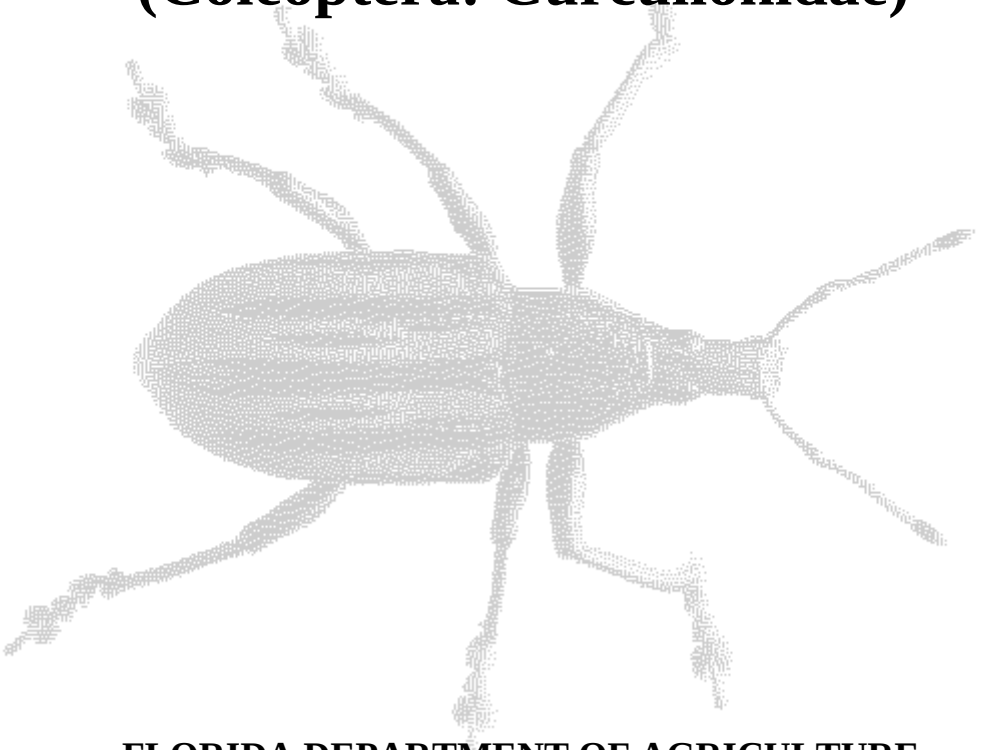
PROCEDURES MANUAL FOR MASS REARING THE SUGARCANE ROOTSTOCK BORER *Diaprepes abbreviatus* (L.) (Coleoptera: Curculionidae)



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BUREAU OF METHODS AND BIOLOGICAL CONTROL
BIOLOGICAL MASS REARING FACILITY

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Introduction

The sugarcane rootstock borer, *Diaprepes abbreviatus* (Coleoptera: Curculionidae), first found in Florida in 1964 (Woodruff 1964), is an extremely destructive pest of many hosts, especially citrus and sugar cane (Woodruff 1968). While the adults feed on leaves, the larva stage is the most harmful. The neonates and grubs feed on the roots, which eventually kills the plant. With this major threat to agriculture, many scientists are researching various controls from insecticides to classical biocontrol methods.

In order to conduct this research, many insects in all stages need to be available, which is possible only by mass rearing. With the assistance of the USDA-ARS in Ft. Pierce, the Florida Department of Agriculture established a colony in the Biological Mass Rearing Facility in February 2000, using techniques developed by (Beavers 1982) and (LaPointe and Shapiro 1999). Some procedures have been modified since to adapt to our rearing conditions.

The *Diaprepes* has and extremely long and variable life cycle, with most of the variability occurring in the larval stage. While several scientists have had various life stage data(Stansley 2000), the egg stage averages about 7 days, larvae about 120-150 days, pupae is about 20 days and the teneral adult about 21 days in the Biological Mass Rearing Facility.

Cage Set Up and Maintenance

Materials Per Cage: Plexiglas cage (and 6" cover; 3 pairs of 1" wax paper strips taped at one end; 5.5" petri dish; 3.5" petri dish; 3 organically grown carrots; 4" slab of prepared weevil diet; 3.75" (8oz) sterile water dish and lid (with 1/4" hole in center); 2 (6" cotton wicks); water; citrus leaves; and 1" masking tape.

Method: Plexiglas cages (12"x12"x16") equipped with an 8" circular 1mm screen mesh side vent and a 2" Plexiglas ring glued around the access opening are set up with approximately 60 adult *Diaprepes*. One new cage per month is started. This will help to avoid dips in egg production and neonate viability due to increasing age of adults. Currently, four cages are maintained in the facility to provide needed life stages for various private industries, academic, and governmental, research projects.

Water is provided in an 8-ounce deli cup with a lid, which is punched with a hole through which 2 cotton dental wicks are moistened and inserted half way. A variety of food is provided. Large pieces of organic carrots are placed in a 5.5" petri dish. A second petri dish (3.5") of prepared *Diaprepes* premix diet is added to the cage, as well as new citrus seedling leaves placed in a 3rd small petri dish. Organically grown green beans can also be used when available. Food is replaced on Mondays, Wednesdays and Fridays. Pairs of one-inch wax paper strips are taped to the top of the cage with masking tape for oviposition. Egg strips are changed daily.

Figure 1. Adult Food

After the cage is assembled, the opening is covered with a cut out, screened 6" coffee can lid and taped with masking tape. Each cage is labeled with I= date initially set, T= date of most recent adult transfer, and # (= current number of insects in the cage).

Figure 2. Adult Cage

To control microorganisms and for aesthetics, cages should be rotated and washed at least once a week. The weevils are placed into new cages with new food and water and egg strips. The dirty cages are washed with a mild dish soap and warm water. Cages are sanitized with 1000ppm bleach solution and rinsed with tap water.

Egg Collection

Materials: 3 pairs of 1"x10" wax paper taped at one end per cage; 1 gallon zipper top freezer bag; 1- 6" cotton dental wick; laundry marker; and 1" masking tape.

Method: In preparation for egg collection, a cotton dental wick is moistened with deionized water with the excess moisture squeezed out. This is placed into a one gallon zipper top plastic bag, labeled with the collection date, to provide water for the newly hatched neonates. New egg strips are prepared by cutting 1" lengths of wax paper the width of the roll (12"), and 2" off of one end. These can be cut ahead of time and stored in a clean Ziploc® bag.

Egg laden wax paper strips are collected once daily, at approximately the same time each day. Due to cross contamination concerns, this step is done after all other work has been performed. Remove the lid from the first cage door, and take the old egg laden wax paper pairs (egg strips) out and place into the bag. When all 3 pairs of egg strips have been removed, tape 3 new pairs of strips to top of cage. Strips should be placed along the sides of the cage top. Continue until all egg strips have been removed, and each cage has 3 new pairs.

Figure 3. Egg Strips

Diet Preparation

Materials: Bioserve® *Diaprepes* diet premix (F1675); agar; benzoic acid; methyl paraben; deionized water; (30 cup diet trays); 1oz souffle cups and lids ; spray bottle containing 1000ppm bleach solution (37.5ml bleach / gal. water); spray bottle containing 70% alcohol; paper towels; steam kettle; 5 liter plastic pitcher; and stainless steel kitchen whisk; 2 gallon Ziploc® plastic bags.

Method: Due to the risk of contamination, traffic into the diet area should be limited and the equipment sanitized (see procedures described below). The following formula yields about 360 cups:

1187.5g	Bioserve® premix
90.0g	Agar
4.5 liters	Dionized water
1.125g	Benzoic acid
1.125g	Methyl paraben

Figure 4. Diet Kettle

Add deionized water to the kettle and turn dial to 10, (highest setting), add agar, and whisk vigorously. Once a rapid boil is observed, and the agar clears, reduce heat to off. Slowly add Bioserve® premix to the hot agar water mixture while whisking until uniform in consistency. Finally add 1.125g benzoic acid and methyl paraben each, stirring to ensure uniformity in the diet mixture.

Place the trays side by side, 2 wide, the length of the clean *Diaprepes* diet table which has been sprayed with 70% isopropyl alcohol and dried. Fill the trays with 1-oz souffle cups. Whisk diet to avoid settling and tilt kettle to fill sterile pitcher 1/3 with the hot diet. Fill each cup with diet to approximately the level of the tray (about ½ full). Empty the remainder of the diet in the pitcher back into the kettle and whisk thoroughly to keep the heavy components from settling out. Repeat the above steps until all the diet has been poured. The filled trays are placed on a bread rack for drying. Allow diet to dry until the desired moisture content of 60%-70% is achieved, which takes about 2 days in our facility (QC3 room). When the desired moisture content is reached, place each tray of 30 cups in a 2 gallon plastic zipper top bag, and seal. The bags of diet are irradiated with E-beam at 1.5 mrads after which it is stored in a clean, cool location (not warmer than 80EF) until needed.

Figure 5. Pouring Diet

Clean up and sanitation: The whisk and pitcher are rinsed of debris and sprayed with 1000ppm bleach solution. The whisk, unused paper towels, and bleach spray bottles are placed in a sealed clear storage bin for the next use. All trash is removed from the diet preparation area. The kettle is washed and sanitized with 1000ppm bleach solution. All items, including the storage bin and contents, kettle, and diet preparation table, should then be moved to storage area.

Cultivation of Citrus For Adult Food

Materials: 300ml citrus seeds, 2.5gal bucket full of water, 20ml 10% bleach, air pump, 1 bag coarse vermiculite, strainer, 32.5" x 22.5" bread tray, 2.5gal watering can, concentrated fertilizer stock solution*, diluted fertilizer solution**.

Step 1. Obtain 300ml of quality seed from refrigerator. Fill bucket with 2.5 gallons water. Set up air pump with pipette on hose end to weigh it down in water. Add 20ml 10% bleach to 2.5 gallons water. Add 300ml Citrus seed to bleach and water solution. Tape end of air pump hose to edge of bucket so pipette is submerged and bubbles are created. Allow seeds to soak in solution with aerator running for approximately 24 hours. This will allow the seed to imbibe water, and will facilitate enzymatic activity.

Step 2. Strain seeds, and rinse with tap water. Fill large tray with approximately 1.5 inches of coarse vermiculite. Sow seeds evenly on top of the vermiculite, cover with more vermiculite and water. Place propagation trays under 4 foot fluorescent grow bulbs, on 16 hours / day duration. Label with date seeds were hydrated, date sowed, variety of citrus, and For *Diaprepes*.

Step 3. Water Monday, Wednesday, and Friday. Plain water should be used until cotyledons begin to shrink and fall away. Once cotyledons have shrunk, water with fertilizer prepared by adding 60 mls of diluted solution to a filled 2.5 gal watering can.

* To prepare the concentrated fertilizer stock solution, fill a 10 L carboy with water and add 330g of 20-20-20 fertilizer.

**To prepare a 5gal carboy of diluted solution, fill the carboy with 5 gal water and add 300ml of previously prepared concentrated fertilizer stock solution.

Citrus Culture Surface Sterilization and Fertilizer Calculations

Parts per million (ppm) = Parts per million is a weight relationship used to express concentration. For irrigation water the term is assumed to be equivalent to milligrams per liter since the specific gravity of such water is approximately 1. The ppm is the unit weight of salts in a million units of water. The metric equivalent is mg/l.

ppm = mg/l

Surface Sterilization Calculation: 10% Bleach =100,000ppm
20ml = .02L
2.5gal × 3.785L / gal = 9.4625L water = the container volume.
100,000ppm = X mg / .02L = 2,000mg pure bleach / container.
ppm = 1mg / L, □ 2,000mg ÷ 9.4625L = 211.36063ppm Used to surface sterilize citrus seeds.
Fertilizer Calculation: 1ppm = 1mg /L
100g × 1000mg / 1g = 100,000mg of 20-20-20 fertilizer added to 3L.
20-20-20 fertilizer is only 20% pure Nitrogen (N-P-K).
.20N × 100,000mg = 20,000mg pure Nitrogen / 3L Carboy.
ppm Concentrate stock Carboy = 20,000mg ÷ 3L = 6666.666 or 6666.7ppm.
6666.7mg / L × 1L / 1000ml = 6.6667 mg / ml of Concentrated Stock

Solution:
150ml × 6.6667mg / ml = 1000.005mg to be Added to 2.5 gallon Watering Can.
1gal = 3.785L, □ 3.785L / gal × 2.5gal = 9.4625L / Watering Can.
ppm Water Applied to Citrus from Can = 1000.005mg pure bleach / 9.4625L water = 105.681ppm

Neonate Transfer

Materials: Büchner funnel; filter paper; air pump; funnel; small 5 dram vial with lid(3 holes drilled off center with 1/32 bit)= "salt shaker" ; 70% alcohol spray bottle; one 7-8 day old bag of neonates; 6.5"x10.75"x2.5"plastic food container; 250ml glass beaker; wash bottle filled with .25% bleach solution.1-oz lids; diet; and surgical gloves (optional).

Method: Prepare the laminar flow hood by spraying and wiping the work space with 70% isopropyl alcohol. Assemble Büchner funnel and place filter paper in top. Place the assembly and the "salt shaker" inside the hood. Place the air pump on a cart with the outflow facing away from the hood. Turn on the UV light for 15 minutes, while preparing 7-8 day old neonates for transfer. For safety, place a sign on the outside door indicating that UV is being used.

Neonates hatch in about 7 days, at which time they are ready to be placed on diet. At this stage, they will begin to crawl toward the corners of the bag. Empty the egg strips by slowly pulling them apart over the sterile dry plastic food container. Remove any debris present in the container with forceps. Force the neonates into the corner of the plastic food storage container by tilting the dish and slightly tapping. Pour neonates into a sterile 250ml glass beaker. Fill the beaker of neonates with approximately 1 inch of .25% bleach solution. Agitate the mixture of neonates by squeezing the bleach bottle forcefully enough to push the neonates under and create bubbles which cause the neonates to float again. Allow the neonates to soak in this solution for 2 minutes.

Figure 6. Hatched Neonates

Spray hands with 70% alcohol or wear surgical gloves. Turn on the air pump and slowly pour the bleach and neonates into the Büchner funnel assembly. Pump until all the bleach solution has been separated from the neonates. Rinse the neonates with deionized water and filter again until completely dry. Pour the dry neonates into the "salt shaker", using a funnel.

Figure 7. Büchner funnel with air pump

Spray the outside of a bag of irradiated diet with alcohol, and place it in the laminar flow hood. Inside the hood, remove the tray of diet from the bag. Shake approximately 7-12 neonate larvae into each cup. Open bag of lids inside hood and handle so that fingers do not touch the lid bottoms. Working from front of the tray toward the back to avoid moving hands over the open cups, place lids on cups, ensuring a tight fit. Place the newly infested tray of diet outside of the hood on an upside down RubberMaid® lid with a trough of mineral oil around the edge.

Figure 8. Neonate Transfer Vial

Once the desired number of trays has been completed, label the tray as follows: E= egg date (the date the eggs were collected), I= Initial transfer (the date the neonates were placed on the diet), the number of individual cups and initials. Record the data on the Larvae Data Form, DA-001 (See Appendix p.A-2) in the notebook kept in the rearing area. Place the stack of trays on the shelf in chronological order (QC3).

Single Larva (Grub) Transfer

Materials: Forceps; 1 stack of 30-35 day old larvae; .25% bleach solution in wash bottle; sterile 1oz lids; 10.75"x13" plastic food container lid, 50ml beaker filled with .25% bleach solution; 2 (6.5"x10.75"x2.5") sterile plastic food containers; 5.5" Petri dishes; and 1mm wire mesh stainless steel cooking strainer, soft forceps.

Method: Thirty to 35 days from the initial transfer date the cups become over-crowded and the larvae tend to fight, creating scarring. Multiple burrows are evident and often grubs are forced to the surface of the diet. At this stage, the grubs need to be separated and placed in individual cups. Contaminated cups should be removed prior to separation of larvae and recorded as losses on the *Diaprepes* discard data form, DA-005 (See Appendix p.A-6). Cups containing no larvae are recorded as dead and cups containing larvae too small to transfer are recorded as slow. The remaining diet "cubes" containing larvae are broken apart into a sterile dish, and healthy, unscarred grubs are placed into another (sterile) dish, and the old diet and cup are discarded.

Place approximately 100 grubs in a metal 1mm mesh screen strainer, and rinse for 1 minute under a gentle stream of tap water. Following rinsing, place grubs in 5.5" sterile petri dish, and rinse with .25% bleach solution from the 500ml wash bottle. Replace the petri dish lid and agitate the grub/ bleach solution with a gentle circular shaking motion. Decant the bleach solution by holding the lid on the petri and tilting the dish over the sink. Rinse once with sterile water and replace cover.

Figure 9. Grubs Ready for Transfer to Diet

Transfer grubs individually to fresh diet inside the laminar flow hood, using soft forceps. Handling of the diet is the same as for initial neonate transfer. A new label is placed on the stack with E = egg date, I= initial transfer date, T = transfer date, the number of cups and initials. These stacks are placed chronologically on shelves on the east wall of QC3 until pupae start to develop. The production data is recorded on the Single Larvae form, DA-002 (See Appendix A-3).

Sorting

Because the *Diaprepes* life cycle is long and variable, developing pupae and contaminated cups are sorted from the grub stacks that are about 3 months older, at least once a week. The egg and the pupae dates are written on the cup lid with a fine tipped Sharpie® marker. This information in recorded on the Pupae Form, DA-003 (See Appendix A-4). The pupae cups are placed in clean tray and stacked on an upside down RubberMaid® lid with a trough of mineral oil. The stacks are stored on a separate shelf in QC2 in chronological order.

While sorting for pupae, contaminated cups and larvae that have chewed through the cup are also taken from the stacks and recorded on the Discard form, DA-005 (See Appendix A-6) with the number and the reason for disposal. As the grub stacks become depleted, the cups are condensed to create room on the shelves.

At least once a week, adults are sorted from the pupae trays as they develop. Data is recorded on the Adult form, DA-004 (See Appendix A-5). The appropriate numbers of adults are held for stocking new cages, when needed and the rest are shipped to various scientists.

Figure 10. Sorting Pupae

Environmental Parameters

Temperature is maintained between 78E F and 82E F in Q.C. 1, 2, and 3.

Relative humidity is not a concern since the larval, pupae and adult stages are kept in closed cups with moist diet. Eggs are protected within wax paper strips and incubation takes place in a closed plastic bag

Photoperiod is controlled using timers set for 14 hours of light in Q.C. 1 and 2 to simulate summer conditions. Q.C. 3 is kept dark to simulate conditions underground.

Rearing Area Sanitation

Contaminant Scouting: *Diaprepes* cultures should be visually checked as often as possible for bacteria (slimy film), fungus (cottony masses), and mites (granular diet surface with "moving specks"). Any visible contaminants should be recorded as losses on the Discard Form, DA-005 (See Appendix A-6) and discarded.

Environmental Microbiology: A sanitation program has been developed to control microbiological contaminants in the rearing areas (See Appendix A-7). Air plating is done to monitor the level of contaminants once a week to ensure that this program is conducted properly. Two types of enriched agars, TGA and PDA, are used to detect mold, bacteria and yeast. One 3.5" petri dish of each type of agar is uncovered for 20 minutes in Q.C.1, Q.C. 2, and Q.C. 3. The TGA and PDA plates are incubated for 2 and 4 days respectively, and the number and type of microorganisms are recorded. This data is kept in the microbiology area.

Daily Duties

Sinks: Wash dishes using a mild detergent and rinse with 37.5mls of bleach per gallon of water. Empty and rinse sinks in all rooms at end of day.

Garbage: Remove garbage and place in freezer for 24 hour duration, after which the material is taken to the dumpster.

Counters and Table Tops: Spray surfaces with 1000ppm bleach at the beginning and end of work schedule.

Floors: Sweep and Mop with bleach

Weekly Duties

Floors: Sweep all rooms (Q.C. 1,2,and 3), and mop with detergent and water.

Monthly Duties:

Ceilings, cupboards and drawers, lights, walls and doors: Apply standard quaternary ammonia solution using swivel head mop to the following surfaces in Q.C. 1, 2, and 3.

AC vents: Remove from the ceiling and spray with water, followed by application of the standard solution of quaternary ammonia.

As the above sanitation duties are completed, it is recorded on the Sanitation Checklist Form, SFL 01-10 (see Appendix A-7).

Rearing Area Organization

Organization and order of duties are directly related. Persistence of contamination in lab reared insect colonies has led to separation of colony stages.
Q.C. 1, has been designated a "dirty area" and as such is reserved for the adult egg laying cages and contaminated cultures held for recovery.
Q.C. 2, has been designated "semi clean", and is reserved for cultures 30 days or older (neonate larvae which have been transferred to single cups, and later stages of the life cycle including pupae and teneral adults).
Q.C. 3, has been designated "clean", and as such is reserved for new cultures only, and these cultures should be moved to Q.C. 2 at the time of transfer.

Order of Duties:
Clean work should be performed in the morning prior to exposure of equipment/ personnel to contaminants. Duties such as neonate transfers and sanitation of Q.C. 3 fall into this category.
Semi-clean work should be performed next. This includes separation of neonate larvae, transfers to fresh diet, and sanitation of Q.C. 2. Dirty work should be performed last. This includes egg collection, cage maintenance, and sanitation of Q.C. 1. Moving from dirty areas to clean ones should be avoided.
Note: It is important to keep in mind where you have been, and what has been handled.

Supplies

Supplies should be checked periodically to ensure that shortages do not occur. A list of needed items should be given to the immediate supervisor or purchaser, based on lead time necessary to obtain these items. (See Appendix A-1 for suppliers.)

APPENDIX

Contents by Page Number

Source Directory for *Diaprepes* Materials

1oz Cups
1oz Cup Lids

1. Heritage Paper
   822 N.W. 107 terr.
   Gainesville, FL 32606 (352-332-1417)

10.75" x 13" Plastic food container lids

1. Merton Restaurant Supplies.
   207 W. Gore St.
   Orlando, FL 32806 (407-425-4557)

Citrus Root Weevil Premix
30 Cup Plastic Trays

1. Bioserve.
   P.O. Box 450
   Frenchtown, NJ 08825 (908-996-2155)

Benzoic Acid
Methyl Paraben
3.5", 5" Petri Dishes

1. Fisher Scientific.
   3970 Johns Creek ct. ste. 500
   Suwannee, GA 30024 (1-800-766-7000)

Agar

1. Coll-Chem Corp.
   P.O. Box 721
   Ridgewood, NJ 07451 (201-445-6662)

Zipper Top Bags (all sizes)
Mineral Oil
Organic Carrots
Dawn Dish Soap
Wax Paper

1. Publix.
   125 S.W. 34th st.
   Gainesville, FL 32607

Bleach

1.Home Depot
  P.O. Box 9903
  Macon, GA 31297-9903

Dental Wicks

1. Richmond Dental.
   P.O. Box 34276
   Charlotte, NC 28234 (1-800-277-0377)

DIAPREPES LARVAE DATA - I

EGG DATE E	INFEST DATE I	NO CUPS	TECH INITIALS	SHIP DATE	NO SHIPPED	EGG DATE E	INFEST DATE I	NO CUPS	TECH INITIALS	SHIP DATE	NO SHIPPED

DA-001
rev. 4/01

year

C:\myfiles\reportforms\diaprepesforms.xls

DIAPREPES LARVAE DATA - T

EGG DATE E	TRANSFER DATE T	NO CUPS	TECH INITIALS	SHIP DATE	NO SHIPPED	EGG DATE E	TRANSFER DATE T	NO CUPS	TECH INITIALS	SHIP DATE	NO SHIPPED

DA-002
rev. 4/01

year_____

C:\myfiles\reportforms\diaprepesforms.xls

DIAPREPES PUPAE DATA

EGG DATE E	PUPAE DATE	NO CUPS	SHIP DATE	NO SHIPPED	EGG DATE E	PUPAE DATE	NO CUPS	SHIP DATE	NO SHIPPED

DA-003
rev. 4/01

year_____

C:\myfiles\reportforms\diaprepesforms.xls

DIAPREPES ADULT DATA

EGG DATE E	PUPAE DATE	ADULT DATE	NO CUPS	SHIP DATE	NO SHIPPED	EGG DATE E	PUPAE DATE	ADULT DATE	NO CUPS	SHIP DATE	NO SHIPPED

DA-004
rev. 4/01

year_____

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DIAPREPES DAILY DISCARDS

Discard Date _____

Egg Date	No Cups	Stage N	S	P	A	Reason for Discard Mold	Mites	Bacteria	Dead	Slow	Elytra	CT's	Other

DA-005
rev. 1/04

C:\myfiles\reportforms\diaprepesforms.xls

SANITATION CHECKLIST
AREA: Diaprepes Rearing Rooms

DATE: FROM_____ TO _____

	SUN	MON	TUES	WED	THU	FRI	SAT
DAILY ITEMS							
Floor - sweep/mop (bl)							
Garbage removed							
Sinks (bl)							
Table tops/carts (bl)							
WEEKLY ITEMS							
Floors - all rooms (dt)							
Horizontal surfaces/cabinet tops (qu)							
MONTHLY ITEMS							
AC vents (qu)							
Ceilings - rooms 1,2,3 (qu)							
Humidifiers (qu)							
Cupboards (int. & ext.) (qu)							
Lights (qu)							
Walls/doors - rooms 1,2,3 (qu)							
Floors stripped/waxed - as needed							

(bl = Bleach, dt = Detergent, qu = Quat)

COMMENTS:

SFL010-10
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