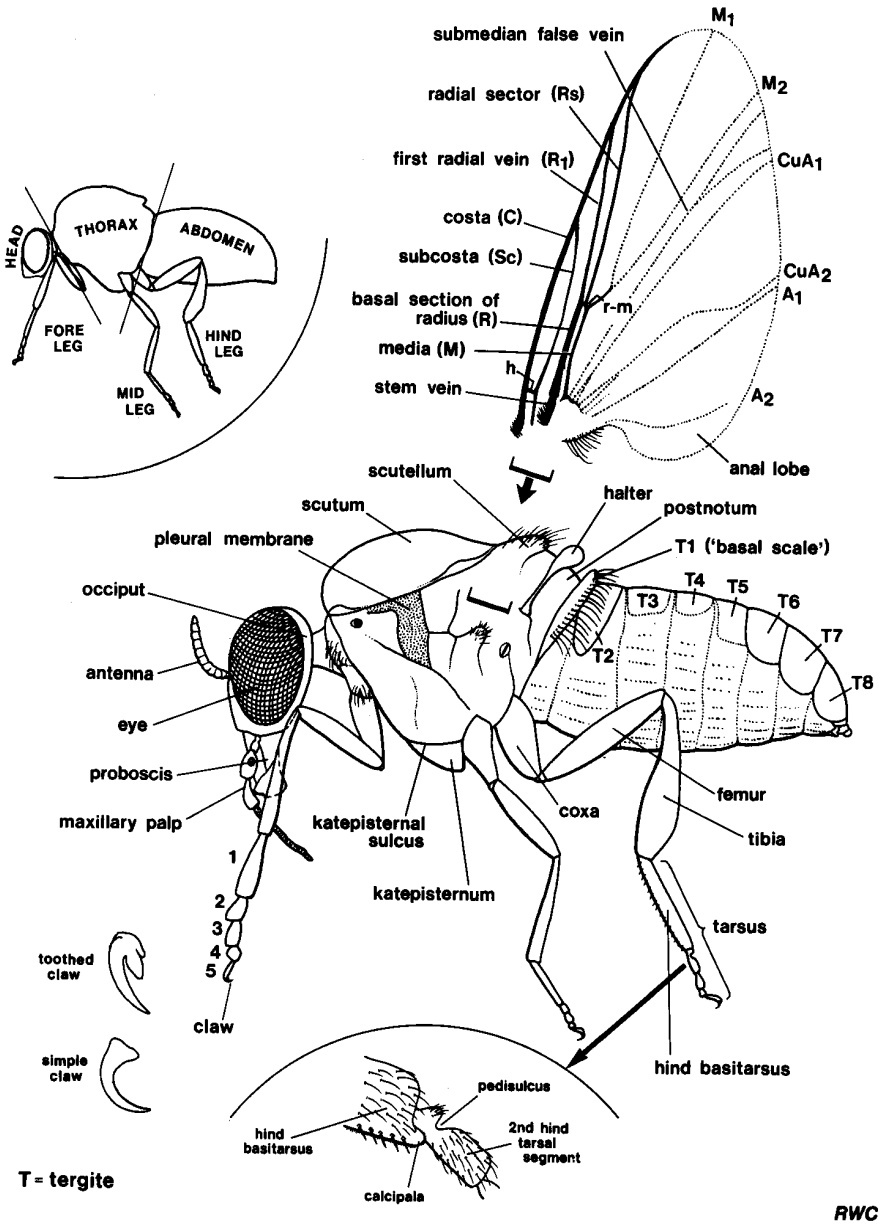


Front view of the heads of (A) male and (B) female *Simulium*
Source: Kettle (1995)

5. Ocelli absent
6. Wings broad at base, narrowing distally, anterior veins (costa, sub-costa and radius) heavy and remaining veins weak



Wing of female *Simulium*
Source: Kettle (1995)



Basic morphology of adult female *Simulium*
Source: Crosskey (1990)

Blackfly egg characteristics

1. Imperfectly ovoid, sub-triangular, in shape without floats
2. Shell surface smooth, micropyle at narrower end
3. Length 0.1 - 0.5 mm

Blackfly larva characteristics

1. Elongate and wormy with two pseudopods (prolegs), one mid-ventral on thorax other terminal on abdomen each with circlet
2. Head prognathous with pairs of cephalic fans, mandibles, and maxillae
3. Later instars with visible histoblasts (developing pupal gills), wing buds and leg buds
4. Abdomen feebly segmented
5. Trilobed rectal organ
6. Mature larvae 3.5 – 12 mm long

Blackfly pupa characteristics

1. Sheathed in cocoon, open at head end
2. Head flexed beneath thorax, face horizontal
3. Thorax convex dorsally, flattened ventrally with pair of pupal gills rooted antero-dorsally and usually with many branches
4. Abdomen elongate with 9 segments and has onchotaxy of hooks or tubercles on at least some of mid-segments
5. Length 2 – 7 mm

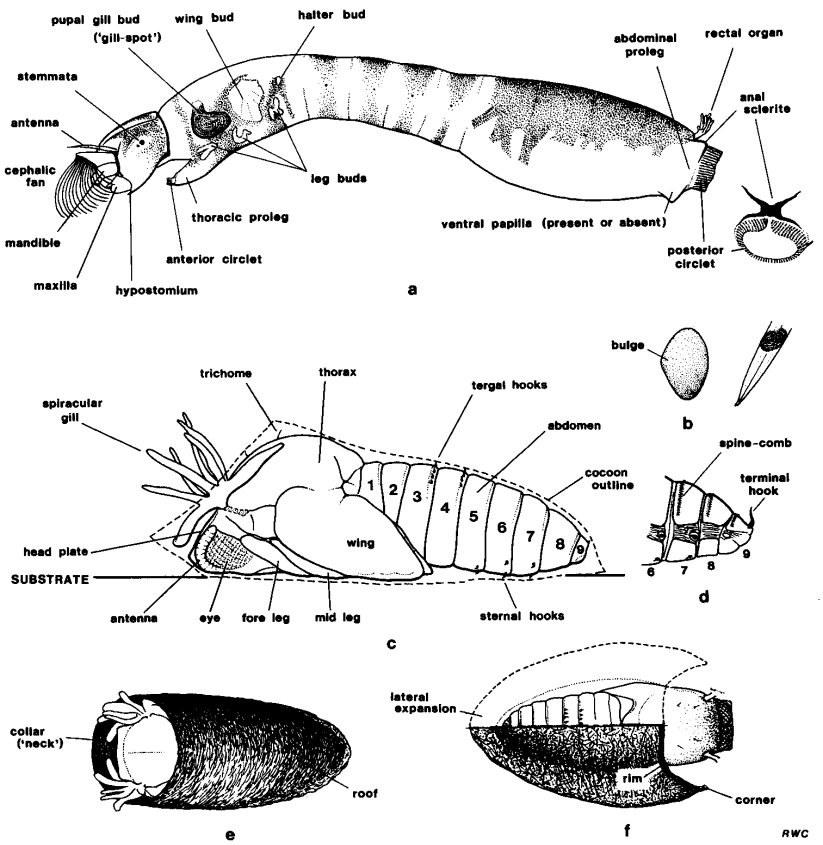


Figure 6.1 Basic morphology of the immature stages of Simuliidae: (*a*) mature larva in left side view (with apex of the abdomen in dorsal view); (*b*) egg, showing typical bulged shape (with small group of layered eggs on leaf tip); (*c*) pupa in left side view with outline of cocoon shown by pecked line (drawn from *Simulium lineatum*); (*d*) tip of pupal abdomen of *Prosimulium*, showing long terminal hooks; (*e*) cocoon of shoe-shaped type, dorsal view (collar present); (*f*) cocoon of slipper-shaped type, dorsal view (collar absent).

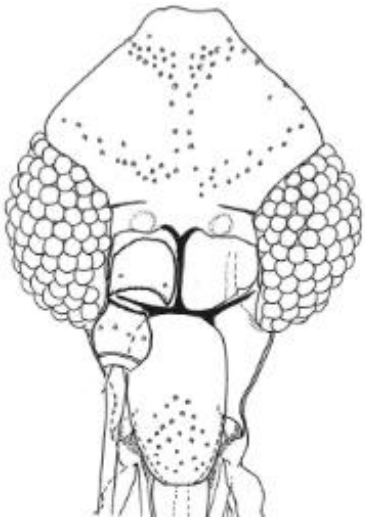
Source: Crosskey (1990)

GN_04: Key characteristics of Phlebotomine sandflies

Distinguishing the subfamily Phlebotominae

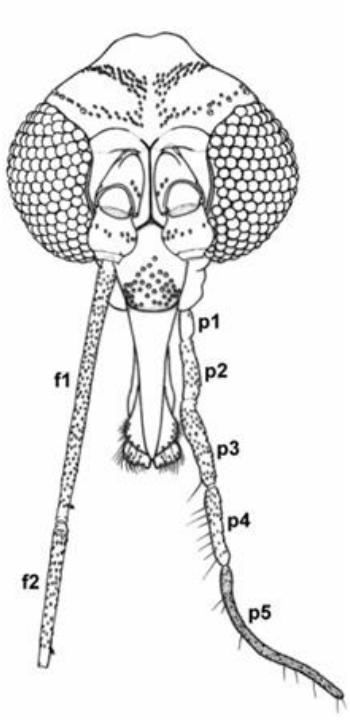
The Phlebotominae are a subfamily of the family Psychodidae, they have the following characteristics:

1. Phlebotominae have the absence of an eye bridge (eyes are separated).



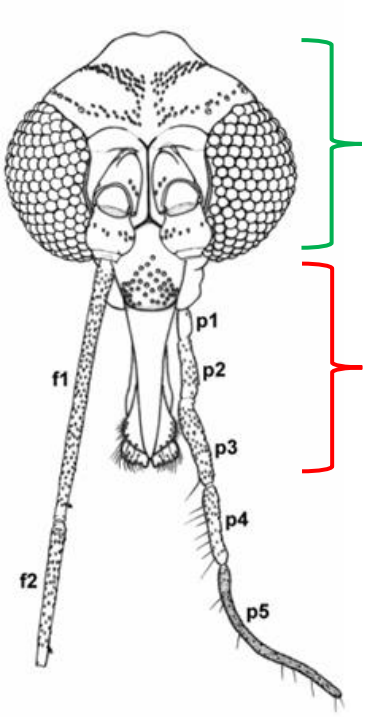
Sandfly (*Lutzomyia tejadai*) with separated eyes.
Source: Galati *et al.* (2017).

2. Phlebotominae have five-segmented palps



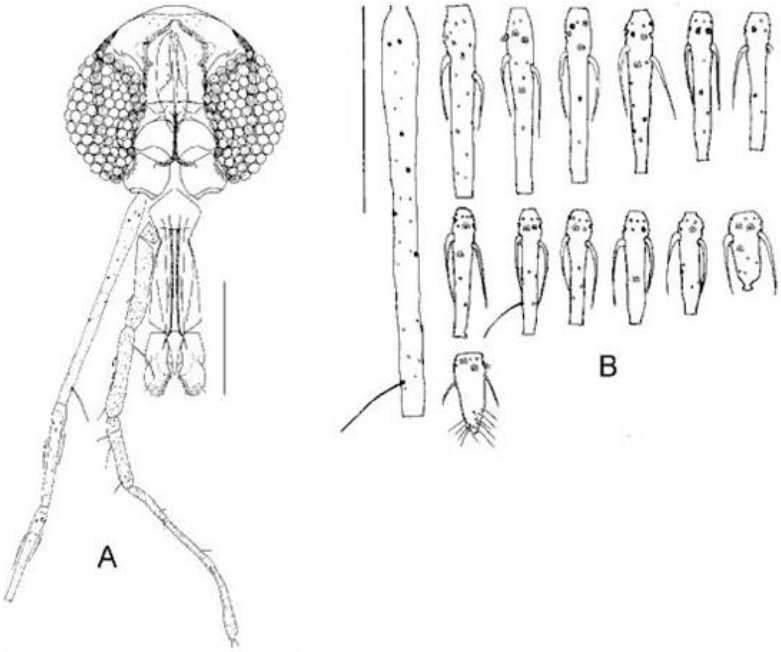
Sandfly (*Lutzomyia olmeca nociva*) with five-segmented palps
Source: Curler (2011)

3. The mouthparts of Phlebotominae are at least as long as the head



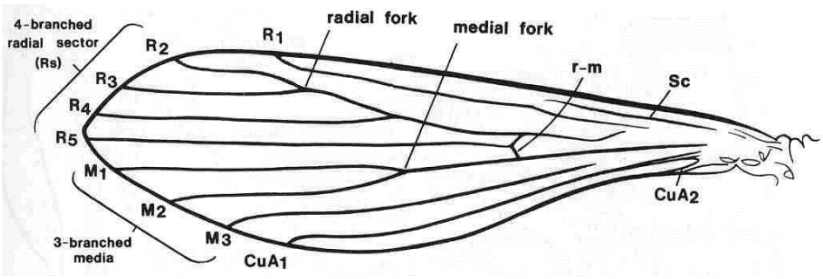
Sandfly (*Lutzomyia olmeca nociva*). Mouthparts (red) are at least as long as the head (green).
Source: Curler (2011)

4. The antennal segments of Phlebotominae are almost cylindrical

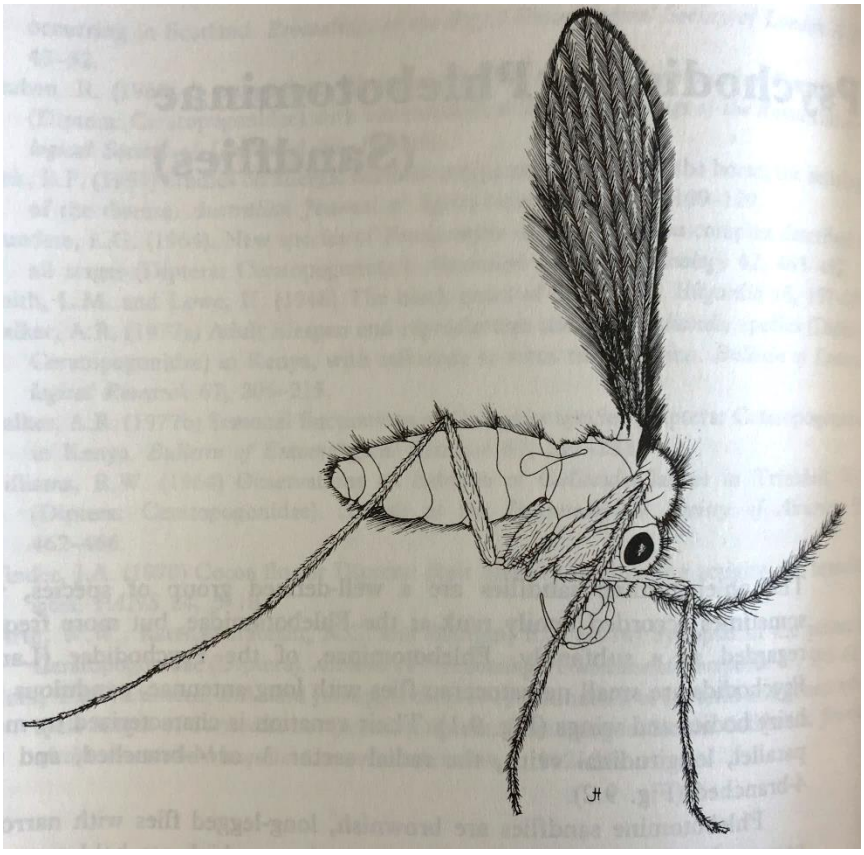


Sandfly (*Lutzomyia falquetoi*). A: head. Bar: 0.2mm; B: antenna segments.
Source: Curler (2011)

5. Phlebotominae have five-branched radial veins on their wings.



Sandfly wing with five-branched radial veins: R1-R5
Source: Lane & Crosskey (1993)



Lateral view of a female Phlebotomus.
Source: Kettle (1995)

GN_05: Sticky trapping

When using this protocol, the following should be referenced:
Cameron MM *et al.* (1994).Comparative activity of phlebotomine sandflies in different crops in the Peruvian Andes. *Bull ent Res*, 84: 461-7.
Cameron MM *et al.* (1995). An association between phlebotomine sandflies and aphids in the Peruvian Andes. *Med Vet Entomol*, 9: 127-32.
Cameron MM *et al.* (1995). Sugar meal sources for the phlebotomine sandfly Lutzomyia longipalpis in Ceará State, Brazil. *Med Vet Entomol*, 9: 263-72.

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A. Selecting Sentinel Houses for Sticky Trap collections

One of the commonest methods for collecting sandflies during ecological or epidemiological studies is the use of sticky traps – sheets of paper coated in castor oil which intercept flying sandflies. These studies require many traps and it is very important that they are labelled correctly so that the site and placement of collections can be identified.

It is very important that the sticky traps are placed in the same houses whenever a village is sampled so that fair comparisons can be made over time. Note that sticky trap sampling is quite labour-intensive so less houses will be sampled by this method than for test tube and aspiration methods. Realistically, each field collector should be able to place 8 traps in each of 4 houses (2 mixed dwelling and 2 houses without cattle), so 12 mixed dwelling and 12 houses without cattle need to be selected from each village.

1. Maps of each village under surveillance should be generated, indicating the positions of houses (brick or thatched), mixed dwellings, cattle sheds and other buildings of interest (schools, poultry houses etc).

2. Using the maps constructed, count how many houses/mixed dwellings there are of each type and record whether they are constructed from brick, straw etc.

3. Allocate a code and number to each house/mixed dwelling e.g. if there are 146 houses, they can be labelled HS001 through to HS146, and if there are 122 mixed dwelling, they can be labelled MD001 through to MD122.

4. Using a random number generator to select 12 houses/mixed dwellings of each type. For example, use the random generator provided at: https://www.random.org/

5. Use the same procedure to generate new numbers for the next village to be sampled.

B. Preparing & Placement of Sticky Traps

Equipment required:

- Plain A4 photocopying paper (need 192 sheets per village per night for sticky traps + 192 sheets for separating the sticky traps the following morning.
- Pencils
- Paintbrushes (around 10 cm diameter)
- Castor oil (need approx. 1.5 litre of clear castor oil for 400 sticky traps).
- 12 Storage boxes for transporting sticky traps (one per field assistant + spares) – need to have lids and need to be larger than A4 dimensions and deep enough to hold 64 sheets of paper.
- String/clips for positioning traps

Method:

1. Label a clean A4 sheet of paper using a pencil with the following information
   a) Village Code
   b) House Code
   c) Trap Placement: e.g. D (for wall of house with door), L (for wall of house left to door) etc, also if it is placed outside (OUT) or inside (IN)
   d) Date (Write date when traps are set up [evening])

2. Using a large paintbrush (e.g. 10 cm wide), brush castor oil on both sides of the paper. Make sure that both sides are coated evenly all over the surface of the paper.

3. Repeat so that each house to be sampled has 8 sticky traps (4 inside, and 4 outside). Place all of the traps for one house on top of each other and store in a polythene box with a lid (the box has to be bigger than the A4 sheets of paper so that they can be stored flat).

4. Each field collector should have a separate box to hold all of the sticky traps that he needs for the 4 houses that he will visit (2 mixed dwellings, and 2 houses). Therefore, he will need 32 traps in total (8 per house x 4 houses). The box needs to be deep enough to store the traps (and the blank sheets of paper he will place to separate sticky traps when he collects them the following morning).

5. Position traps either outside of the house or inside of the house according to the code written on the trap, Select an opening (e.g. window, or eave) if possible.

6. Hold the sticky trap firmly in place using either string/clips as appropriate.

7. You need to fix 8 traps per house (4 outside, and 4 inside: 1 on each wall: door, left, right, back).

8. On the following morning, collect all traps. Make sure that there is a blank sheet at the bottom of the box, and place a blank sheet of paper between each trap.

9. Cover the box with the lid as soon as you have placed the traps inside the box (to prevent any flies in flight from landing on the traps).

C. Processing Sticky Trap Collections

Equipment required:

- Soap detergent solution
- Entomological paintbrushes (small)
- Petri dishes for processing (can be re-used)
- Small polythene tubes (eppendorfs/Nunc tubes) – allow for 96 tubes/village/night
- Parafilm (to seal tubes)
- Permanent marker pens
- Holders for keeping tubes upright (e.g. polystyrene boxes)
- 70% ethanol

Method:

1. Make a 20% soap detergent solution (e.g. 50 ml detergent + 200 ml water – mix well).

2. Using a small paintbrush, pick off all the insects from both sides of one sticky trap and place into a labelled Petri dish (or similar container) containing 20% soap detergent solution (to remove oil). Note that the label should contain the village code, house code, where the trap was placed, and date of collection.

3. Cover the container (when you are not examining it under a microscope) to stop any insects flying in the laboratory from landing inside).

4. Note that traps contain insects other than sandflies. Your first step is to separate biting insects, which may be of medical importance, from non-biting insects. Examine the specimens under the dissecting microscope and separate biting insects (those with piercing/chewing mouthparts) from non-biting insects (discard non-biting insects).

5. Separate the biting insects into vector groups: mosquitoes, midges, and sandflies.

6. Record the number, sex and status (unfed, blood-fed, gravid) of the specimens of interest.

7. Transfer the specimens of interest to a Petri dish containing water and wash for 5 minutes – repeat twice.

8. For storage, place all of the specimens of interest from one sticky trap collection in a tube containing 70% ethanol. Make sure that the tube is labelled (with a permanent marker pen) and has the same code as the petri dish (contains the village code, house code, where the trap was placed, and date of collection). If available, place parafilm around the top of the tube to prevent evaporation of the alcohol or spillage.

GN_06: Slide mounting

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

Equipment Required

- Stereomicroscope (x10 – x40 magnification)
- Timer
- Watchmaker No. 3 fine-point stainless steel forceps
- Pipette (20-200 µl)
- 2x microscalpel (e.g. Interfocus Micro Knife Angled 22.5˚ (part number 10316-14)
- Angular or straight needle (optional)
- 6 Glass and/or plastic 250-500 ml bottles (50:50 clove oil : ethanol mix must be kept in a glass bottle, ethanol dilutions can be kept in plastic or glass bottles)
- 1-2 mm outside diameter glass rod between 5-10 cm long, toothpick or other suitable implement

Consumables Required (Figure 1)

- 76 mm x 26 mm x 1 mm ground edge high-optical clarity microscope slides
- 5-10 mm wide round or square No (0.13-0.16 mm thick) or No. 0 (0.08-0.10 mm thick) glass coverslips
- Slide labels
- Solvent resistant fine-point pen
- Paper towel
- Reaction tubes, PCR plate micro centrifuge tube or other suitable container
- 1ml glass bijous or other tube/container compatible with clove oil
- No.1 fine paintbrush (optional)

Reagents Required

- Euparal mountant
- Analytical grade 100% ethanol
- Autoclaved distilled water
- Clove oil
- 10% (w/v) or 2 M acetic acid Solution (optional only if additional specimen clearing required)
- 10% (w/v) potassium hydroxide solution (optional only if additional specimen clearing required)

Source. Equipment, consumables and reagents required (*stereo microscope, 10% (w/v) or 2 M acetic acid solution and 10% (w/v) potassium hydroxide solution not shown)

Preparation

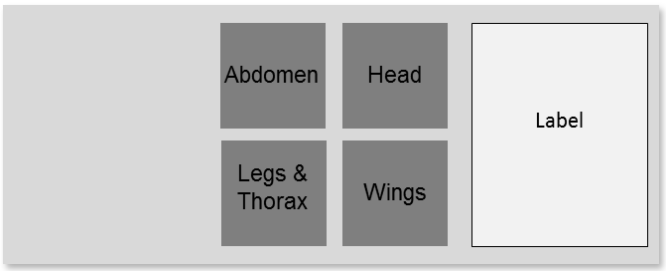
1. Prepare stock of 50%, 70%, 80% and 90% dilutions of ethanol (dilutions made with sterile distilled water).

2. Prepare specimens for mounting by incubating specimens individually in 200 µl tissue digest solution as per steps C1 through C14 of protocol GN_08 (non-destructive DNA extraction of Dipteran specimens).

3. Under a stereomicroscope at between 10-40x magnification, place the specimen to be mounted in a drop of ethanol on a petri dish or microscope slide. If further clearing of the specimen is required follow method A, if no further clearing is required follow method B (typically specimens cleared by incubating in tissue digest solution as per protocol GN_08 do not require additional clearing).

A. IF CLEARING IS NEEDED

1. Label a clean microscope slide with the following information:
   a) Species (if known)
   b) Sex (Male; Female)
   c) If female, the status (Non-Pigmented; Pigmented; Blood-fed; Gravid)
   d) Geographical coordinates of collection site (Latitude; Longitude)
   e) Type of trap used to make collection
   f) Name and affiliation of identifier
   g) Type of mounting media used
   h) Sample ID.



Source. Standard layout for slide mounted specimens

2. Under a stereomicroscope approximately 10-15x magnification, using the toothpick/glass rod or another suitable implement place one drop of Euparal on the microscope slide at the bottom right corner of a square approximately 1.5 cm wide on the central area of the microscope slide.

3. Prior to further clearing the specimens wings must be removed and mounted as they are too delicate to survive intact additional cleaning). Hence, in a drop of 50% clove oil 50% ethanol solution to the right hand side of the drop of Euparal position the specimen dorsal side up and starting with the left wing using the microscalpel separate the wing from the thorax and place it dorsal side up in the centre of the top half of the bottom right-hand square of Euparal i.e. with the basal potion of the wing to the right. Repeat for the left wing placing it dorsal side up below the left wing in the bottom half of the bottom right-hand square of Euparal i.e. with the basal portion of the wing to the left.

4. Using your toothpick/glass rod or another suitable implement, spread the Euparal drop until it is very thin and covering an area about the size of the cover slip. Using forceps select a cover slip and ease the cover slip down from right to left over the coverslip. Without crushing the specimen, gently press the coverslip to flatten. Minor adjustments to the position of the specimen under the coverslip can be made by gently tapping in the appropriate area; the vibrations in the Euparal will help the specimen flow away from the source of the tapping.

5. To complete clearing, place the remaining part of the specimen (now minus its wings) in a 500 µl microcentrifuge or other suitable container with 200 µl 10% KOH overnight (approximately 16 hours). Label the tube/container with the appropriate Sample ID using a solvent resistant pen.

6. Neutralise the KOH by moving the specimen to a new 500 µl microcentrifuge tube or other suitable container containing 200 µl of 10% acetic acid for 30 minutes. Label the tube/container with the appropriate sample ID using a solvent resistant pen.

7. Proceed with protocol B step 8.

B. AFTER CLEARING OR IF NO CLEARING IS NEEDED

1. Label a clean microscope slide with the following information (as above):
   a) Species (if known)
   b) Sex (Male; Female)
   c) If female, the status (Non-Pigmented; Pigmented; Blood-fed; Gravid)
   d) Geographical coordinates of collection site (Latitude; Longitude)
   e) Type of trap used to make collection
   f) Name and affiliation of identifier
   g) Type of mounting media used
   h) Sample ID.

2. Dehydrate the specimens to be mounted individually in a series of ethanol washes at 50%, 70%, 80%, 90% and 100% ethanol for 60 minutes per concentration if mounting directly after removing from a tissue digest solution (Protocol GN_08). If specimens have been stored in 70% ethanol following treatment in tissue digest solution (Protocol GN_08), only ethanol washes at 80%, 90% and 100% for 60 minutes per concentration are required. Use 200 µl of each ethanol concentration in a reaction tube, microcentrifuge tube, microplate or any other suitable container. Label tube/container appropriately with sample ID using solvent resistant pen.

3. Transfer specimens individually to a glass tube/container containing 200 µl of a 50:50 solution of clove oil : 100% ethanol for 60 minutes. (clove oil will 'melt' the majority of plasticwares e.g. Elisa microplates, in general PCR plates have been found to be compatible with clove oil however, before use check the plasticware is compatible with clove oil) Label tubes/containers with appropriate sample ID's using a solvent resistant pen.

4. Transfer specimens individually to a tube/container containing 200 µl clove oil for at least three days. Label tubes/containers with appropriate sample IDs using solvent resistant pen.

5. Under a stereomicroscope at between 10-15x magnification, using the glass rod place four drops of Euparal on the microscope slide at the corners of a square approximately 1.5 cm wide on the central area of the microscope slide.

6. Transfer the individual specimen to be mounted from the clove oil, briefly touching the individual to a clean paper towel to remove excess clove oil, and place in the lower left drop of Euparal on the appropriate microscope slide.

7. In the lower left drop of Euparal position the specimen dorsal side up and starting with the left wing using the microscalpel separate the wing from the thorax and place it dorsal side up in the centre of the top half of the bottom right-hand square of Euparal i.e. with the basal portion of the wing to the right. Repeat for the right wing placing it dorsal side up below the left wing in the bottom half of the bottom right-hand square of Euparal i.e. with the basal portion of the wing to the left.

8. With a micro-scalpel remove the head and place it in the centre of the upper left-hand square of Euparal, with the antennae to the left. Specimens cleared using tissue digest solution (Protocol GN_08) cannot support the weight of a cover slip so place 3 or 4 small pieces of broken cover slip around the head to support the cover slip.

9. Reposition the remainder of the specimen and either:

    a. Using the microscapel place the abdomen ventral side up at the centre of the top left square of Euparal, reposition if required and mount the remainder of the thorax and legs laterally in the bottom left-hand square of Euparal.

    Or

    b. With extreme care (and only if the mesonoteum is required for taxonomic purposes) position the remainder of the specimen ventral side up and using the microscalpel slice off the mesonotum together with the scutellum to separate the abdomen and place the abdomen ventral side up at the centre of the top left square of Euparal. Leave the mesonotum, dorsal side up, with the rest of the thorax and legs in the bottom left-hand square of Euparal.

10. Check the Euparal has not set too much in any of the four areas, add more using the toothpick/glass rod or other suitable implement before placing coverslips if required. Coverslips may also be applied immediately after each section is dissected and positioned, this is recommended if drying of the Euparal is experienced due to time take to dissect/mount other portions of the specimen.

11. Using forceps select a cover slip and starting with the upper right-hand drop of Euparal ease the cover slip down from right to left, so that the antennae flow out and away from the head to ease identification. Without crushing the specimen, gently press the coverslip to flatten. Minor adjustments to the position of the specimen under the coverslip can be made by gently tapping in the appropriate area; the vibrations in the Euparal will help the specimen flow away from the source of the tapping. Repeat for the remaining two drops of Euparal.

12. Reduce the magnification so that all four coverslips can be easily seen and check there is sufficient Euparal to cover to the edges of the coverslip if not use a glass rod with a very small amount of Euparal on the end to carefully touch the edge of the coverslip, capillary action will pull the Euparal under the coverslip.

13. Clean any excess Euparal from the slide using a fine paintbrush and 100% Ethanol.

14. Position the slide horizontally and allow slides to dry/set completely before handling. Check the slides about one week after mounting to ensure the Euparal has not receded from the edges of the coverslips. If shrinkage of the Euparal has occurred, add a small volume of fresh Euparal to the edge of the affected cover slip using a toothpick/glass rob (or other suitable implement) and capillary action will draw the Euparal into the gap, recheck the affected slides again in a about one week, repeat if required.

15. For storage place the slide in the appropriate cardboard slide tray and place the slide tray back in its box. Ensure the slides are stored in the dark (i.e. the slide tray is kept in its box) and flat, as the Euparal may take several weeks to fully harden). Once slide are completely dry and hardened slides may be transferred to slide storage boxes where they are stored vertically.

16. Check slides about 1 week after mounting to ensure Euparal has not receded from the edges. If this occurs, add some fresh Euparal to the edge of the cover slip and capillary action will draw the Euparal into the gap.

Tips & Troubleshooting

- Regularly wipe forceps, microscalpels etc to prevent build-up of Euparal while mounting specimens.
- If labels must be hand-written two labels, one either end of the slide, may be used to allow all the required information to be included. Round coverslips can be easier to achieve an edge to edge coverage of Euparal than square coverslips.
- Labels with permanent adhesive should be used, if non-laminated paper labels are used they can be sealed by brushing a fine layer of PVA adhesive over the label(s) and at the edges of the label(s).
- If pre-cut 5 to 10 mm square coverslips are not available, larger coverslips may be cut using a diamond knife to the required size. Round coverslips can be easier to achieve an edge-to-edge coverage of Euparal than square coverslips.
- If the glass rods for dispensing mounting media are not available, toothpicks or the plunger of a 1 mm syringe (with the rubber stopper removed) can be used.
- Unlike in other mounting media types small air bubbles trapped in slide preparations are absorbed by the Euparal during drying, although this may take several days.
- Significant shrinkage of Euparal may occur during drying. In moderately thick preparations, this results in shrinkage away from the edges of the cover slip. This is counteracted by using a wider area of mountant and a slightly larger 10 mm cover slips when Euparal is used in comparison to when specimens are slide mounted with Canada Balsam (where 7 mm diameter coverslips are typically usually used), but an excess of any medium around the edge of the cover slip is undesirable. It may also be necessary to top up the Euparal mountant where required as described in step 17.
- Remounting of specimens should be avoided wherever possible, however, if required, Euparal mounted specimens may be removed by soaking in absolute Ethanol, Euparal essence or xylene.
- The Euparal mounting medium is relatively fast-drying. Allowing the slide to remain overnight in a drying oven set at about 35° C, over a slide hot plate or in the open at room temperature for a few days will yield usable and permanent preparations. Slides should however always be stored horizontally at least for the first few months to prevent slippage of any media which has not fully set. The drying progress can be checked by very lightly pressing on the centre of the coverslip and watching to see if there is any movement of the specimen in the medium.
- Purchase pre-washed/pre-cleaned microscope slides to limit the dirt, dust etc on slides, a microfiber cleaning cloth or propan-2-ol (isopropanol) applied using a lint-free foam/cotton swabs may be used to further clean slides as required.

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

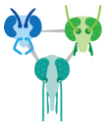
D.1 Results and sample details should be recorded in the appropriate laboratory notebook.

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.

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.

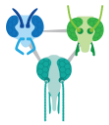
C.8 Dipteran Samples:

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.

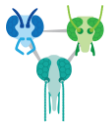
C.20 Pipette the mixture including any precipitate from each tube into the relevant DNeasy® spin column. Use a new tip per tube to prevent cross-contamination, discard used tips.

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).

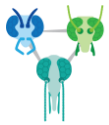
C.38 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential DNA contamination.

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

GN_10: PCR Amplification of the DNA Barcode Segment of the mitochondrial DNA Cytochrome Oxidase Subunit I (COI) gene

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 amplification of the DNA Barcode (Hebert *et al.* 2003) segment of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COI) gene from genomic DNA (gDNA) or total nucleic acid extractions.

B. Materials required

Equipment

- PCR laminar flow cabinet (or microbiological safety cabinet Class II) (ideally two cabinets, to allow separation of mastermix preparation and template addition activities)
- Thermal cycler
- Fridge (+4°C)
- Freezer (-20°C)
- Reaction tube spinner, plate spinner, or centrifuge with plate rotor (appropriate to the plasticware to be used)
- Vortex
- Pipettes (0.1-2.0 µl, 1-20 µl, 20-200 µl, 100-1000 µl)
- Multichannel pipette (5-50 µl) (optional)
- Disposable pipette tips containing hydrophobic filters (2 µl, 20 µl, 200 µl, 1000 µl)
- Thin-wall reaction tubes as individual tubes, 8 or 12 tube strips or PCR plates plates (of an appropriate size (e.g. 200 µl) and type for the brand and model of thermal cycler to be used (see the thermal cycler's manufacturers guidance for further information on compatible plasticware)
- For primers: 1.5 ml screw cap or flip-top (e.g. Eppendorf® Safe Lock Tubes #0030 123.328) microcentrifuge tubes
- For mastermix preparation: 1.5 ml flip-top microcentrifuge tube or 15 ml centrifuge tubes (dependent upon volume of mastermix required to be prepared)
- PCR adhesive plate sealers (*only if using 96-well PCR plates)
- Solvent resistant marker pen
- Blue roll
- Label printer (*optional)
- Microcentrifuge tube labels
- Solvent resistant cryopen
- 96 well PCR tube rack
- Microcentrifuge tube rack
- Low volume reagent reservoir (or equivalent)
- Benchtop cooler for microcentrifuge tubes or ice bucket & ice (optional)

Reagents

- DNAZap™ (AM9890, ThermoFisher Scientific, UK) (or equivalent)
- Nuclease free water (NFW)
- 1 M Tris-HCL pH 8.0 (e.g. ThermoFisher Scientific, #15568025)
- Forward & reverse primers (see Table 1 & 2)
- Selected Mastermix Components (see below)

Platinum™ Taq Mastermix

- 10 mM dNTP Mix (e.g. D7295 Sigma, UK; 18427-088: ThermoFisher Scientific, UK)
- Platinum™ Taq DNA Polymerase (kit contains Platinum™ Taq polymerase, 10x reaction buffer and 50 mM Magnesium Chloride ($MgCL_2$) (10966018 120rxs; 10966026 300rxn; 10966034 600 reactions: ThermoFisher Scientific, UK)
- Bovine serum albumin (BSA) (20mg/ml molecular grade ultrapure non-acetylated) (e.g. 10711454001: Sigma Aldrich)

TopTaq Mastermix

- TopTaq mastermix kit (Qiagen: 200403)

C. Method

C.1 Review Table 1 and select the appropriate primer set, mastermix and thermal profile for the target taxa.

C.2 Ultraviolet (UV) sterilise the PCR laminar flow hood (or microbiological safety cabinet (MSC)) which is designated for mastermix preparation and if required also clean the working area, pipettes etc. with DNAZap™ (or equivalent). Once cleaned complete the following steps within the PCR laminar flow hood (or MSC).

C.3 Prepare a working stock of 10 mM Tris-HCL pH 8.0 (e.g. for 50 ml add 500 µl 1M Tris-HSL pH 8.0 to 49.5 ml nuclease free water), label with the name and concentration of the solution, your initials and the date made [store at 4°C when not in use].

C.4 Resuspend lyophilised primers to 100 µM with 10 mM Tris-HCL pH 8.0 using the volume indicated by the supplier (10 µl 10 mM Tris-HCL pH 8.0 per 1 nmole of primer e.g. 36.4 nmole primer add 364 µl 10 mM Tris-HCL pH 8.0 to each lyophilised primer and vortex briefly). Store at 4°C for approximately 20 minutes to allow primers to fully resuspend.

C.5 Make a working stock of each primer at the concentration required by the mastermix by briefly vortexing resuspended 100 µM primers, then make an appropriate dilution in a second appropriately labelled 1.5 ml microcentrifuge tube [store both re-suspended 100 µM primer and working stock 10 µM primer at -20°C when not in use]. For example:

- For 100 µl of 10 µM working primer stock add 10 µl re-suspended 100 µM primer to 90 µl 10 mM Tris HCL pH 8.0).

C.6 Appropriately label individual reaction tubes, tube strips or PCR plates so that tubes can be linked to each specimen/samples' unique identifier code (ensure plasticware is appropriate for the thermal cycler to be used).

C.7 Thaw if needed, then vortex briefly forward and reverse primers and other mastermix components. The mastermix components used in this protocol do not need to be prepared over ice.

C.8 In a microcentrifuge or centrifuge tube as appropriate to the volume require, make sufficient of the selected mastermix for the number of wells required for samples and positive and negative controls (volume per reaction x number of reactions required +10% (to account for wastage during pipetting, use of reagent reservoirs etc.)).

C.9 Vortex the mastermix and aliquot 20 µl into each well. For large numbers of wells, distribute the mastermix into a low volume reagent reservoir, eight-well tube strip or other appropriate plasticware and use a multichannel pipette to distribute the mastermix.

C.10 If separate mastermix preparation and template addition areas/hood are not available proceed directly to step C.11, if separate areas are available seal tubes/plates containing mastermix and transfer the plate/tubes containing mastermix to the designated template addition area then proceed to step C.11.

C.11 Thaw if needed, then briefly gently mix/vortex DNA extractions (DNA template) (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.12 Using a new pipette tip per well to prevent cross-contamination transfer 5 µl of each DNA template into the corresponding labelled 200 µl reaction tube and if using individual tubes or strips seal securely after each template addition to reduce the potential for cross-contamination.

C.13 Using a new pipette tip per well transfer 5 µl of the nominated positive control sample into the designated positive control well(s), if using individual tubes or strips seal securely after each template addition to reduce the potential for cross-contamination.

C.14 Using a new pipette tip per well transfer 5 µl of nuclease free water into the designated negative control well(s). If using individual tubs or strips of tubes seal securely after the addition of nuclease free water.

C.15 If using PCR plates seal the plate securely with PCR adhesive film/foil. Or if using individual reaction tubes or tube strips ensure they are all securely sealed.

C.16 Briefly vortex tubes/plate.

C.17 Spin reaction tubes/plate for approximately 10 seconds in the reaction tube spinner / centrifuge to concentrate reaction mix at the base of the wells.

C.18 Place reaction tubes in the thermal cycler and carry out PCR amplification using the thermal profile appropriate to the primers used see Table 1.

C.19 UV Sterilise the PCR Laminar flow hood(s)/MSCs and other working areas used and if required also clean working area, pipettes etc. with DNAZap™ (or equivalent).

C.20 When the thermal cycle is finished, remove reaction tubes from the thermal cycler and store PCR product at 4°C if to be used with 24 hours or store at -20°C until required for downstream analysis.

Table 1 PCR Primer sets for different taxonomic groups

Primer Set Name	PCR Primers		Sequencing Primers		Amplicon size (base pairs-bp) excluding primers	Mastermix & Thermal Profile
	Forward	Reverse	Forward	Reverse		
COIpanCUL	COIpanCULF1_t1	COIpanCULR1_t1	M13F(-21)	M13R(-27)	658 bp	Table 3 or 4
Lep/Fol Cocktail	LCO1490_t1 + LepF1_t1	HCO2198_t1 + LepR1_t1	M13F(-21)	M13R(-27)	658 bp	Table 5 or 6

Table 2 Primer Sequences (_t1 suffix indicates primer has M13 tail, M13F(-21) sequence shown in italics, M13R(-27) shown underlined)

Oligo Name	Sequence (5'-3')	Size (bp)	Reference
Sequencing			
M13F(-21)	TGTAAAACGACGGCCAGT	17	Messing, 1983
M13R(-27)	CAGGAAACAGCTATGAC	17	Messing, 1983
mtDNA COI			
COIpanCULF1_t1	TGTAAAACGACGGCCAGTTChACwAAyCAyAAArwTATTGG	41	Harrup, 2018
COIpanCULR1_t1	CAGGAAACAGCTATGACTAwACyTCdGGrTGICCrAArAATCA	43	Harrup, 2018
LCO1490_t1	TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG	43	Folmer *et al.*, 1994
HCO2198_t1	CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA	43	Folmer *et al.*, 1994
LepF1_t1	TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG	43	Herbert *et al.*, 2004
LepR1_t1	CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA	43	Herbert *et al.*, 2004

Table 3 COIpanCUL Platinum™ Taq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water			14.9
PCR Buffer	10x	1x	2.5
$MgCL_2$	50 mM	1.5 mM	0.75
dNTPs	10 mM	0.2 mM each	0.5
Bovine Serum Albumin	20 mg/ml	0. 2 mg/ml	0.25
Platinum™ *Taq* DNA Polymerase		1 U/rxn	0.1
Forward Primer			
COIpanCULF1_t1	10 µM	0.2 µM	0.5
Reverse Primer			
COIpanCULR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	2 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	50˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Soak	∞	4˚C

Table 4 COIpanCUL TopTaq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water			4.0
TopTaq Mastermix	2x	1x	12.5
CoralLoad Concentrate	10x	1x	2.5
Forward Primer			
COIpanCULF1_t1	10 µM	0.2 µM	0.5
Reverse Primer			
COIpanCULR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	3 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	50˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Soak	∞	4˚C

Table 5 Lep/Fol Cocktail Platinum™ Taq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water (NFW)			13.9
PCR Buffer	10x	1x	2.5
$MgCL_2$	50 mM	1.5 mM	0.75
dNTPs	10 mM	0.2 mM each	0.5
Bovine Serum Albumin	20 mg/ml	0.2 mg/ml	0.25
Platinum™ *Taq* DNA Polymerase		1 U/rxn	0.1
Forward Primers			
LCO1490_t1	10 µM	0.2 µM	0.5
LepF1_ t1	10 µM	0.2 µM	0.5
Reverse Primers			
HCO2198_t1	10 µM	0.2 µM	0.5
LepR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	2 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	46˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

Table 6 Lep/Fol Cocktail TopTaq mastermix and thermal profile

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
Nuclease Free Water (NFW)			3.0
TopTaq Mastermix	2x	1x	12.5
CoralLoad Concentrate	10x	1x	2.5
Forward Primers			
LCO1490_t1	10 µM	0.2 µM	0.5
LepF1_ t1	10 µM	0.2 µM	0.5
Reverse Primers			
HCO2198_t1	10 µM	0.2 µM	0.5
LepR1_t1	10 µM	0.2 µM	0.5

Initial denaturation	3 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	40 seconds	46˚C
Extension	1 minute	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

D. Results

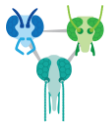
D.1 Where appropriate associated experiment results should be recorded in the relevant laboratory notebook.

D.2 Confirm successful amplification of the DNA Barcode segment of the COI gene via agarose gel electrophoresis on a 2% (*w/v*) agarose gel. If using the 2% 96-well E-Gel™ run the gel for 8 minutes on program EG.

D.3 Successful amplification of the DNA Barcode segment of the COI gene using this protocol is indicated by the presence of a band at the appropriate size for the target amplicon (see Table 1) plus the primer size (see Table 2), results are recorded as the presence or absence of a band at the correct size. No band should be present in the negative control lane(s).

E. Tips and troubleshooting

- Addition of between 1 ng to 1 µg of gDNA template per reaction is optimal, hence depending on the DNA yield of the DNA extractions the volume of DNA template added to each reaction may need to be increased or decreased as required, increasing or decreasing the volume of NFW to ensure the total volume including template is constant at 25 µl to maintain the concentration of other reagents in the mastermix.
- If after the PCR process the samples have appeared to condense, the caps or sealing film may not have been attached correctly or the heated lid may not have been set to be on during the thermal cycle. Correct and repeat as required.
- Platinum™ Taq DNA polymerase (ThermoFisher Scientific) is a recombinant Taq DNA polymerase complexed with an antibody that inhibits polymerase activity at ambient temperature. It is therefore stable for room temperature reaction setup hence reaction setup



over ice is not required, however it should be stored at -20°C when not in use. The polymerase is activated in a temperature-dependent manner (at 94°C) during the start of the PCR thermal cycle, once the antibody is dissociated, the Taq DNA polymerase regains its full activity.

- The TopTaq Mastermix Kit (Qiagen) is a ready to use PCR mastermix, containing PCR buffer, $MgCL_2$ and dNTPs reducing pipetting steps during mastermix setup. The TopTaq master mix may be stored at 4°C, reducing the requirement to thaw reagents prior to use and reactions do not need to be prepared over ice. The TopTaq Mastermix Kit also includes CoralLoad Concentrate a gel loading reagent with two gel-tracking dyes which can be included directly in the PCR mastermix reaction mix, reducing pipetting steps prior to agarose gel electrophoresis.
- This protocol may be adapted for use with a variety of other mastermixes and Taq DNA polymerases from other suppliers. Care must be taken, however, when selecting alternative reagents that cost-savings do not negatively influence the quality of resulting data. We have found that Qiagen TopTaq™ mastermix offers a good compromise between, cost-saving, ease of use and efficient and accurate amplification. The alternative Platinum™ Taq DNA Polymerase together with the inclusion of BSA is preferable with more difficult/low yield DNA templates.
- Bovine serum albumin (BSA) when used as an additive in PCR acts to enhance enzyme activity e.g. Taq and increases PCR yields from low purity templates. As an additive BSA is also helpful in reducing the loss of enzyme through non-selective adsorption to reaction tube walls, pipette tips and other plasticware.
- D-(+)-Trehalose may also be added to the Platinum™ Taq mastermixes listed in Table 3 and 5 at a final concentration of 5% as a PCR enhancer (reduce the volume of nuclease free water included in the reaction to ensure the final reaction volume remains at 25 µl). The addition of D-(+)-Trehalose when used with Invitrogen Platinum™ Taq DNA Polymerase also allows mastermixes to be aliquoted out into PCR plates and frozen ready for use. Ready-made frozen plates of this type should be used within 3 months. D-(+)-Trehalose can reduce the optimal annealing temperature of the primers a gradient PCR should be utilised to check that the inclusion of D-(+)-Trehalose in the reaction mastermix has not decreased the efficiency of the reaction due to changes in the optimal primer annealing temperature.
- The primer pairs listed in this protocol have been found to perform best with a relatively low annealing temperature producing a high abundance of the target PCR product with minimal non-specific amplification. However if non-specific products are observed when PCR products are visualised following agarose gel electrophoresis, try reducing the number of cycles (between 30-40 cycles is suitable for most DNA templates) and/or increasing the annealing temperature.
- The Platinum™ Taq and TopTaq mastermix recipes given in this protocol can be adjusted to include alternative primer pairs (see the literature for relevant primers details). Optimal concentration and annealing temperatures of new primer pairs should be confirmed using positive control samples prior to being utilised with field samples. If alternative primer concentrations are required, the volume of nuclease free water included in the reaction should be adjusted as required to ensure the final reaction volume remains at 25 µl.
- Platinum™ Taq DNA polymerase is also available as a 2x mastermix (13000013 colourless 200 reactions; 13001013 with tracking dyes 200 reactions) which can be substituted at 12.5 µl per reaction for the Platinum™ Taq DNA polymerase, 10x PCR Buffer, 50 mM $MgCl_2$ and the 10 mM dNTP components of the mastermix listed in Table 3 and 5, adjust the volume of NFW to ensure total reaction volume including mastermix, BSA, forward and reverse primers and DNA template remains at 25 µl.

GN_11: Agarose Gel Electrophoresis using SYBR Safe DNA gel stain

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

A. Introduction

This method sheet describes the procedure for the separation and visualization of DNA fragments by agarose gel electrophoresis using SYBR® Safe DNA gel stain.

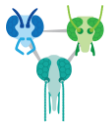
B. Materials

Equipment

- Pipettes (0.2-2 µl, 2-20 µl)
- 50 ml conical flask
- 100 ml measuring cylinder
- Microwave
- Balance (≤0.01 g accuracy)
- Electrophoresis gel tank with power pack
- Electrophoresis gel comb
- Electrophoresis gel tray
- 500 ml wash bottle
- 10 litre carboy or 1 litre glass or plastic bottle for 1x TAE solution
- Transilluminator (both UV transillumination and blue-light transillumination is suitable for use with SYBR® Safe DNA Gel Stain)
- Safe Imager™ viewing glasses (ThermoFisher Scientific: S37103) (or equivalent) (optional if dedicated gel documentation system is used)
- Image capture device
- Heat-proof glove or similar
- Fridge (+4°C)
- Freezer (-20°C)
- Sterile disposable pipette tips containing hydrophobic filters (0.2-2 µl, 2-20 µl)
- Parafilm® or 200 µl reaction tubes (not required if PCR product already contains a loading dye e.g. Qiagen CoralLoad Concentrate)
- Autoclave tape or gel tray end blocks
- Blue roll
- Nitrile Gloves

Reagents

- 50x tris-acetate-EDTA (TAE) (2 M tris-acetate, 0.05 M EDTA, pH 8.3, nuclease free)
- Deionised water (or equivalent) for buffers
- DNA loading dye (e.g. Qiagen Gel Pilot Loading Dye 5x (Qiagen 239901) or Invitrogen™ BlueJuice™ Gel Loading Buffer (10X) (ThermoFisher Scientific: 10816015) (not required if PCR product already contains a loading dye e.g. Qiagen CoralLoad Concentrate)
- DNA Ladder (e.g. E-Gel® Low Range Quantitative DNA Ladder (ThermoFisher Scientific: 12373-031); ThermoFisher Scientific: TrackIt™ 50 bp DNA Ladder (ThermoFisher Scientific: 10488-043); Qiagen GelPilot 100bp Plus DNA Ladder (Qiagen: 239045))
- Agarose for electrophoresis
- SYBR® Safe DNA Gel Stain (ThermoFisher Scientific: S33102)
- DNAZap™ Solutions (ThermoFisher Scientific: AM9890) (or equivalent)



C. Method

C.1 Put on nitrile gloves.

C.2 Use DNAZap™ (or equivalent) to clean all working areas and 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 Prepare 1x tris-acetate-EDTA (TAE) buffer (20 ml 50x TAE per 980 ml deionized water).

C.5 Fill gel tank with sufficient 1x TAE to cover gel.

C.6 Fill labelled wash bottle with 1x TAE buffer.

C.7 Add 3 µl SYBR® Safe DNA Gel Stain to the positive end of the gel tank.

C.8 Select an appropriately sized and percentage gel (see Table 1) to cast.

C.9 Prepare casting tray by sealing ends using autoclave tape (or use appropriate gel tray blocks) and fit the gel comb(s) to the appropriate position(s) in the tray.

C.10 Add the required amount of agarose and 1x TAE to a conical flask (see Table 1) and heat to dissolve agarose in a microwave oven (beware of boiling agarose it can easily overflow and burn, use a heat proof glove or similar when handling the hot conical flask).

C.11 Allow the agarose to cool to approximately 60˚C (so that the conical flask feels warm but not hot to the touch).

C.12 Add SYBR® Safe DNA Gel Stain to a 1x concentration to the melted agarose (e.g. 3 µl SYBR® Safe DNA Gel Stain to 30 ml agarose) and gently swirl solution to mix (avoid making bubbles in the solution).

C.13 Gently pour melted agarose into the prepared casting tray (pop any bubbles in the poured agarose using a pipette tip).

C.14 Allow the gel to set at room temperature for approximately 30 minutes or until set.

C.15 If the PCR product does not already include a loading dye (e.g. Qiagen CoralLoad Con-centrate), add an appropriate amount to a subsample of the PCR product either in separate drops on a sheet of parafilm or in new 200 µl reaction tubes (if the concentration of PCR product is too high reduce the volume added to the dye and replace volume with nuclease free water). For example:

- Qiagen Gel Pilot Loading Dye (contains xylene cyanol (light blue), bromophenol blue (dark blue and Orange G (orange)): add 1 volume Qiagen Gel Pilot Loading Dye 5x to 4 volumes PCR product i.e. for 10 µl wells add 2 µl Qiagen Gel Pilot Loading Dye 5x to 8µl PCR product, to-tal volume 10 µl.
- BlueJuice™ Gel Loading Buffer (10x): add 1 µl BlueJuice™ Gel Loading Buffer (10x) to 9 µl PCR product, total volume 10 µl.

Table 1 Agarose gel concentrations

Percentage of Gel (*w/v*)	Efficient Range of Separation of Linear DNA molecules (base pairs (bp))	Volume of 1x TAE (ml)	Amount of Agarose (g)
0.5	1000-30,000	30	0.15
		50	0.25
		100	0.50
0.7	800-12,000	30	0.21
		50	0.35
		100	0.70
1.0	50-10,000	30	0.30
		50	0.50
		100	1.00
1.2	400-7,000	30	0.36
		50	0.60
		100	1.20
1.5	200-3,000	30	0.45
		50	0.75
		100	1.50
2.0	50-2,000	30	0.60
		50	1.00
		100	2.00

Table 2 Loading dye mobility rates (values indicate the size of DNA fragments with which the dye will co-migrate at that particular gel concentration)

Agarose Gel Concentration (%)	Xylene Cyanol FF	Bromophenol Blue	Cresol Red	Orange G
0.7	8000	600	3000	100
1.0	4000	400	1500	50
1.5	2000	250	900	20
2.0	900	120	300	<10
3.0	400	50	>100	<10

C.16 Reseal tubes containing remaining PCR product immediately and store if required for further applications at 4˚C if to be used with 24 hours otherwise store at -20˚C.

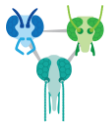
C.17 Remove the autoclave tape or gel tray end blocks from the end of the gel tray and then carefully remove the comb from the gel itself.

C.18 Load the gel with ladder and PCR product by either:

i. Placing the gel on a sheet of blue roll and using the wash bottle wash a small amount of 1x TAE buffer over the surface of the gel to fill the wells. Add an appropriate volume of DNA ladder to at least one well on each roll of the gel (volume dependent upon comb size). Then load one sample per well (use a new tip for each well to prevent cross-contamination) (no not load a sample into the well containing the DNA ladder). Then carefully lower the gel into the electrophoresis tank with the wells at the negative (black) end of the tank (DNA fragments will migrate from negative to positive.

Or

ii. Lower the gel into the electrophoresis tank with the wells at the negative (black) end of the tank (DNA fragments will migrate from negative to positive). Add an appropriate volume of DNA ladder to at least one well on each roll of the gel (volume dependent upon comb size). Then load one sample per well (use a new tip for each well to prevent cross-contamination) (no not load a sample into the well containing the DNA ladder).



C.19 Place the lid on the gel tank and connect all wires required between the power pack and the gel tank.

C.20 Run the gel at approximately 5 V/cm (distance as measured between the gel tank electrodes) for 45-60 minutes or as required (monitor the progress based on the migration distance of the gel tracking dyes in the loading dye (see Table 2 for examples).

C.21 When sufficient time has elapsed turn off and disconnect the power supply and carefully remove the gel from the electrophoresis tank.

C.22 Visualize the gel on UV transilluminator or blue-light transilluminator.

C.23 Save a copy of an image of the gel and record details in the relevant laboratory notebook including the agarose concentration used, the DNA ladder used, which wells correspond to which samples, the presence and size of bands present and details of any wells where bands are not present, ensure copy of an unedited gel is saved i.e. no contrast enhancements, cropping etc.

C.24 Unlike ethidium bromide-based gels, SYBR® Safe gels require no specialist disposal. Dispose of gel as per local rules.

C.25 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential DNA contamination.

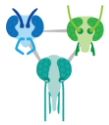
C.26 Remove and discard gloves.

D. Results

Appropriate details of all samples used with this protocol including details of their location on the gel should be recorded in the appropriate lab book in addition to details of what percentage agarose gel was used, which DNA ladder was used, how the gel was interpreted referencing other method sheets where appropriate, and if an image capture device is available, a copy of an image of the gel, including an unedited copy i.e. no contracts enhancements, no cropping.

E. Tips and troubleshooting

- SYBR® Safe DNA Gel Stain is a highly sensitive stain for visualization of DNA in agarose or acrylamide gels. SYBR® Safe DNA gel stain is specifically formulated to be a less hazardous alternative to ethidium bromide that can utilize either blue light or UV excitation. While SYBR® Safe DNA Gel Stain is considered safer/less mutagenic than either ethidium bromide and its alternatives such as Biotium GelRed™ it is still a DNA-binding material, and appropriate personnel protective equipment must be used (as detailed in this protocol) with reference to the appropriate local risk assessments.
- When bound to nucleic acids, the SYBR® Safe DNA gel stain has a fluorescence excitation maxima at 280 and 502 nm, and an emission maximum at 530 nm. When used with blue light illumination, SYBR® Safe DNA gel stain has less background fluorescence than ethidium bromide–stained gels when illuminated with UV light.
- SYBR® Safe DNA gel stain should ideally be stored at 4°C, however, can be stored at between 2°C to 25°C. Undiluted SYBR® Safe in DMSO freezes at low temperatures; therefore, if frozen the product must be completely thawed and mixed before using. Repeated freeze-thawing has minimal impact on product performance.



- If step C.7 was missed and the SYBR® Safe DNA Stain was not added prior to the gel before setting and being run, the gel may be stained post-electrophoresis. Add 3 µl of SYBR® Safe DNA stain to 50 ml of 1x TAE (50 ml of agarose is sufficient to cover most minigel trays, adjust volumes as required for larger gels), place the gel to be stained in a plastic container (do not use a glass container, as the dye in the staining solution may adsorb to the walls of the container, resulting in insufficient gel staining). Then gently pour sufficient 1x TAE with SYBR® Safe DNA Stain into the plastic contained such that the gel is fully immersed. Cover the container with aluminium foil and incubate at room temperature for 30 minutes, placing the container on an orbital shaker at ~50 rpm to agitate the solution. After the 30 minutes has elapsed, turn off the shaker and remove the gel, continuing with the protocol at step C.22 no destaining is required.
- If the agarose gel is observed to melt during an electrophoretic separation it is a sign that either the electrophoresis buffer has been omitted in the preparation of the gel or has become exhausted during the course of the run. For high-voltage electrophoresis over long time periods, Tris-Borate-EDTA (TBE) buffer should be used instead of TAE buffer as it has a greater buffering capacity. However, borate is an enzyme inhibitor hence using TBE is not recommended if you will be isolating the DNA for downstream enzymatic steps (e.g. via gel excision). For example, borate carry-over can affect ligations, therefore use TAE in preference for these samples.
- If poor resolution of DNA fragments are observed following electrophoresis the most frequent cause is an inappropriate choice of agarose concentration. Low percentage agarose gels should be used to resolve high-molecular-weight DNA fragments and high percentage gels for low-molecular-weight DNA (see Table 1). Low melting point agarose may be more appropriate to low-molecular weight DNA fragments, check agarose supplier's recommendation. Fuzzy bands, encountered particularly with small DNA fragments, result from diffusion of the DNA through the gel, this is especially true when gels are run for long periods of time at low voltages. Reassess the choice of agarose, gel time, running voltage and time the gel is run for and repeat as required.
- Trailing/smearing of DNA bands is most frequently observed with high-molecular-weight DNA fragments (or degraded DNA), this is often caused by overloading the DNA sample or running gels at high voltages. DNA samples loaded into torn sample wells will also cause extensive smearing, as the DNA will tend to run in the interface between the agarose and the gel support. Do not use torn gels, if trailing/smearing of band is observed in an untorn gel reassess the volume of DNA sample added per well and the voltage the gel is run at, and re-run a new gel as required.
- The dyes in the DNA loading buffers migrate at different rates depending on the dye and the concentration of the agarose gel (see Table 2). Loading buffers typically contain two or three different dyes, allowing the user to monitor DNA migration as the gel is running. The loading buffer are available with a range of different combinations of dyes with different migration rates (Table 2). Loading buffer containing dyes with migration rates most suited to monitoring the size of DNA fragment(s) to be analysed/visualised should be selected for use.

GN_12: Agarose Gel Electrophoresis using Pre-Cast High Throughput SYBR Safe E-Gels

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

A. Introduction

This method sheet describes the procedure for the separation and visualization of DNA fragments using a pre-cast E-Gel® 96 gels containing SYBR® Safe DNA gel stain (ThermoFisher Scientific) and the High-Throughput E-Gel® Electrophoresis System (ThermoFisher Scientific).

B. Materials

Equipment

- Single channel pipette (2-20 µl)
- Multichannel pipette (5-50 µl 12-channel multichannel) (optional)
- Invitrogen™ E-Gel® High-Throughput DNA Electrophoresis System E-Base™ Integrated Power System (EBM03)
- Transilluminator (both UV transillumination and blue-light transillumination is suitable for use with SYBR® Safe DNA Gel Stain)
- Gel documentation system
- Computer with Invitrogen™ E-Editor™ Software installed
- Fridge (+4°C)
- Freezer (-20°C)
- Sterile disposable pipette tips containing hydrophobic filters (2-20 µl)
- Blue roll
- Nitrile Gloves
- Parafilm® or 200 µl reaction tubes (not required if PCR product already contains a loading dye e.g. Qiagen CoralLoad Concentrate)

Reagents

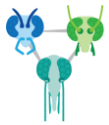
- Nuclease free water
- DNAZap™ Solutions (ThermoFisher Scientific: AM9890) (or equivalent)
- DNA Ladder (e.g. E-Gel® Low Range Quantitative DNA Ladder (ThermoFisher Scientific: 12373-031))
- E-Gel® sample loading buffer (ThermoFisher Scientific: 10482055) (ThermoFisher Scientific: 10482-055) (not required if PCR product already contains a loading dye e.g. Qiagen CoralLoad Concentrate or if the optimised run time is already known for the PCR product)
- E-Gel® 96 gels with SYBR® Safe (ThermoFisher Scientific)

C. Method

C.1 Put on nitrile gloves.

C.2 Use DNAZap™ solutions to clean all working surfaces and 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 Unseal package and remove the E-Gel®, carefully remove the comb from the gel and place E-Gel® on the white back of the packet (to aid visualisation when loading the gel.

C.5 Add 10 µl of nuclease free water and 10 µl DNA ladder to each marker lane.

C.6 If no loading buffer is required (i.e. optimised run time for PCR product is already known, or the PCR product already contains a loading buffer e.g. Qiagen CoralLoad Concentrate) add 15 µl of nuclease free water into each lane a sample is to be run in, then add 5 µl of sample per well. Reseal tubes containing remaining PCR product and store at 4˚C. Then proceed to step C.8, otherwise proceed to step C.7

C.7 If the PCR product does not already include a loading dye e.g. Qiagen CoralLoad Concentrate and/or the optimised run time for PCR product is not already known add E-Gel® Sample Loading Buffer to a subsample of the PCR product in new reaction tubes or a PCR plate such that the total volume is 20 µl i.e. 5 µl PCR product, 15 µl E-Gel® sample loading buffer. Reseal tubes containing remaining PCR product and store at 4˚C.

C.8 Add 20 µl nuclease free water into all remaining unused wells.

C.9 Plug in the E-Base™ and select the EG program (The EG program is to run E-Gel® 96 and 48 gels, while the EP program is to run the E-PAGE® 96 and 48 gels) then adjust the run time setting as required. Maximum run time for E-Gel® 96 well gels is 12 minutes.

C.10 Clip the loaded gel into the E-Base™, if the gel is properly inserted the fan in the E-Base™ will start to run and the red-light illuminates.

C.11 To begin electrophoresis press the pwr/prg button on the E-Base™, the red light will change to green and the time start counting down.

C.12 When the gel has finished running the E-Base™ will beep and the green light will change to a red flashing light.

C.13 Turn off the E-Base™ and remove the E-Gel®.

C.14 Visualize the gel on UV Transilluminator or blue-light transilluminator.

C.15 Save copy of an image of the gel, then align and save a copy of the aligned gel image using E-Editor™ software. Record details in the relevant laboratory notebook including agarose concentration used, DNA ladder used, which wells correspond to which samples, the presence and size of bands present and details of any wells where bands are not present, ensure copy of both the aligned and unedited gel is saved i.e. no contrast enhancements, cropping etc.

C.16 Unlike ethidium bromide-based gels, SYBR® Safe gels require no specialist disposal. Dispose of gel as per local rules.

C.17 Discard PCR product if no longer required for further downstream applications, otherwise store PCR product at 4°C if to be used in the next 24 hours, or store at -20°C.

C.18 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential DNA contamination.

C.19 Remove and discard gloves.

D. Results

Appropriate details of all samples used with this protocol including details of their location on the gel should be recorded in the appropriate lab book in addition to details of what percentage agarose gel was used, which DNA ladder was used, how the gel was interpreted referencing other method sheets where appropriate, and if an image capture device is available, a copy of an image of the gel.

E. Tips and troubleshooting

- SYBR® Safe DNA Gel Stain is a highly sensitive stain for visualization of DNA in agarose or acrylamide gels. SYBR® Safe stain is specifically formulated to be a less hazardous alternative to ethidium bromide that can utilize either blue light or UV excitation. While SYBR® Safe DNA Gel Stain is considered safer/less mutagenic than either ethidium bromide and its alternatives such as Biotium GelRed™ it is still a DNA-binding material and so should be regarded as potentially mutagenic, and appropriate personnel protective equipment must be used (as detailed in this protocol) with reference to the appropriate local risk assessments.
- When bound to nucleic acids, the SYBR® Safe DNA gel stain has a fluorescence excitation maxima at 280 and 502 nm, and an emission maximum at 530 nm. When used with blue light illumination, SYBR® Safe DNA gel stain has less background fluorescence than ethidium bromide–stained gels illuminated with UV light.
- If poor resolution of DNA fragments are observed following electrophoresis the most frequent cause is an inappropriate choice of agarose concentration or insufficient running time to allow bands to separate. Low percentage agarose gels should be used to resolve high-molecular-weight DNA fragments and high percentage gels for low-molecular-weight DNA. Reassess the choice of gel concentration and time the gel is run for and repeat as required. E-Gel® with SYBR® DNA Stain are suitable for the resolution of 100 bp to 2 kb fragments dependent upon the E-Gel® choose (see suppliers recommendation). The detection sensitivity is 1ng per band of DNA.
- Trailing and smearing of DNA bands is most frequently observed with high-molecular-weight DNA fragments, this is often caused by overloading the DNA sample or running gels at high voltages. DNA samples loaded into torn sample wells will also cause extensive smearing, as the DNA will tend to run in the interface between the agarose and the gel support. Do not use torn gels, if trailing/smearing of band is observed in an untorn gel reassess the volume of DNA sample added per well and the voltage the gel is run at, and re-run a new gel as required.
- Each E-Gel® should be loaded within 30 minutes of removing the gel from the package and run within 15 minutes of loading.
- Adjust the run time for the E-Gel® as appropriate for the fragments being used such that they are well distributed across the length of the run but not such that they overrun into the next well.
- The E-Gel® 96 loading format is compatible with multi-channel pipettors, and the most commonly used 8-, 12-, and 96-pin liquid handling robots.
- See the E-Gel® Technical Guide for further information on the E-Gel® system or contact a ThermoFisher Scientific technical representative.

F. References

E-Gel® Technical Guide

GN_13: PCR product purification using MinElute 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 for the purification of PCR products prior to sequencing and other downstream applications.

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
- Fridge (+4°C)
- Freezer (-20°C)
- Disposable pipette tips containing hydrophobic filters (2-20 µl, 20-200 µl, 100-1000 µl)
- 1.5 ml flip-top microcentrifuge tubes (e.g. Eppendorf Safe Lock Tubes: 0030 123.328)
- Label printer
- Microcentrifuge tube labels
- Solvent resistant cryopen
- Nitrile gloves

Reagents

- Analytical grade 100% ethanol
- DNAZap™ Solutions (ThermoFisher Scientific: AM9890) (or equivalent)
- 3 M pH 5.0 sodium acetate (NaOAc) (e.g. Sigma-Aldrich: S7899-100ML)
- Qiagen MinElute® PCR Purification Kit (Qiagen 50 columns: 28004; 250 columns: 28006), includes:
    - MinElute® spin columns
    - 2ml collection tubes
    - pH Indicator
    - Buffer PB
    - Buffer PE
    - Buffer EB (10 mM Tris HCL pH 8.0)

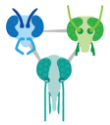
C. Method

C.1 Put on nitrile gloves.

C.2 Use DNAZap™ (or equivalent) to clean the working area and any equipment e.g. pipettes as required, 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 Qiagen MinElute Buffer PE (see bottle label for volume), tick the box on the Buffer PE Label to indicate ethanol has been added. Label the bottle with your initials and the date. Shake bottle to mix, store at room temperature.



C.5 Add 1:250 volume pH indicator to Qiagen Buffer PB (120 µl pH indicator to 30 ml Buffer PB). The yellow colour of Buffer PB with pH indicator I indicates a pH ≤ 7.5. Label the bottle with your initials and the date. Store at room temperature.

C.6 Pre-cool the centrifuge to 21°C.

C.7 Aliquot five volumes of Buffer PB to one volume of PCR product (i.e.100 µl per 20 µl PCR product). Use a new tip per well to prevent cross-contamination. Seal tubes and vortex briefly.

C.8 PCR product/Buffer PB mix should be pale yellow indicating a pH ≤ 7.5 if the solution is orange or violet add 10 µl 3 M sodium acetate (NaOAc) pH 5.0 then reseal the tubes and vortex briefly (solution should turn pale yellow). If CoralLoad Concentrate has been used in the PCR mastermix add 10 µl 3 M NaOAc pH 5.0 to all tubes.

C.9 Arrange MinElute® collection tubes in a microcentrifuge tube rack and place one appropriately labelled MinElute® PCR purification column per collection tube.

C.10 Transfer the PCR product/Buffer PB mix to the corresponding MinElute® PCR purification column and seal the cap on the spin column. Use a new tip per tube to prevent cross-contamination.

C.11 Centrifuge all MinElute® PCR purification columns in their collection tubes at 18,500 x g (~13,000rpm) at 21°C for 1 minute.

C.12 Discard flow through in each collection tube, returning each column to its original collection tube to prevent cross-contamination.

C.13 Aliquot 750 µl of Buffer PE to each MinElute® PCR purification column. Use a new tip per column to prevent cross-contamination. Seal the cap on the column.

C.14 Centrifuge all MinElute® PCR purification columns with their collection tubes at 18,500 x g (~13,000 rpm) at 21°C for 1 minute.

C.15 Discard flow through in each collection tube and return each column to its original collection tube to prevent cross contamination.

C.16 To dry the column, centrifuge all MinElute® PCR purification columns with their collection tubes at 18,500 x g (~13,000 rpm) at 21°C for 1 minute.

C.17 Place double-labelled 1.5 ml microcentrifuge tubes in a microcentrifuge tube rack.

C.18 Transfer each MinElute® PCR purification column to the corresponding appropriately labelled microcentrifuge tube, discard Qiagen collection tubes.

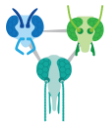
C.19 Add 10 µl Buffer EB (10 mM Tris-HCL, pH 8.5) to the centre of each MinElute® PCR purification column membrane. Use a new tip per column to prevent cross-contamination.

C.20 Allow MinElute® PCR purification columns to stand for 1 minute at room temperature (~21°C).

C.21 Centrifuge all MinElute® PCR purification columns with their microcentrifuge tubes at 18,500 x g (~13,000 rpm) at 21°C for 1 minute.

C.22 Discard MinElute® PCR purification columns and seal all microcentrifuge tubes containing purified PCR product.

C.23 Store purified PCR product at 4°C if to be used within 24 hours, otherwise store at -20°C.



C.24 Use DNAZap™ (or equivalent) to clean all surfaces used to reduce/remove any potential DNA contamination.

C.25 Remove and discard gloves.

D. Results

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

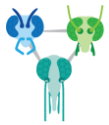
D.2 PCR product concentration may be checked using a NanoDrop spectrophotometer or using a Qubit® Fluorometer with either the Qubit® High Sensitivity dsDNA Assay kit or the Qubit® Broad Range dsDNA Assay Kit (choice of kit is dependent upon expected PCR product yield, see manufacturers guidance for further information).

E. Tips and troubleshooting

- PCR products purified using the method described in this protocol are ready to be either sent to a commercial sequencing facility for Sanger sequencing or for preparation for Sanger sequencing in-house using an appropriate kit and protocol e.g. the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific).
- See the Qiagen MinElute® PCR Purification Kit handbook for further information on this purification kit.

F. References

Qiagen MinElute® PCR Purification Kit handbook



GN_14: DNA Barcoding introduction to good laboratory practice guide

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

A. Introduction

During DNA Barcoding studies cross-contamination between samples in a batch and between sample-batches can arise from several different sources including the repeated isolation of nucleic acids (DNA and RNA templates) and the handling of amplified PCR products (amplicons) in an area. With DNA templates typically more prone to contamination than RNA templates as they are more stable, hence contaminants are more persistent in the environment. Consistently observing a network of protocols focused on maintaining laboratory areas in a contamination-free condition is essential for the operation of a successful molecular biology laboratory and for the completion of a successful DNA Barcoding study. When arranged in a linear fashion the steps involved in a DNA barcoding studies can be separated into three major groups, the pre-PCR activities (sample preparation and PCR preparation), the post-PCR activities (PCR execution and agarose gel electrophoresis) and sequencing. The essential parts of a contamination control program should include space and time separation of pre-PCR, post-PCR and sequencing activities, use of physical aids to reduce and/or eliminate contamination of work areas, the use of aliquoted reagents and the incorporation of negative controls. Further specific suggestions for controls to reduce the risk of cross-contamination occurring are given below.

B. Personnel Protective Equipment

Personnel protective equipment (PPE) should include 'Howie'-style laboratory-coats and single-use disposable nitrile (or equivalent) laboratory gloves, these items protect you from contaminating your samples and protect yourself from your samples or reagents being handled which may contain hazardous reagents. 'Howie'-style laboratory coats are part of an international recognised codified standard of protective wear. The coat style is named after J.W Howie who chaired the UK department of Health and Social Security committee whose 1978 report codified standards of clinical laboratory practice. The 'Howie'-style laboratory coat has buttons on the left flank, full length sleeves, elasticated wrists and a mandarin collar. It is designed to minimize pathogen/hazardous material contact with street clothes. Laboratory coats should be washed regularly, and not shared between pre-PCR, post-PCR and sequencing work areas and/or laboratories. Disposable plastic oversleeves worn over the laboratory coat sleeves can also provide additional protection from contaminating laboratory coat sleeves.

C. Laboratory Equipment

Dedicated laboratory equipment, in particular pipettes, should be used for the pre-PCR, post-PCR and sequencing procedures and not shared between these areas or between laboratories. Pipettes should always be used with sterile disposable pipette tips with hydrophobic filters to prevent the carryover of aerosols created during pipetting and transfer of contaminants between reactions.

PCR reactions should be setup in a dedicated PCR laminar flow cabinet (or equivalent). Ideally two cabinets should be available such that mastermix preparation and template addition activities can be physically separated. Working areas including cabinets used for mastermix preparation and/or template addition and equipment in particular pipettes in PCR laboratories should be wiped with a DNA degradation solution e.g. DNAZap™ (ThermoFisher Scientific) or 10% hypochlorite (bleach) solution, before and after use. Where available, ultraviolet (UV) sterilisation of PCR laminar flow cabinets and equipment before and after use can further reduce the risk of cross-contamination. Ultraviolet radiation is effective for reducing contamination of PCR process for amplicons >300 bp. Manufacturer's instructions regarding the use of UV decontamination of laminar flow hoods should be followed to prevent exposure of staff and students to UV radiation.

Steam autoclaving of items at 121˚C for 20 minutes at 15 psi is generally sufficient to destroy DNA contamination and DNases from most items (RNases cannot be removed by autoclaving), and can be used to provide an additional level of routine protection from cross-contamination or in response to a spillage contaminating an item. However, times, temperatures and pressures of autoclave cycles can vary depending on the type and settings of the autoclave used. Ensure items are suitable and will not be damaged by autoclaving at the temperature setting of the autoclave to be used.

D. Reagents and Samples

Sterile, nuclease (RNase and DNase) free water should be used for all pre-PCR, post-PCR and sequencing reactions. Reagents including PCR mastermixes, primers, nuclease free water should be aliquoted in appropriate volumes for the assay and to minimise the number of freeze-thaw cycles. Primers are particularly sensitive to contamination and aliquots of primers should never be open when tubes containing DNA extractions, PCR products etc. are open in the work area. New batches of reagents e.g. primers, Taq DNA polymerase etc. need to be assessed for performance against well-characterized positive control material.

E. Samples

The steps of the PCR protocol should be streamlined to minimise manipulation of samples during DNA extraction and PCR setup, reducing the number of occurrences during which cross-contamination could occur. A negative or 'no template' amplification control, e.g. nuclease free water, should always be included in each reaction batch to check for potential contamination of the reagent mastermix and equipment used to prepare the PCR reactions. In addition a positive amplification control should be used for every batch of PCR reactions set-up. The positive control should normally be a DNA extract that amplifies consistently to a moderate level, use of a strong positive is an unnecessary risk due to high amplicon copy number it would contain.
The maintenance of an identifiable chain between voucher specimen, DNA extraction, PCR product and COI sequence is essential for accurate DNA Barcoding studies. All samples should be double labelled with their unique identifying code using either solvent-resistant cryo-labels or solvent-resistant cryopen. Accurate records of work undertaken should be made by user in laboratory notebooks on a daily basis and on all forms required for sample management. The Barcode of Life Data System (BOLD) hosts an extensive online workbench of data management and analysis pipelines for DNA Barcode data http://v4.boldsystems.org/. Including pipelines for free analysis and the hosting of specimen collection data together with matched morphological and molecular identification data, and pipelines for submission of data to GenBank and provision of DOI numbers for datasets facilitating the public open access release of datasets. Further information on guidelines and minimum quality standards for DNA Barcode data can be found at www.boldsystems.org/docs/dwg_data_standards-final.pdf. Further information on the International Barcode of Life Initiatives see the International Barcode of Life Project (iBOL) www.ibol.org.

F. Training

Maintaining a clean and contamination-free laboratory is the responsibility of all users. All staff and students using the pre-PCR, post-PCR and sequencing areas should read, understand and receive appropriate training in all relevant protocols and local rules for using the laboratory areas and be aware of the issue raised above and the specific preventative measures and understand how they play an important role in the prevention of contamination.

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