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Title of resource
GN_15: Identification of blood meal source from dipteran specimens
Authored by
When using this protocol, the following should be referenced: Garros, C., Gardès, L., Allène, X., Rakotoarivony, I., Viennet, E., Rossi, S. and Balenghien, T. (2011). Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses. *Infection, Genetics and Evolution*, 11(5): 1103-1110.
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Description
Three multiplex species-specific PCR-based assays to identify the blood meal source of engorged female dipteran specimens for veterinary interest. Protocol from the Gnatwork Brazil workshop, November 4-8th 2019.
Intended use
Scientific research use and training purposes.
Restrictions on use
Content is not to be redistributed in the public domain (e.g. presentation, lecture, online or in publications).
Resource history
Protocol taken from Garros *et al*. (2011) for identifying the blood meal source of *Culicoides* females.

GN_15: Identification of blood meal source from dipteran specimens

Citation:
Garros, C., Gardès, L., Allène, X., Rakotoarivony, I., Viennet, E., Rossi, S. and Balenghien, T. (2011). Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae: Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses. *Infection, Genetics and Evolution*, 11(5): 1103-1110.

A. Introduction

This method details the protocol for the Identification of blood meal source from dipteran specimens.

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)
- PCR reaction tubes either as individual tubes, tube strips or PCR plates
- 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)
- Disposable pipette tips containing hydrophobic filters (2µl, 20µl, 200µl, 1000µl)
- For primers: 1.5 ml screw cap or flip-tops (e.g. Eppendorf® Safe Lock Tubes #0030 123.328) microcentrifuge tubes
- For mastermix preparation: 1.5 ml flip-top microcentrifuge tube or 15ml centrifuge tubes (dependent upon volume of mastermix required to be prepared)
- PCR adhesive plate sealers (*only if using 96-well PCR plates)
- Blue roll
- 96 well PCR tube rack
- Microcentrifuge tube rack

Reagents

- DNAZap™ (AM9890, ThermoFisher Scientific, UK) (or equivalent)
- Nuclease free water (NFW)
- Forward & reverse primers (see Table 1)
- NucleoSpin Tissue Extraction Kit (740952, Magerey-Nagel, Düren, Germany)
- Selected Mastermix Components (see below)

Taq DNA Polymerase

- 10 mM dNTP Mix (e.g. D7295 Sigma, UK; 18427-088: ThermoFisher Scientific, UK)
- Taq DNA Polymerase (201203: Qiagen; kit contains 10x PCR Buffer, 10x CoralLoad PCR Buffer, 5x Q-Solution, 25 mM $MgCl_2$)

C. Method

C.1 Separate the abdomens of engorged female specimens from the body.

C.2 Extract the DNA from the abdomen using NucleoSpin Tissue kit, following the manufacturer's instructions.

C.3 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.4 Make a working stock of each primer at the concentration required by the mastermix by briefly vortexing resuspended 100µM primers, then make an appropriate dilution in a second appropriately labelled 1.5ml microcentrifuge tube [store both re-suspended 100µM primer and working stock 10µM primer at -20°C when not in use].

C.5 Because multiplexing numerous primer sets can lead to cross-reactivity, this protocol defines three multiplex combination assays (see Table 1 for primers, Table 2 for assay conditions):

- Multiplex combination 1: dog, pig.
- Multiplex combination 2: chamois, red deer, roe deer, cow, goat, sheep.
- Multiplex combination 3: cat, horse.

Table 1 Primer set used for the identification of blood meal origin in dipteran abdomens

Reverse primers	Oligo Name	Sequence (5'-3')	Size (bp)
Multiplex combination 1			
Forward primer UNIV1		GACCAATGATATGAAAAACCATCGTTGT	
Dog *Canis lupus familiaris*	CANIS	CAAGCATACTCCTAGTAAGGATCCG	170
Pig *Sus scrofa*	SUS	TCTGATGTGTAATGTATTGCTAAGAAC	219
Multiplex combination 2			
Forward primer UNIV2		TGAGGACAAATATCATTYTGAGGRGC	
Chamois *Rupicapra rupicapra*	RUPI	TGAGGGTAGCCTTGTCTACCGAGAAGCCT	90
Red deer *Cervus elaphus*	CERVUS	AGTAAGTGTACTATAGCGAGTGCTGCG	188
Roe deer *Capreolus capreolus*	CAPREOLUS	TTGTCCGCGTTTGATGGGATTCCTATC	220
Cow *Bos taurus*	BOS	TAAGATGTCCTTAATGGTATAGTAG	287
Goat *Caprus hircus*	CAPRA	TTAGAACAAGAATTAGTAGCATGGCG	313
Sheep *Ovis aries*	OVIS	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336
Multiplex combination 3			
Forward primer UNIV3		TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAG	
Cat *Felis catus*	FELIS	GATTCATGTTAGGGTTAGGAGATCC	180
Horse *Equus caballus*	EQUUS	TACGTATGGGTGTTCCACTGGC	208

C.6 Thaw if needed, then vortex briefly forward and reverse primers and other mastermix components.

C.7 Make sufficient of the mastermix (Table 2) 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.8 Vortex the mastermix and aliquot into each well.

C.9 If separate mastermix preparation and template addition areas/hood are not available proceed directly to step C.10, 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.10.

C.10 Thaw if needed, then briefly gently mix/vortex DNA extractions (caution excessive vortexing can increase DNA fragment shearing decreasing the quality of the DNA extraction).

C.11 Using a new pipette tip per well to prevent cross-contamination transfer DNA template into the reaction wells.

C.12 Using a new pipette tip per well transfer the nominated positive control sample into the designated positive control well(s). See troubleshooting tip one for an appropriate positive control.

C.13 Using a new pipette tip per well transfer nuclease free water into the designated negative control well(s).

C.14 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 sealed securely.

C.15 Briefly vortex tubes/plate.

C.16 Spin reaction tubes/plate for approximately 10s in the reaction tube spinner / centrifuge to concentrate reaction mix at the base of the wells.

C.17 Place reaction tubes in the thermal cycler and carry out the appropriate PCR amplification using the thermal profile appropriate to the primers used (see Table 2).
Note. For Multiplex combination 2 primer set, touchdown PCR cycles are used to reduce nonspecific amplification.

Table 2 PCR master mix and thermal profiles

Component	Initial Concentration	Final Concentration	Volume Per Reaction in µl
10x Reaction Buffer	10x	1x	2.5
dNTPs	10 mM	200 µM each	0.5
Toptaq	5 units/µl	1.25 U/rxn	0.25
Primers	10 µM stock	0.4 µM (10 µM/rxn)	1
Host DNA			25 – 50 ng
Nuclease free water			To 25 µl final volume

Multiplex combination 1 or 3 primer set		
Initial denaturation	5 minutes	94˚C
3-step Cycling (40 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	30 seconds	53˚C
Extension	30 seconds	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

Multiplex combination 2 primer set		
Touchdown PCR cycles used to reduce nonspecific amplification		
Initial denaturation	5 minutes	94˚C
3-step Touchdown PCR Cycling (20 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	30 seconds	61˚C, decrease by 0.5 ˚C each cycle
Extension	30 seconds	72˚C
3-step Cycling (15 Cycles)		
Denaturation	30 seconds	94˚C
Annealing	30 seconds	53˚C
Extension	30 seconds	72˚C
Final Extension	10 minutes	72°C
Hold	∞	4˚C

C.18 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.19 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.

C.20 Visualise PCR products on a 2% (w/v) agarose gel (see Gnatwork protocol GN_11: Agarose Gel Electrophoresis using SYBR Safe DNA gel stain).

D. Results

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

D.2 Confirm successful amplification 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. Successful amplification using this protocol is indicated by the presence of a band at the appropriate size for the target amplicon plus the primer size (Table 1), 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

- For positive controls, where possible use EDTA-blotted blood collected in vet clinics or by wildlife institutions and extract DNA using a commercial kit (DNeasy Tissue & Blood, Qiagen [see GN_09: DNA extraction from dipteran specimens, whole blood and cultured cells using Qiagen DNeasy spin columns]).
- Addition of between 25 – 50 ng of DNA 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 25µl to maintain the concentration of other reagents in the mastermix.
- 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