

BIOLOGICAL CONTROL OF RHINOCEROS BEETLE IN THE PACIFIC USING *ORYCTES VIRUS* OPERATIONAL PROTOCOLS (VERSION A, APRIL 2010)



Trevor Jackson¹, Sada Nand Lal^{2,3}, Kuateman Tuapola⁴, Shareen Prasad², Jana Monk¹, Nicola Richards¹ and Sean Marshall¹

¹ AgResearch Limited, Private Bag 4749, Christchurch 8140, New Zealand

² Secretariat of the Pacific Community (SPC), Plant Protection Division, Suva, Fiji

³ 11 Johnstone Drive, Palmerston North 4410, New Zealand

⁴ Ministry of Agriculture and Fisheries, Samoa



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These ***Operational Protocols*** were developed as part of the project “***Capacity building for improved biocontrol of food crop pests in the South Pacific***” funded by the NZ State Sector Development Partnerships Fund (DFP) administered by NZAID. We have put together information in the protocols which we hope will help researchers address the needs of biological control of rhinoceros beetle and other pests in the Pacific. This version has been developed within the project, but we hope that it can be improved and upgraded with feedback from users. Please send us any ideas for improvement;

To; Trevor Jackson, AgResearch Ltd, Lincoln Research Centre, Private Bag 4749, Christchurch 8140, New Zealand.

Email trevor.jackson@agresearch.co.nz

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1 Introduction

This manual assembles a number of operational protocols developed during the AgResearch/SPC programme “Capacity building for improved biocontrol of food crop pests in the South Pacific” carried out with funding from NZAID/ODA between 2004 and 2009. A key aspect of the programme was to establish an improved capability for management of rhinoceros beetle within the Pacific through better use of the *Oryctes* virus for control of the pest beetle.

The background to rhinoceros beetle (*Oryctes rhinoceros*) and its control by *Oryctes* virus is described in a number of reviews including those of Huger (2004)¹ and Jackson (2009)². This manual includes methods for collection and handling of beetles for assessment of virus infection, which were developed and used during the programme. The protocols are often based on those of earlier researchers but are intended to be a practical guide of techniques that have worked well for the project team and a guide to new users.

The manual includes sections on collection of beetles from the field, dissection of beetles to provide samples for diagnosis of virus infection, diagnosis of virus infection by (i) PCR, (ii) histological methods and/or (ii) bioassays, and release of infected adults into the field. We do not mean to suggest that these are the only way that these procedures can be carried out, but they are methods that have worked for us and we hope will help others in their tasks to reduce the impact of rhinoceros beetle and other pests in the Pacific and other regions.

These methods have been developed within the research programme and would not have been possible without the help of our colleagues at AgResearch and SPC, Paul Scotti and the enthusiastic support of staff from LAES, Papua New Guinea and MAFF, Samoa.

References

- ¹Huger, A.M. (2005) The *Oryctes* virus: Its detection, identification, and implementation in biological control of the coconut palm rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). *Journal of Invertebrate Pathology* 89: 78-84.
- ²Jackson, T.A. (2009). The use of *Oryctes* virus for control of rhinoceros beetle in the Pacific Islands. In: Hajek AE, Glare TR, O’Callaghan M eds. Use of microbes for control and eradication of invasive arthropods. Springer Science & Business Media BV, Netherlands. Pp 133-140.

2 Collection of rhinoceros beetle adults and larvae (*Trevor Jackson, Sada Nand Lal and Shareen Prasad*)

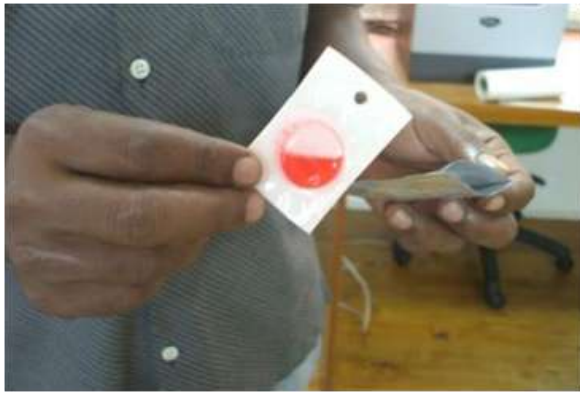
Rhinoceros beetle adults and larvae must be collected from the field in order to assess virus levels or to obtain individuals for bioassay, artificial infection and release. In all cases it is important to collect robust living specimens and to avoid cross contamination in the presence of virus. Adult beetles can be collected in traps baited with the synthetic pheromone ethyl 4-methyloctanoate, which acts as an aggregation pheromone for both sexes. Larvae, pupae and young adults can be collected from either natural or artificial breeding sites with a large mass of organic matter.

2.1 Pheromone traps

The pheromone trap can be constructed from a plastic bucket with a lid. Two large holes (diameter = 2.8 cm) and 2 small holes (in the centre) should be made in the lid as illustrated below. The holes can be cut using hot wire or hot rod.



The pheromone sachet should be opened and attached with a wire under the bucket lid. The operator should make sure the sachet is not damaged and that it is placed in an upright position inside the bucket.



The lid should be placed on the bucket (= pheromone trap). In the field, the pheromone trap should be placed on a strong branch with the bucket hanging upright.



For diagnostic purposes (PCR, histology) beetles should be removed at least once per week and stored individually in plastic containers. If used for monitoring adult beetle numbers, the bucket should be emptied at 3-week intervals and the collected beetles destroyed. Pheromone sachets need to be replaced every 4 weeks.

2.2 Breeding heaps/traps

In nature, the rhinoceros beetle lays its eggs in decaying coconut trunks, stumps, heaps of fronds, husks and other parts of coconut tree or decaying heaps of vegetative matter, sawdust, compost and animal dung. Compost and heaps of decaying vegetative matter are favourable breeding sites and can produce extremely large numbers of beetles in a short time. Artificial breeding traps can be established by laying sections of coconut trunk in quadrats of 2–3 meters across and filling the centre with decaying sawdust. The breeding traps should not be exposed to direct sunlight and should be covered with dried fronds or dried grass.



2.3 Collection and storage of beetles

After beetles are collected from pheromone traps or breeding sites, they should be placed into individual containers that are marked with the collection number. The containers should be transported in a cool bin (so that they do not overheat) to the laboratory where they must be stored in cool conditions until needed (e.g. for gut extractions). Beetles can be stored at 4°C for 2-3 days or frozen and kept at -20°C for long term storage. Storing beetles individually reduces the risk of virus contamination.



3 Dissection (*Trevor Jackson*)

Adult beetles must be dissected before disease diagnosis can be carried out. The method of storage of the dissected sample will depend on the diagnostic test being carried out.

3.1 Equipment for extraction of beetle gut

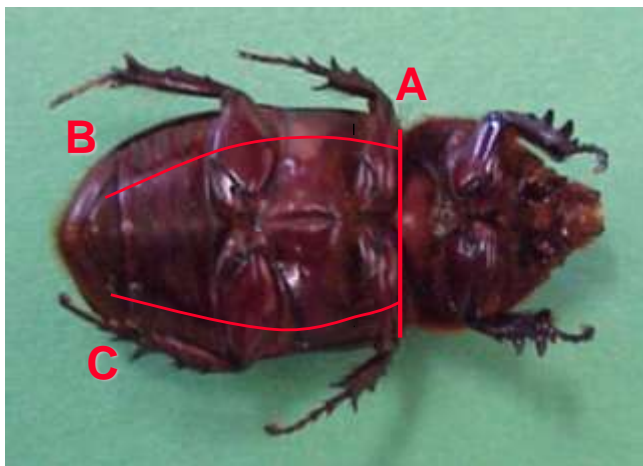
- Heavy and fine dissecting scissors
- Fine forceps
- Tissues
- Gloves
- 0.1 M HCl* (wash bottle or spray bottle)
- 70-99% ethanol
- 2-ml tubes with O-ring
- Rubbish bag or bin for used tissues etc.

*inactivates DNA (e.g. on your equipment, bench surface), ethanol doesn't inactivate DNA.

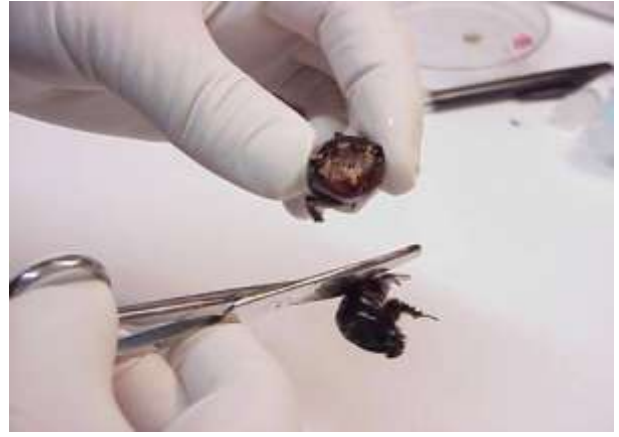
3.2 Extraction of mid gut

Before starting, the bench surface and equipment should be wiped with 0.1 M HCl to inactivate any DNA and with 70% ethanol to remove any residues.

To open the body cavity and expose the gut, the head and pronotum are removed by cutting across Line A, slightly to the posterior of the junction between the pronotum and the second thoracic segment with heavy scissors. The posterior section is then opened by cutting with fine scissors along lines C and D separating the dorsal and ventral cuticle.



Heavy dissecting scissors are used to cut off the head and pronotum along line A.



To open up the abdomen it will be necessary to carefully insert the fine dissecting scissors along the line of the alimentary tract in order to cut through the wing muscles. The abdomen can then be opened along lines C and D.



Fingers are used to pull apart the dorsal and ventral cuticle to expose the gut. Fine forceps can then be used to lift out the midgut.



Diseased (left) and healthy gut (right). Record the characteristics of the gut on the data sheet. The key aspects are: dark or milky, thin or swollen, empty, or full of organic matter.

3.3 Recording of characteristics and other data

One line of the data sheet (see example below) should be filled in for each individual beetle. An insect identification number, the location and trap number, the date of collection, gender of the beetle, dissection date, and the gut characteristics should be recorded. A copy of this sheet should be sent to the analytical facility, e.g. SPC Suva, that carries out the diagnosis.

Insect ID	Site	Collection date	gender	Dissection date	Insect and gut condition	PCR result	Histology

3.4 Storage of gut for diagnostics

The method of storage of the gut sample will depend on the diagnostic method being used. The midgut can be divided into two pieces to provide samples for both types of analysis.

1. For DNA/PCR analysis, the gut sample should be stored in 70-99% ethanol.
2. For histology, the gut sample can be stored in 10% neutral buffered formalin (10% NBF) or FAA (5 ml formalin, 2.5 ml glacial acetic acid, 45 ml 99% ethanol). The advantages of FAA fixative are:
 - Tissues are fixed with minimal cell disruption and samples can be further processed for light or electron microscopy if required.
 - Tissues can be stored in the fixative at room temperature without the samples becoming brittle or the need to change fixative.

The disadvantage of FAA is that it does not preserve DNA.

3.5 Transport of samples

3.5.1 Packaging

Transportation of samples containing flammable and corrosive liquids by air requires a special container, LabPak-1, which can be obtained from Thermofisher Scientific NZ Ltd (3 Atlas Place, Mairangi Bay, Auckland, New Zealand; <http://www.biolab.co.nz>). The gut sample should be placed in a 1.7-ml screw-top tube (O-ring) containing 0.5 ml 70-99% ethanol (for PCR) or 0.5 ml FAA (5 ml formalin, 2.5 ml glacial acetic acid, 45 ml 99% ethanol) for



histology. Tubes should be labelled with beetle number and the solution (ethanol or FAA).

The International Air Transport Association (IATA) Dangerous Goods Regulations permits the transport of small volumes of flammable and corrosive liquids in approved containers (LabPak-1). See web site: www.iata.org.

Use permanent marker pen to label tubes with short codes. Record detailed information about each sample on a data sheet (see section 3.3).

Shipping containers protect against potential leakage of alcohol from the sample tubes. Wrap samples in absorbent material (e.g. cellulose packing, thick paper towel or cotton wool) before placing inside shipping container. Bubble wrap can be used to stop movement of sample package inside the container. A warning label 'Dangerous Goods in Excepted Quantities' must be displayed on the outside of the package. Complete the label with appropriate details as follows and glue on one side of the container that shows "infectious substance" biohazard label.



The image shows a standard IATA Dangerous Goods in Excepted Quantities label. It features a red and white striped border and a dashed blue outline. The text on the label includes: 'DANGEROUS GOODS IN EXCEPTED QUANTITIES', a disclaimer, fields for 'Signature of Shipper' and 'Date', a section for 'Name and address of Shipper', a section for 'This package contains substance(s) in Class(es) (check applicable box(es))' with checkboxes for classes 1 through 9, and a field for 'UN Number'. At the bottom, there are fields for 'PROPER SHIPPING NAME' and 'UN NUMBER'.

When sending ethanol samples you only need to tick class 3 and write UN1170. For FAA samples you need to tick classes 3 and 8 and write UN1170, UN2209 and UN2789.

Under "documentation" attach the information listed in the box (see right).

3.5.2 Biosecurity documentation

All samples that are to be sent to Fiji should be accompanied by a Biosecurity Import permit or the required certificate or document from the point of origin. Further information may be obtained from http://www.quarantine.gov.fj/c_importing.aspx.

Description of contents:

- Samples for research purposes
- Dead, non-infectious insect guts preserved in ethanol as follows:
 - 1.7ml screw-top tubes each containing 0.5ml ethanol + insect gut

IATA approved container for transporting flammable liquid

*IATA = International Air Transport Association.

4 Diagnosis of virus infection by DNA methodology (*Jana Monk*)

The extraction of virus DNA from the beetle gut is the first step for the molecular, DNA-based diagnosis of a virus infection. There are different methods for extracting DNA, and the simplest approach is to use special pre-prepared kits. This chapter describes three extraction procedures – two are based on commercial kits and the third uses a novel technique:

- Qiagen DNeasy Blood and Tissue Kit (which is very good but quite expensive)
- Zymo Research Genomic DNA Tissue MiniPrep kit (which is currently giving good results)
- FTA cards

These are described in more detail in the sub-sections below. The selection of method will mainly depend on cost. Both the Qiagen and Zymo kits have been tested and work equally well, but the Zymo kit can significantly reduce the costs per DNA extraction.

Once pure DNA has been obtained, the polymerase chain reaction (PCR) is used to detect the presence of *Oryctes* virus. PCR is a technique to amplify a few copies of a specific piece of DNA (in this case virus DNA) across several orders of magnitude, generating thousands to millions of copies of the target DNA. To check whether the PCR generated the anticipated DNA fragment (and thus confirming the presence of the virus), agarose gel electrophoresis is employed for separation of the PCR products. The size of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

4.1 DNA extraction

Three alternative methods for collecting DNA for PCR analysis are described below.

4.1.1 DNA extraction (DNeasy Blood & tissue kit, Qiagen)

1. Prepare a 56°C heating block
2. Use a sterile pipette tip to transfer the gut sample (preserved in 70-99% ethanol, as described in section 3.4) into a sterile Petri dish
3. Wipe scalpel blade with 99% ethanol and excise approx. 25 mg of insect gut (2-3mm)
4. Place piece in a 1.5 ml tube (use scalpel blade and/or a sterile pipette tip)
5. Wipe blade twice with 99% ethanol and new tissues. From here on use Qiagen DNeasy Blood & Tissue Kit. Buffers ATL and AL may form precipitates upon storage, if so place at 37°C until crystals dissolve.
6. Add 180 µl ATL buffer and use sterile micropestle to macerate tissue
7. Add 20 µl proteinase K, vortex to mix
8. Incubate at 56°C for 1-3 hours (alternatively, this step can be done at 37°C overnight)
9. After removing samples turn heating block up to 70°C for step 12
10. Vortex samples for 15 sec
11. Add 200 µl buffer AL, vortex to mix
12. Incubate at 70°C for 10 min
13. Add 200 µl ethanol (99%), vortex to mix
14. Centrifuge if sample is not completely lysed: 1 min, 8,000 rpm
15. Transfer sample/supernatant into a DNeasy mini spin column placed in a 2 ml collection tube
16. Centrifuge 1 min at 8,000 rpm
17. Discard flow through
18. Add 500 µl Buffer AW1
19. Centrifuge 1 min at 8,000 rpm
20. Discard flow through
21. Add 500 µl Buffer AW2
22. Centrifuge 1 min at 8,000 rpm
23. Discard flow through
24. Centrifuge 2 min at 13,000 rpm (to dry column)
25. Place column in new 1.5 ml tube
26. Add 50 µl Buffer AE directly onto column membrane
27. Incubate at room temp for 1 min
28. Centrifuge 1 min at 8,000 rpm to elute DNA
29. Repeat steps 26-28
30. Store DNA at -20°C or keep at 4°C for PCR set up.



4.1.2 DNA extraction with the ZR Genomic DNA™-Tissue MiniPrep kit, Zymo Research)

1. Prepare a 55°C heating block
2. Use a sterile pipette tip to transfer the gut sample into a sterile Petri dish
3. Wipe scalpel blade with 99% ethanol and excise approx. 25 mg of insect gut (2-3 mm)
4. Place piece in a 1.5 ml tube (use scalpel blade and/or a sterile pipette tip)
5. Wipe blade twice with 99% ethanol and new tissues
6. From here on use Zymo ZR Genomic Tissue MiniPrep kit.
7. Add 95 µl H₂O, 95 µl 2x Digestion buffer, and 10 µl proteinase K to the gut sample
8. Macerate tissue with micro pestle
9. Incubate tube for 3 hours at 55°C
10. Add 700 µl Genomic Lysis buffer to the tube and mix
11. Centrifuge at 10,000 x g for 1 min to remove insoluble debris
12. Transfer supernatant into a Zymo-Spin IIC column in a collection tube
13. Centrifuge at 10,000 x g for 1 min
14. Add 200 µl of DNA Pre-Wash Buffer to the spin column in a new collection tube
15. Centrifuge for 1 min
16. Add 400 µl of g-DNA Wash Buffer to the spin column
17. Centrifuge for 1 min
18. Transfer spin column to a clean tube and add 100 µl DNA Elution buffer or PCR water

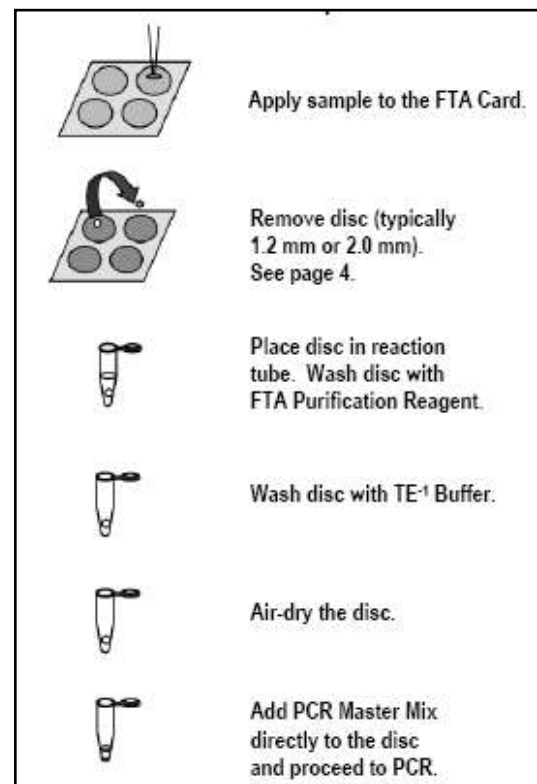


4.1.3 FTA® Cards

FTA® technology is a novel method designed to simplify the collection, shipment, archiving and purification of nucleic acids from a wide variety of biological sources. FTA® Cards are impregnated with a patented chemical formula that lyses cell membranes and denatures proteins upon contact. Nucleic acids are physically entrapped, immobilized, and stabilized for storage at room temperature. FTA® Cards protect nucleic acids from nucleases, oxidation, UV damage and microbial and fungal attack. Infectious pathogens in samples applied to FTA® Cards are rendered inactive upon contact. Samples collected on FTA® Cards can be stored at room temperature; the nucleic acids are stable for years. Samples stored on FTA® Cards and enclosed in a multi-barrier pouch can be shipped through the Postal Service with no special handling restrictions, making them an extremely useful tool for field collection.

The protocol for preparing beetle gut samples for FTA® card storage and analysis is listed below.

1. Press fresh insect gut onto the card and allow drying completely.
2. Punch a few disks out of the FTA® matrix impregnated with gut material.
3. Place discs in 1.5 ml tube and wash by adding 200 µl FTA® purification reagent
4. Incubate 5 min at room temperature
5. Remove reagent with pipette
6. Repeat the washing step once
7. Add 200 µl of TE buffer
8. Incubate 5 min at RT
9. Remove TE buffer with pipette
10. Dry discs at RT for ca 1 hour or at 50-60°C in thermo block
11. Use one disc for PCR (25 µl reaction)



4.2 Polymerase Chain Reaction (PCR)

In molecular biology, the polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

4.2.1 Materials and set-up

The following materials are needed for the PCR method:

- ABgene 1.1x Reddy Mix PCR Master Mix (contains 2.5 mM MgCl₂)
- PCR water (sterile)
- Primers OrV15a (5'-ATTACGTCGTAGAGGCAATC-3') and OrV15b (5'-CATGATCGATTCGTCTATGG-3'), which give a PCR product of 945 bp
- Bovine serum albumine (BSA)
- 0.2 ml thin-walled reaction tubes
- Thermo cycler
- DNA samples
- Agarose
- DNA dye SYBR safe
- 0.5x TBE buffer

Primers 15a and 15b have been specifically designed for *Oryctes* virus and can be ordered from www.invitrogen.com. To make the setup easier and to avoid mistakes with the calculation of the individual compounds it is helpful to create an Excel file as shown here:

OrV PCR		
Compounds	microliter	x
	1x25 μ l	16
Master Mix	22.3	356.8
Primer 15a	0.5	8
Primer 15b	0.5	8
BSA	0.2	3.2
H ₂ O	0.5	8
Volume	24	a 24
DNA	1	1

The yellow cell represents the total number of samples prepared for one PCR run (e.g. 14 unknown samples, 1 positive control, 1 negative control = 16 samples). A virus positive DNA sample can serve as positive control, whereas PCR water can be used as the negative control.

4.2.2 PCR parameters

Once the reaction tubes have been pipetted out, they should be placed in the thermo cycler to carry out the PCR. The thermo cycler should be set to carry out the following program:

1. 5 min	94°C (denaturation)	
2. 30 sec	94°C (denaturation)	} 35 cycles
3. 45 sec	50°C (annealing)	
4. 1 min	72°C (extension)	
5. 8 min	72°C (final extension)	
6. "forever"	12°C	

4.2.3 Preparing, loading and running an agarose gel

To visualise the DNA fragments produced by PCR, an agarose gel should be prepared as follows:

1. For a 1% gel, add 1 g agarose to 100 ml of 0.5x TBE (for small gels) or 0.5 g to 50 ml of 0.5x TBE into 250ml flask labelled with "DNA gels".
2. Mix by swirling and microwave for about 1-2 minutes (depending on your microwave). Stop and mix the solution once or twice during the micro waving.
3. Cool to 60-65°C and add the DNA stain SYBR safe (10 µl for 100 ml or 5 µl for 50 ml gels).
4. Pour your agarose/dye mix into the prepared gel tray (with comb) avoiding bubbles.
5. Multiple combs can be used on a gel to accommodate additional samples.
6. When gel solidifies (after 20-30 min at 20°C), transfer gel and tray into the electrophoresis chamber filled with 0.5x TBE so that it covers gel completely. Remove the comb carefully or you may rip out the bottom of the wells.

Following are instructions for loading and running the gel:

- Load the samples carefully into the wells.
- Load 10 µl of a size marker on the left or right side
- Write down what samples are in each well.
- Close the lid of the gel chamber and connect voltage (80-100 V)
- Run the gel for 30-50 min (depending on your system)

4.2.4 Documentation and interpretation of results

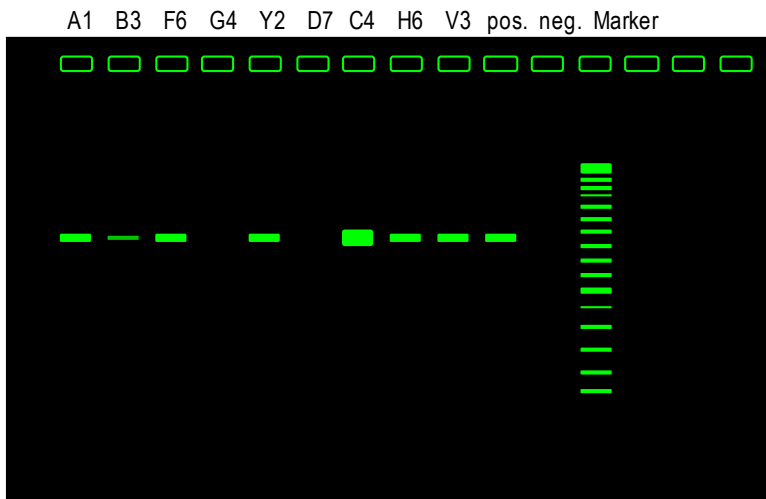
Place the gel on the surface of a transilluminator (wear gloves!). Place the hood with LCD monitor and digital camera on top of the transilluminator and turn on the UV light. Photograph the picture with the camera.



Transilluminator, with set up for viewing and photographing the gel.



The following is an example of a gel that displays the DNA fragment obtained from a PCR with samples from nine different beetle guts, along with a positive and negative control and the molecular weight marker, as outlined in the Table below.



lane	μ loaded	sample ID	PCR result
1	10	A1	+
2	10	B3	(+)
3	10	F6	+
4	10	G4	-
5	10	Y2	+
6	10	D7	-
7	10	C4	++
8	10	H6	+
9	10	V3	+
10	10	pos.	+
11	10	neg.	-
12	5	100bp marker	

The first thing to check is that the positive control contains a band of the expected size, and that there is no band in the negative control. Next look at the bands generated for the individual beetle samples. Two (G4 and D7) of the nine samples did not produce bands and therefore, it can be concluded that those gut sections were not infected with virus. The other seven beetle samples gave a fragment of the expected size, indicating that virus was present in those gut sections. The lighter intensity band for B3 indicates that less virus was present (e.g. at an early stage in the infection process) than the other samples, while for beetle C4, the high intensity band indicates a large amount of virus DNA was present (e.g. heavily infected beetle).

4.2.5 Disposal of agarose gels and gloves

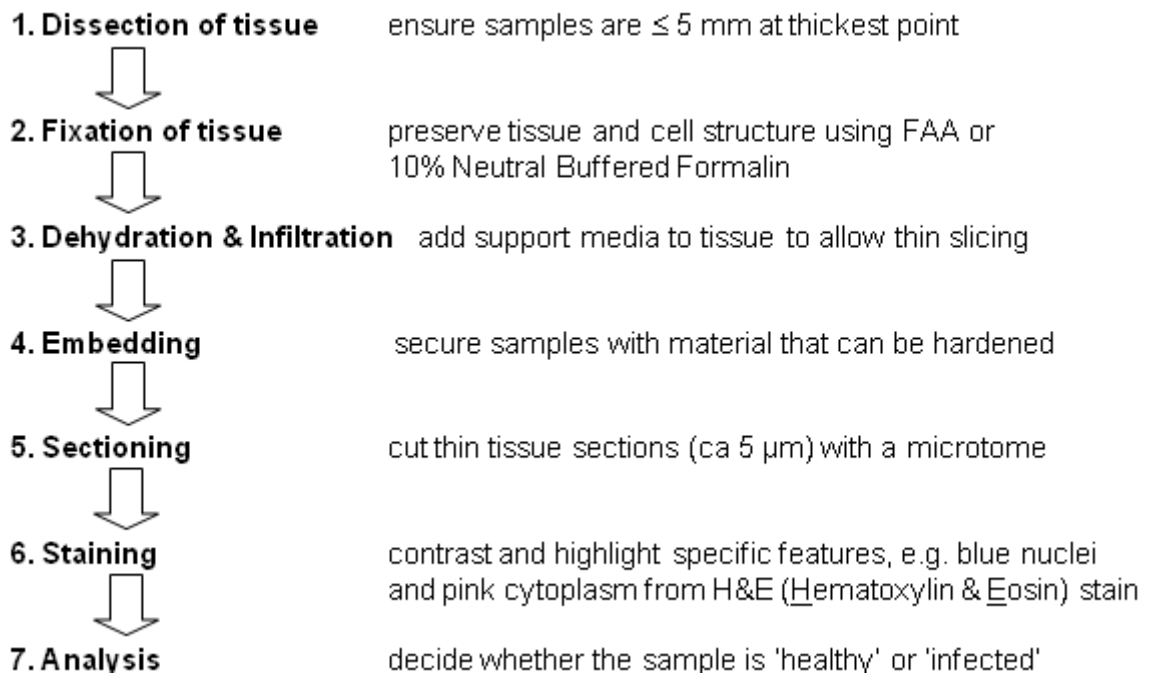
SYBR safe stained agarose gels should be treated as toxic waste. The gels should be placed in a plastic colander that sits inside a rubbish bin (with lid). When the colander is full, empty it into a biohazard bag and dispose of the bag according to the local requirements for toxic waste. Contaminated gloves should be disposed in a separate bin but can be disposed with general rubbish.

5 Diagnosis of virus infection by histology (*Sean Marshall*)

Histology is the study of the anatomical features of cells from tissue samples, which are generally in the format of thin slices or sections. The various combinations of histological stains employed (e.g. hematoxylin and eosin) are used to provide contrast within the tissue and can highlight specific structures of interest. The information provided from histological staining is an essential part of investigating the presence/absence of disease or infection. A chapter outlining the use of histology has been included in this manual because the combined use of PCR and histopathology methods is currently the most reliable procedure for identifying and confirming *Oryctes* virus infection in rhinoceros beetles. PCR-based screening is useful to identify samples negative for the presence of the virus, but the high sensitivity of the PCR method is a drawback for being able to reliably distinguish infected beetles from beetles whose gut samples have just been contaminated with a small amount of the virus. Visual inspection of histological sections is a complimentary method to assist in identifying beetles that have the mid to late stages of virus infection, although it is not sensitive enough to reliably identify early stages of infection.

5.1 Overview of basic histology processing steps

The following diagram illustrates the steps required to diagnose a virus infection using histology. Steps 1, 2 and 7 are done by the user, while steps 3-6 can be done through a laboratory service.



5.2 Step 1: Dissection of beetle midgut sample

See Chapter 3 for details on dissecting midgut tissue from beetles. Before starting the dissections prepare FAA fixative – see directions below. FAA fixative can be pipetted (1 ml aliquots) into 1.7 ml sample tubes before dissections begin if a large number of samples are to be processed.

5.3 Step 2: Fixation of beetle midgut sample

This section describes two alternate fixative solutions, FAA and 10% NBF. By nature these solutions contain harmful chemicals and should be handled with special precautions.

Formaldehyde is a dangerous chemical that is found in both the FAA and 10% NBF fixatives. Formaldehyde can be purchased as a liquid or in solid form. More information can be found at: <http://swehsc.pharmacy.arizona.edu/exppath/resources/formaldehyde.php>.

Liquid forms of formaldehyde should be stored tightly sealed; since exposure to air encourages the oxidation of formaldehyde to formic acid (37% formaldehyde is usually shipped with 10-15% methanol to inhibit this change). Formaldehyde should be stored at room temperature (cold temperatures encourage the formation of trioxymethylene with a resulting white precipitate). The shelf life of an unopened 37% formaldehyde stock solution is ca 1 year if stored in the dark at 20-25°C. Once opened, the solution should be used within 3-4 months. Therefore if possible purchase in small volumes. Solutions containing ca 4% formaldehyde should be used within 1-2 months, if not sooner. Suggested sources of formaldehyde include Sigma-Aldrich: #533998-500ML Formaldehyde histological grade, 37 wt. % in H₂O or Fluka: #47629 Formaldehyde solution.

An alternative to formaldehyde solutions is crystalline paraformaldehyde, which can be used to make up the required solutions, but it is hazardous to workers if recommended safety procedures are not followed. If you are not familiar with making solutions from the crystalline form, it is recommended that a premade formaldehyde solution is purchased.

Acetic acid is a component of the FAA fixative. Acetic acid is a strong acid and should be handled with care. It is often purchased as glacial (meaning concentrated), e.g. Scharlau: #AC0352. Diluted acetic acid solutions can also be used as long as the final concentration in FAA is 2.5%

5.3.1 FAA fixation

1. We have found that FAA (5% formaldehyde : 2.5% acetic acid : 50% ethanol : 35% water) provides good fixation of midgut tissue. Prepare FAA in small volumes as needed (see Table). Store at room temperature (20-25°C) and use within ca 2 months. The formaldehyde should be gently shaken before use to avoid a concentration gradient in the bottle.

Table: Directions for making 200 ml of FAA fixative.

Solution	Volume
37% formaldehyde solution	25 ml
Glacial acetic acid	5 ml
95% ethanol	100 ml
distilled water	70 ml

2. Place dissected tissue into a 1.7 ml screw cap sample tube containing 1 ml of FAA fixative. To make sure the fixative reaches all areas of the tissue sample, the gut fragment should not be bigger than 5 mm at the thickest point (this should not be a problem for midgut). Also, the volume of fixative should be at least 10 times more than the volume of sample tissue (based on approximate volumes). Initial fixation should be at room temperature since the penetration of formaldehyde is related to the temperature of the solution.

3. Use a permanent marker pen to label tubes with short codes. Record detailed information about each sample on a data sheet (see section 3.3).

4. Leave samples in fixative for a minimum of 24 hours. Samples may be left in fixative for several days to weeks prior to downstream processing.

5. Sample may be sent off to a histology service for further processing after 24-48 hours. You may be requested to either send the tissue in the original sample tubes they were fixed in, or be requested to place the tissue in a histology embedding/processing cassette. Further details on the cassette are provided in the sections describing steps 3-6 of the histology process.

5.3.2 10% NBF fixation

An alternative fixative to FAA is 10% neutral buffered formalin (10% NBF; 10% formalin is an old name for a 4% formaldehyde solution). It may be easier and safer to buy premade 10%

neutral buffered formalin. Use within printed expiry date if purchased premade or 3 months if self-made. Store 10% NBF at room temperature.

Table: Directions for making 1000 ml of 10% neutral buffered formalin fixative.

Solution	Volume/weight
37-40% formaldehyde	100 ml
Sodium phosphate (monobasic) $\text{NaH}_2\text{PO}_4 \times 1\text{H}_2\text{O}$	4.0 g
Sodium phosphate dibasic (anhydrous) Na_2HPO_4	6.5 g
Distilled water up to 1000 ml	ca 900 ml

5.4 Steps 3-6: Dehydration & Infiltration, Embedding, Sectioning, Staining

Dehydration and infiltration involves several processing steps that remove water from the tissue sample to allow the introduction of a support media (e.g. paraffin) into the tissue, which helps prevent ripping or tearing of the tissue during sectioning. The embedding procedure (also referred to as blocking) surrounds and secures the tissue within a support material that can be hardened (typically paraffin) to allow easy handling of the sample. Sectioning refers to the cutting of thin slices of tissue (ca 5 μm thick) using a blade microtome. The tissue slices are then stuck onto glass slides and are ready for staining. Staining is done to provide contrast and highlight specific features of interest in the tissue. These steps require specific training and skills to carry out properly. If they are not readily available to you, there are a number of places that can be contacted to enquire about use of their histology services.

5.4.1 Histology Services

Prior to directly contacting a histology service, please contact Shareen Prasad at the Secretariat of the Pacific Community (SPC) in Fiji. They will advise you on the most current procedure for requesting histology results for dissected midgut samples related to *Oryctes* biocontrol management.

Possible sources of histology services include the local university, medical or veterinary laboratories. Make enquires about whether or not you are able to use their histological services. If they agree, they will instruct you on how they wish to receive your samples, and how they typically process tissue samples.

Alternatively, it may be possible to use the histology services of Gribbles Veterinary Pathology in Christchurch, New Zealand. Since Gribbles Veterinary Pathology normally processes veterinary samples, it will be necessary to first check with to ensure they are still

2. Remove fixed tissue sample from the 1.7 ml tube and place into the labelled histology processing/embedding cassette and close. A biopsy foam pad (blue in photo above) may be required to help hold the sample in place if midgut tissue is particularly thin.
3. Place cassette containing tissue sample into a plastic jar (with a good tight seal!) filled with enough fixative to completely cover all cassettes in the jar (a size of 100 or 250 ml is often sufficient).
4. Package jar in an appropriate shipping container for local delivery to the histology service. Note that for international shipments it may be necessary to ship the individual sample vials, and request the histology service provider to place the tissue samples in the cassettes (this may incur further costs). This is due to restrictions on shipping dangerous goods (see below for more details).
5. From the service, request hematoxylin and eosin (H&E) staining, and explain that the midgut is a tube and you are trying to capturing sections located at the midpoint of the tube (i.e. want to see the 2 gut epithelial sides with the central luminal space). If possible, ask for 3-4 sections per slide and obtain 3 slides.
6. After processing and H&E staining, slides are returned and are ready for analysis.

5.4.2 Couriers and sending samples for histology processing

It will be necessary to contact the local international courier company to ensure they are willing to deliver the fixed samples. The fixative used to preserve the samples contains chemicals that are subject to international shipping restrictions (see chapter 3.5 above and the website: www.iata.org for more details).

Currently we have been advised that a maximum of 30 ml of the fixative per package can be safely shipped. Check with your chosen courier that this is an allowable quantity to ensure your package can be delivered to the desired location.

Depending on the shipping destination, an import permit from the destination country may also be required.

It is important to include a detailed description of contents (see chapter 3.5) on the courier package. In particular, state that formaldehyde is present in the fixative used for the samples. Depending on the shipping destination, you may be required to specify the insect species name, e.g. *Oryctes rhinoceros* (coconut palm rhinoceros beetle).

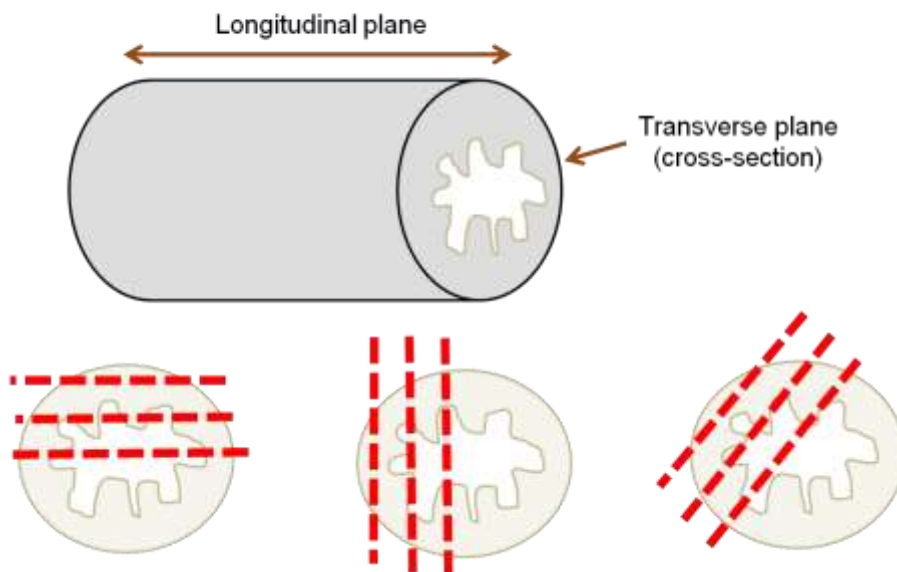
5.5 Step 7: Analysis

5.5.1 Interpretation of results

Hematoxylin and eosin (H&E) stain is a common staining method in histology. The staining method involves application of the basic dye hematoxylin, which colours basophilic structures with blue-purple hue, and alcohol-based acidic eosin Y, which colours eosinophilic structures bright pink. The basophilic structures are usually the ones containing nucleic acids, e. g. the nucleus itself, bacteria found in the midgut or the *Oryctes* virus. The eosinophilic structures are generally composed of intracellular or extracellular protein. Most of the cytoplasm is eosinophilic.

Depending on the exact location of each section, pictures of the same gut sample may appear different. You will need to think in 3-dimensional space, as illustrated in the diagram below.

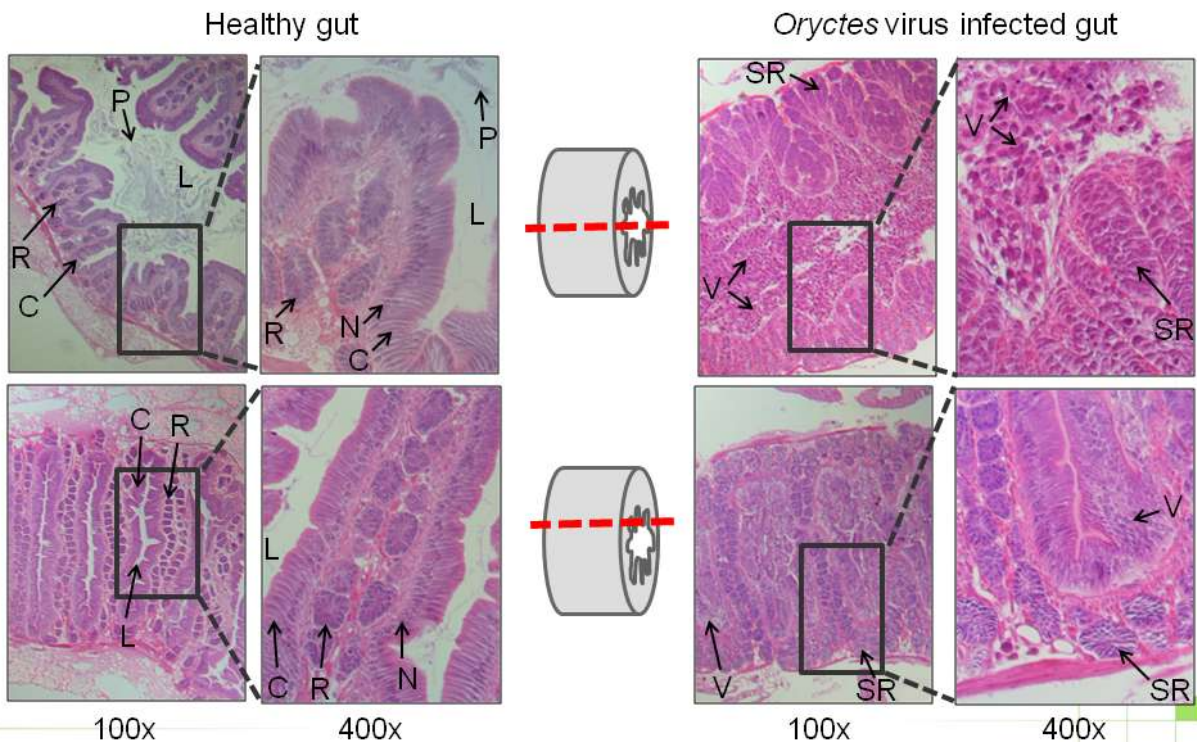
Section orientation



5.5.2 Examples of stained histological tissue sections

The figure below shows two examples of H&E stained tissue sections as seen under a light microscope. Compact dark blue/violet staining highlights DNA material (e.g. nucleus), while the cytoplasmic material stains pink with a diffuse blue tint.

The series of pictures on the left shows two sections of a healthy gut, both viewed at magnifications of 100-fold and 400-fold. In the top row, the columnar cells (C) of the healthy gut epithelium form ordered 'wavy' columns and at 400x magnification the rows of nuclei (N) are arranged at the basal end of the columnar cells (small dark blue staining). The cells of the regenerative crypts (R) appear as distinct large circular clusters (dark blue stain). The lumen (L) of healthy guts appears free of cell-like vesicles, with portions of peritrophic membrane (P; containing food particles) being generally easily observed. These same features can also be seen in the healthy gut photos on the bottom row, but they are not as distinct. This is due to the section being cut slightly off centre from the midpoint of the gut, which results in a distorted appearance.



The series of pictures on the right show a virus-infected gut that is full of *Oryctes* virus particles. The top row displays a gut epithelium where, although the 'waviness' is still present, the neat columns of columnar cells have disappeared and the regenerative crypt clusters appear swollen (SR; compare with healthy gut). Additionally, the gut lumen is completely filled with individual round cell-like vesicles (V) that have been sloughed from the gut epithelium; neither 'clear' space nor peritrophic membrane can be observed. Again the bottom row is less distinct because the section has been cut off centre from the midpoint of

the gut.

It is worth remembering that further sections of a given sample may be necessary to provide a more definitive answer. This may be done by requesting further sections from the embedded sample block.

An excellent review and a more detailed description of the *Oryctes* virus pathology may be found in: Huger, A.M. (2005) The *Oryctes* virus: Its detection, identification, and implementation in biological control of the coconut palm rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). *Journal of Invertebrate Pathology* 89: 78-84.

6 Diagnosis of virus infection by bioassay (*Trevor Jackson and Kuateman Tuapola*)

This bioassay method for treating beetles with purified cultures of *Oryctes* virus was established by AgResearch/MAFF Samoa during the rhinoceros beetle research programme.

The bioassay was designed to compare a pure culture of virus prepared by AgResearch with a preparation of the virus collected from Samoan beetles. Virus-free adult beetles will be fed sugar cane dosed with virus and held in individual containers. A control treatment will be fed sugar cane without virus. There should be 10 beetles for each treatment. The experiment should be repeated with three different batches (collections) of beetles. The beetles should be examined every 3 to 4 days when the section of sugar cane should be replaced. The state of the beetle (live or dead) and the amount of feeding should be recorded. The results should be analysed to determine time to death of the treated beetles and amount of sugar cane consumed.

6.1 Collection of beetles

Beetles for virus tests should be collected from breeding sites, as sampling and PCR have shown that levels of virus are generally low within the logs. The beetles or pupae should be extracted carefully from the rotting logs and placed individually in 50 ml containers (pink tops) and placed in the cool bin. The cool bin should contain a cold freezer pad or a bottle of frozen water to keep the samples cool until returned to the laboratory.

In the laboratory beetles and pupae should be placed carefully into 75 ml containers (yellow tops) and surrounded with clean sawdust to about 2/3 full. The containers should be labelled with the site and date of collection on the outside of the container. The containers should be kept on their sides in a dark area of the insectary until there are enough fresh beetles for testing. To indicate that the beetles are ready for testing add a small piece of sugar cane to the tube. The beetles are ready for testing once they have started feeding on the sugar cane.

Collect beetles from breeding sites (log traps).



Place beetles in individual plastic containers and transport to the lab as quick as possible.



To infect beetles with *Oryctes* virus inoculate sugar cane pieces with the virus solution (1 droplet). Place beetle, sugar cane and saw dust into a container.



6.2 Preparing a virus sample from fresh beetle guts

Adult beetles should be collected from pheromone traps. The beetles should be dissected and guts removed from those showing swollen guts indicating virus infection (see picture).

The swollen guts from five beetles should be mixed together in 5 ml of clean water (bottled drinking water) and macerated (mashed) together to form a suspension of virus. The mixture should be left to settle for 5 minutes and the suspension removed by pipette placing about 0.5 ml into screw top tubes. The tubes should be labelled with the date of preparation and placed in the freezer (-20°C) until use.



6.3 Bioassay of beetles for effect of virus

When thirty feeding beetles have been collected they should be placed individually in the 150 ml containers with fresh damp sawdust (It is important that this sawdust is clean and not contaminated by virus from infected beetles).

The beetles in their containers should be divided randomly into three groups of ten. The containers should be marked on the side with the letter of the treatment group. Treatments will be applied to a split section of sugar cane.

The three treatments are:




- A. Control - fresh water
- B. Fresh virus from beetles
- C. Pure virus from AgResearch.

First treat the sugar cane for Group A by adding a droplet of fresh water to the cut side of the cane so that it absorbs into the core. Place the lids on the containers securely (The lids should have holes to allow air movement in and out of the container).

Allow the frozen sample container with sample B to thaw. Fill the tube to the 1.5ml mark with clean water (2/3 full). Transfer two droplets of water onto the cut surface of the sugar cane and place in each of the containers for the B group. Place on the lids.

Take a sample of the pure virus (Sample C) from the refrigerator. Fill the tube to the 1.5 ml mark with clean water. Transfer two droplets onto the sugar cane and place in each of the containers for the C group. Place on the lids.

Samples should be kept in the insectary without being exposed to direct sunlight. The samples should be assessed twice weekly (at 3-4 day intervals) when the state of the beetle is noted (Live or dead) and the amount of feeding on the piece of sugar cane is noted as the percentage consumed (For example, light feeding = 5%; totally consumed = 100%). The results from each assessment should be written down on a data sheet and transferred to an Excel file in the computer as soon as convenient. The experiment should be monitored for 60 days or until most of the insects are dead.

	
<p>Containers with sawdust for keeping individual beetles</p>	<p>Treating sugar cane with a droplet of water or virus</p>
	
<p>Place 2 drops of the virus suspension on the cut surface of the food source</p>	

The experiment should be repeated three times with different batches of insects (i.e. different beetle collections) at each time.

7 Release of *Oryctes* virus for biocontrol (*Trevor Jackson and Sean Marshall*)

7.1 Beetles

Beetles collected from pheromone traps are not suitable for treating with virus as they are often too old and will only survive for a few days in the laboratory. Some may already be infected with virus or contaminated from other infected beetles. For these reasons we recommend collecting neonate beetles from the breeding sites or rearing them from larvae and pupae before treating with virus.

7.1.1 Selection of sites for beetle collection

Beetles for virus release should be collected from sites where virus incidence is thought to be low. Sampling and PCR have shown that levels of virus are generally low within the logs at breeding sites. Beetles can be collected from special “Log traps” (see chapter 1.2) provided these have not been treated with virus for beetle control. Areas where PCR is showing low viral incidence in the general population are particularly useful for collection.

7.1.2 Collection of beetles

Freshly emerged beetles and pupae should be collected from breeding sites in the field. The beetles or pupae should be extracted carefully from the rotting logs and placed individually in 50 ml containers and placed in the cool bin. The cool bin should contain a cold freezer pad or a bottle of frozen water to keep the samples cool inside until returned to the laboratory. The containers should be labelled with the site and date of collection on the outside of the container. (Larvae can also be placed in groups in containers into the cool bin for transport back to the laboratory).

7.1.3 Handling of pupae

Place the pupae carefully into 75 ml containers and surrounded with sawdust to about 2/3 full. The containers should be labeled with the site and date of collection on the outside of the container. The containers with pupae should be kept on their sides in a dark area of the insectary. The containers should be checked every 3-4 days and the date when the beetle is first observed should be noted.

7.1.4 Treating the beetles with virus

Pure virus (strain X2B) preparations should be provided by AgResearch or SPC Suva (see picture). Maintain the virus in the specified conditions until use (see chapter 6.). Beetles selected



as ready for treatment should be provided with a drop of water (control) or a drop of virus suspension to the mouthparts. (A droplet is about 100 µl in size). The experimenter should ensure the droplet is absorbed into the gut.

7.1.5 Release of virus treated beetles

Infected beetles should be released into the field in the late afternoon prior to sundown. They should be placed in shaded positions under trees or covered with leaves or fronds. On release the beetles should be active and rapidly bury themselves under loose vegetation.

7.2 Virus Storage

Pure cultures of virus prepared by AgResearch are supplied in sealed vials containing 1 ml of virus suspension (other volumes can be arranged). Upon receiving a shipment, the virus tubes should be stored in their container in a dark refrigerator (4°C). Do not freeze pure virus, as virus titer will be reduced. Short periods at room temperature (such as when setting up the experiment) will not harm the virus, but if the virus is left at room temperature (20°C or higher) for extended periods the virus will lose its ability to infect.

Once a vial has been opened, the contents should be used on the same day to be sure the virus has not been affected by growth of microbial contaminants. However, it is possible to store the remaining portion of the virus at 4°C for use within the next 1-2 weeks, but the quality of the virus will be unknown, due to the slow growth of bacterial and fungal contaminants. The exception to this would be if the vial contents have been kept sterile (i.e. aseptic technique used). Deterioration occurs because the pure virus is stored in cell culture media, which is a rich solution that will allow bacteria and fungi to grow.

7.3 Ordering cell culture produced virus

7.3.1 Production capacity (time and volume)

AgResearch is able to supply the X2B strain of *Oryctes* virus at a standardized titre of 1×10^6 infectious units per millilitre (IU/ml), with virus supplied in 1 ml aliquots (other volumes are also feasible). Other strains of *Oryctes* virus can also be supplied, on a case by case basis.

The minimum dose recommended to establish infection in a susceptible beetle is 5000 IU. It is recommended that an excess of virus is applied to ensure all treated beetles will be infected when released. If 50 µl of virus solution is used to dose an adult beetle, a 10 ml volume of cell culture-produced virus titred at 1×10^6 IU/ml will provide enough material to infect about 200 adult beetles.

7.3.2 Notice period

For production of 20 ml AgResearch requires approximately 4-6 weeks advance notice to be able to prepare a shipment. For volumes larger than 20 ml, please contact us to discuss a suitable timeframe.

Please keep in mind that this is a biological system and occasionally it behaves in unexpected ways that cannot always be explained or predicted – a good reminder that ongoing research is important, particularly in the area of biocontrol.

7.3.3 Import Permits for live virus

An import permit may be required to allow shipments of virus into the country, though may vary with each country. Generally, you should contact your government department responsible for importation of biological materials (e.g. Ministry of Agriculture).

Alternatively, if you are unsure of whom to contact, Shareen Prasad from SPC (ShareenP@spc.int) will be able to advise you. If a permit is required and AgResearch is shipping directly to the requesting country, please forward a copy of the import permit to AgResearch (contact Tracey Nelson, tracey.nelson@agresearch.co.nz), so that we can attach it to the shipment. Please be sure to include all instructions on where and how the permit should be displayed and any other details that may be required by the requesting country. Packages will be shipped by the fastest possible route, which is generally via courier.

7.3.4 Costs

The cost of a virus order will depend on the quantities and strain(s) requested, and this is subject to regular review. For the latest pricing, please contact AgResearch to discuss your needs. Pricing reflects our need to recover the basic costs of consumables, staff time, plus maintenance of the cell line, virus strains and associated equipment. The costs of shipping and handling are in addition to the virus production and will be added to the final price.

Please direct enquires regarding *Oryctes* virus to either:

Sean Marshall (sean.marshall@agresearch.co.nz) or

Trevor Jackson (trevor.jackson@agresearch.co.nz)

Fax +64-3-325-9946

Mail AgResearch, Lincoln Research Centre, Private Bag 4749, Christchurch 8140, New Zealand.

Appendix 1: Suppliers of lab equipment and consumables used in 2009

Lab supplies	Catalogue number	Supplier	Price
1ml filtered tips 6 boxes of 96	LC1187-965	Interlab	\$90.00
1-100ul filter tip, 10 boxes of 96 (Labcon)	LC 1184-965	Interlab	\$100.00
0.1-10ul filter tips, 10 boxes of 96 (Labcon)	LC 1181-965	Interlab	\$95.00
latex gloves (10 pks of 100)	#105-108	Interlab	\$85.00
1.5 ml tubes (bag of 500)	AXMCT175C	Global Sciences	
Agarose, DNA grade, low EEO (Applichem)	ApLa-2114-0500	Global Sciences	\$994.00
Flipper racks	3570	Global Sciences	
Flipper freezer racks	3575	Global Sciences	
1litre 10xTBE	15581044	Invitrogen	\$54.40
Dnase/Rnase-free distilled water (500ml)	10977015	Invitrogen	\$31.00
primers (3a, 15a, 15b)	online	Invitrogen	\$40.00
SYBR Safe DNA gel stain (10,000 fold concentrate)	SKU# S33102	Invitrogen	\$119.00
1x TE buffer (100 ml)	12090-015	Invitrogen	\$40.00
1M Tris-HCl (1 litre)	15568-025	Invitrogen	\$69.00
Thermoprime plus PCR Master Mix (2.5mM MgCl ₂)	AB-0619	Innovative Sciences	\$135.00
0.2ml thin-walled tubes with flat caps (x1000)	AB-0620	Innovative Sciences	\$75.00
Qiagen DNeasy Blood and Tissue Kit (x50)	QIA69504	Biolab Scientific	\$435.40
BSA - 60mg 10mg/ml	NEBB9001S	Biolab Scientific	\$40.65
Biohazard bags (50)	LAB3500/504	Biolab Scientific	\$71.00
Zymo Research Genomic DNA Tissue MiniPrep	D3050	Ngaio Diagnostics Ltd	\$259.00

Interlab Ltd, PO Box 13018, Johnsonville, Wellington
Ph: +64 4 972 2330; Fax: +64 4 939 6170
Email: info@labconnect.co.nz

Biolab Scientific Ltd (Akl), Private Bag 102 922, North Shore Mail Centre, Auckland
Ph: +64 9 980 6700; Fax: +64 9 980 6788
<http://www.biolab.co.nz>

Global Science & Technology (Lower Hutt), PO Box 101 253, North Shore Mail Centre, Auckland NSMC. URL: www.globalscience.co.nz
Tel: +64 9 443 5867; Fax: +64 9 444 7314
Email: sales@globalscience.co.nz

Invitrogen New Zealand Ltd (Ecom), PO Box 12-502, Penrose, Auckland
Ph: +64 9 579 3024; Fax: +64 9 579 3119
Email: nzmail@invitrogen.com
URL: <http://www.invitrogen.com>

Innovative Sciences Ltd, PO Box 13108, Dunedin
Phone: +64 3 488 3832; Fax: +64 3 488 3837
Email: isl@molbio.co.nz

Ngaio Diagnostics Ltd, PO Box 4015, Nelson, New Zealand
Ph: +64 3 548 4727; Fax: +64 3 548 4729