Ceriodaphnia dubia Chronic Toxicity Testing Method

DEPARTMENT OF ENVIRONMENTAL PROTECTION LABORATORY
BIOLOGY PROGRAM
TALLAHASSEE, FLORIDA

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Ceriodaphnia dubia Chronic Toxicity Testing Methods

1. SCOPE AND APPLICATION

1.1. This method is based on EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, 4th Edition, Test Method 1002.0. It describes the procedure used to estimate the chronic toxicity of whole effluent and receiving waters (freshwater aqueous samples) on <24-hour old (and ≤ 8-hour range in age) *Ceriodaphnia dubia* (water flea) neonates during a 7-day static renewal exposure. Tests covered by this procedure are BSY-CS-FCD and BSY-CD-FCD.

2. SUMMARY OF METHOD

2.1. Samples are received and prepared in the laboratory. Sample physical-chemical (phys-chem) parameters are measured and samples are adjusted as needed to meet permit or method test requirements. Sample is filtered through a 60 µm screen to remove debris and/or indigenous organisms. Sample dilutions and control waters are placed into test chambers. Test organisms are collected, fed, and held for observation prior to loading into test chambers. Organisms are then loaded into test vessels. Tests are incubated in laboratory incubators. While tests are in progress, survival/mortality, reproduction, and phys-chem parameters are measured and recorded daily before and after renewal. Tests are checked at ~24-hour intervals. The test endpoints are survival and reproduction. Results are entered into the Laboratory Information Management System (LIMS).

2.2. Test acceptability criteria: 80% or greater survival in the controls, 60% or more of control females must have produced their third brood, and the average number of young per surviving female must be 15 or greater (presumes all criteria for a valid test are met). Test duration is until a minimum of 60% of surviving females have three broods (maximum test duration of 8 days). (This presumes all criteria for a valid test are met).

2.3. Adjust sample as needed to meet permit requirements. Adjust pH (TA-08.06) and dissolved oxygen concentration; dechlorinate (TA-09.18), etc.

2.4. Interferences: Refer to EPA 821-R-02-013 Section 12.3. Includes toxic substances introduced by contaminants in dilution water or on hands, glassware, sample hardware, and testing equipment. Dissolved oxygen <4.0 mg/L or >9.0 mg/L; pH <6.0 SU or >9.0 SU; salinity >1 part per thousand; ammonia, chlorine, debris, indigenous organisms that may attack or be confused with the test organisms.

2.5. Definitions:

2.5.1. Neonates: Organisms less than 24-hours old

2.5.2. Diluent: Control water

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2.5.3. Sample 1: Original (1st) sample taken from the facility; used for initial test set-up (0-hr and 24-hr)

2.5.4. Sample 2: Sample collected 48 hours after the original sample; used for renewals (48-hr and 72-hr after test initiation)

2.5.5. Sample 3: Sample collected 96 hours after the original sample; used for renewals (96-hr, 120-hr, 144-hr and 168-hr (if needed) after test initiation)

3. EQUIPMENT AND SUPPLIES

3.1. 30 mL disposable plastic cups filled with deionized (DI) water and soaked for a minimum of 12 hours

3.1.1. (Ten replicate cups) + (one parameter cup) =11 cups per test concentration.

3.2. 60-place Plexiglass tray to hold test cups and one smaller tray to hold parameter cups. One tray per sample or facility tested.

3.3. 250 mL Autoclaved, graduated cylinder for making test dilutions

3.4. 1000 mL Autoclaved, Erlenmeyer flask for mixing test dilutions

3.5. 6000 mL Autoclaved, Erlenmeyer flask to hold effluent sample

3.6. 60 µm mesh to filter sample

3.7. 1 mL and 10 mL Serological pipettes, graduated

3.8. Pipette pump for serological pipettes

3.9. Plastic transfer pipette

3.10. Magnetic stir bars and stirrer

3.11. 0.2 mL Calibrated pipettor

3.11.1. Pipette tips

3.12. YCT and algal suspension combined 1:1 for daily feeding (YCT prepared per SOP TA-01.01; algal suspension of *P. subcapitata* prepared per SOP TA-01.04.)

3.13. Dissecting microscope or magnifying glass

3.14. Random loading sheet and a die

3.15. Effluent samples to be tested (recommended to be warmed to 25 ± 1°C)

3.16. Meters to measure:

3.16.1. pH calibrated per SOP TA 06.43

3.16.2. Dissolved oxygen (DO) calibrated per SOP TA-06.39

3.16.3. Temperature using digital thermometer calibrated per SOP TA-06.13

3.16.4. Conductivity/salinity calibrated per SOP TA-06.36

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3.17. Hach® kits to measure:

3.17.1. Total residual chlorine (TRC) per SOP TA-06.22

3.17.2. Alkalinity per SOP TA-06.08

3.17.3. Hardness per SOP TA-06.08

3.17.4. Ammonia per SOP TA-06.35

3.18. *C. dubia* neonates meeting age requirements, obtained per SOP TA-05.02

3.19. Thermo Scientific Incubator (Incubator #1 or #5)

3.19.1. Incubator should maintain test temperature at 25 ± 1°C and a photoperiod of 16 hours light: 8 hours dark. Light intensity should be ~50-100 ft. candles. Sample temperatures in cups must not deviate more than 3°C for the duration of the test.

3.20. Computer with access to LIMS

3.21. *Bioassay Sample log-in* logbook

3.22. *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia* logbook

4. REAGENTS AND STANDARDS

4.1. Moderately Hard Freshwater (MHWCD), prepared per SOP TA-03.15.

5. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

5.1. See SOP FS 1000 for sample collection and preservation.

5.1.1. Sample criteria for use in a test: a) the sample should be held at 0-6°C prior to test initiation or be cooling if hand delivered on day of collection; and b) sample holding time should not exceed 36-hr from time of collection (last sample time when 4 grab samples are collected over a 24-hour period, or the end of the 24-hour collection period for a composite sample) to test initiation.

5.1.2. A series of three samples (designated as samples 1, 2, and 3) are collected and used upon receipt. Sample 1 is used for 2 days, sample 2 is used for 2 days, and sample 3 is used for 3 days, if needed.

5.1.2.1. The remainder of each sample is stored in the walk-in cooler for solution renewal the following day or days.

5.1.2.2. Any variation in sample collection (from above) is not necessarily cause for test invalidation but the DEP toxicity coordinator should always be notified/consulted for approval/acceptability.

5.2. See SOP TA-06.14 for bioassay sample log-in procedure.

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6. TEST INITIATION

6.1. Using tape and a waterproof marker, label the Plexiglass tray containing soaked 30mL test cups with test concentration and facility sampled.

6.2. Calibrate meters and record readings in appropriate LIMS logbook daily during test. Also, check meters at end of day and record values.

6.3. Filter sample through 60 µm mesh into 6000 mL Erlenmeyer flask.

6.4. Fill a 30 mL disposable plastic cup with effluent sample and take an initial pH measurement per SOP TA-06.43. Record value in *Bioassay Sample log-in logbook* and LIMS TOX_PH logbook.

6.4.1. If pH is outside acceptable range of 6.0–9.0, then an additional test with a pH-adjusted aliquot will be run. Adjust pH of sample per SOP TA-08.06. Record pH adjustment and/or measurement in the appropriate benchsheet. The test with the non-adjusted sample is then analyzed as a screening test, regardless of whether the pH adjusted aliquot is used for a screening or definitive test.

6.5. In same sample cup, take an initial DO measurement per SOP TA-06.39. Record value in *Bioassay Sample Log-in logbook* and LIMS TOX_DO logbook.

6.5.1. If the D.O. is ≤ 4.0 mg/L or ≥ 9.0 mg/L, aerate the entire sample at a rate of ~100 bubbles per minute to as near saturation as possible prior to test initiation. Record aeration (initial and final DO, aeration rate, and duration) in the appropriate benchsheet. Samples with a potential DO problem generally show a downward trend in DO during the first few hours of the test. Monitor the DO closely; if the DO begins to fall, set up gentle aeration (<100 bubbles per minute) to ALL test solutions for the duration of the test using micropipettes and the aeration manifold. No action is required if DO increases after test initiation. In same sample cup, measure the conductivity, and temperature of the sample.

6.6. Measure total residual chlorine (TRC) in sample per SOP TA-06.22. Record value in the LIMS TOX_CHLORINE logbook and on the appropriate bioassay benchsheets. Check field data sheet supplied by sampler for field TRC and atypical field measurements.

6.6.1. If TRC ≥0.1 mg/L, and non-chlorine toxicity is to be measured, dechlorinate a subsample (volume needed to initiate the test) of the sample using sodium thiosulfate per SOP TA.9.18. Setup a concurrent screening test using the 100% sample that has not been dechlorinated and a control. Record chlorine result, amount of sodium thiosulfate used and final chlorine result after dechlorination in the logbook and in the designated section in LIMS.

6.7. Measure alkalinity, hardness, and total ammonia of sample per SOP TA-06.08 and TA-06.35. Record in the appropriate LIMS logbook.

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6.8. Preferred method for Definitive tests:

6.8.1. Warm sample to 25 ± 1ºC.

6.8.2. Empty DI water from soaking cups prior to filling with diluents (MHWCD) and sample test concentrations.

6.8.3. Using a graduated cylinder, pour 20 ± 1 mL of diluent into each of the eleven control replicate cups, including the parameter cup.

6.8.4. Pour 20 ± 1 mL aliquots of the 100% sample into each of the eleven 100% sample concentration cups, including the parameter cup.

6.8.5. Measure the desired volumes of diluent and sample into a 1000 mL Erlenmeyer flask using two graduated cylinders. Swirl flask by hand to mix until homogenized. Measure 250 mL of the mixture back into a 250 mL cylinder. Transfer 20 mL from the 250 mL cylinder into test replicate cup A; repeat for the remaining replicates for that concentration, including the parameter cup. Rinse cylinders with deionized water between concentrations. Repeat for each test concentration

6.8.5.1. For each sample tested, use ten replicates per concentration (plus a parameter cup) in a dilution series based on the Facility's NPDES permit, instruction from the appropriate DEP ROC or, if applicable, previous knowledge of toxicity level. Usually, a minimum of 5 test concentrations plus the control are used.

6.8.6. Place the eleventh cup of each concentration in a separate, small tray to measure physical parameters at a later time. A second tray is used to prevent possible probe contamination of the test cups.

6.8.7. Example of sample volume (0.5 dilution) below for ten 20mL replicates and one parameter cup per replicate of each concentration in the *C. dubia* chronic toxicity test:

Concentration	Diluent	Sample	Total Volume
Control	250 mL	0 mL	250 mL
6.25%	250 mL	250 mL of 12.5%	500 mL
12.5%	250 mL	250 mL of 25%	500 mL
25%	250 mL	250 mL of 50%	500 mL
50%	250 mL	250 mL of 100%	500 mL
100%	0 mL	250 mL	250 mL

6.9. Perform QA standard reference toxicant (SRT) tests monthly or concurrently if bioassays are preformed less frequently. The SRT must be done 30 days prior to the start of a facility bioassay.

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6.9.1. DEP Laboratory uses Potassium chloride as the standard toxicant. Other toxicants can be used. An example of sample volumes for acute definitive potassium chloride SRT test, using the toxicant made per SOP TA-09.01, is below. Concentrations and toxicants can be modified as needed. Diluent is synonymous with control water.

6.9.2. Example of KCl (1 g/L toxicant) concentrations and diluent volumes for chronic SRT test.

Concentration	Diluent	Toxicant	Total Volume
Control	250 mL	0 mL	250 mL
0.3 g/L	210 mL	90 mL of 1 g/L KCl	300 mL
0.4 g/L	180 mL	120 mL of 1 g/L KCl	300 mL
0. 5 g/L	150 mL	150 mL of 1 g/L KCl	300 mL
0.6 g/L	120 mL	180 mL of 1 g/L KCl	300 mL
0.7 g/L	90 mL	210 mL of 1 g/L KCl	300 mL

6.10. Using calibrated 0.2 mL pipettor, or calibrated dropper, feed 0.2 mL of combined 1:1 YCT and algal suspension per test cup (recommended).

6.10.1. Feeding is done every day before transfer of organisms. Record feeding time in bench sheets comment section or in other designated space.

6.11. Measure pH, temperature, DO, and conductivity of the solutions in the parameter cups (eleventh cup) in the separate tray.

6.11.1. Phys-chem parameters on “new sample” should be taken before transfer of organisms to ensure that concentrations were poured correctly. This allows the chance to interpret and correct mistakes without causing harm to the organisms.

6.11.2. Record all readings, and any comments pertaining to the condition or appearance of the sample, in their appropriate columns on the Parameter page of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Definitive Tests* logbook.

6.11.2.1. Conductivity measurements are reported to 3 significant figures. pH, DO, and temperature measurements should be read to one decimal place. Take measurements from lowest to highest toxicant/sample concentration.

6.11.3. Empty the phys-chem cups into the sink, rinse cups, and flush the drain with water.

6.12. <u>Test initiation</u> (0-hour and Day-0)

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6.12.1. With a plastic pipette, randomly load one *C. dubia* neonate from one chronic culture cup (collected specifically for chronic test from adults for neonates) into replicate A of the control and replicate A of each test concentrations. Repeat for the remaining 9 sets of replicate cups using a different cup of neonates per set of replicate cups (generally total of 6 cups/set). If necessary, same cup of neonates can be used to load more than 1 set of replicates/concentration.

6.12.1.1. Loading is done at a light table.

6.12.2. Use a random loading pattern found in the lab. It is based on the one found in the EPA chronic manual.

6.12.2.1. Use one cup to load one row randomly (usually 6 cups). Loading is done across concentrations using randomization template.

6.12.2.2. In the logbook, record which cup was used to load a row.

6.13. A person other than the loader should verify that there is one neonate in each replicate (test initiation).

6.14. Place the tray of cups in the incubator and cover the tray with clear Plexiglass.

Note: Covers prevent accidental contamination and minimize evaporation.

6.15. Record counts and the date and time of test initiation on the appropriate pages of the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Screening Tests* logbook.

7. INTERIM READINGS AND RENEWALS

7.1. Every day at 24-hour (± 3 hours) intervals, perform applicable Steps 6.2-6.12.3 to prepare new test cups/concentrations, transfer *C. dubia,* take parameters, feed, etc.

7.1.1. On days when a new set of effluent sample water arrives, follow Steps 6.2 through 6.8 for phys/chem readings. When two or more jugs of sample water were collected on the same day, only temperatures must be recorded from all jugs in *Bioassay Sample Log-in* logbook. The remaining phys/chem parameters (pH, DO, and conductivity) should be performed and recorded from only one jug in *Bioassay Sample Log-in* logbook.

7.1.2. Parameters of the “before renewal” and “after renewal” solutions are taken daily. All before-renewal parameters should be taken using “A” replicates. If the “A” replicate is not available (removed due to mortality), measure the next available, alphabetically sequential aliquot of the same concentration.

7.2. On days 1 until test termination (see 2.2), retrieve the previous day’s cups from the incubator count and record of surviving *C. dubia* and record the number of neonates on the Reproduction pages of the logbook.

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7.2.1.1. If necessary, use the lowest power of a dissecting scope (magnifying glass) or the light table.

7.2.1.1.1. Young will typically be present on days 3-8.

7.3. With a plastic transfer pipette, transfer ONE *C. dubia* adult from previous tray into the corresponding test cup on the new tray.

7.3.1.1. Transfers MUST be performed prior to measuring physical parameters in cups from the previous day.

7.3.1.2. Verify count under the lowest power dissecting scope (magnifying glass) or the light table.

7.3.2. Place new tray of cups into incubator and cover tray with clear Plexiglass.

7.4. Repeat applicable steps 6.2 -7.3.2 daily until test termination criteria (60% of surviving control females have 3rd brood) is met, then proceed to Step 8. DO NOT CONTINUE TEST BEYOND 8 DAYS.

8. TEST TERMINATION (WHEN 60% OF SURVIVING CONTROL FEMALES HAVE 3RD BROOD):

8.1. Count number of young produced and record in appropriate columns on bench sheet.

8.1.1. Adults and young MUST be counted prior to measuring physical parameters in cups from the previous day, but they don't need to be removed to take the final measurements.

8.1.2. At test termination, all observations on numbers of offspring should be completed within 2 hours. The rapid rate of development of *C. dubia* means that an extension of more than a few hours in the test period could be a significant part of the brood production cycle of the animals and allow production of additional broods.

NOTE: Adults not producing young by end of the test are placed on a slide and examined to determine if they are male or female usually by a member of the invertebrate taxonomy group. Refer to EPA-821-R-02-013 Section 13.10.9-13.13.2.2.

8.2. Measure pH and DO of the solutions and record in appropriate columns on bench sheet.

8.3. Record any comments pertaining to the condition of the test animals.

8.4. Record test termination date and time.

8.5. Pour contents of all test cups into sink and flush drain with water.

8.6. Dispose of cups in trashcan and put tray in storage area.

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8.7. Enter test results into the EPA WET Excel spreadsheet to obtain the NOEC, LOEC, or IC25. Refer to SOP TA-07.17. Data transcription (paper to electronic) should be verified by another analyst (Windows *speak* /*read aloud* function can be used).

9. DATA ARCHIVAL

9.1. Use a black, waterproof pen for all bench sheets and logbooks.

9.2. To correct raw data entries, place a single line through the incorrect or illegible entry, write the corrected entry near the error, date and initial the correction. Do not overwrite entries.

9.2.1. The corrected entry is written as near as possible to the error or in the comment box on the appropriate benchsheet.

9.2.2. If the correction requires an explanation, write a comment and reference the error with a letter. Initial and date the explanation. Do not attempt to put all information (correction and comments) into a small space.

9.2.2.1. Use sequential alphabetical annotation to identify errors and corresponding comments (use A for first error and use A for corresponding explanatory comment; use B for second error, etc.).

9.3. Upon sample receipt, log sample receipt, temperature, and condition into Chronic Bioassay Bench Sheets logbook. Fill in information requested at top of bench sheets as completely as possible prior to test initiation. Include full name of facility, address, contact person, date and time collected, field TRC, LIMS sample and job numbers, test chamber ID, test chamber size, test solution volume, food type, and food batch numbers. Access scanned Field Sheets via \\fldep1\dear\Labs\BizhubScans\Receiving to obtain sampling information. Initial TRC, alkalinity, and hardness measurements should be recorded in their respective LIMS logbooks.

9.4. Record meter calibration readings in the LIMS logbooks and appropriate bench sheets.

9.5. During sample prep and analysis, parameters and organism data should be entered in the *Chronic Bioassay Bench Sheets for Ceriodaphnia dubia Screening Tests* logbook.

9.6. Enter the NOEC, LOEC, and IC25 results from the EPA WET Excel spreadsheet (Refer to Step 8.7.) into the appropriate logbook, computer file, and LIMS results editor. A second person must verify data prior to uploading into LIMS.

10. QUALITY CONTROL

10.1. Refer to DEP Laboratory Quality Manual for corrective action and handling out-of-control data. Refer to EPA 821-R-02-013 Test Method 1002.0 for test conditions and acceptability criteria. Sample should be held at 0-6°C prior to test initiation and

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between renewals. (Exception for hand delivered sample on day of collection - sample should be cooling relative to collection temperature.)

10.2. Sample holding time should not exceed 36 hours from time of collection until initial use of sample.

10.3. Incubator maintained at the selected test temperature of 25 ± 1°C.

10.4. Test temperature must not deviate more than 3°C in the test cups for the duration of the test.

10.5. Incubator light intensity maintained at ~50-100 ft-candles for a photoperiod of 16-hour light**:** 8-hour dark.

10.6. Perform QA standard reference toxicant (SRT) tests monthly since the *C. dubia* are cultured in-house (see Step 6.10).

10.7. If a problem occurs during the sample processing or testing, the following steps are performed: 1) Lab supervisor is notified. 2) Client is notified (client makes decision for additional testing). 3) Program administrator is notified. 4) A non-conformance report (NCR) is submitted describing the problem and the corrective action.

10.7.1. To create NCR: open LIMS and go to Laboratory/Administrative/Non-Conformance Report.

10.8. Refer to https://floridadep.gov/dear/quality-assurance/content/dep-sops for sample collection and preservation, and the Laboratory Quality Manual for corrective action and handling out-of-control data.

11. SAFETY/HAZARDOUS WASTE MANAGEMENT

11.1. Proper laboratory protection, including lab hood or ventilation system, lab coat, gloves, and safety glasses, is required when working with chemicals and unprocessed samples.

11.2. Refer to DEP Laboratory Quality Manual for sample disposal .

11.3. Refer to the Laboratory Health and Safety Plan.

11.4. Read MSDS information for reagent handling and disposal.

12. REFERENCES

12.1. EPA 821-R-02-013 *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th Edition, Test Method 1002.0.*

12.2. DEP SOP TA-01.01, *YCT (Yeast, Cereal Leaves, Flake-Fish Food, or Trout Chow) Preparation*

12.3. DEP SOP TA-01.04, Pseudokirchneriella subcapitata *Culture for* Ceriodaphnia dubia *Food*

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12.4. DEP SOP TA-03.15, *Preparation of Synthetic Moderately Hard Freshwater*

12.5. DEP SOP TA-05.02, *Production of* Ceriodaphnia dubia *for Chronic Toxicity Testing*

12.6. DEP SOP TA-06.08, *Measuring Total Alkalinity and Hardness*

12.7. DEP SOP TA-06.13, *Thermometer Accuracy Check and Use*

12.8. DEP SOP TA-06.14, *Bioassay Sample Log-In Procedure*

12.9. DEP SOP TA-06.22, *Measuring Total Residual Chlorine using the Hach Pocket Colorimeter II Test Kit*

12.10. DEP SOP TA-06.43, *Calibration and Use of Fisherbrand Accumet AB315 pH/mV Meter*

12.11. DEP SOP TA-06.36, *Calibration and Use of the Fisherbrand Traceable Bench Conductivity meter for Conductivity and Salinity*

12.12. DEP SOP TA-06.35, *Measuring Total Ammonia using the Hach Ammonia Nitrogen Pocket Colorimeter II Test Kit*

12.13. DEP SOP TA-06.39, *Thermo Scientific Orion Star A213 Benchtop RDO/DO Meter Calibration and DO Measurement*

12.14. DEP SOP TA-07.17, *Using EPA Whole Effluent Toxicity (WET) Statistics for Acute and Chronic Bioassay Tests*

12.15. DEP SOP TA-09.01, *Potassium Chloride Reference Toxicant Stock Solutions*

12.16. DEP SOP TA 09.18, *Dechlorination Procedure using Sodium Thiosulfate*

12.17. DEP Laboratory Quality Manual

12.18. DEP Laboratory Health and Safety Plan

APPENDIX OF CHANGES

07/25/2008 Moved *Scope and Application, Summary of the Method* and *Interferences* from comments to body of SOP. Added *References* and *Appendix of Changes*. Changed SOP TA-01.05 to TA-01.04 in Methods 6 comment. Removed SOP TA-06.06 form Materials # 11 comment.

07/13/2009 Inserted “surviving female” in the initial Materials comment. Changed “know” to ‘known” in *Daily* Step 5 comment. Changed “5” to “2” in Day 7 Step 5 comment (pg 3). Added “NOTE: If test termination criteria (60% of surviving control females have 3rd brood) proceed to Day 7” to Day 5 and Day 6 comment. Updated Quality Manual reference.

08/14/2009 Replaced reference to SOP TA -07.08 to SOP TA-07.16

07/21/2010 Updated reference to Biology Quality Manual. Removed reference to vinyl gloves in the Safety section comment and added a Caution statement.

05/18/2011 Updated reference to Quality manual and updated SOP link. Changed IC20 to IC25. Changed logbook names to match actual titles.

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04/11/2013 Reformatted the SOP.

02/11/2015 Formatted for 508 compliance.

03/15/2016 Updated links. Changed Table total volume. Re-arranged text.

03/08/2017 Updated links. Re-arranged text.

01/10/2018 Clarified test duration. Modified text to reflect clarification.

01/09/2018 Updated links and SOP references. Re-arranged text.

01/02/2020 Updated links. Re-arranged steps for section 6.9.

12/17/2020 Updated with universal language

12/27/2021 Checked grammar, re-arranged indents to follow format, and updated Links. Added Title page and table of contents due to SOP length.

12/22/2022 Updated links, checked for grammar, and updated SOP list to remove ones that are no longer in use and add new relevant ones.

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