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LAMP Protocol for Entomopathogenic Serratia spp. in Insect Frass V.1

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We use this protocol and it's working

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Abstract

A protocol for the detection of insect-associated *Serratia* spp. in the frass of live insects, using a LAMP assay targeting the *ure*D gene of urease-positive species from the *Serratia marcescens* complex. In order to improve the DNA extraction from folivorous insects a bacterial separation and concentration protocol has been developed prior to the DNA extraction, but this part of the protocol is not required for carnivorous and omnivorous insects. To improve accuracy the LAMP assay is conducted in triplicate, and a pH-linked calorimetric assay changes from pink to yellow for positive results. This protocol is potentially suitable for field applications with minimal equipment required.

Image Attribution

Rohan Cleave, Zoos Victoria

Materials

Equipment Required:

-Pipettors and pipette tips in a range of sizes (1000 µL 100 µL 10 µL)

-Qiagen Tissue Lyser II, alternatively a high-speed horizontal vortex

-Low speed swing bucket centrifuge (500 x g) (ideally but can be done without)

-High speed microcentrifuge (16000 x g)

-Equipment for measuring DNA concentration and quality (e.g. Thermo Fisher Nanodrop or Implen Nanophotometer)

-Heating element fitted to the tube size used for LAMP (PCR block, heating block, hot water bath)

-Access to a fridge (4 °C) and freezer (-20 °C)

Materials Required (per extraction):

-5 x 1.5 mL microcentrifuge tubes

- 5 mL sterile phospate buffered saline

-Sterile surface for handling frass (e.g. parafilm or petri dish)

-1 x sterile forceps (or reusable forceps sterilised between each pooled sample)

-1 x sterile pestle (Axygen Cat. AX-PES-15-B-SI-1)

-1 x 10 mL graduated centrifuge tube (ideally screw-top)

-1 x 2.0 mL round-bottomed microcentrifuge tube

-DNeasy PowerSoil Pro kit (Qiagen Cat. 47014/6)

-Molegular Grade water (MG H20)

-WarmStart® Colorimetric LAMP 2X Master Mix with UDG (NEB Cat. M1804S/L)

-LAMP Primers (IDT):

Serr_ureD_F3: ATCGCCTGACGCTCGA

Serr_ureD_B3: TAGAGCAGCGTCGCCGT

Serr_ureD_FIP: CTGCATCTGCGCCGCGTA-GCCTGCGGGCATGTCA

Serr_ureD_BIP: TCTGGAGATGATGCCCGATCCG-CGTAATGCGGGTATCGGTAA

Serr_ureD_LF: TGGCGTGGATCTTGGTCG

Serr_ureD_LB: CTGCGGTTCGCGCTTCA

-3 x 0.2 mL PCR tubes

Equipment and Materials Required

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-Qiagen Tissue Lyser II, alternatively a high-speed horizontal vortex
-Low speed swing bucket centrifuge (500 x g) (ideally but can be done without)
-High speed microcentrifuge (16000 x g)
-Equipment for measuring DNA concentration and quality (e.g. Thermo Fisher Nanodrop or Implen Nanophotometer)
-Heating element fitted to the tube size used for LAMP (PCR block, heating block, hot water bath)
-Access to a fridge (4 °C) and freezer (-20 °C) or ice to achieve these temperatures

2 Materials Required (per extraction):
-5 x 1.5 mL microcentrifuge tubes
- 5 mL sterile phospate buffered saline
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-3 x 0.2 mL PCR tubes

Bacterial Separation and Concentration

3 Collect 300 mg fresh Sample per DNA extraction and store at -20 °C until processing.

Note

This protocol has been developed for folivorous insects such as phasmids and larval lepidopterans. Carnivorous and omnivorous insects are not likely to require bacterial separation and concentration, so the protocol can commence with 300 mg of crude frass added directly to the PowerBeads tube (Step 13) in the DNA Extraction section.

Note

The following protocol is for one pooled frass sample and one DNA extraction. Scale up as required. You may choose to do one or more DNA extractions for each pooled frass sample.
If you conduct multiple extractions on one pooled sample, you may use the same surface, forceps and pestle without sterilising between.

4 Tip out Sample onto a sterile surface (e.g. parafilm or petri dish) and using sterile forceps add approximately 60 mg frass (for *D.australis* approximately 2 adult, 8 subadult or 17 juvenile frass pieces) to each of five 1.5 mL microcentrifuge tubes (300 mg total).

Note

Different life stages of insect produce frass pieces of significantly different sizes. For maximum accuracy count the frass pieces in 1 gram of your pooled sample, then calculate the average mass / frass peice and calculate the number of frass peices in 60 mg. We previously calculated that frass from adult *D. australis* is approximately 30 mg/piece, from subadults 8 mg/piece, and juveniles 3.6 mg/peice. Therefore, for *D. australis* you can use the above estimates for 60 mg frass added to each microcentrifuge tube.

Note

If using reusable forceps disinfect them between each of the pooled frass samples you are testing. We generally scrubbed with Virkon if there was gross contamination, then rinsed with tap water, dried, and sterilised with 70-80% ethanol and flame.

5 Add 1.0 mL sterile phosphate buffered saline (PBS) to each of the five microcentrifuge tubes containing Sample .

6 Using one sterile pestle for each pooled frass sample crush the Sample in each tube until well homogenised in the PBS. A 100 µL pipette tip can be used to seperate crushed frass from the bottom of the tube and assist with homogenization.

7 Transfer the contents of all five microcentrifuge tubes into one 10 mL graduated centrifuge tube.

8 Mix the 10 mL tube well by shaking. Using a large swing bucket centrifuge spin down the graduated centrifuge tube at 600 x g for 5 mins.

Note

This slow centrifugation should separate most of the plant and insect material in the frass into the pellet while leaving most of the bacterial cells suspended in the supernatant.

Note

If a swing bucket centrifuge is not available stand the mixed 10 mL tube upright at 4 °C for 60 mins to allow the pellet to settle, then proceed with the following steps but without mixing again between transfers of supernatant.

9 Using a 1000 µL pipette tip transfer up to 1.8 mL (2 x 900 µL) of supernatant to a clean 2.0 mL round-bottom microcentrifuge tube. To maximise bacterial yield, aspirate close to the pellet without disturbing it.

10 Pellet the supernatant in the 2.0 mL tube using a standard microcentrifuge at 16000 x g, Room temperature for 5 mins. Carefully discard most of the supernatant from the 2.0 mL tube, taking care not to disturb the bacterial pellet.

11 Repeat Steps 8 -10 go to step #8 , adding to the same 2.0 mL tube. If significant supernatant is still present in the 10 mL tube can repeat a third time to maximise bacterial cell yield.

DNA Extraction

12 Briefly spin the PowerBead Pro tube to ensure the beads have settled at the bottom of the tube.

Note

This Protocol is adapted from the DNeasy PowerSoil Pro Kit Handbook
Perform all steps of the DNA extraction at Room temperature.
Solution CD2 must be stored at 4 °C, all other reagants in the PowerSoil Pro kit should be stored at Room temperature.
If Solution CD3 has precipitated, heat it at 60 °C until the precipitate dissolves.

13 Using a 1000 µL pippette tip re-homogenise the bacterial cell pellet in 800 µL of Solution CD1, then transfer all the contents of the 2.0 mL tube to a PowerBead tube. Shake briefly to mix.

14 Conduct cell disruption and lysis by shaking the PowerBead tube in the Tissue Lyser II at 25 Hz for 10 mins.

Note

If no Tissue Lyser is available a horizontal vortex at max speed for at least 10 mins may be used.

15 Centrifuge the PowerBead tube at 15000 x g for 1 min. Transfer the supernatant to a clean 2.0 mL microcentrifuge tube. Expect 500 µL to 700 µL supernatant, which may contain some frass particles.

16 Add 200 µL Solution CD2 and mix well by gentle pipetting.

17 Centrifuge the 2.0 mL tube at 15000 x g for 1 min. Avoiding the pellet, transfer up to 700 µL supernatant to a clean 2.0 mL microcentrifuge tube.

18 Add 600 µL of Solution CD3 and mix well by gentle pipetting.

19 Load 650 µL of lysate into an MB Spin Column. Centrifuge at 15000 x g for 1 min.

20 Discard the flow-through and repeat the step above go to step #19 to ensure all of the lysate has passed through the spin column.

21 Carefully place the spin column into a clean 2.0 mL collection tube. Take care not to splash any of the flow-through onto the spin column.

22 Add 500 µL of Solution EA to the spin column. Centrifuge at 15000 x g for 1 min.

23 Discard the flow-through and place the spin column back into the same 2.0 mL collection tube.

24 Add 500 µL of Solution C5 to the spin column. Centrifuge at 15000 x g for 1 mins.

25 Discard the flow-through and place the spin column into a clean 2.0 mL collection tube. Centrifuge at 16000 x g for 2 min.

26 Carefully place the spin column into a clean 1.5 mL elution tube, without allowing any flow through to be transferred to the new tube on the spin column. Carefully add 100 µL of MG H20 to the centre of the white filter membrane in the spin column. Centrifuge at 15000 x g for 1 min.

27 Discard the spin column. The DNA is now contained in the flow-though and ready to be used. If not using immediately store at -20 °C. Measure the concentration and quality of extracted DNA using spectrophotometry (e.g. Thermo Fisher Nanodrop or Implen Nanophotometer).

LAMP Assay

28 Prepare a positive control of *Serratia ureilytica* AM923 DNA at a concentration of app. 50 ng/µL. Can be stored at 4 °C for up to 7 days.

29 Make up stock solutions of the LAMP primers using MG H20. Add the same amount of MG H20 to the dried primers as the primer amount in moles to make [M] 1000 micromolar (µM) stock solutions. Dillute the F3, B3, LF and LB primers 1:10 to make [M] 100 micromolar (µM) working stocks.

30 Prepare a 25X LAMP Primer Mix using the LAMP worksheet. The Primer Mix can be stored at -20 °C for use in multiple assays.

LAMP_Spreadsheet_Master.xlsx 13.4KB

31 Prepare the working Master Mix using the LAMP worksheet, with MG H2O, 1 X LAMP Master Mix and 1 X LAMP Primer Mix. Use 2 or 3 LAMP assays per extraction (duplicate or triplicate) to ensure confidence in the results.

LAMP_Spreadsheet_Master.xlsx 13.4KB

32 Vortex the working Master Mix thoroughly and spin briefly. Transfer 24 µL to each of PCR tube. Add 1 uL MG H2O to the negative control tubes and close caps on negative controls.

33 Add 1 uL DNA sample to each of the tubes, including the positive controls loaded last to minimise false positives. Close all caps snugly and spin down PCR tubes to ensure DNA sample is in contact with the Master Mix.

Note

We add the 1 uL DNA samples to the open caps of the PCR tubes, then close the caps and spin down the tubes. This helps to visualise that the sample has definitely been added to the tubes, but do whatever you are most comfortable with.

34 Incubate the LAMP assays for 30 mins at 65 °C .

Note

In a laboratory this is most easily done on a PCR block, but could be done on a heat block sized correctly to the microcentrifuge tubes being used, or in a hot water bath.

35 At the end of the 30 min incubation, the results of the LAMP assays can be interpreted and photographed.

Expected result

Yellow = Positive
Pink = Negative
Orange = Inconclusive

36 The LAMP products can be stored at 4 °C for several months, but over time the amplification products will break down, affecting the pH and changing the colour. If left at Room temperature the products will break down and cause colour change much faster.

Note

Any opening of the tubes containing LAMP products (i.e. to run on an agarose gel) should be done with caution, as the very high yield of amplification products by LAMP can easily contaminate a workspace with LAMP products.