

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

TOS PROTOCOL AND PROCEDURE: GROUND BEETLE AND MOSQUITO SPECIMEN PROCESSING

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See configuration management system for approval history.

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Change Record

REVISION	DATE	ECO #	DESCRIPTION OF CHANGE
A_DRAFT	01/10/2014	ECO-01138	Draft release
B	03/26/2014	ECO-01673	Production release, template change, and other changes as detailed in Appendix C

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

TABLE OF CONTENTS

1 DESCRIPTION.....1

1.1 Purpose 1

1.2 Scope..... 1

1.3 Acknowledgements..... 1

2 RELATED DOCUMENTS AND ACRONYMS2

2.1 Applicable Documents 2

2.2 Reference Documents..... 2

2.3 Acronyms 2

2.4 Definitions..... 3

3 BACKGROUND AND OBJECTIVES3

3.1 Background 3

3.2 NEON Science Requirements 3

3.3 NEON Data Products 4

4 PROTOCOL4

5 QUALITY ASSURANCE AND CONTROL4

6 SAFETY4

7 PERSONNEL REQUIREMENTS.....4

8 TRAINING REQUIREMENTS.....5

9 SAMPLE FREQUENCY AND TIMING5

9.1 Sampling Frequency and Timing 5

10 STANDARD OPERATING PROCEDURES.....6

SOP A: Preparing for Laboratory Processing and Analyses 6

SOP B: Laboratory Processing..... 9

SOP C: Processing for Genetic Analysis 22

SOP D: Photographing Insect Specimens..... 29

11 REFERENCES35

APPENDIX A QUICK REFERENCES FOR LAB PROCESSING36

APPENDIX B CHECKLISTS FOR LAB PROCESSING37

APPENDIX C CHANGE NOTES.....38

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

APPENDIX D	INSECT LOCALITY LABELS	39
APPENDIX E	INSECT DETERMINATION LABELS	46
APPENDIX F	INSECT UNIQUE NUMBER LABELS.....	50
APPENDIX G	CUTTING LABELS	54

LIST OF TABLES AND FIGURES

Table 1: Insect pinning and pointing equipment list	7
Table 2 Genetic analysis preparation equipment list	22
Table 3: Photography equipment list	29
Table 4. Data required for locality labels.....	40
Table 5. Accepted site name abbreviations for locality labels	40
Table 6. Number of labels needed for the different activities requiring insect locality labels.	45
Table 7. Data required for labels	46
Figure 1. Technician preparing pinned and pointed specimens.....	6
Figure 2. Pinned (left) and pointed (right) beetle specimens.....	9
Figure 3. Materials for pointing including a schematic of a point showing where to place pin	10
Figure 4. A pinning block with a point and the various labels that will be affixed	11
Figure 5. A ground beetle on its side in preparation for pointing	12
Figure 6. Examples of bending a point tip to maintain a level body position of the specimen	13
Figure 7. Steps for gluing a specimen to a point including dipping, attaching and drying.....	13
Figure 8. Diagram of ventral side of a beetle, showing where to place point.....	14
Figure 9. A well-pointed specimen with associated labels	14
Figure 10. A poorly pointed specimen.....	15
Figure 11. Attaching a locality label below a pointed beetle.	15
Figure 12. Mosquito pointing steps.....	16
Figure 13. Pointed mosquitoes.....	16
Figure 14. Attaching a locality label below a pointed mosquito.	17
Figure 15. Ground beetle pinning and dorsal side schematic showing where to place the pin	18
Figure 16. Demonstration of correct and incorrect pinning alignment.	18
Figure 17. Attaching a locality label below a pinned ground beetle.	19
Figure 18. A leg is inserted back into its socket using forceps and a dab of glue.....	19
Figure 19. Proper orientation of mounted specimens in a Schmitt box.	20
Figure 20. Ground beetle showing the priority in which legs are removed for DNA barcoding	25
Figure 21. Anatomy of a beetle leg.....	25
Figure 22. Placing tissue in a well.	26
Figure 23. Filled 96 well plates	26

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

Figure 24. A 96-well microplate prepared for shipping 28

Figure 25. Translucent box with light setup. 31

Figure 26. Examples of beetle and mosquito photos 32

Figure 27. How to measure beetle specimens 34

Figure 28. Example labels for CO2 light trap and pitfall trap samples 39

Figure 29. Spreadsheet after step 1 for 8 plots 41

Figure 30. Dimensions for creating a locality label template..... 42

Figure 31. Selecting recipients from an Microsoft Excel file during a Mail Merge..... 42

Figure 32. Inserting merge fields into label template 43

Figure 33. Example determination labels; size shown is larger than size of actual label..... 46

Figure 34. Spreadsheet after step 1 for beetle and mosquito species..... 47

Figure 35. Dimensions for creating a locality label template..... 48

Figure 36. Selecting recipients from a Microsoft Excel file during a Mail Merge..... 48

Figure 37. Inserting Merge Fields into label template..... 49

Figure 38. Individual ID label (a mosquito label from Domain 10)..... 50

Figure 39. Mosquito pooled sample vial label..... 50

Figure 40. Mail Merge pop-up window 53

Figure 41. Inserting Merge Fields into label template..... 53

Figure 42. Example of a strip of labels..... 54

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

1 DESCRIPTION

1.1 Purpose

The primary purpose of this document is to provide change controlled version of Observatory protocols and procedures for Plot Establishment. This document provides the content for training and field-based materials for NEON staff and contractors. Content changes (i.e. changes in particular tasks or safety practices) occur via this change controlled document, not through field manuals or training materials.

This document is a detailed description of the field establishment process, relevant pre- and post-field tasks, and safety issues as they relate to this procedure and protocol.

1.2 Scope

This document relates the tasks for a specific field sampling and directly associated activities and safety practices. This document does not describe:

- General safety practices
- Site-specific safety practices
- General equipment maintenance

It does identify procedure-specific safety hazards and associated safety requirements such as safe handling of small mammals or safe use of required chemicals and reagents.

1.3 Acknowledgements

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

2 RELATED DOCUMENTS AND ACRONYMS

Cara Gibson and Patrick Travers contributed to earlier versions of these protocols.

2.1 Applicable Documents

Applicable documents contain information that shall be applied in the current document. Examples are higher level requirements documents, standards, rules and regulations.

AD [01]	NEON.DOC.004300	EHS Safety Policy and Program Manual
AD [02]	NEON.DOC.004316	Operations Field Safety and Security Plan
AD [03]	NEON.DOC.000724	Domain Chemical Hygiene Plan and Biosafety Manual
AD [04]	NEON.DOC.001155	NEON Training Plan
AD [05]	NEON.DOC.050005	Field Operations Job Instruction Training Plan
AD [06]	NEON.DOC.000909	TOS Science Design for Ground Beetle Abundance and Diversity
AD [07]	NEON.DOC.000910	NEON Science Design for Mosquito Abundance, Diversity, and Phenology
AD [08]	NEON.DOC.014055	FSU Operations and Maintenance Plan
AD [09]	NEON.DOC.014049	TOS Protocol and Procedure: Mosquito Sampling
AD [10]	NEON.DOC.014050	TOS Protocol and Procedure: Ground Beetle Sampling
AD[11]	NEON.DOC.004309	NEON Field Site Information
AD [12]	NEON.DOC.005003	NEON Level 0 Data Products Catalog
AD [13]	NEON.DOC.001582	Datasheets for TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing

2.2 Reference Documents

Reference documents contain information complementing, explaining, detailing, or otherwise supporting the information included in the current document.

RD [01]	NEON.DOC.000008	NEON Acronym List
RD [02]	NEON.DOC.000243	NEON Glossary of Terms
RD [03]	NEON.DOC.005003	NEON Scientific Data Products Catalog
RD [04]	NEON.DOC.014051	Field Audit Plan
RD [05]	NEON.DOC.000824	Data and Data Product Quality Assurance and Control Plan

2.3 Acronyms

SDS	Safety Data Sheets
PG	Propylene Glycol

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

2.4 Definitions

A **protocol** is a formal summary description of a procedure and its related rationale, and includes information on knowledge and resources needed to implement the procedure. A **procedure** is a set of prescribed actions that must take place to achieve a certain result, and can also be called a method. It differs from a science design in that science designs provide a more complete description of the rationale for selecting specific protocols. It differs from a training manual in that training manuals provide materials in support of skills acquisition in the topic areas including information on how to best train staff rather than detailing only the steps of the procedure.

3 BACKGROUND AND OBJECTIVES

3.1 Background

This protocol contains instructions for processing beetle and mosquito specimens for archival storage and for DNA barcoding. NEON has adopted standardized archiving methods, including label formats and pinning and pointing styles. For mosquitoes, NEON is following the Walter Reed Biosystematics Unit standard for mounting (Gaffigan & Pecor, 1997). For beetles, NEON is following general community standards (Johnson & Triplehorn, 2004) which are described, for example, in *Borror and DeLong's Introduction to the Study of Insects* (page 755).

The majority of ground beetle specimens and a small subset of mosquito specimens are pinned/pointed prior to being sent to an archive facility. In addition to being a community standard for specimen preservation, this allows NEON to provide a voucher collection that demonstrates which specimens were classified as particular species. Not all ground beetles and mosquitoes are pinned/pointed. Detailed instructions for selecting beetles for pinning and pointing are provided in AD[10]. Only mosquitoes that will be sent for DNA barcoding will be pointed.

DNA barcoding is a molecular taxonomic technique that uses a short genetic marker to classify an organism to species. This technique was recommended to NEON at an NSF-sponsored Bioarchive meeting in order to provide confirmation of technician identifications, but also alleviate the burden on expert taxonomists of identifying common and abundant species.

3.2 NEON Science Requirements

This protocol fulfills Observatory science requirements that reside in NEON's Dynamic Object-Oriented Requirements System (DOORS). Copies of approved science requirements have been exported from DOORS and are available in NEON's document repository, or upon request.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

3.3 NEON Data Products

Execution of this protocol procures samples and/or generates raw data satisfying NEON Observatory scientific requirements. These data and samples are used to create NEON data products, and are documented in the NEON Scientific Data Products Catalog (RD [04]).

4 PROTOCOL

This protocol follows field collection of ground beetles AD[10] and mosquitoes AD[09] and describes laboratory processing of insects including pinning (SOP A: Preparing for laboratory processing and analyses), photographing (SOP C: Processing for Genetic Analysis), preparing and submitting samples for DNA barcoding (**SOP B. Laboratory Processing**).

5 QUALITY ASSURANCE AND CONTROL

The procedures associated with this protocol will be audited according to the Field Audit Plan (RD[04]). Additional quality assurance will be performed on data collected via these procedures according to the Data and Data Product Quality Assurance and Control Plan (RD[05]).

6 SAFETY

Personnel working at a NEON site must be compliant with safe field work practices as outlined in the Operations Field Safety and Security Plan (AD[02]) and EHS Safety Policy and Program Manual (AD[01]). Additional safety issues associated with this field procedure are outlined below. The Field Operations Manager and the Lead Field Technician have primary authority to stop work activities based on unsafe field conditions; however, all employees have the responsibility and right to stop their work in unsafe conditions.

Safety Data Sheets (SDS) are available for the following chemicals used in this work:

- Propylene glycol
- Ethanol
- Paradichlorobenzene (moth crystals)

7 PERSONNEL REQUIREMENTS

Prior experience collecting ground beetles or working with related insects (i.e., entomological fieldwork) is desirable but not required. Personnel should have good fine manual coordination for handling individual specimens and pinning/pointing. Personnel should also have excellent attention to detail and good handwriting in order to fill out and correctly associate labels with a small font size and detailed information with each specimen.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

8 TRAINING REQUIREMENTS

All technicians must complete required safety training as defined in the NEON Training Plan (AD[04]). Additionally technicians complete protocol specific training for safety and implementation of the protocol as required in Field Operations Job Instruction Training Plan (AD[05]).

9 SAMPLE FREQUENCY AND TIMING

9.1 Sampling Frequency and Timing

To ensure expedient delivery of data products, insect lab processing must occur in the fall months after field collection each year. Sorting and pinning should commence as soon as possible after field activities have ended. All beetle specimens should be sent to expert taxonomists and all tissue samples submitted for DNA barcoding by the end of the calendar year. This will allow time in the spring for taxonomists and barcoding facilities to process and return NEON samples. Subsequently, morphospecies identifications can be resolved to the species level and teaching collections can be updated before the start of the next field season.

During processing, the following steps should be completed in order:

1. Pin and point all beetles and/or mosquitoes to be barcoded (**SOP B. Laboratory Processing**)
2. Record specimens to be barcoded in Barcode Plate datasheet (**SOP B. Laboratory Processing**).
3. Photograph each specimen to be sent for barcoding (**SOP C: Processing for Genetic Analysis**).
4. Measure length of each beetle to be barcoded (may occur before/during step 3). **SOP D: Photographing Insect Specimens.**

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

10 STANDARD OPERATING PROCEDURES

SOP A: Preparing for Laboratory Processing and Analyses

Sample Processing Background

- 1) Prepare workspace
 - a) Prepare lab bench space in the clean lab to sort and prepare specimens (see **Figure 1**). You will need access to at least one dissecting microscope.
 - b) Ensure storage space is available in Cornell cabinets and/or Schmitt boxes, shipping mailers for pinned specimens, and cold storage space for frozen mosquitoes and ethanol preserved bycatch and beetles.



Figure 1. Technician preparing pinned and pointed specimens

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Laboratory Equipment and Materials

Table 1: Insect pinning and pointing equipment list

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Insect Pin, Black, #3, 100/Pack	Mounting insects	10	N	N
Required	Storage for pinned specimens (unit tray/Schmitt box)	Storing mounted specimens	Variable	N	N
Required	Point Punch, Square Base	Creating points	1	N	N
Required	Marker, Permanent, Archival, Pigma Micron Brand, size 01, 0.25mm, Black	Marking on specimen labels	5	N	N
Required	Paper, 100 pct rag cotton, Smooth, 9 x 12 pad; Strathmore 342 Bristol or Equivalent.	Creating points	1 sheet	N	N
Required	Insect Pinning Block, Anodized Aluminum, 12, 16 and 20 mm Dimension Holes for Labels, 26 mm Dimension Holes for points	Positioning point & labels on pin	1	N	N
Required	Jewelers forceps (fine point)	Handling insects	3	N	N
Required	Microscope, stereo-zoom, 10x widefield eyepieces, 0.7x to 4.5x zoom lens for a total magnification of 7x to 45x	Used during pointing	2	N	N
Required	Paradichlorobenzene - moth crystals	Pest prevention	1 lb, ~6.5 g per box	N	Y
Suggested (may use foam)	Balsa wood (approximately .25" x .5" x 2.5")	Temporarily hold specimens or pins/points & attaching points/labels to pins	4	N	N
Required	Dual Gooseneck Light	Used with	1	N	N

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
		microscope			
Required	Elmer's white glue	Attaching specimens to points	2	N	N
Required	Fumigant boxes	Storing moth crystals within containers for storing specimens	1 per cornell drawer and 10 extra	N	N
Required	Locality labels (see Appendix D)	Labeling specimen collection data	1 per specimen	N	N
Required	Determination labels (see Appendix E)	Labeling specimen identification	1 per specimen	N	N
Required	Plain white paper	Pointing specimens under microscope	1	N	N

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

SOP B: Laboratory Processing

- 1) Select specimens to mount.
 - a) All ground beetles will be pinned/pointed except abundant and easily identifiable species (noted on site-specific lists in AD [10]). Only a subset of abundant and easily identifiable species will be pinned/pointed. Detailed instructions for selecting this subset and species lists for each site are provided in AD[10]. If you encounter a very abundant species (e.g., >100 individuals), contact the staff insect ecologist at HQ about adding it to the site-specific list of abundant and easily identifiable ground beetles.
 - b) Only mosquitoes that are submitted for DNA barcoding will be pointed. Mosquitoes will either be pointed at the identification facility or selected individuals to be pointed will be returned in vials to the domain where they were trapped (guidance will be provided to domain staff via a Jira ticket once contracts have been established).
- 2) Determine whether to pin or point the specimen.
 - a) Generally specimens 5 mm and greater are pinned, and specimens smaller than 5 mm are pointed (see Figure 2).
 - b) Pointing is a good default option for any specimen you are concerned will be destroyed by pinning.
- 3) Summary of Insect Mounting Criteria

Insect	Criteria	Mounting Method
Ground Beetle	≥ 5mm	Pinning
Ground Beetle	≥ 5 mm but likely to be damaged by pinning	Pointing
Ground Beetle	< 5 mm	Pointing
Mosquito	Any size	Pointing



Figure 2. Pinned (left) and pointed (right) beetle specimens

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- 4) Prepare points (if pointing). Pointing consists of attaching specimens with adhesive (Elmer’s white glue) to the tip of a small triangle, known as a ‘point’.
 - a) Prepare a batch of points prior to beginning pointing specimens.
 - b) Using 100% rag cotton paper (Bristol or equivalent), prepare ~40 triangles using the point punch.
 - c) Over a balsa wood surface, for each point, press a No. 3 archival quality pin into the point equidistant from the two corners and the center of the base (see x on Figure 3). Ensure that the point is at a 90 degree angle from the pin and neither angled up nor down.



Figure 3. Materials for pointing including a schematic of a point showing where to place pin

- d) A pinning block consists of a series of sequentially shallower pin holes and is used to position insects and labels at uniform heights on a pin. Using a pinning block, push the point up the pin until it is 15 mm from the top (non-pointed end) of the pin. This leaves enough space above the insect for the pin to be a useful handle and below for data and other labels. Specimens at a uniform height are easier to examine and compare with one another.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

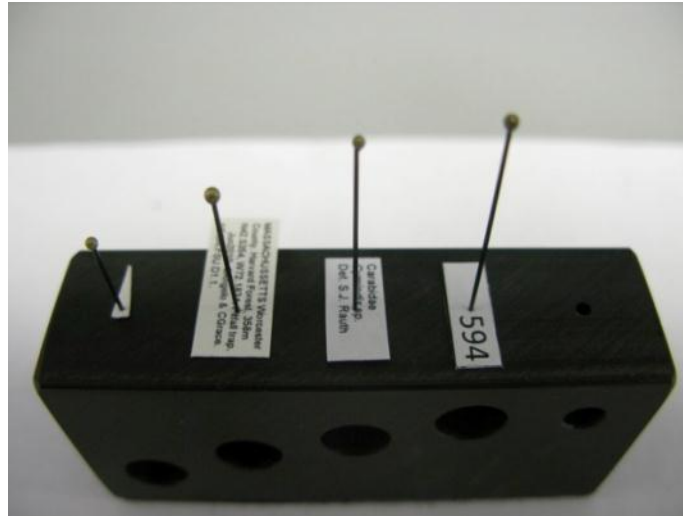


Figure 4. A pinning block with a point and the various labels that will be affixed to a pinned or pointed specimen. This shows the heights at which these items should be placed on a pin.

- 5) Prepare labels (Figure 4)
 - a) See Appendix D for instructions on creating locality labels. These will be created for all mounted beetle and mosquito specimens at the time of preparation and will be placed on the pin below each specimen.
 - b) See Appendix E for instructions on creating determination labels. These will be created for all mounted beetle and mosquito specimens at the time of preparation and will be placed on the pin below the locality label below each specimen.
 - c) See Appendix F for instructions on creating insect individual ID labels. These will be created for all mounted beetle and pointed mosquito specimens at the time of preparation and will be placed on the pin below the determination label below the locality label below each specimen.
 - d) It is important to consider how you will attach the correct locality and determination labels to specimens as you are mounting them. **A specimen dissociated from its labels is worthless.**
- 6) Mount specimens.
 - a) Point **beetle** specimens ≤ 5 mm (or larger specimens if you are not confident they can be pinned without damage).
 - i) Pointing should be done under the dissecting microscope so that the point is attached to the correct place on the beetle. For efficiency, work with several specimens (from the same pitfall trap or sampling bout) at a time.
 - ii) Allow specimens to dry for a few minutes. It is easier to work with dried specimens when pointing.
 - iii) Use forceps to put the beetle on its left side on a slip of plain white paper so that its head is facing the right and its legs are toward the edge of the bench (Figure 5)

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B



Figure 5. A ground beetle on its side in preparation for pointing

- iv) Slide the slip of paper with the beetle to the center of the microscope stage.
- v) Examine the slope of the ground beetle's thorax to determine if the tip of the point needs to be bent to ensure that the specimen will lie flat on the point. Use forceps to bend the point if needed (Figure 6).
- vi) **NOTE:** The sides of the thorax can slope inward to differing degrees, therefore, the tip of the point can be bent down with forceps to accommodate the size and shape of each particular specimen. For example, a specimen that has a flat-bottomed thorax can be mounted on a flat (unbent) point. A specimen that has the sides of the thorax vertical must have the tip of the point bent downward at a right angle. See Figure 6 for thorax slope scenarios and Figure 9 and Figure 10 for examples of well-pointed and poorly-pointed specimens, respectively.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

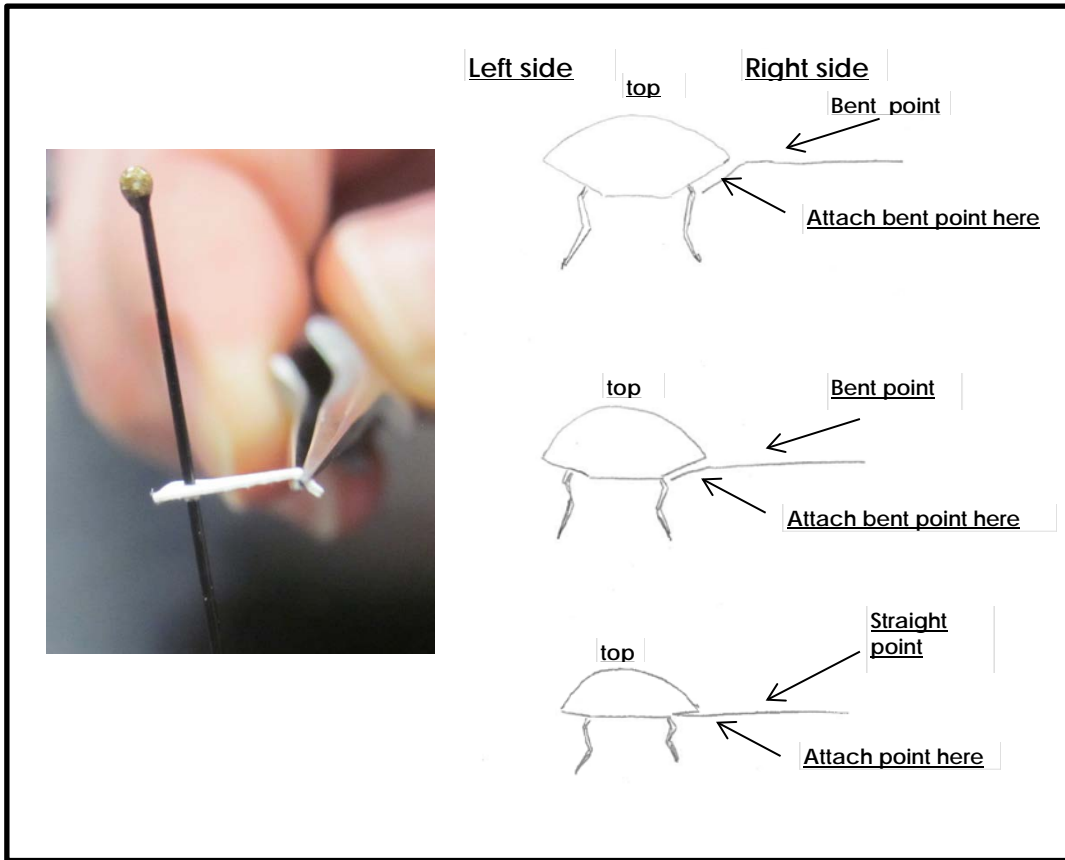


Figure 6. Examples of bending a point tip to maintain a level body position of the specimen

- vii) Take the prepared point and touch the tip of the point in a bit of glue (Figure 7). Use the minimum amount of glue that will attach the specimen firmly to the point. Avoid obscuring any part of the beetle with large amounts of glue. Wipe off excess glue.



Figure 7. Steps for gluing a specimen to a point including dipping, attaching and drying

- viii) Under a microscope, narrow in on the target spot for the point on the thorax between the bases of the 2nd and 3rd pair of legs on the beetle's right side (from dorsal perspective) and gently press the glue to the beetle's body (Figure 7, Figure 8). **Note:** Although the point and glue will obscure one side of the thorax, the other side will be free for examination. Neither the point nor the glue should extend onto the top or the bottom of the beetle, nor touch the head, abdomen, or wings.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

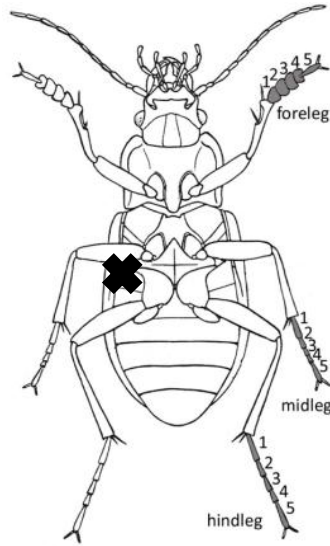


Figure 8. Diagram of ventral side of a beetle, showing where to place point

- ix) Wait for the glue to set a bit so that the beetle stays relatively still when the pin is moved. Then lift and angle the pin so that the weight of the specimen is leaning directly into the point (Figure 8).
- x) Place the pin in a block of styrofoam such that gravity is holding the specimen down on the point at the correct angle.
- xi) Adjust the specimen as needed with forceps or a pin to ensure the specimen binds firmly to the point. A heavy specimen that rotates on the point should be straightened as the glue thickens but before it completely hardens.

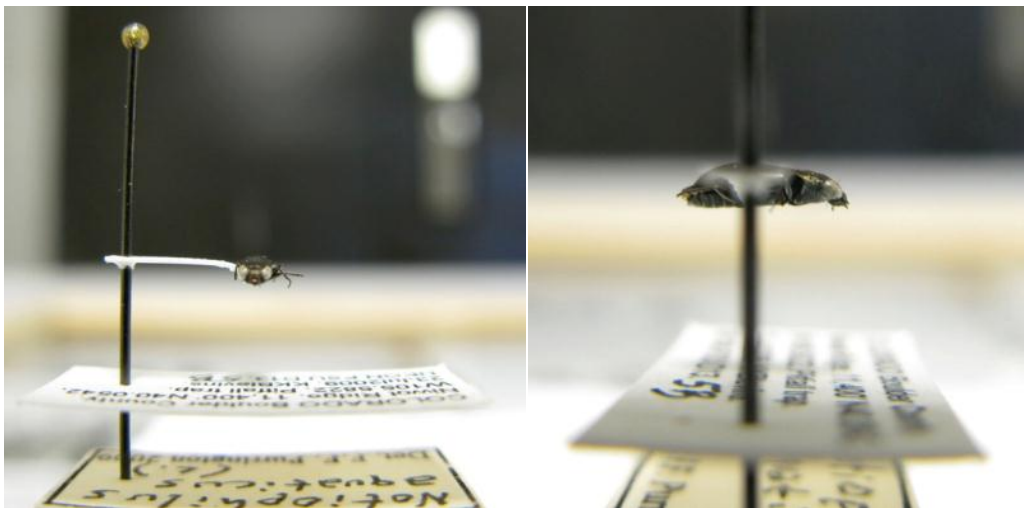


Figure 9. A well-pointed specimen with associated labels

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

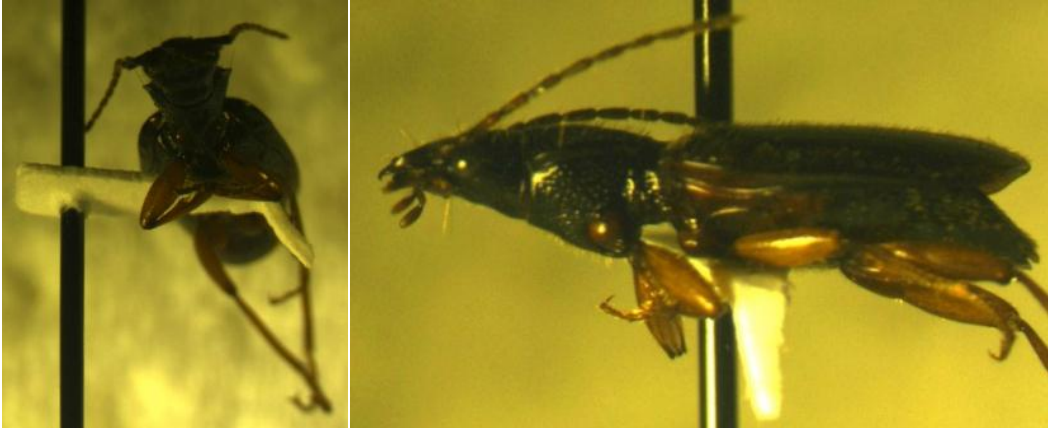


Figure 10. A poorly pointed specimen

- xii) Attach the locality label. Use the second-deepest hole in the pinning block to position the label at the correct height (Figure 11). Ensure that the label information is correct. Think of the locality label as protection for the specimen. Position the specimen perpendicular to the text on the label, with the back edge of the point slightly inset from the short edge of the label (Figure 10). Ensure that the label information is correct
- xiii) Attach the determination label below the locality label. Use the third-deepest hole in the pinning block to position the label at the correct height (Figure 9).. Orient the label in the same manner as the locality label.
- xiv) Attach the insect individual ID label below the determination label using the fourth-deepest (most shallow) hole. Orient the label in the same manner as the locality label.



Figure 11. Attaching a locality label below a pointed beetle.

- xv) When all of the specimens are mounted and labeled, examine them under the microscope to confirm that they are all attached securely to the points. To test, place the specimens in a Schmitt box or unit tray and tap the box or tray. The specimens should remain fixed. Re-attach any specimens (as long as it is clear which locality label it belongs with).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- b) Point **mosquito** specimens. Pointing mosquitoes is very similar to pointing beetles, except for the orientation and point of attachment.
- i) Using forceps, put the mosquito or mosquitoes on their left side on a slip of plain white paper so that their heads are facing the left and their legs are toward the edge of the bench (Figure 12)
 - ii) Slide the slip of paper with the mosquitoes to the center of the microscope stage.
 - iii) Take the prepared point and touch the tip of the point in a bit of glue (Figure 12). Use the minimum amount of glue that will attach the specimen firmly to the point. Avoid obscuring any part of the mosquito with large amounts of glue.
 - iv) Touch the glue-dabbed point against the right pleuron (side of thorax) with the legs pointing toward the pin (Figure 12)
 - v) Lift and angle the pin so that the weight of the specimen is leaning directly into the point.

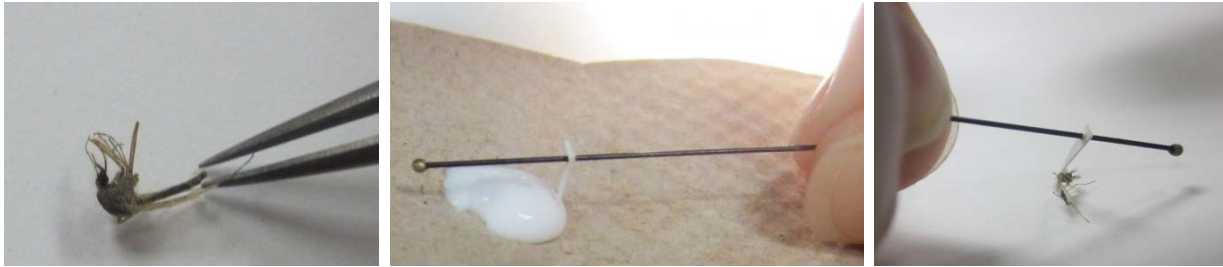


Figure 12. Mosquito pointing steps.

- vi) Place the pin in a block of styrofoam such that gravity is holding the specimen to the point.
- vii) Adjust the specimen with forceps or a pin (Figure 13). Adjusting the specimen should cause it to bind firmly to the point. Specimen should be straightened as the glue thickens but before it completely hardens.



Figure 13. Pointed mosquitoes.

- viii) Attach the locality label. Use the second-deepest hole in the pinning block to position the label at the correct height. Ensure that label information is correct. Pointed specimens are positioned perpendicular to the text on the label, with the back edge of the point slightly

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

inset from the short edge of the label and the specimen centered between the two longer edges of the label (Figure 14).



Figure 14. Attaching a locality label below a pointed mosquito.

- ix) Attach the determination label below the locality label using the third-deepest hole. Ensure that the label information is correct. Orient the label in the same manner as the locality label.
 - x) Attach the insect individual ID label below the determination label using the fourth-deepest (most shallow) hole. Orient the label in the same manner as the locality label.
 - xi) When all of the specimens are mounted and labeled, examine them under the microscope to confirm that they are all attached securely to the points. To test, place the specimens in a Schmitt box or unit tray and tap the box or tray. The specimens should remain fixed. Re-attach any specimens (as long as it is clear which locality label it belongs with).
- c) Pin beetles > 5 mm.
- i) Note: if you are new to pinning, start with larger specimens as they are easier. Keep specimens in ethanol until ready to pin.
 - ii) Use only No. 3 archival quality pins. Finer pins are too flexible and vibrate in the specimen, destroying them from within. Thicker pins damage specimens as they are inserted.
 - iii) Insert the pin vertically through the top layer of the body (Figure 15). **It is important to insert the pin in the correct location in the beetle's body.** A properly placed pin is positioned on the right side of the beetle (viewed dorsally), halfway between the midline of the beetle's body and the edge of the beetle where the elytra (wing shells) begin to round. Holding the beetle on its sides, use your hands (not forceps) to gently slide the beetle onto the pin. Push pin slowly and with constant pressure.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

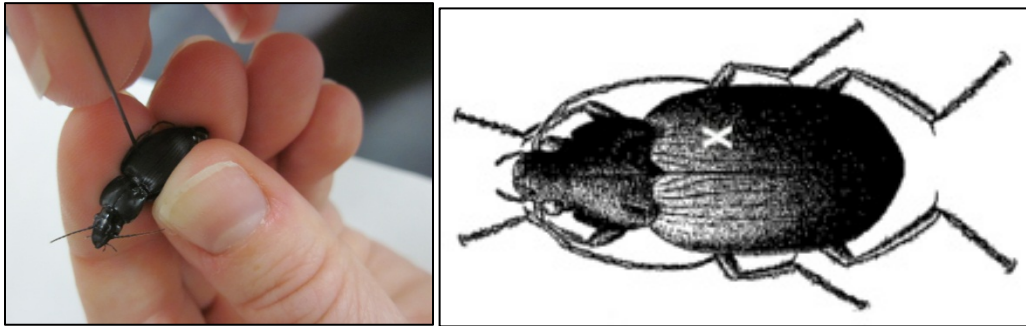


Figure 15. Ground beetle pinning and dorsal side schematic showing where to place the pin

- iv) Pause and look at the specimen from the side **BEFORE** pushing the pin all of the way through.
- v) Ensure that the pin is at a right angle to the beetle's body (Figure 16).
- vi) Push the specimen up the pin until its top surface is 15 mm from the top of the pin. This leaves enough space above the insect for the pin to be a useful handle and below for data and other labels. Specimens at a uniform height are easier to examine and compare with one another.

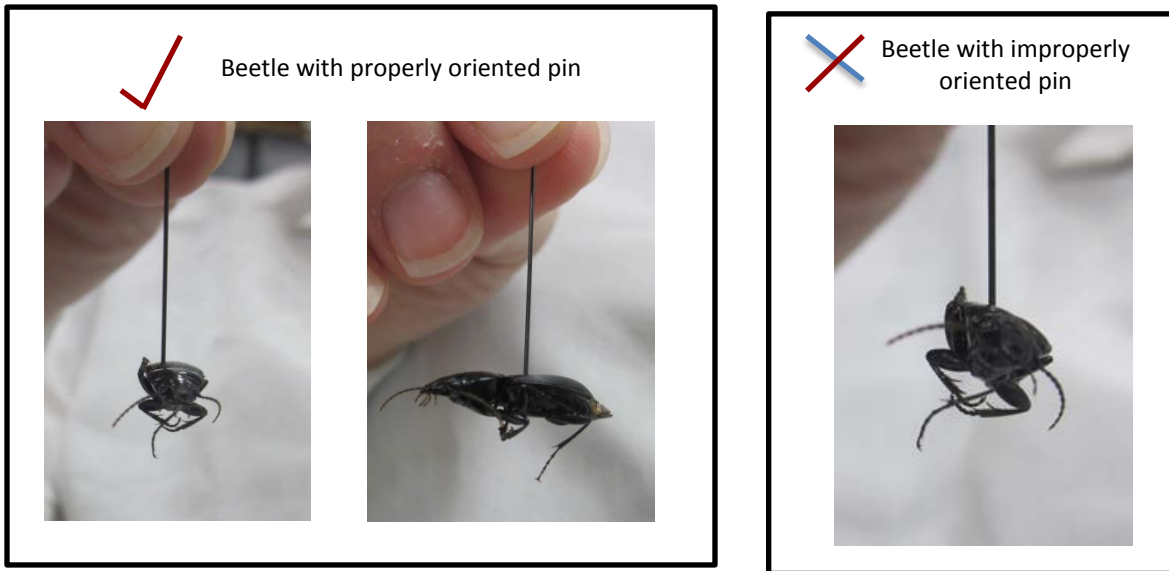


Figure 16. Demonstration of correct and incorrect pinning alignment.

- vii) Attach the locality label. Use the second-deepest hole in the pinning block to position the label at the correct height. Ensure that label information is correct. Think of the locality label as protection for the specimen. Pinned specimens are placed in the middle of the locality label with the head on the left side. The specimen should be lined up with the long axis of the body parallel with the print on the label (Figure 17).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B



Figure 17. Attaching a locality label below a pinned ground beetle.

- viii) Attach the determination label below the locality label using the third-deepest hole in the pinning block to position the label at the correct height. Ensure that the label information is correct. Orient the label in the same manner as the locality label.
- ix) Attach the insect individual ID label below the determination label using the fourth-deepest (most shallow) hole. Orient the label in the same manner as the locality label.
- d) Loss of body parts
 - If a part of a specimen, such as a leg, falls off during pinning, save it and re-attach it to the beetle (provided you are certain which specimen the body part belongs to) (Figure 18).
 - i) Hold the part being reattached with forceps.
 - ii) Touch the top of the body part to a bit of glue.
 - iii) Place the body part back into correct location and orientation.



Figure 18. A leg is inserted back into its socket using forceps and a dab of glue.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Sample Preservation

Organizing mounted specimens

1. Place specimens in box or tray so that locality labels are oriented in the same direction (Fig. 19).
2. Organize specimens by species or morphospecies, leaving space to place specimens mounted at a later date/from different sampling bouts.

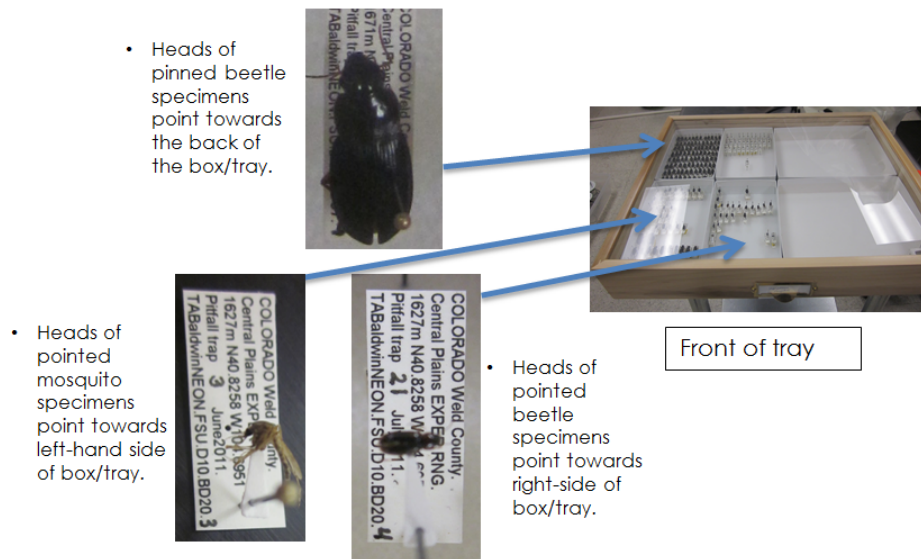


Figure 19. Proper orientation of mounted specimens in a Schmitt box.

The following are best practices that should be followed for best preservation of samples.

- Store pinned and pointed specimens in airtight Schmitt boxes or in unit trays in Cornell cabinet drawers with ~6.5 g of paradichlorobenzene (moth crystals) in a cardboard fumigant box.
- Secure the fumigant box in the corner of the Schmitt box or unit tray with pins so that it does not slide around and damage specimens.
- Check/replace paradichlorobenzene monthly.
- Samples can be stored in this manner indefinitely.
- Maintain fumigant in every drawer, including those without specimens. The fumigant acts as a repellent, but will not kill pests if they get into the collection.
- Never leave drawers sitting out, put them away when you are not working on them
- Keep counters clean, always wipe down with ethanol before putting a drawer on a countertop.
- Minimize amount of time lids are off drawers; never put drawers or lids on the floors
- Keep all dead insects out of lab, e.g. lights & windowsills. If sticky traps are used, replace them frequently.
- Specimen storage containers and workspaces should be kept clean to prevent pests. Dermestid beetles are common pests in insect collections. If you notice dermestid beetle activity, such as

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

debris under a specimen or larval skins of dermestid beetles, freeze the drawer/box for two weeks and clean up the damage.

Sample Shipping

Beetle samples will be shipped after specimens have been pinned/pointed, photographed, and had tissue removed for DNA barcoding purposes. Some mounted ground beetle specimens will be maintained at the domain lab for inclusion in the teaching collection. Other mounted specimens will be shipped to archive facilities. More details on this are provided in AD[10]. Pointed mosquito specimens can be shipped to the archive facility after they have been photographed and tissue has been removed for DNA barcoding.

Data Handling

Specimen-level data must be recorded for all mounted specimens, including collection details and species identification. These datasheets will be part of NEON’s data publication and will also be submitted to the DNA barcoding facility. For some specimens only morphospecies designations will be available when shipping to DNA barcoding facilities.

Equipment Maintenance, Cleaning and Storage

1) Microscope

- Keep covered when not in use
- Lenses may be cleaned with lens cleaning wipes such as Kimwipes as necessary

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

SOP C: Processing for Genetic Analysis

Sample Processing Timing

Once all beetle specimens have been processed, mounted and identified to species or morphospecies, a subset of specimens will be prepared for DNA barcoding. Similarly, once all mosquitoes have been identified at external facilities, a subset of those returned to the domain lab will be mounted and prepared for DNA barcoding. All specimen-level data for mounted specimens should be recorded into the Domain Lab ID and Pinning spreadsheet before beginning barcode processing.

DNA barcode samples must be submitted (shipped to a barcoding facility) by January of the following year in which they were collected.

Equipment and Materials

Table 2 Genetic analysis preparation equipment list

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
required	95% Ethanol	Sterilizing bench space and gloves	1 L	N	Y
suggested	Spray bottle for ethanol	Sterilizing bench space and gloves	1	N	N
required	Jewelers forceps (fine point)	Removing tissue	3	N	N
required – provided by CCDB	96-well microwell plates	Storing tissue to be barcoded	Variable	N	N
required – provided by CCDB	96-well microwell caps	Covering microwell plates	To fit plates	N	N
required	Microscope, stereo-zoom, 10x widefield eyepieces, 0.7x to 4.5x zoom lens for a total magnification of 7x to 45x	May be used to remove legs, particularly from small specimens	2	N	N
required	Packing tape	Shipping	variable	N	N
required	Shipping supplies (boxes, padded envelopes)	Shipping	Variable	N	N
required	Cardboard specimen mailers	Organizing specimens	Variable	N	N
required	nitrile gloves	Handling specimens	Variable	N	N
required	Shipping labels	Shipping	Variable	N	N
required	Dual Gooseneck Light	Used with microscope	2	N	N
required	Lighter, Butane, Refillable, Stainless Steel Extension, Adjustable Flame Control	Sterilizing forceps	1	N	N

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Preparation

- 1) Select the beetle and mosquito specimens to be barcoded.
 - a) Use the Domain Lab ID and Pinning Datasheet to determine how many beetle species or morphospecies were collected during the field season. The number will likely be on the order of 25 different species per site. Use data from external facilities to determine the number of mosquito species collected during the field season. The number will likely be on the order of 15 different species per site.
 - b) Select 570 individuals for 6 DNA barcode plates per domain (95 per plate). These numbers may change from year to year. The following rationale should be used to select specimens based on our confidence in their species identification:
 - i) After identification at an external facility, mosquitoes will be returned to the domain facility for barcode processing. For expertly identified mosquitoes, submit up to 5 specimens per species per domain. If there are fewer than 5 specimens for a given species, send all specimens per species per domain. Select individuals to represent as many sites within the domain as possible.
 - ii) Common beetle species that can reliably be identified by parataxonomists are listed in AD[10]. For species on this list, submit 10 individuals or 11% of individuals for that species (whichever is higher) for DNA barcoding.
 - iii) Beetle types/species not on this list are more difficult to identify and errors in identification are more likely to occur. For these species, submit either 20 individuals or 21% of the individuals for that type/species (whichever is higher). If types/species are present at multiple sites in the domain, include representatives from all possible sites. If multiple parataxonomists have identified the beetles, include representative individuals from all parataxonomists.
 - c) Before processing, calculate how many DNA samples will be generated by the number of available specimens, compare this number to the target and adjust sample size accordingly. If you need to add more specimens, add by increments of one specimen per species, unless there is only one specimen of a given species. If you need to remove specimens, reduce the total number of individuals per species, starting with the most abundant species so that the rarest species are still represented. You may contact the insect ecologist at HQ (David Hoekman, dhoekman@neoninc.org) for assistance if necessary.
 - d) Where possible, select specimens from a variety of sampling dates and locations within a site. Additionally, if there is obvious morphological variation within a species, select specimens that represent that variation.
- 2) Print one of the 96-well datasheets.
- 3) Prepare a workspace with 2 cardboard shipping mailers (to be used for organizing specimens during processing, not for shipping), 96-well microplate with row caps loosely attached, forceps, lighter, pencil or ethanol-safe pen, and gloves.

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

Sample Processing in the Lab

Microplates can contain any combination of beetle and mosquito specimens. Each microplate that is sent for DNA barcoding costs the same regardless of how many specimens are present. Fill each microplate completely (95 specimens) before shipping.

- 1) Wipe down the work area with 95% ethanol.
- 2) Prepare 95 specimens for barcoding. Work with a single microplate at a time and enter all data before proceeding to the next microplate.
 - a) Pin the 95 specimens into the foam bottom of one of the cardboard shipping mailers. Think of this as the staging area for specimens to have a leg removed for barcoding.
 - b) Fill out a 96 well datasheet with the plate number, sample location in the plate, barcode lab and catalog ID.
 - c) Pin a hardcopy of the 96 well datasheet into the foam bottom of the second cardboard shipping mailer and secure the corners into the styrofoam using pins. This is where specimens will be placed after having their leg removed. The specimen should be pinned into the box on the datasheet corresponding to which well its leg was placed in. The pinned beetles on the data sheet are a map of the 96-well plate.
- 3) Subsample specimens.
 - a) DO NOT place any foreign objects (e.g. labels) into sampling wells. If something falls into a well (e.g. eyelash), note it, and do not place a specimen sub-sample into that well. Move to the next well. The goal for the number of specimens barcoded will be reduced by the number of contaminated wells (e.g. if 1 well is contaminated, the goal will be reduced from 570 to 569).
 - b) Cover wells that are not in use with row caps. Note that strip caps are numbered and correspond to numbering on plates.
 - c) Prior to beginning, and between each specimen, flame-sterilize forceps for at least 2 seconds to ensure that no residual tissue is present.
 - d) The prioritized order for leg removal is as follows (if first priority leg is missing, move to the second and so on):
 - i) Beetles:
 - (1) Right side midleg
 - (2) Right hindleg
 - (3) Right foreleg
 - (4) Left side midleg
 - (5) Left hindleg
 - (6) If you encounter a beetle with only one leg and it is the left foreleg, then take the left foreleg (Figure 20).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

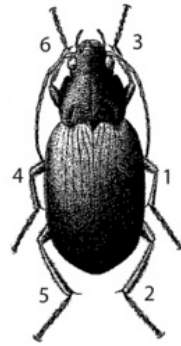


Figure 20. Diagram of a ground beetle showing the priority in which legs are removed for DNA barcoding

- ii) Mosquitoes:
 - (1) Right side midleg
 - (2) Left side midleg
 - (3) Right foreleg
 - (4) Left foreleg
 - (5) Right hindleg
 - (6) Left hindleg
- e) Excise a leg sample using forceps.
 - i) For small beetles (2-5 mm), excise the whole leg.
 - ii) For large beetles (>5 mm), take a subsample of the leg (tibia and tarsus only or femur only, 2–4 mm length) (Fig. 21).
 - iii) For mosquitoes, excise the entire leg.

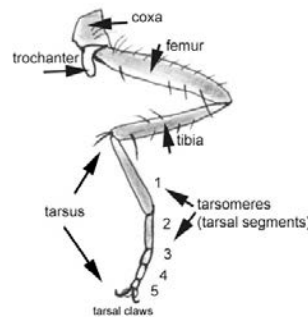


Figure 21. Anatomy of a beetle leg

- f) Place the specimen’s leg tissue sample in the well (Figure 22).
 - i) Gently pull open the row caps to the row you need to access. Minimize time that row caps are off.
 - ii) Static from the plate and on the forceps can make it difficult to get the leg into the well. BE PATIENT, it may take some time to get the leg into the well.
 - iii) Be gentle with the leg so that it does not end up in a well you did not intend.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- iv) It can be helpful to gently tap the forceps to shake the leg into the well. If the leg flies out of the well, look carefully to find it. If it has gone into a different well, do not remove it. Note where it is located and keep that sample if it fell into an empty well. Gently replace the row caps.

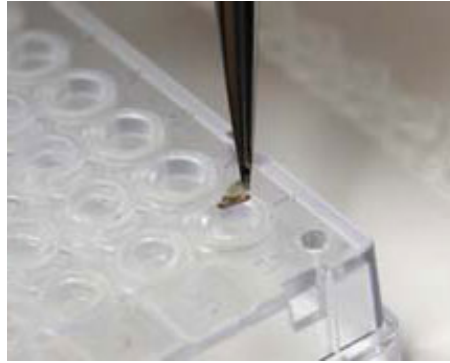


Figure 22. Placing tissue in a well. Note the row caps on all the rows not currently being utilized. Be sure to keep wells covered at all times, except when actively placing tissue into them.

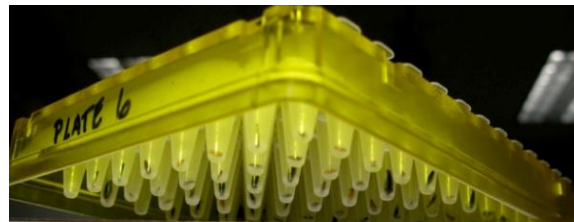


Figure 23. Filled 96 well plates

- g) Immediately after placing the leg in a well, pin the specimen into the box in the 96-well datasheet (that is attached to the shipping mailer) corresponding to the well its leg was placed in. This provides a map of the spatial arrangement of tissue samples in the 96-well plate. For example, if the leg of a specimen was placed in well A1, you would pin that specimen into the top left box of the datasheet (labeled as well number one).
- h) Using a lighter, flame-sterilize the forceps between each sample.
- i) Repeat until you have filled all wells in the microplate, **EXCEPT** for well H12 which always remains empty as a negative control (fill 95 wells).

Complete data entry prior to filling a new plate.

Sample Preservation

Store well plates inside a plastic bag in a location where they will be protected from jostling (e.g. inside a cabinet). Plates should be stored at room temperature until shipping.

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

Data Handling

STOP after each plate and enter the Plate number and Sample location into the Barcode Plate datasheet. Plate number is the unique plate number recorded on the 96-well datasheet. Sample location refers to the position each specimen occupies in the plate (A01-H12). Have another technician double-check data entry.

There are additional processing steps for pinned specimens (identification, adding determination and individual ID labels). This workflow is described in AD [06] and AD [07].

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Sample Shipping

Once the Beetle Sorting (described in AD [06]), Domain Lab ID and Pinning (described in AD [06] and AD [07]), Barcode Plate, and Barcode Image Data (described in SOP D) spreadsheets are complete, notify the insect ecologist at HQ (David Hoekman, dhoekman@neoninc.org) so that quality checking of these data can be expedited. Once the data have been quality checked, you will ship the plates. Ship 96-well microplates dry at room ambient temperature (20°C) to external facilities. Some facilities may require specific documentation (e.g., BMAA form for Canadian Centre for DNA Barcoding).



Figure 24. A 96-well microplate prepared for shipping.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

SOP D: Photographing Insect Specimens

Equipment and Materials

Table 3: Photography equipment list

Maximo Item No.	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Color separation guide (Color and measurement scale)	Providing color scale	1	No	No
suggested	box, opaque white plastic 1/2 gallon, 6"x6"x6"	Positioning & diffusing light around specimen	1	No	No
Required	styrofoam for photo box	Positioning specimen for photo	1	No	No
Required	Green tissue paper	Covering Styrofoam for background	1	No	No
Required	Dual Gooseneck Light	Lighting specimen	1	No	No
Suggested	Vellum as light diffusers (photos)	diffusing light around specimen	1	No	No
MX104227	Memory Card, SDHC, 32GB, Class 10; Promaster #5940 or Equivalent.	Store images	1	No	No
MX104649	Camera, Body Only, DSLR, 20+ Megapixel Resolution, multi-point focus, ISO-6400+, Water and Dust Resistant.	Photographing mounted insects	1	No	No
MX104660	Camera Lens, For DSLR Camera, Macro100/105mm focal length, f/2.8, 1x zoom, Autofocus, must be same brand as DSLR camera body	Photographing mounted insects	1	No	No
MX104653	Camera Battery, Lithium-Ion, Rechargeable, For DSLR Camera, Replacement, Must be compatible with DSLR camera body	Photographing mounted insects	1	No	No
MX104657	Memory Card Reader, For DSLR Camera, USB 3.0/2.0 Compatible, CompactFlash, SDHC, and SDXC Format Compatible	Transferring images	1	No	No
MX104381	TriPod, compact, non-magnetic, 1/4 x 20 ball head	Photographing mounted insects	1	No	No

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

Preparation

- 1) Organize specimens to be photographed
- 2) Check settings on DSLR camera with 100mm macro lens. Follow all manufacturer directions for proper set up.
- 3) Set up camera on tripod and position height of the camera so that it points straight down into the work space where the specimens will be photographed. The tripod helps eliminate any shaking or movement that will negatively affect the quality of the photograph.
- 4) Prepare the light diffusing box in which specimens will be positioned for photographing.
 - a. Set up a translucent Tupperware container to be used to stage specimens and diffuse light (Fig. 25). Cut Styrofoam to a size that will fit in the box. The size should be large enough to hold the specimen, its individual ID label, and the color and measurement scale but small enough to be easily removed from the Tupperware.
 - b. Cover the Styrofoam with green tissue paper.
 - c. Cut additional pieces of Styrofoam just smaller than the bottom of the Tupperware to raise the level of the specimen up so that it can easily be placed about 15 cm from the camera lens. These do not need to be covered with tissue.
- 5) Set up the dual gooseneck lights so that they shine directly on the specimen or on the two sides of the box (Fig. 25).
- 6) Cut strips of vellum paper to 11-14 inches long and 3-4 inches tall to be used for additional light diffusion if necessary. Tape the short ends of these strips together to form a circle with a diameter of 3-5 inches. These can be placed around the specimen with the light shining through these to create more light diffusion if there is glare on the specimen obscuring its features.
- 7) Pin the color and measurement scale into the green Styrofoam block.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

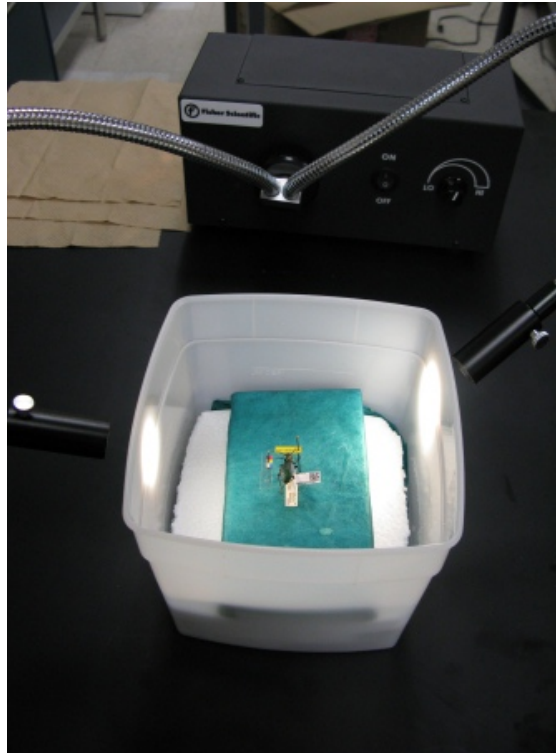


Figure 25. Translucent box with light setup.

Sample Processing in the Lab

Use the DSLR camera with 100mm macro lens on a tripod for all photographs. Photographs may be taken with only ambient light, however if features of a specimen are obscured by glare on its body, place the specimen inside a light-diffusing box (see Figure 24). If glare is still a problem, you can use the vellum paper rings to further diffuse light. The box can alternatively be positioned on its side if that is more convenient for the tripod/camera set-up at the domain lab.

Process one specimen at a time. Select the first specimen and remove its individual ID label and place it on a second pin. Set up each specimen as shown in Figure 26, with the number at the top of the frame perpendicular to the color and measurement scale. Each of these items should be pinned at the same height so that they are all in the same depth of field. BE VERY CAREFUL to replace the individual ID label after taking the picture.

1) Orient the specimen properly according to its taxon.

- i) Beetles: position the specimen on the styrofoam block so that its dorsal side (top shell or rounded back) is facing up (Figure 26).
- ii) Mosquitoes: position the specimen on the styrofoam block so that the lateral (side view) is facing up (Figure 26).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

2) Adjust camera settings.

- iii) Set the camera to capture JPEG images.
- iv) Keeping the camera in automatic focus, set to aperture priority.
- v) Adjust the ISO and f-stop settings to allow the depth of field to capture the entire body of a beetle or mosquito, including wings in focus.
 - (1) A good range for the ISO settings is 200-1600. The camera has automatic increases in the settings, but good test points are 200, 400, and 1600.
 - (2) Aperture is adjusted via the f-stop, with a good target around f/16. As you increase upwards, you will lose sharpness but gain depth of field. As you decrease the f/stop, you will lose depth of field. Make adjustments accordingly, taking into account your particular specimen.
- b) The specimen should be approximately 15 cm from the front of the camera lens. If necessary, adjust the specimen height until the entire specimen, as well as scale bar, color scale, and specimen ID label, is included in the field of vision of the camera lens.
- c) Take photograph and review. If necessary, take additional photographs until you are satisfied with the quality.



Figure 26. Examples of beetle and mosquito photos. Note that individual ID format includes an indication of whether the specimen is a beetle or mosquito but is not precluded with “NEON”.

3) Save images.

- a) Upload all images to a computer.
- b) Review images and select one image per specimen for processing (identified as the best picture of a specimen).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- c) Name the file with the individual ID label of that specimen and then the letter 'r' to indicate it is the raw file (e.g. BET.D03.000001r.). Raw files can be accumulated and periodically processed as a group.
- 4) Process images.
 - a) After the raw image has been saved it must be processed. When editing groups of images, copy the original raw files of the group to a separate folder.
 - b) Crop each image to a 4x6 aspect ratio and eliminate as much extraneous space as possible without cropping any part of the insect, color scale, scale bar, or unique number.
 - c) Once the image has been cropped, save with a new file name, replacing the r with an e (e.g. BET.D03.000001e.jpg)

Data Handling

- 1) Once all images have been processed for a season, assemble them into a single folder so that you can create a list of all the image file names.
 - a) Generate a .txt file with the list of images.
 - i) Open command prompt: Start -> Run & type in **cmd**.
 - ii) Navigate to the correct drive within the command prompt: Prompt will show which drive you are in currently (e.g. if in H drive will say H:\>). To change to a different drive, type the name of that drive (e.g. **C:**) and hit enter.
 - iii) Navigate to desired folder: After you are in the correct drive, type in the command **cd** followed by a space and then the complete file path to the folder containing the images.
e.g. C:\> cd Insect_Images\2013_Images
 - iv) Type in the command **dir /b *.jpg>list.txt** - this will create a text file with a list of the file names in the folder.
 - v) Hints: Special characters (&,%,\$,#, etc.) are not recognized and should not be in the file name. Avoid using spaces in folder and file names – use an underscore (“_” in place of spaces). Spaces are required after **dir** and **b** in the command.
 - b) Copy the list of image files from the .txt file into Image data spreadsheet
 - c) Fill in the remaining data in the Image data spreadsheet
- 2) Record lengths of specimens. Prior to submitting images to BOLD, the length of each beetle is recorded in the Image data spreadsheet.
 - a) Visually estimate the body length to the nearest millimeter using the scale bar (units are mm) in the image. Measure specimens from the tip of the elytra (wings) to the end of the clypeus (head) (see Fig. 27). This does not include the mandibles, which can be in variable positions during death. It may be helpful to use a straight edge on the screen to line up these parts of the specimen with the scale bar. Record each measurement in the Barcode Image Data datasheet.
 - b) Do not measure mosquitoes.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B



Figure 27. How to measure beetle specimens

- 3) Check 20 of the rows in the Image datasheet against the Domain Lab ID and Pinning datasheet to verify that the correct information is associated with each image file.
- 4) Submit images to HQ using the common drive/FieldOperations/domainfolder(cxx)/photos. Headquarters staff will upload images to the appropriate BOLD project once files have been quality checked.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

11 REFERENCES

- Gaffigan, T., & Pecor, J. (1997). *Collecting , Rearing , Mounting and Shipping Mosquitoes* (pp. 1–8).
- Johnson, N. F., & Triplehorn, C. A. (2004). *Borror and {DeLong’s} Introduction to the Study of Insects* (7th ed.). Belmont, {CA}: Brooks Cole.

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

APPENDIX A QUICK REFERENCES FOR LAB PROCESSING

None available.

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

APPENDIX B CHECKLISTS FOR LAB PROCESSING

None available.

<i>Title:</i> TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		<i>Date:</i> 03/26/2014
<i>NEON Doc. #:</i> NEON.DOC.001100	<i>Author:</i> D. Hoekman	<i>Revision:</i> B

APPENDIX C CHANGE NOTES

The following changes have been made between Rev A and Rev B of this protocol:

- Individual ID labels have been updated to distinguish between beetles and mosquitoes.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

APPENDIX D INSECT LOCALITY LABELS

Locality labels identify when and from where a sample was collected. Locality labels are used in the field to label traps and in the laboratory to label whole samples and individual mounted specimens. Locality labels include particular information in order to be consistent with collection records from other global campaigns. At NEON we are using locality labels for samples containing ground beetles, mosquitoes and ticks.

Equipment List

Maximo Item No.	Item Description	Quantity	Purpose	Habitat-Specific	Special Handling
Required	Ethanol-safe archival paper	Variable	Printing labels	No	No

The locality label-sheet has 8 columns, each with 27 labels. Each column of labels is identical and contains the information for one plot and one month. If more than 27 labels per plot are needed, print additional copies of this document.

GEORGIA Baker County. Jones Ecological Res Ctr. 38m N31.1874 W84.4707 CO2 light trap Jul2013. RNelson NEON.JERC.562.	GEORGIA Baker County. Jones Ecological Res Ctr. 47m N31.2034 W84.4631 Pitfall trap Jul2013. Rnelson NEON.JERC.406.
--	--

Figure 28. Example labels for CO2 light trap and pitfall trap samples; note that each label is 5 lines long; size shown is larger than size of actual label.

- 1) Compile the label data for all relevant plots in a Microsoft Excel spreadsheet
 - a) Enter label data following order and formatting guides (see Table 4) into a Microsoft Excel spreadsheet with one column for each item.
 - i) Plot number, latitude, longitude, and elevation will be provided by NEON Headquarters. Formatting may need to be updated. For mosquito traps, use the centroid of the plot.
 - ii) Complete remaining information based on your location and sites.
 - iii) Each row in your spreadsheet should correspond to one plot code and its associated data (state, county, site, lat/long, elevation).

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Table 4. Data required for locality labels

Column	Format	Example
State	All capital letters	GEORGIA
County	See example	Baker County
Site	Abbreviated following list provided in Table 5, no punctuation	Jones Ecological Res Ctr
Elevation	In meters, to the nearest meter	38m
Latitude	In decimal degrees, to 4 decimal places	N31.1874
Longitude	In decimal degrees, to 4 decimal places	W84.4707
Trap Type	See example*	CO2 light trap OR Pitfall trap OR for ticks drag/flag*
Month	Abbreviated to 3 letters	Jun
Year	4 digit year the trap was collected	2013
Collector	Field Operations Manager first initial and last name	RNelson
Plot code	NEON.Site Code.Plot Number.	NEON.JERC.562.

*For tick locality labels, indicate collection method as either “drag” for drag sampling, “flag” for flagging or “drag/flag” for a combination of those two collection methods.

- b) Fill in the appropriate trap type.
- c) Fill in the appropriate month and year according to either:
 - i) when the field collection will take place in the case of labels printed for field use, or
 - ii) when the field collection took place in the case of labels printed for pinning/pointing insects.
- d) Add additional columns for punctuating in the labels as shown in Figure 28.

Table 5. Accepted site name abbreviations for locality labels

Domain	Site Code	Site Name	Site Name Abbreviation	County or Counties
1	HARV	Harvard Forest	Harvard Forest LTER	Worcester OR Franklin
10	RMNP	Rocky Mountain National Park	Rocky Mtn Natl Park	Larimer
10	STER	North Sterling, CO	Sterling	Logan
10	CPER	Central Plains Experimental Range	Central Plains Expm Rng	Weld
3	OSBS	Ordway-Swisher Biological Station	Ordway-Swisher Biol Stn	Putnam
3	DSNY	Disney Wilderness Preserve	Disney Wilderness Preserve	Polk OR Osceola

Reference the NEON Field Site Information document AD[12] for appropriate site name abbreviations for locality labels.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- 2) Format data for 8 plots in Microsoft Excel.
 - a) From your full list, copy the header row plus 8 rows of data (9 rows total) into a new sheet of your workbook. Rename the sheet. Use a meaningful name such as ‘MosqLocalityJun2012’ to easily identify it later. Save and close the workbook – if you do not close the file, the Mail Merge may not work correctly.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	State	County	Period	Site	Period	Elevation	Lat	Long	Trap Type	Month	Year	Period	Collector	Plot code	Peric
2	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	38m	N31.1874	W84.4707	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.562	.
3	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	41m	N31.1862	W84.4817	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.630	.
4	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	33m	N31.1827	W84.4781	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.822	.
5	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	36m	N31.2006	W84.4729	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.654	.
6	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	39m	N31.1975	W84.4771	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.986	.
7	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	37m	N31.1982	W84.4742	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.542	.
8	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	38m	N31.2061	W84.4746	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.006	.
9	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	48m	N31.1891	W84.4610	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.186	.
10	GEORGIA	Baker County	.	Jones Ecological Res Ctr	.	47m	N31.2034	W84.4631	CO2 light trap	Jul	2013	.	Rnelson	NEON.JERC.406	.
11	GEORGIA	Baker Countv	.	Jones Ecological Res Ctr	.	43m	N31.2068	W84.4567	CO2 lieht trap	Jul	2013	.	Rnelson	NEON.JERC.526	.

Figure 29. Spreadsheet after step 1 for 8 plots

- 3) Export the data into Microsoft Word using Mail Merge
 - a) NOTE: It will likely be helpful to review documentation specific to the version of Microsoft Office you are using on creating labels using a Mail Merge. Here we describe the process using Microsoft 2010.
 - b) Open a Microsoft Word document and under **Mailings**, select **Start Mail Merge** and then select **Labels**.
 - c) In the **Labels** menu select **New Label** and enter the dimensions exactly as in Figure 29. Name the label (e.g. ‘insect locality labels’) to easily find it when creating a new sheet of labels.

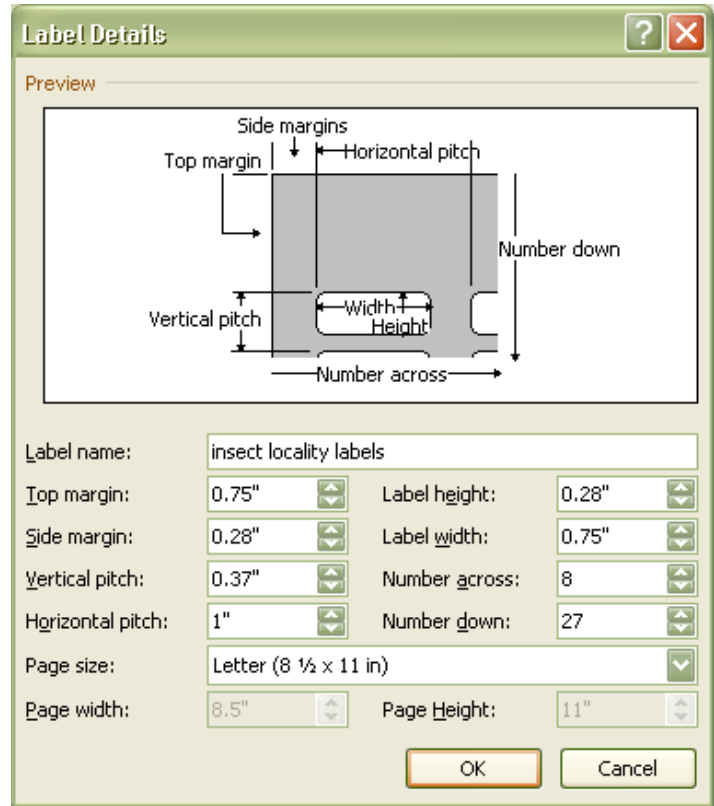


Figure 30. Dimensions for creating a locality label template in Microsoft Word

- d) Once label dimensions are specified, a blank template document will appear. In the **Mailings** tool bar, click on **Select recipients** and select **Use existing list**. A file navigation window will appear. Navigate to and select the Microsoft Excel spreadsheet with the label information.
- e) A pop-up window will appear like the one in Figure 30 below. There will be a list of the sheets within the workbook. Select the worksheet with the list of 8 trap codes into. Make sure to check the box that says 'First row of data contains column headers'.

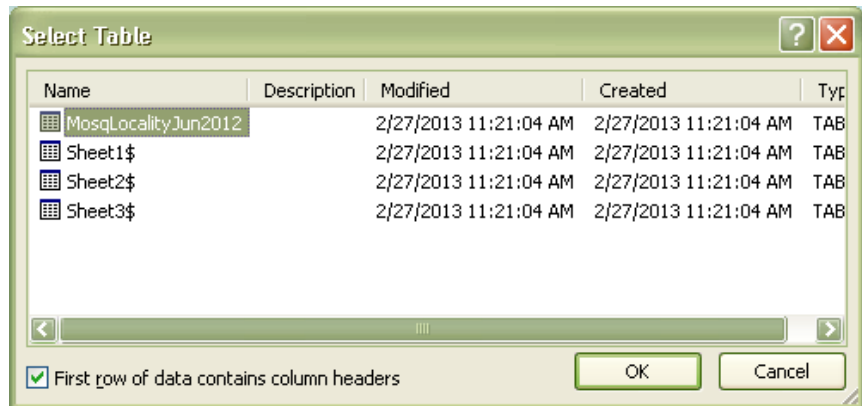


Figure 31. Selecting recipients from an Microsoft Excel file during a Mail Merge

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- f) Perform final formatting in Microsoft Word.
 - i) Place your cursor in the topmost left cell of the label sheet.
 - ii) Populate the labels by clicking on **Insert Merge Field** in the **Mailings** menu. A drop-down menu will appear with all of the column headings from the Microsoft Excel spreadsheet. Enter the fields as shown in Figure 31, making sure to include the punctuation at this stage.
 - (1) Before “Month”, put in 3 underscores and then make the font color for the underscores white so they are not visible. This will ensure that there is space to write in the day of the month.
 - (2) Include single spaces (indicated by *space* below) between each field except between Month and Year and before the periods.
 - (3) Set font for the document to Arial with font size 3.5.

<STATE>*space*<County><Period>*space*<Site><Period>*space*Elevation_m>*space*<Lat>*space*
<Long>*space*<Trap_Type>___<Month><Year>*space*<Period><Collector>*space*
<Plot_code><Period>

Figure 32. Inserting merge fields into label template

- iii) After finishing the first cell, click on **Update Labels** – this will copy formatting into remaining labels.
 - iv) Preview the actual information from the Microsoft Excel sheet that will populate the labels by clicking on **Preview Results**.
 - v) In the Preview Results, confirm that the first label in each column is complete, each column should represent one trap code and all 8 of the trap codes from the list you created should be present (one per columns).
 - vi) In the **Mail Merge** menu, select **Finish & Merge** and select **Edit Individual Documents**. A pop-up menu will appear. Choose the ‘All’ option. This will create a Microsoft Word document with the labels. Save the document. Use a meaningful name such as ‘MosqLocalityJun2012Field’.
 - vii) In the Microsoft Word document you just saved, copy the label in the first row of each column down through the rest of that column. Save the document after you have made these edits.
 - viii) Scan down each column in your Microsoft Word document and ensure that each column has identical labels with only one trap code.
- 4) Print your labels
 - a) Print the labels on ethanol-safe archival paper (e.g., Bioquip 1223RA).
 - b) Determine how many labels are needed for each plot for the selected activity (see Table 6). If more than 23 labels per plot are required, print the document multiple times until enough labels are printed.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- c) **NOTE:** The calculations and quality checks listed below for field work are based on current plans. If the schedule or frequency of trapping changes, the numbers will have to be recalculated using the following formula:

$$A * B * C = D$$

$$D/27 = E$$

A = # of labels/trap

B = # of traps/plot

C = # of trap collections/plot during a month

D = # of labels needed/plot during a month

E = # of strips of labels needed

(for fractions, round up to the nearest whole number)

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Table 6. Number of labels needed for the different activities requiring insect locality labels.

Activity	Variables	Label Calculation	Label Sheet Calculation
Mosquito field work (year 1)	A = 3 labels/trap B = 1 trap/plot C = 3 trap collections (2 nights and 1 day) per bout 30 plots/site	D = 3 labels per trap x 1 trap/plot x 3 collection events = 9 labels per plot E = $9/27 = .33$ print 1 strip of labels per plot (30 plots = 270 labels per bout)	Each label sheet contains 8 strips (columns). Using 1 strip per plot, for 30 plots you would need 4 sheets of labels (30 strips / 8 strips per sheet = 3.75 sheets).
Mosquito field work (years 2-30)	A = 3 labels/trap B = 1 trap/plot C = 3 trap collections (2 nights and 1 day) per bout 10 plots/site	D = 3 labels per trap x 1 trap/plot x 3 collection events = 9 labels per plot E = $9/27 = .33$ print 1 strip of labels per plot (10 plots = 90 labels per bout)	Each label sheet contains 8 strips (columns). Using 1 strip per plot, for 10 plots you would need 2 sheets of labels (10 strips / 8 strips per sheet = 1.25 sheets).
Beetle field work	A = 3 labels/trap B = 4 traps/plot C = 2 trap collections per month** 10 plots/site	D = 3 labels per trap x 4 traps/plot x 2 collection events = 24 labels per plot E = $24/27 = .88$ print 1 strip of labels per plot Print 1 set of labels (10 plots = 120 labels per bout)	Each label sheet contains 8 strips (columns). Using 1 strip per plot, for 10 plots you would need 2 sheets of labels (10 strips / 8 strips per sheet = 1.25 sheets). There are enough labels in each strip to complete 2 bouts (at the core site).
Mosquito pinning	1 label/specimen	Based on number of mosquitoes to be barcoded from each plot	
Beetle pinning	1 label/specimen or pooled sample	Print labels as needed. Refer to field datasheet.	

*Bouts every 2 weeks at core sites and every 4 weeks at relocatable sites.

**Most months will include 2 bouts at the core site and one bout at each relocatable

5) Prepare labels for use (see Appendix G)

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

APPENDIX E INSECT DETERMINATION LABELS

Determination labels are used in lab work to label individual pinned or pointed specimens or pooled beetle sample with a species or morphospecies identification.

Equipment List

Maximo Item No.	Item Description	Quantity	Purpose	Habitat-Specific	Special Handling
Required	Ethanol-safe archival paper	Variable	Printing labels	No	No

<i>Aedes triseriatus</i> det. J. S. Smith 2013	D3morph_DA det. J. S. Smith 2013
--	--

Figure 33. Example determination labels; size shown is larger than size of actual label

- 1) Compile the label data in an Microsoft Excel spreadsheet
 - a) Enter label data following order and formatting guides (see Table 7) into a Microsoft Excel spreadsheet with one column for each item as shown in Figure 33.
 - b) Each row in your spreadsheet should correspond to one species identification. For labels printed prior to insect sorting, leave the identifier blank so that it can be filled in as sorting occurs.
 - c) Add an additional column for the abbreviation 'det' in the labels as shown in Figure 33.
 - d) Rename the sheet using a meaningful name such as 'MosqDet2013'. Save and close the workbook – if you do not close the file, the Mail Merge may not work correctly.

Table 7. Data required for labels

Column	Format	Example
Species	Species name - Italicized, first letter capitalized Morphospecies – not italicized	<i>Aedes vexans</i> D3morphA
Identifier	First initial, middle initial, last name	J.S. Smith
Year Identified	4 digit year	2013

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

	A	B	C	D
1	Species	det.	Identifier	Year Identified
2	<i>Aedes triseriatus</i>	det.	J. S. Smith	2013
3	<i>Aedes fulvus pallens</i>	det.	J. S. Smith	2013
4	<i>Anopheles perplexens</i>	det.	J. S. Smith	2013
5	<i>Cicindela punctulata punctulata</i>	det.	J. S. Smith	2013
6	<i>Clivina</i>	det.	J. S. Smith	2013
7	<i>Coquillettia perturbans</i>	det.	J. S. Smith	2013
8	<i>Culex erraticus</i>	det.	J. S. Smith	2013
9	<i>Culex nigripalpus</i>	det.	J. S. Smith	2013
10	<i>Culiseta melanura</i>	det.	J. S. Smith	2013
11	D3morph_DA	det.	J. S. Smith	2013
12	D3morph_JB	det.	J. S. Smith	2013
13	D3morph_JC	det.	J. S. Smith	2013
14	<i>Mansonia dyari</i>	det.	J. S. Smith	2013
15	<i>Mansonia titillans</i>	det.	J. S. Smith	2013
16	<i>Pasimachus sublaevis</i>	det.	J. S. Smith	2013
17	<i>Pasimachus punctulatus</i>	det.	J. S. Smith	2013
18	<i>Psorophora ciliata</i>	det.	J. S. Smith	2013
19	<i>Scarites subterraneus</i>	det.	J. S. Smith	2013
20	<i>Tetracha carolina</i>	det.	J. S. Smith	2013
21	<i>Uranotaenia sapphirina</i>	det.	J. S. Smith	2013
22	<i>Uranotaenia lowii</i>	det.	J. S. Smith	2013

Figure 34. Spreadsheet after step 1 for beetle and mosquito species

- 2) Export the data into Microsoft Word using Mail Merge
 - a) **NOTE:** It will likely be helpful to review documentation specific to the version of Microsoft Office you are using on creating labels using a Mail Merge. Here we describe the process using Microsoft 2010.
 - b) Open a Microsoft Word document and under **Mailings**, select **Start Mail Merge** and then select **Labels**.
 - c) Dimensions for these labels are the same as for locality labels, so the correct label may already exist in the list. If not, in the **Labels** menu select **New Label** and enter the dimensions exactly as in Figure 34. Name the label (e.g. 'insect locality labels') to easily find it when creating a new sheet of labels.
 - d) Once label dimensions are specified, a blank template document will appear. In the **Mailings** tool bar, click on **Select recipients** and select **Use existing list**. A file navigation window will appear. Navigate to and select the Microsoft Excel spreadsheet with the label information.
 - e) A pop-up window will appear like the one in Figure 35 below. There will be a list of the sheets within the workbook. Select the worksheet with the list of species. Make sure to check the box that says 'First row of data contains column headers'.

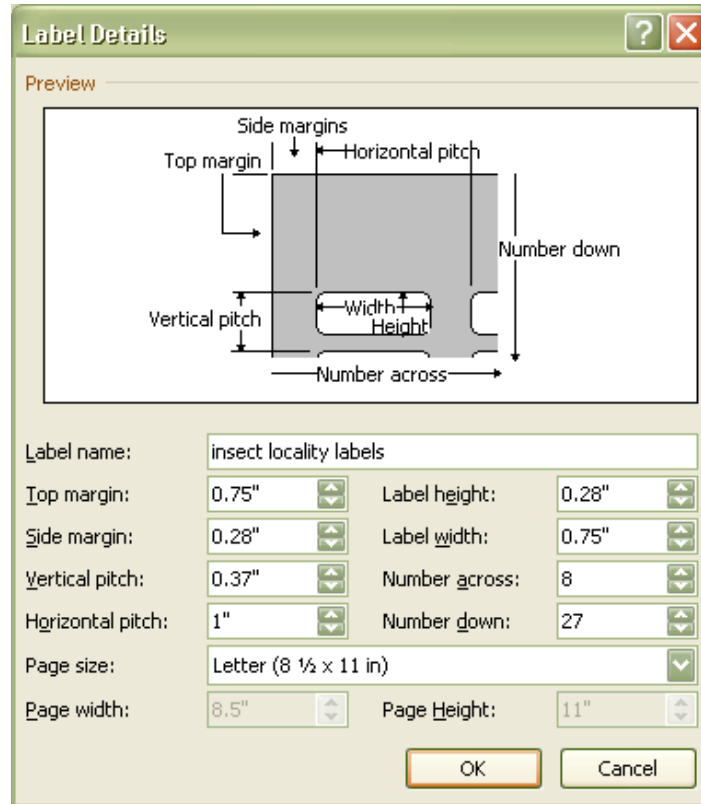


Figure 35. Dimensions for creating a locality label template in Microsoft Word

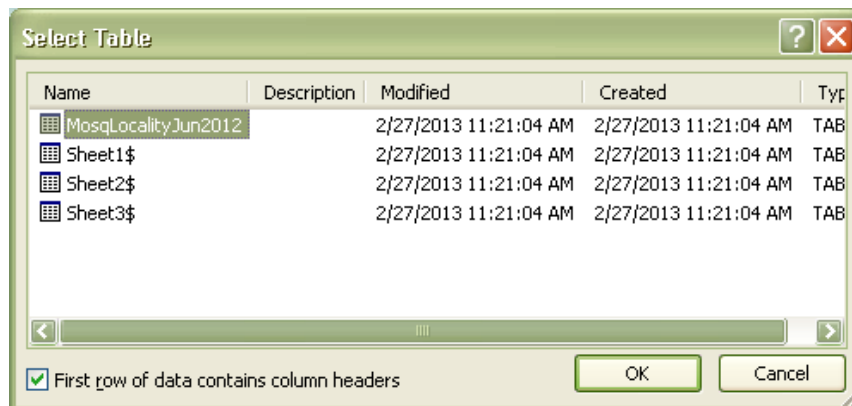


Figure 36. Selecting recipients from a Microsoft Excel file during a Mail Merge

- 3) Perform final formatting in Microsoft Word
 - a) Place your cursor in the topmost left cell of the label sheet.
 - b) Populate the labels by clicking on **Insert Merge Field** in the **Mailings** menu. A drop-down menu will appear with all of the column headings from the Microsoft Excel spreadsheet. Enter the fields as shown in Figure 36.
 - i) Include a hard return (Enter) where indicated so that species is on the first line of the label, 'det.' and identifier are on the second line, and year is on the third line..

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- ii) Include a space between 'det.' and identifier (indicated by space below).
- iii) Select Arial font size 3.5.
- iv) Note: Formatting such as italics does not get transferred from Microsoft Excel to Microsoft Word during a Mail Merge; italics will have to be applied to the species name field (but not the Morph designations).

<Species>Enter<det.> spaceIdentifier>Enter <Year>

Figure 37. Inserting Merge Fields into label template

- c) After finishing the first cell, click on **Update Labels** – this will copy formatting into remaining labels.
 - d) Preview the labels by clicking on **Preview Results**.
 - e) In the Preview Results, confirm the first row in each label has a species name, the second line has 'det.' and an identifier's name, and the third row has the year.
 - f) In the **Mail Merge** menu, select **Finish & Merge** and select **Edit Individual Documents**. A pop-up menu will appear. Choose the 'All' option. This will create a Microsoft Word document with the labels. Save the document. Use a meaningful name such as 'MosqDet2013'.
 - g) In the Microsoft Word document, italicize species (but not Morph designations).
 - h) Copy and paste each label to generate the required number of labels for specimens. Group all labels for the same species within the same column. Save the document after you have made these edits.
- 4) Print the labels on ethanol-safe archival paper (e.g., Bioquip 1223RA).
- 5) Prepare labels for use (see Appendix G)

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

APPENDIX F INSECT UNIQUE NUMBER LABELS

Unique number labels are used in lab work to label individual pinned or pointed specimens and mosquitoes from a given plot from a given sampling period.

Equipment List

Maximo Item No.	Item Description	Quantity	Purpose	Habitat-Specific	Special Handling
Required	Yellow cardstock	Variable	Printing individual ID labels	No	No
Required	Adhesive shipping labels equivalent to Avery 18167	Variable	Printing mosquito pooled sample vial label	No	No

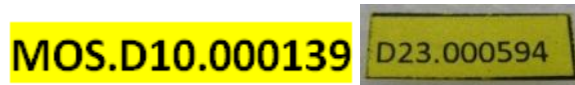


Figure 38. Individual ID label (a mosquito label from Domain 10).



Figure 39. Mosquito pooled sample vial label

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

Individual ID Specimen Labels

Note that the individual ID **label** omits the “NEON” prefix that is part of an individual ID. This is to save space on the label.

- 1) Use the Microsoft Excel file titled “Insect Individual ID Labels”
 - a) First tab: “Domains” contains the correctly formatted Domain codes, which are the first component of the individual ID labels. Change domain number to location for which labels are being printed.
 - b) Second and third tabs (one for beetles and one for mosquitoes): Each page in these tabs contains 12 columns and 47 rows, for a total of 564 labels per page. The column width is 0.56”, row height is 0.21”, font is Calibri size 7. All the borders are turned ON around each cell – borders will help keep straight lines when cutting out the labels.
 - i) The first 6 pages (through label #1974) have been created for both beetles and mosquitoes.
 - ii) Be sure to only print one copy of each page. Start with the first page and only print the second page after you have used up the labels from the first page. For beetles it is reasonable to expect you may break onto a second page within one year.
 - iii) Keep careful track of your page of labels and pay close attention to the last number used during the previous bout of printing individual ID labels.
 - iv) More label pages will be added to the “Insect Individual ID Labels” spreadsheet in future years as needed.

- 2) Insert a sheet of yellow cardstock into the manual feed tray of the printer. Select all the labels on the page you have prepared of your Microsoft Excel file. Under the print menu, make sure to select “Print Selection”. Under Printer Properties, set the Paper source to Manual Feed.

- 3) Prepare labels for use (see Appendix G)

Generate & Print Mosquito Pooled Sample Labels

- 1) Use the Microsoft Excel file titled “MosquitoPooledSampleLabels”
 - a) First tab: “Domains” contains the correctly formatted Domain codes, which are the first component of the labels.
 - b) Change domain number to location for which labels are being printed.
 - c) Second tab:
 - i) The first time labels are printed, delete the contents of the second column. For subsequent printings, follow these directions:
 - ii) Save a copy of the spreadsheet with the range of numbers you will be printing (e.g. Mosquito Pooled Sample Labels 2257-4512) for the second set of numbers. Be sure to include the range of label numbers in the file name.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

- iii) Manually enter the information in the second column for the first label only. After entering the information for the first one, you can then click in the bottom right corner of the cell and drag down until you have filled cells for 1040 labels. Refer to the chart below for the label number range for each set that you print.

Each printing bout includes 13 pages of 80 labels, for a total of 1040 labels:

First printed set: 1 - 1040
 Second printed set: 1041 - 2080
 Third printed set: 2081 - 3120
 Fourth printed set: 3121 - 4160
 Fifth printed set: 4161 - 5200
 Etc, etc.

- 2) Before you start the Mail Merge Process in Microsoft Word, make sure to save the above Microsoft Excel file in place where you will be able to easily navigate to it and close the workbook – if you do not close the file, the Mail Merge may not work correctly.
- 3) NOTE: It will likely be helpful to review documentation specific to the version of Microsoft Office you are using on creating labels using a Mail Merge. Here we describe the process using Microsoft 2010.
- 3) Open a Microsoft Word document and under **Mailings**, select **Start Mail Merge** and then select **Labels**.
 - a) In the **Labels** menu select **Avery US Letter** in the Label Vendors drop down box.
 - b) In the **Product** Number box, scroll down until you see the product number of the labels you are using (18167 Return Address Labels).
 - c) Name the label so that you can find it every time you create a new sheet of labels. Once you hit OK, a blank template document will appear with properly sized boxes.
 - d) In the **Mailings** tool bar, click on **Select recipients** and select **Use existing list**.
 - e) A file navigation window will appear. Navigate to the Microsoft Excel spreadsheet with the label information and select this file.
 - f) A pop-up window will appear (Figure 40).
 - i) Select the workbook that contains the domain and label information.
 - ii) Check the box that says ‘First row of data contains columns headers’.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
NEON Doc. #: NEON.DOC.001100	Author: D. Hoekman	Revision: B

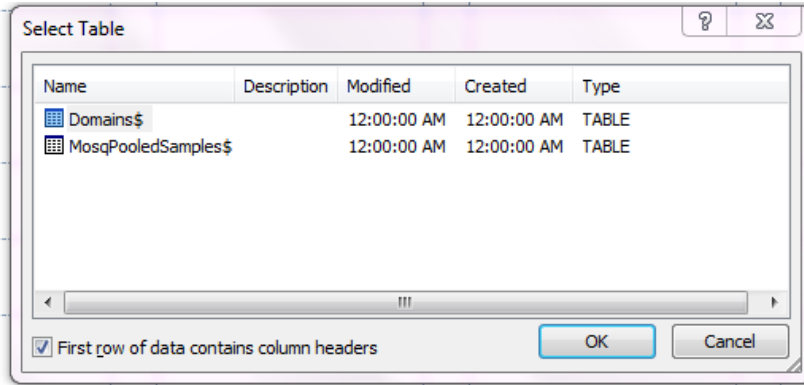


Figure 40. Mail Merge pop-up window in which you select the tab within the Microsoft Excel file you saved that contains the information for the mosquito pool sample labels.

- 4) Perform final formatting in Microsoft Word
 - a) Place your cursor in the topmost left cell of the label sheet.
 - b) Begin to populate the labels by clicking on **Insert Merge Field** in the **Mailings** menu. A drop-down menu will appear with all of the column headings from your Microsoft Excel spreadsheet. Enter the fields as shown in Figure 41. Center the text in field both horizontally and vertically. Select Arial Bold font with size 11.
 - c) After you have finished with the first cell, click on **Update Labels** – this will copy your formatting in the rest of the labels. You can preview the actual information from the Microsoft Excel sheet that will populate the labels by clicking on **Preview Results**.
 - d) In the **Mail Merge** menu, select **Finish & Merge** and select **Edit Individual Documents**. A pop-up menu will appear, Choose the 'All' option. This will create a Microsoft Word document with the labels you just created. Save the document with an informative file name, including the range of label numbers for future reference, such as 'MosqPooledSample_0-1040'.

<Domain><Label>

Figure 41. Inserting Merge Fields into label template

- e) Your final labels are formatted as:

D23.Cul.000001

- 5) Print your labels.
 - a) Insert 13 sheets of Avery 18167 labels (or equivalent, 0.5" x 1.75"; MX104982) into the manual feed tray of the printer. Under Printer Properties, set the Paper source to Manual Feed.

Title: TOS Protocol and Procedure: Ground Beetle and Mosquito Specimen Processing		Date: 03/26/2014
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APPENDIX G CUTTING LABELS

Cut labels into long columns using a snap-off blade knife and ruler. There should be no obstructions on the cutting mat or the ruler while doing this. Using a snap-off blade is essential to making clean cuts. Using scissors to make a cut of this length is highly prone to error.

Equipment List

No	Item Description	Purpose	Quantity	Habitat-Specific	Special Handling
Required	Scissors	Cutting apart labels	1	No	No
Required	Self-healing cut mat (30 cm x 46 cm)	Cutting apart labels	1	No	No
Required	Exacto knife	Cutting apart labels	2	No	No
Required	Ruler, Metal, 18 inch	Cutting apart labels	1	No	No

- Cut labels with as little white space on all sides of the text as possible (Figure 42).
- These cuts result in many labels in a vertical strip.

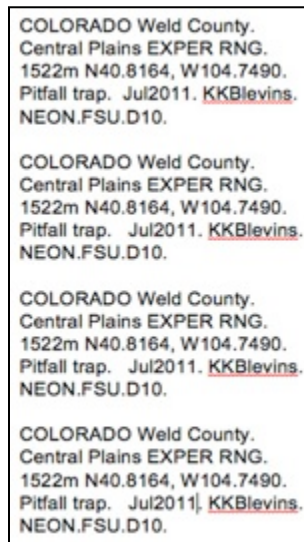


Figure 42. Example of a strip of labels

- Store labels in strips. When needed, cut individual labels across the row with scissors, leaving as little white space around the text as possible.
- Use ethanol-safe pens whenever adding information to labels to ensure longevity of markings.