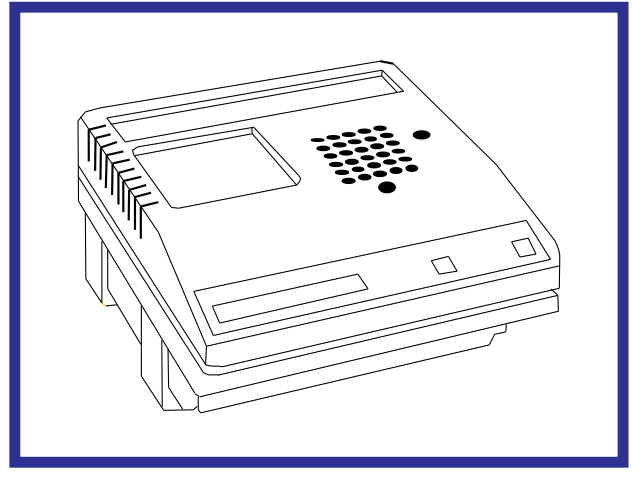
Microtox®
Acute Toxicity
Basic Test
Procedures



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6-19-1995

Click the drawing of the Model 500 to return to the Manual Directory.

Microtox Acute Toxicity Test
Materials Required:

Microtox Acute Toxicity Test Reagent
Reconstitution Solution
Diluent
Cuvettes

10 - 100 µL Pipettor & tips
0.25 - 2.5 mL Pipettor & tips
Repeat pipettor & syringes

Table of Contents

MICROTOX®
ACUTE TOXICITY BASIC TEST FUNDAMENTALS

PRINCIPLE OF OPERATION

The test exposes luminescent test organisms in Microtox Acute Toxicity Test Reagent to test samples, and measures the increase or decrease in light output by the test organisms.

Reagent contains living luminescent bacteria that have been grown under optimal conditions, harvested, and then lyophilized (freeze-dried). The lyophilized bacteria are rehydrated with Reconstitution Solution to provide a ready-to-use suspension of organisms.

The test system measures the light output of the luminescent bacteria after they have been challenged by a sample and compares it to the light output of a control (reagent blank) that contains no sample. A difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms.

PRECISION

Each test cuvette contains roughly a million individual test organisms that are challenged by the test sample. Variations among individual organisms become statistically insignificant. The system measures a single parameter, the simultaneous light output of all of the organisms.

Each batch of Reagent, containing lyophilized test organisms, is prepared under conditions within very rigidly controlled limits.

TIME AND TEMPERATURE

Different chemicals affect living organisms at different rates, reflecting differences in mechanism of action.

For some classes of chemicals, the effect on light output is complete in 5 minutes.
For other classes of chemicals, the light output is still decreasing rapidly at 5 minutes. In these cases, 15-minute data may be more reliable.

We recommend that 5-minute and 15-minute data be taken routinely when dealing with unknown samples. Some laboratories elect to collect data routinely at five, fifteen and thirty minutes. The software will automatically process data taken at any one, two, or three times you select.

The temperature during exposure to various materials will affect the response of the living organisms. For the Microtox Acute Toxicity Test procedures the Incubator Wells and READ Well temperature is 15°C.

CONTROL (BLANK)

Reagent Control is required for each test and is run concurrently with the test. Light levels normally change with time, for reasons other than the bioreactivity of the test sample.

Use of the Reagent Control allows the operator to compensate for some of these variables when reducing the data. In all variations of the Basic Test procedure, the responses of all the test cuvettes are normalized to that of the Control test response, which compensates for errors up to 20% in the 10 µL pipetting of reagent.

SUPPLIES & ACCESSORIES

Testing requires several special items in addition to those commonly found in testing laboratories.

REAGENT

The Microtox Acute Toxicity Test Reagent is specially formulated for bioreactivity testing with sensitivity to a broad range of toxicants.

The reagent is a freeze-dried preparation of a specially selected strain of the marine bacterium *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum*, NRRL number B-11177). A vial of reagent contains roughly one hundred million test organisms.

Shelf life is one year when stored at minus (-) 20ºC to (-) 25ºC. Do Not store the Reagent below minus (-) 25ºC.

Storage at normal refrigerator temperature greatly reduces shelf-life.

Reagent should not be stored in a self-defrosting freezer, which defrosts by warming up periodically.

Use the reagent within one to two hours after reconstitution. The sensitivity of the reagent is essentially unchanged for 1-2 hours after reconstitution. Changes in sensitivity may become significant after that time for some samples.

Some laboratories reconstitute a vial of Reagent at the beginning of the day, store it in the REAGENT Well, then use the rehydrated reagent as needed through the day until it is consumed.

For many applications, this is acceptable practice, but if Reagent is to be used 90 minutes or more after reconstitution, its performance should be monitored periodically with a standard such as PHENOL, to indicate changing sensitivity.

RECONSTITUTION SOLUTION AND REFERENCE WATER

Reconstitution Solution and Reference Water are alternate names for specially prepared nontoxic Ultra Pure Water.

Shelf life is one year when stored at room temperature.

DILUENT

The Diluent is a specially prepared nontoxic 2% Sodium chloride (NaCl) solution, used for diluting the sample and the reagent.

The marine bacterium in the reagent requires osmotic protection that is provided by the 2% NaCl.

Shelf life is one year when stored at room temperature.

OSMOTIC ADJUSTMENT SOLUTION

Osmotic Adjustment Solution (OAS) is a specially prepared nontoxic 22% Sodium chloride (NaCl) solution, used to adjust the osmotic pressure of the sample to approximately 2% NaCl.

To adjust a sample osmotically, add one part of OAS to ten parts of sample.

(X mL sample x 0.1 = mL OAS)

Example:

2.5 mL sample x 0.1 = 0.25 mL OAS

The sample can also be osmotically adjusted by the addition of solid AR grade NaCl to a final concentration of 2.0%.

Shelf life is one year when stored at room temperature.

DO NOT PREPARE SOLUTIONS

Do not make Diluent, Osmotic Adjustment Solution or Reference Water yourself or use substitutes. The production of uncontaminated solutions is difficult, and some laboratories have created problems using their own Diluent and Osmotic Adjustment Solution.

CUVETTES

Cuvettes are used to contain samples, controls, and Reagent during testing. They are nontoxic and disposable.

Used cuvettes cannot reliably be cleaned for reuse. Traces of detergent or sample contaminants interfere with later tests. The risk of interference from contamination is unacceptably high.

PIPETTORS AND PIPETTOR TIPS

Test protocols require repeated precise transfer of small amounts of liquid, as little as 10µL. High precision adjustable micro-pipettors are necessary. It is highly recommended to have and use the following pipettors:

10 - 100 µL adjustable volume pipettor

0.25 - 2.5 mL adjustable volume pipettor

repeat pipettor

Sample Collection and Preparation

SAMPLE COLLECTION

Use new clean, borosilicate, screw cap containers (30 to 50 mL) with Teflon® lined caps. Fill the container completely to the top with sample, leaving NO airspace.

Completely filling the container helps keep volatile material in solution.

SAMPLE STORAGE

Test the sample as soon as possible after collection. If testing is delayed, store samples at normal refrigerator temperature.

The toxicity of the sample can change with time, so testing the sample within 1-2 hours after collection is best, but this is not always possible. Try to test the sample within 24-48 hours after collection.

COLORED SAMPLE

Perform the test protocol, and determine the sample's ECXX (ICXX). Check the sample concentration at the ECXX (ICXX) for visible color. If it contains obvious color, perform the Color Correction Protocol.

TURBID SAMPLE

If the sample is turbid and this is objectionable, centrifuge the sample at an adequate speed and time to remove the turbidity.

The turbity is objectionable when the toxicity from the turbid material is not wanted. If the toxicity is desired from the material that causes turbity, do not centrifuge the sample. If the sample is too turbid to perform a Basic Test use the Solid-Phase Test.

CHLORINE CONTENT

The Reagent (organism) is sensitive to chlorine as are all microorganisms.

Municipal Water Treatment (drinking water) or Waste Water Treatment Plants have a common problem with the water they are producing, bacterial contamination. Chlorination of the water is generally used to solve this problem. Chlorine is toxic: that is what it is being used for killing microorganisms.

Most of the time when either a Municipal Water Treatment or Waste Water Treatment Plant wants to check for acute toxicity, they do not want to know the effect of chlorination.

Collect the samples before chlorination, unless you are testing for the effect of chlorination. Do not collect a chlorinated sample for testing, unless you want to know the effect of chlorination or can not collect a sample that is not chlorinated. If knowing the effect of chlorination is desired, it is recommended to test the sample just before chlorination and just after chlorination.

When the effect of chlorination is desired or can not be avoided, dechlorinate the sample with Sodium Thiosulfate.

SODIUM THIOSULFATE DECHLORINATION

To dechlorinate a sample add 1 part of Sodium Thiosulfate Stock Solution to 10 parts of Sample.
Example: 100 uL Sodium Thiosulfate Stock Solution added to 10 mL Sample.
Final Sodium Thiosulfate concentration in the sample is 100 mg/L.

SODIUM THIOSULFATE STOCK SOLUTION PREPARATION

1. Weight out 1.0 g Sodium Thiosulfate ($Na_2S_2O_3$).
2. Add 100.0 mL Diluent.

   The Sodium Thiosulfate will last for 1-2 months when stored in this manner.

SAMPLE pH

1. Measure the pH of the sample, and record the pH as part of the information in the sample Description.
   The bacterial Reagent is sensitive to pH. There is a minimal pH effect between 6.0 and 8.0. When the pH is higher than 8.0 or lower than 6.0, and the sample has buffering capacity the effect can be dramatic. Example: distilled water adjusted to pH 5.0 with HCl, has NO pH effect because there are not enough hydrogen ions to affect the pH of the Reagent. Another sample at the same pH (5.0) may show considerable toxicity if it does have buffering capacity.
2. If the pH of the sample is below 6.0 or above 8.0 and pH adjustment is required, adjust the pH as shown below.
   Adjustment is required when the toxic effect of pH is not wanted.
   A. If the pH is below 6.0, adjust the pH to 6.0 with NaOH. If over-titration occurs, discard sample and start again.
   B. If the pH is above 8.0, adjust the pH to 8.0 with HCl. If over-titration occurs, discard sample and start again.
      When the sample is below pH 6.0, titrating the sample above 6.0 may precipitate the sample, thus changing the effective toxicity.
      When the sample is above pH 8.0, titrating the sample below 8.0 may precipitate the sample, thus changing the effective toxicity.
      When the sample has been over-titrated, back titrating may not resolubilize the sample, if precipitation occurred.
      Use a strong concentration of acid or base (5 N) for coarse pH adjustment of a sample with a high/strong buffering capacity to minimize sample dilution.
      Use a weak concentration of acid or base (0.5 N) when the sample has a low/weak buffering capacity.

PURPOSE OF A STANDARD

Testing a standard, whose test results are well characterized, confirms your understanding of a test protocol and checks the performance of the complete test system (e.g. analyzer, Reagent, Diluent and Reconstitution Solution).

The Basic Test Protocol (with two controls, eight sample dilutions in duplicate) is the best procedure for testing "Standards," as it provides the highest confidence level, precision and flexibility. For Zinc Sulfate Standard use 1:1.5 serial dilutions with the Basic Test Protocol.

Although the Abbreviated Basic Test Protocol can be used, by substituting the "Standard" for the sample, it is not recommended. If you use the Abbreviated Basic Test for Zinc Sulfate Standard use 1:2 serial dilutions.

When testing "Standards" never except an EC50 derived from extrapolated data, always retest using the appropriate "Standard" dilutions.

PHENOL STANDARD

Phenol $EC50_{5\ minutes}$ = 13 to 26 mg/L

1. Weigh out ~50 mg (~0.050 g) of crystalline Phenol, add it to a 500 milliliter (mL) amber volumetric flask.
   Do not try to weigh out the exact amount of sample, calculate sample concentration for the amount weighed out.

   If an amber volumetric flask is not available, cover the entire flask with aluminum foil to protect the Phenol Standard from light.
2. Add Diluent to the 500 mL mark on the volumetric flask.
3. Seal the flask, and mix well by inverting the volumetric flask.
4. Label the flask and store at normal refrigerator temperature (2-8°C).
   The Phenol standard will last for 3-4 months when stored in this manner.

ZINC SULFATE STANDARD

EC50 $_{15\ minutes}$
$ZnSO_4 \cdot 7H_2O$ = 3 to 10 mg/L
Zn^{++} = 0.6 to 2.2 mg/L

1. Weigh out ~ 50 mg (~ 0.050 g) of ZnSO4·7H2O, add it to a 500 milliliter (mL) volumetric flask.
   Do not try to weigh out the exact amount of sample; calculate sample concentration for the amount weighed out.
2. Add Diluent to the 500 mL mark on the volumetric flask.
3. Seal the flask and mix well by inverting the volumetric flask.
4. Label the flask and store at normal refrigerator temperature (2-8°C).
   The Zinc sulfate standard will last for 3-4 months when stored in this manner.

MICROTOX ACUTE TOXICITY BASIC TEST VERSATILITY

The table below shows the combinations of numbers of tests, controls and dilutions that can be performed at one time. The Basic Test protocol with slight modifications can take readings on one to three controls, plus three to fourteen serial dilutions of the same sample (a combined maximum of fifteen cuvettes).

No. tests	No. Controls	No. dilutions
1	1	3-14
1	2	3-13
1	3	3-12
2	1	3-4
3	1	3-4
1 D	1	3-9
1 D	2	3-8
1 D	3	3-7
2 D	1	3-4
2 D	2	3

D = Test in Duplicate

BASIC TEST

Two controls, eight sample dilutions in duplicate.

This procedure measures the relative acute toxicity of a sample, producing data for calculation of the EC50 or IC50 value or other ECXX or ICXX values.

It is highly recommended that the first test you perform be the Detailed Basic Test Protocol with a Phenol or Zinc Standard as the sample.

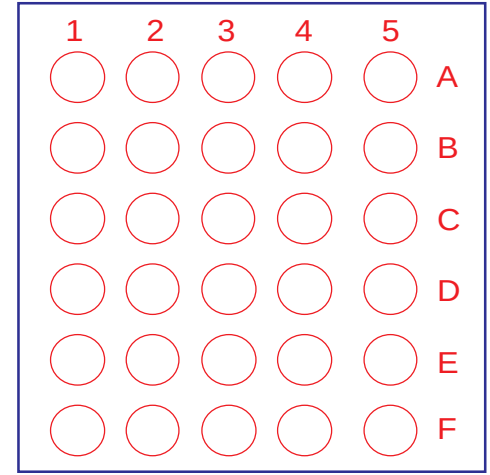
This protocol is the best procedure for testing samples of unknown toxicity or when the test results must provide the highest confidence and precision. This Basic Test provides the most flexibility, along with the highest confidence level and test precision.

Recommended for testing:

Pure Compounds
Septage
Waste Water Treatment Influent
Waste Water Treatment Digester

ANALYZER PREPARATION

1. Place clean, unused cuvettes in the REAGENT Well & all incubator block wells.
   M500 incubator block layout:



2. Pipette 1.0 mL Reconstitution Solution into the cuvette in the REAGENT Well.

µL = microliter 1000 µL = 1 mL
mL = milliliter 1000 mL = 1 L
L = Liter

Place the appropriate tip very firmly on the pipettor, then twist. Observe the pipettor tip for liquid leaks during its use. If there is a leak, the tip is not on firmly enough.

The unit of measurement will be mL when the 0.25 to 2.5 mL adjustable volume pipettor (250 µL, 500 µL or 1000 µL fixed volume pipettors) is to be used and µL when the 10 to 100 µL pipettor (10 µL fixed volume pipettor or repeat pipettor) is to be used.

3. Pipette 0.5 mL Diluent into each cuvette in wells:
   B1 through B5
   C1 through C5
   E1 through E5
   F1 through F5
4. Pipette 1.5 mL Diluent into each cuvette in wells:
   A1 through A5
   D1 through D4

   Do not add diluent to D5.

SAMPLE PREPARATION

1. Add 2.5 mL of sample to cuvette D5.

Primary Dilution of Sample

When all the sample dilutions have gamma's greater than 1.0 and the EC50 derived from extrapolated data, a primary dilution of the sample is required.
See Primary Dilution table on the next page.

A primary dilution is a predilution of the sample before following the Basic Test Protocol. If a primary dilution is used, remember to enter the appropriate initial concentration in Computer Preparation. For further information on how to make primary dilutions, see the Dilutions discussion in the Data Quality Section of the Manual.

If Osmotic Adjustment Is Required Perform Steps 2-4 Below.

Do not osmotically adjust Phenol or Zinc standards since they have been prepared in Diluent.

Osmotic adjustment is required when the salt content is below 2%. The amount of OAS used above is for when the sample salt content is 0.0%.
Solid Sodium chloride can be used for osmotic adjustment.

2. Add 0.25 mL OAS to D5.
3. Mix the sample in D5 using the pipettor (set to 1.5 mL), by filling and dispensing the pipettor 3-4 times.
4. Discard 300 µL from D5.

Primary Dilution Table

Primary Dilution	Sample / Diluent	Initial Concentration
none		45.0
1:10	Use For Septage & Waste Water Treatment Plant Digester Samples sample / Diluent 250 µL / 2.25 mL	4.5
1:100	(250 µL / 2.25 mL) + (250 µL / 2.25 mL)	0.45
1:1000	(250 µL / 2.25 mL) + (250 µL / 2.25 mL) + (250 µL / 2.25 mL)	0.045

SAMPLE DILUTION

1. Make 1:2 serial dilutions of the sample by transferring 1.5 mL from cuvette to cuvette as shown below. Mix using the pipettor (by filling and dispensing the pipettor 1 time).

cuvette	to	cuvette
D5	to	D4
D4	to	D3
D3	to	D2
D2	to	D1
D1	to	A5
A5	to	A4
A4	to	A3

2. Discard 1.5 mL from cuvette A3.
3. Wait 5 minutes for temperature equilibration.

REAGENT PREPARATION

1. Remove and discard the seal and stopper from a vial of Microtox Acute Toxicity Test Reagent.
   Keep the Reagent in the freezer until ready to use.
2. If the reagent pellet is not seated on the bottom of the vial, tap the vial until the pellet is seated.
3. Take the cuvette containing the Reconstitution Solution from the REAGENT Well. Place the lip of the cuvette on top of the Reagent vial. Then, as Quickly as you can, Dump the Reconstitution Solution into the reagent vial.
   Do not use a pipettor for reconstitution.

   Slow reconstitution (pouring reconstitution solution onto the reagent pellet) causes low reagent light levels.
4. Swirl the reagent vial 3-4 times, pour the reagent into the cuvette, then place the cuvette back into the REAGENT Well.
   Do not overmix, or use a vortex mixer, as this will warm the reagent, and can affect test results.

5. Mix the reconstituted reagent with the pipettor set to 0.5 mL (using a new pipettor tip), by filling and dispensing 10 times.
   The reagent will contain microscopic clumps after reconstitution. The mixing breaks up these clumps, helping in the uniform dispersal of the reagent.
6. Transfer 10 µL reconstituted reagent into each cuvette in wells:
   B1 through B5
   C1 through C5
   E1 through E5
   F1 through F5
   Use the 10-100 µL pipettor, 10 µL pipettor or a repeat pipettor.

Adjustable Volume 10-100 µL Pipettor

When despensing reagent, using the 10-100 µL pipettor, be as quick as possible, remove a cuvette from the incubation well & add 10 µL of reagent, then place cuvette back into its incubation well. Add reagent to all the appropriate cuvettes before going to the next step.

Fixed Volume 10 µL Pipettor

When dispensing (adding) reagent into a cuvette, do not remove the cuvette from its incubator well. Place the pipettor tip under the surface of the liquid but not against the bottom of the cuvette. This can be accomplished easily by placing the pipettor tip inside the cuvette and resting it against the side wall. Slide the pipettor down until you feel the ridge on the pipettor tip touching the rim of the cuvette. Stop there, the tip is in a good position.

Repeat Pipettor

When using the repeat pipettor, add the reagent to all the cuvettes while not allowing the syringe tip or reagent come into contact with the tube walls.

Do not let the syringe tip touch the sample.

HOW TO USE A REPEAT PIPETTOR

1. Move the piston of the syringe up and down several times to ensure smooth operation in the dispenser.
2. Push the piston lever to its lowest position.
3. Place syringe into the syringe holder and the piston holder respectively.
4. Set the repeat pipettor Volume Adjustment Dial.
5. Place the pipettor tip into the reagent.
6. Pull the piston lever up slowly, carefully filling the syringe with reagent, keeping the tip below the liquid level.
7. Discard the first aliquot before pipetting.

7. Mix the reagent in each cuvette, by shaking 2-3 times holding the top of the cuvette and moving your wrist back and forth:

   B1 through B5
   C1 through C5
   E1 through E5
   F1 through F5

   Do not cover the top of the cuvette and mix by shaking up and down, or by inversion.
8. Wait 15 minutes for reagent stabilization.

COMPUTER PREPARATION

1. Turn on the computer.
2. Call up the Basic Test program.
3. Select: Start Testing
   Set number of tests: 1
   Enter a file name for test
   Enter sample description
   (pH, etc...)
4. Set Test Parameters:
   Number of controls (2)
   Number of dilutions (8)
   Replications (duplicate)
   Test Time (5, 15, X)

   Initial Concentration (45 if OAS, 50 if none*)
   "Initial concentration" is the actual highest sample concentration after transfer to a cuvette containing reagent. The operator must calculate the initial concentration manually, including consideration for any "primary dilution".

   * None when osmotic adjustment is with solid Sodium chloride or no osmotic adjustment was required.

   Dilution Factor (2)
   Units (%)
   (For standard Phenol or Zinc sulfate, enter mg/L)
   Osmotic adjustment (X)
   (OAS, solid NaCl, none)

Liquid Sample Initial Concentration

The following example shows how to calculate the sample Initial Concentration (iC) in cuvette E5, F5 in percent, after osmotic adjustment with OAS, and with a Primary Dilution Factor (P).

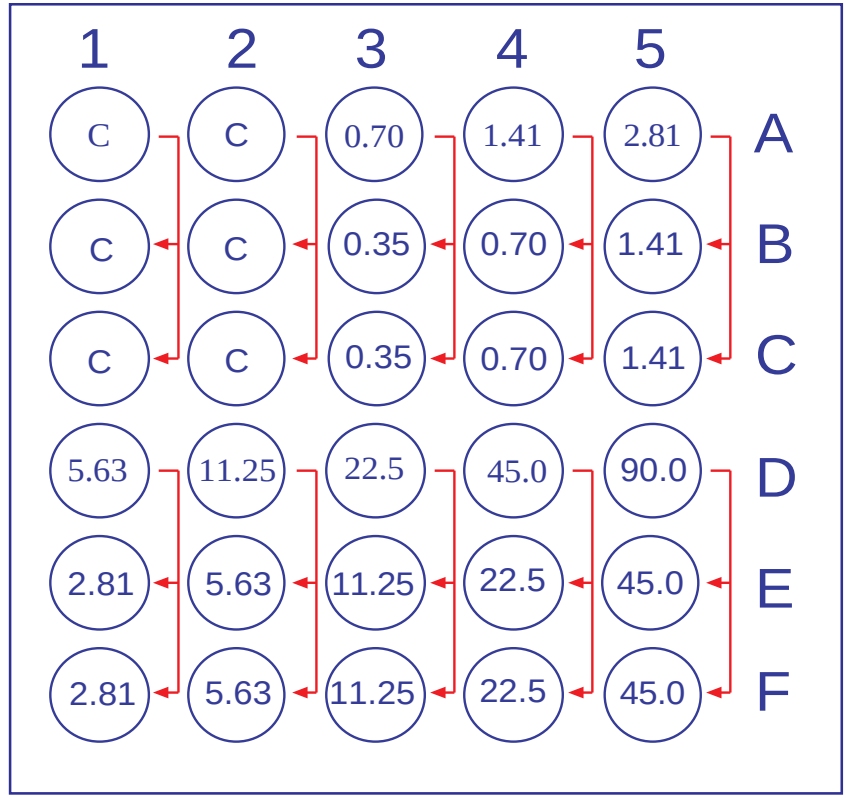
Cuvette D5 concentration (C).

((% sample ÷ P) X µL sample) ÷ (µL OAS + µL sample) = C
((100% ÷ 1) X 3000) ÷ (300 + 3000) = C
300000% ÷ 3300 = C
90.909% = C

Cuvette E5, F5 concentration (iC) after sample transfer from D row to E & F row.

(% sample X µL sample)÷(sample + diluent + reagent)µL = iC
90.909% X 500 ÷ (500 + 500 + 10) = iC
45454.5% ÷ 1010 = iC
45.004% = iC

45.004%, rounded off to 45% ("initial concentration" entered in Computer Preparation)


1
2
3
4
5
C
C
0.70
1.41
2.81
A
C
C
0.35
0.70
1.41
B
C
C
0.35
0.70
1.41
C
5.63
11.25
22.5
45.0
90.0
D
2.81
5.63
11.25
22.5
45.0
E
2.81
5.63
11.25
22.5
45.0
F

C = Control

TEST PROCEDURE

1. After the 15 minute waiting period, place cuvette B1 in the READ Well. Press the M500 SET button. The green READY light will go off. Leave cuvette B1 in the READ well.
    1. Usually the green READY light will be on when the set button is pressed.
    2. If a red warning panel light is on, see Analyzer Trouble-shooting section of the Manual.
    3. Check to see what the red warning means.
    4. If it was one of the two light level warning light proceed with the test after pressing the SET button.
    5. If it was any of the other warning lights follow the instructions in the Analyzer Troubleshooting section.
    6. A pause of up to six seconds will follow before the cuvette is lowered into the READ well. This pause does not indicate a malfunction. Do not press the SET button again if the panel lights are off. The cuvette will be lowered and raised one or two times or three times.
    7. When the green READY light comes back on, the M500 has been auto-calibrated.
    8. Do not press the SET button again during this test.
2. When the green READY light comes back on touch the computer <space bar> key.

    Io = light level (I) at incubation zero time (o). The computer timer will automatically start when the last Io is read.

    Do not drop the cuvettes into the wells, Diluent/Reagent can be lost, affecting the test results.
3. Read initial (zero time, Io) light level in each cuvette as prompted by the flashing "ENTER" on the computer screen, by placing the cuvette in the READ well and pressing the READ button. Then immediately...
4. ... using a new pipettor tip, make the following 500 µL transfers:

A1 to B1 & C1	D1 to E1 & F1
A2 to B2 & C2	D2 to E2 & F2
A3 to B3 & C3	D3 to E3 & F3
A4 to B4 & C4	D4 to E4 & F4
A5 to B5 & C5	D5 to E5 & F5

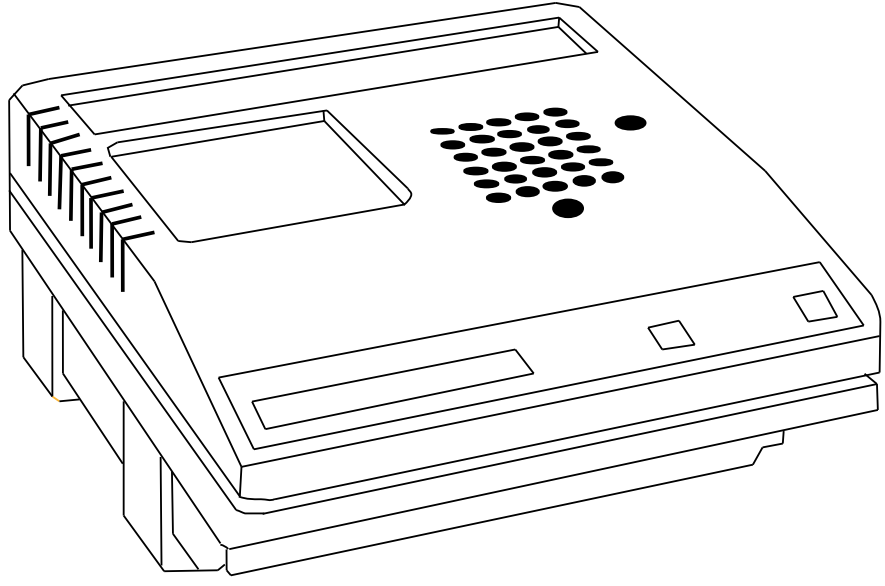
5. Touch the computer <space bar> key.

    This notes the elapsed time spent making the sample transfers. This information is used by the system to set the timing of the I_t reading intervals to the operator's personal pace of work.

6. When the computer timer sounds, READ light levels as prompted by the computer monitor, by placing the cuvette in the READ well and pressing the READ button.

DATA REPORT

1. Select the data file.



BASIC TEST

Two controls, eight sample dilutions in duplicate.

This procedure is a condensed version of the Detailed Basic Test Protocol.

This protocol is the best procedure for testing samples of unknown toxicity or when the test results must provide the highest confidence and precision. This Basic Test provides the most flexibility, along with the highest confidence level and test precision.

Recommended for testing:

Pure Compounds
Septage
Waste Water Treatment Influent
Waste Water Treatment Digester

ANALYZER PREPARATION

1. Place cuvettes in all incubator wells and REAGENT Well.
2. Add 1.0 mL Reconstitution Solution to REAGENT Well.
3. Add 0.5 mL Diluent to:
   B1 through B5
   C1 through C5
   E1 through E5
   F1 through F5
4. Add 1.5 mL Diluent to:
   A1 through A5
   D1 through D4

SAMPLE PREPARATION

1. Add 2.5 mL sample to D5.
2. If osmotic adjustment is required add 0.25 mL OAS to D5 and mix.

   Osmotic adjustment is required when the salt content is below 2%. The amount of OAS used above is for when the sample salt content is 0.0%.

SAMPLE DILUTION

1. Make 1:2 serial dilutions by transferring 1.5 mL, mixing after each transfer:

cuvette	to	cuvette
D5	to	D4
D4	to	D3
D3	to	D2
D2	to	D1
D1	to	A5
A5	to	A4
A4	to	A3

2. Discard 1.5 mL from A3.
3. Wait 5 minutes.

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.
3. Transfer 10 µL reagent to:
   - B1 through B5
   - C1 through C5
   - E1 through E5
   - F1 through F5
4. Mix cuvettes (in step 3) by shaking.
5. Wait 15 minutes after reagent dilution.

COMPUTER PREPARATION

1. Call up the Basic Test program.
2. Select: Start Testing
   Set number of tests: 1
   Enter a file name for test
3. Set Test Parameters:

   Layout

   Number of controls (2)
   Number of dilutions (8)
   Replications (duplicate)
   Test Time (5, 15, X)

   Sample information

   Initial Concentration (45 if OAS, 50 if none)
   Dilution Factor (2)
   Units (%) (For standard Phenol or Zinc sulfate, enter mg/L)
   Osmotic adjustment (X)

TEST PROTOCOL

1. Place B1 cuvette in READ well. Press the SET button.
2. Touch the computer <space bar> key.
3. READ zero time I_o light levels as prompted by the computer monitor. Then immediately...
4. ... make the following 0.5 mL transfers:
   - A1 to B1 & C1
   - A2 to B2 & C2
   - A3 to B3 & C3
   - A4 to B4 & C4
   - A5 to B5 & C5
   - D1 to E1 & F1
   - D2 to E2 & F2
   - D3 to E3 & F3
   - D4 to E4 & F4
   - D5 to E5 & F5
5. Touch the computer <space bar> key.
6. When timer sounds, READ light levels as prompted by the computer monitor.

DATA REPORT

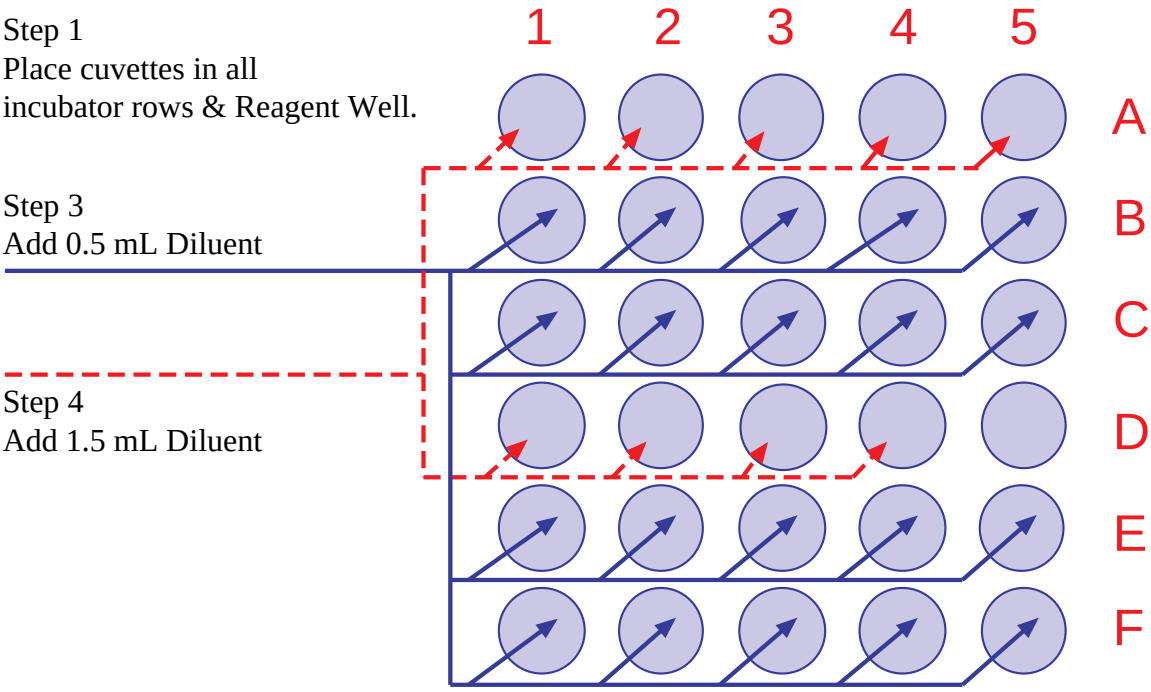
1. Reduce the data.

Basic Test template 1

Step 1
Place cuvettes in all
incubator rows & Reagent Well.

Step 3
Add 0.5 mL Diluent

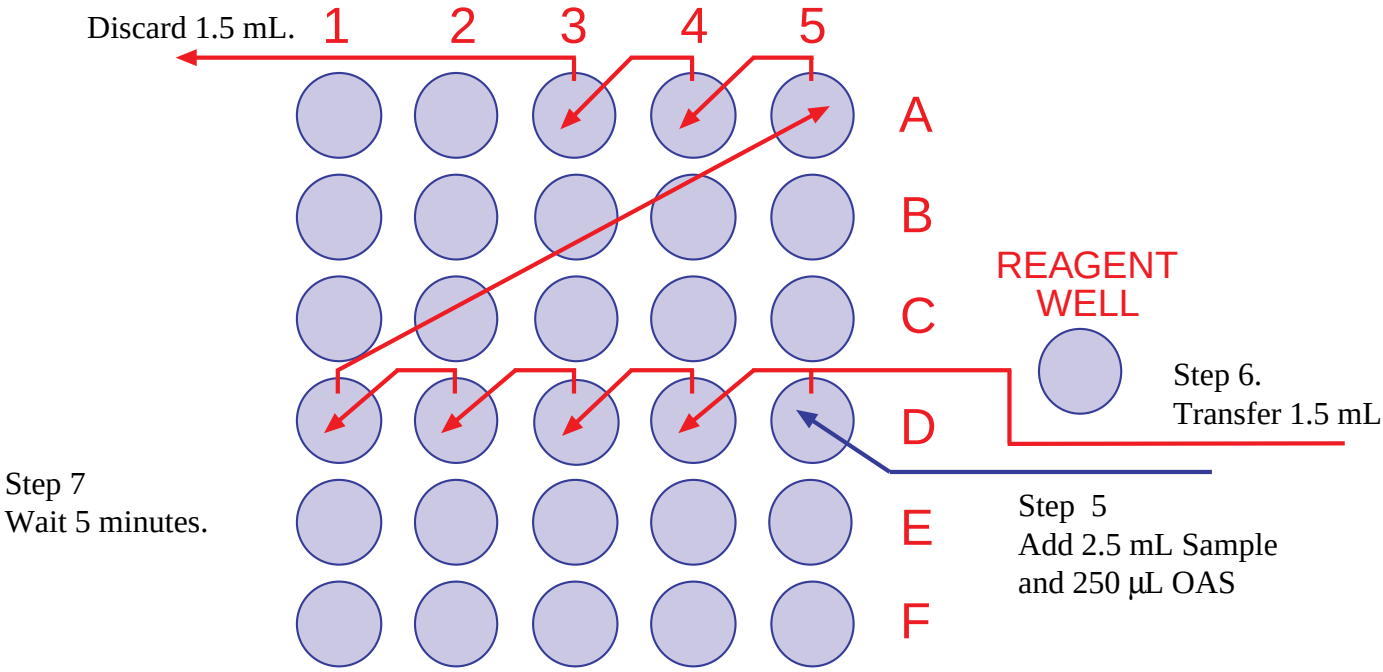
Step 4
Add 1.5 mL Diluent



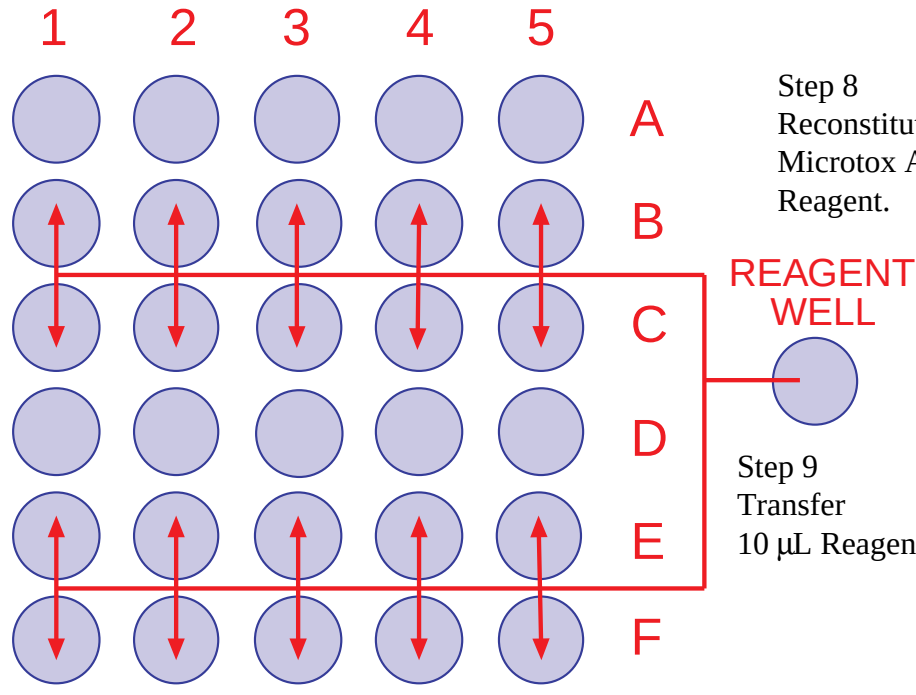
REAGENT WELL

Step 2
Add 1.0 mL
Reconstitution
Solution

Basic Test template 2



Basic Test template 3



Step 8
Reconstitute a vial of Microtox Acute Test Reagent.

Step 9
Transfer
10 µL Reagent.

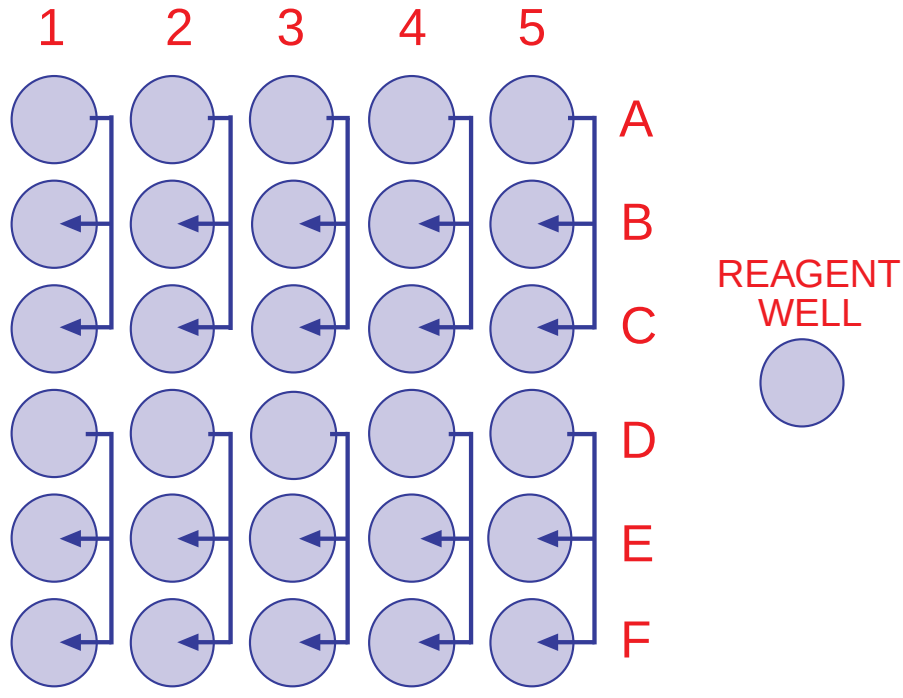
Step 10
Wait 15 minutes.

Basic Test template 4

Step 11
Read Io Light Levels

Step 12
Make 0.5 mL Transfers and mix.

Step 13
Read It Light Levels



ABBREVIATED BASIC TEST

One control, four sample dilutions.

Use this protocol for routine, daily testing at a sample site (when the flexibility, high confidence level and test precision is not required), where the general toxicity level is well known. This is the protocol that you will use most of the time for testing samples.

The first time to analyzing a sample use the Basic Test protocol. Use the data to decide if a primary dilution is required for the Abbreviated Basic Test. If you are having a hard time finding the EC50 with the Abbreviated Basic Test use the Basic Test.

Recommended for testing:

Pure Compounds
Septage
Waste Water Treatment Influent
Waste Water Treatment Digester

ANALYZER PREPARATION

1. Place cuvettes in REAGENT Well and incubator wells:
   A1 through A5
   B1 through B5
2. Add 1.0 mL Reconstitution Solution to REAGENT Well.
3. Add 0.5 mL Diluent to:
   B1 through B5
4. Add 1.0 mL Diluent to:
   A1 through A4

SAMPLE PREPARATION

1. Add 2.5 mL sample to A5.
2. If osmotic adjustment is required add 250 µL OAS to A5 and mix.
   Osmotic adjustment is required when the salt content is below 2%. The amount of OAS used above is for when the sample salt content is 0.0%.
3. Discard 750 µL from A5.

SAMPLE DILUTION

1. Make 1:2 serial dilutions by transferring 1.0 mL, mixing after each transfer:
   A5 to A4
   A4 to A3
   A3 to A2
2. Discard 1.0 mL from A2.
3. Wait 5 minutes.

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.
3. Transfer 10 µL reagent to:
   B1 through B5
4. Mix cuvettes (in step 3) by shaking.
5. Wait 15 minutes after reagent dilution.

COMPUTER PREPARATION

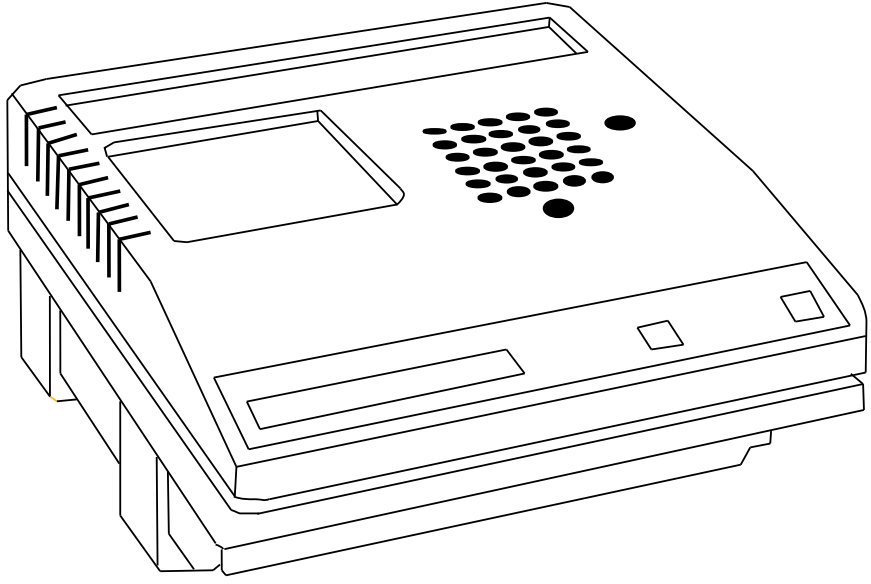
1. Call up the Basic Test program.
2. Select: Start Testing
   Set number of tests: 1
   Enter a file name for test
3. Set Test Parameters:
   Layout
   Number of controls (1)
   Number of dilutions (4)
   Replications (single)
   Test Time (5, 15, X)
   Sample information
   Initial Concentration
   (45 if OAS, 50 if none)
   Dilution Factor (2)
   Concentration table
   Units (%)
   Osmotic adjustment (X)

TEST PROTOCOL

1. Place B1 cuvette in READ well. Press the SET button.
2. Touch the computer <space bar> key.
3. READ zero time I_o light levels as prompted by the computer monitor. Then immediately...
4. ... make the following 0.5 mL transfers:
   A1 to B1
   A2 to B2
   A3 to B3
   A4 to B4
   A5 to B5
5. Touch the computer <space bar> key.
6. When timer sounds, READ light levels as prompted by the computer monitor.

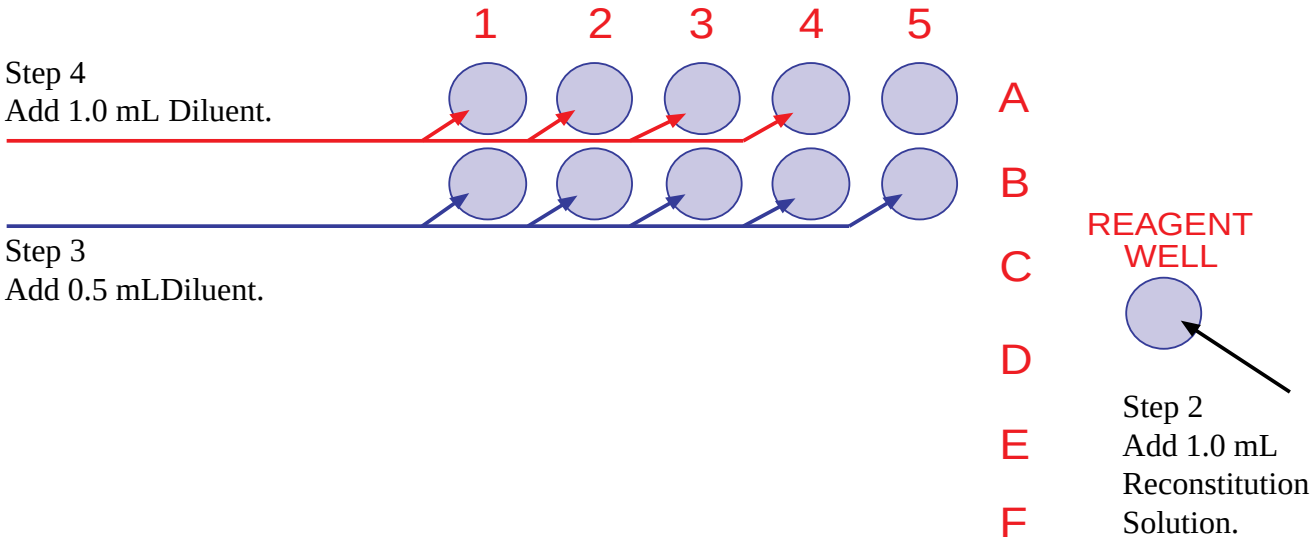
DATA REPORT

1. Reduce the data.



Abbreviated Basic Test template 1

Step 1 Place cuvettes in A & B rows & Reagent Well.

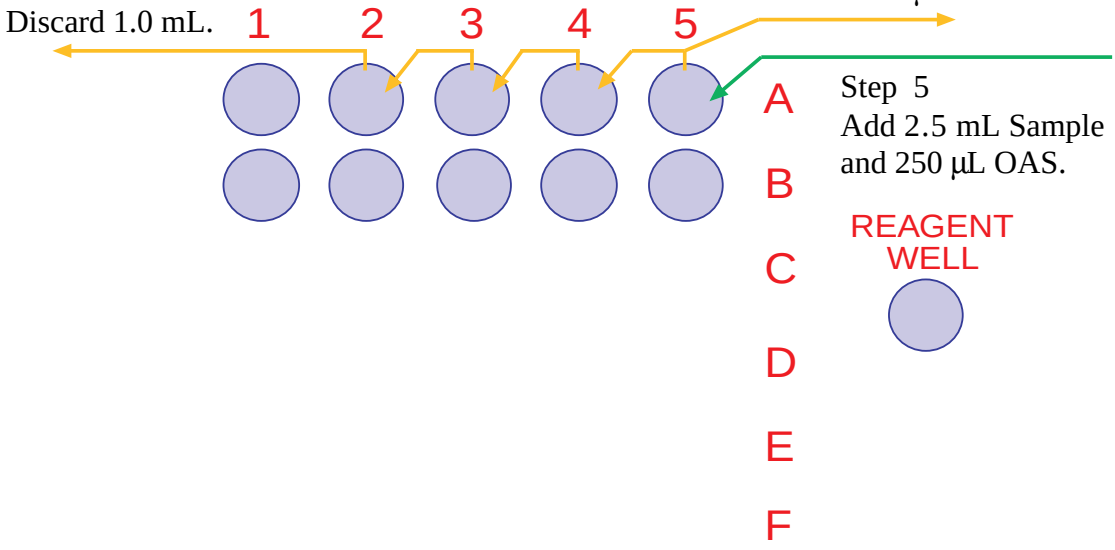


Abbreviated Basic Test template 2

Step 6
Transfer 1.0 mL.

Discard 750 µL.

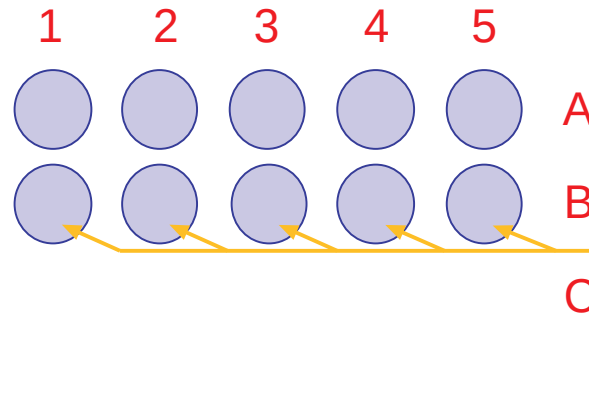
Discard 1.0 mL.



Step 5
Add 2.5 mL Sample and 250 µL OAS.

Step 7
Wait 5 minutes.

Abbreviated Basic Test template 3



Step 9
Transfer
10 µL Reagent.

Step 10
Wait 15 minutes.

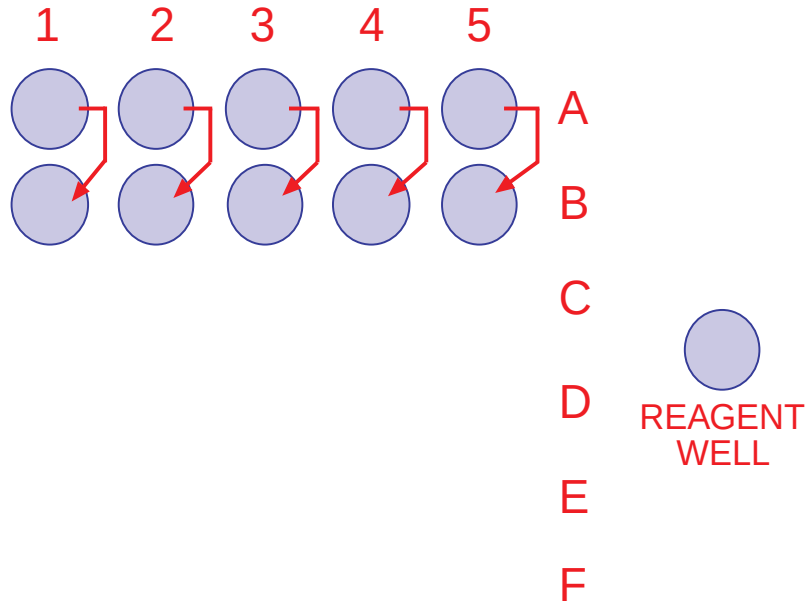
Step 8
Reconstitute a vial of Microtox Acute Test Reagent.

Abbreviated Basic Test template 4

Step 11
Read Io Light Levels

Step 12
Make Transfers and mix.

Step 13
Read It Light Levels



3 SAMPLES SIMULTANEOUSLY

Three samples (one control, four sample dilutions).

This condensed protocol tests three samples at the same time using the "Abbreviated Basic Test" procedure.

Use this protocol for routine, daily testing at a sample site (when the flexibility, high confidence level and test precision is not required), were the general toxicity level has been found.

The first time to analyzing a sample use the Basic Test protocol. Use the data to decide if a primary dilution is required for the Abbreviated Basic Test.

Recommended for testing:

Pure Compounds
Septage
Waste Water Treatment Influent
Waste Water Treatment Digester

ANALYZER PREPARATION

1. Place cuvettes in all incubator wells and REAGENT Well.
2. Add 1.0 mL Reconstitution Solution to REAGENT Well.
3. Add 0.5 mL Diluent to:
   B1 through B5
   D1 through D5
   F1 through F5
4. Add 1.0 mL Diluent to:
   A1 through A4
   C1 through C4
   E1 through E4

SAMPLE PREPARATION

1. Add 2.5 mL sample # 1 to A5.
2. Add 2.5 mL sample # 2 to C5.
3. Add 2.5 mL sample # 3 to E5.
4. If osmotic adjustment is required add 250 µL OAS to A5, C5 and E5 and mix.
   Discard 750 µL from A5, C5 and E5.
   Osmotic adjustment is required when the salt content is below 2%. The amount of OAS used above is for when the sample salt content is 0.0%.

SAMPLE DILUTION

1. Make 1:2 serial dilutions by transferring 1.0 mL, mixing after each transfer:
   A5 to A4
   A4 to A3
   A3 to A2
2. Discard 1.0 mL from A2.
3. Make 1:2 serial dilutions by transferring 1.0 mL, mixing after each transfer:
   C5 to C4
   C4 to C3
   C3 to C2
4. Discard 1.0 mL from C2.
5. Make 1:2 serial dilutions by transferring 1.0 mL, mixing after each transfer:
   E5 to E4
   E4 to E3
   E3 to E2
6. Discard 1.0 mL from E2.
7. Wait 5 minutes.

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.
3. Transfer 10 µL reagent to:
   B1 through B5
   D1 through D5
   F1 through F5
4. Mix cuvettes (in step 3) by shaking.
5. Wait 15 minutes after reagent dilution.

COMPUTER PREPARATION

1. Call up the Basic Test program.
2. Select: Start Testing
   Set number of tests: 3
   Enter a file name for each test.
3. Set Test Parameters:
   Layout
   Number of controls (1)
   Number of dilutions (4)
   Replications (single)
   Test Time (5, 15, X)

Sample information
Initial Concentration
(45 if OAS, 50 if none)
Dilution Factor (2)
Concentration table
Units (%)
Osmotic adjustment (X)

When testing multiple samples the following test parameters must be the same for all the samples: Number of Controls, Number of Dilutions, Replications, Test Time.

The following parameters may change with each sample: initial concentration, Dilution Factor, Concentration table, Units, Osmotic adjustment.

TEST PROTOCOL

1. Place B1 cuvette in READ well. Press the SET button.
2. Touch the computer <space bar> key.
3. READ zero time I_o light levels as prompted by the computer monitor. Then immediately...
4. ... make the following 0.5 mL transfers:

   A1 to B1
   A2 to B2
   A3 to B3
   A4 to B4
   A5 to B5

   Change pipettor tip.

   C1 to D1
   C2 to D2
   C3 to D3
   C4 to D4
   C5 to D5

   Change pipettor tip.

   E1 to F1
   E2 to F2
   E3 to F3
   E4 to F4
   E5 to F5

5. Touch the computer <space bar> key.
6. When timer sounds, READ light levels as prompted by the computer monitor.

DATA REPORT

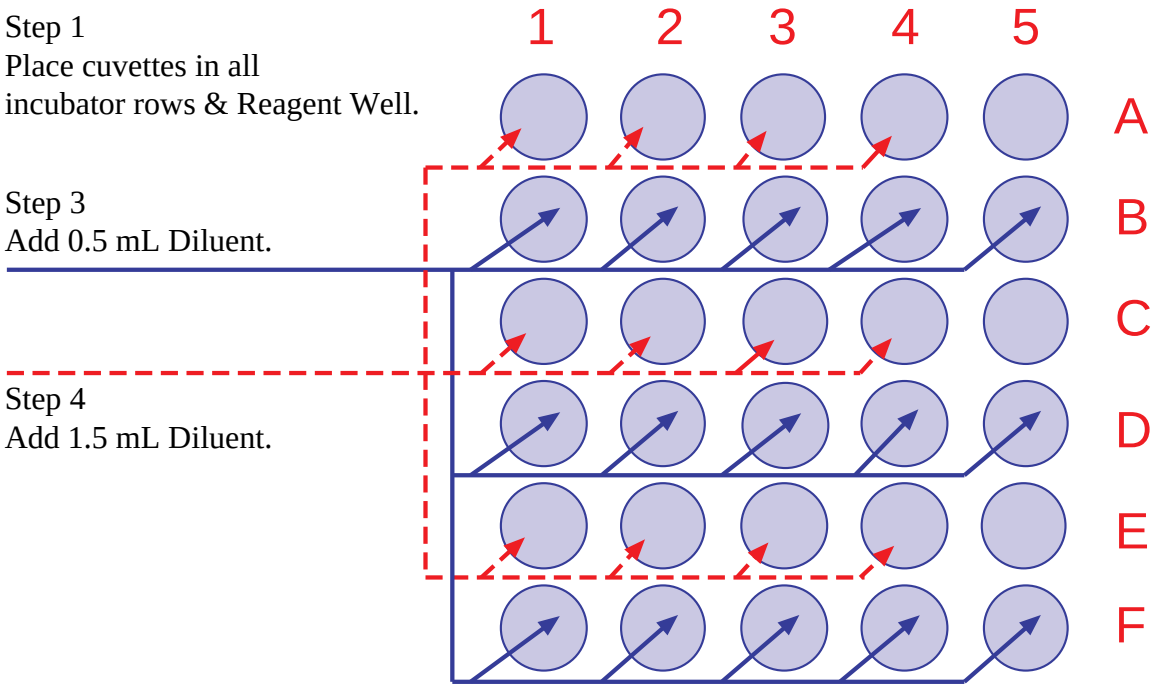
1. Reduce the data.

3 Samples Simultaneously template 1

Step 1
Place cuvettes in all incubator rows & Reagent Well.

Step 3
Add 0.5 mL Diluent.

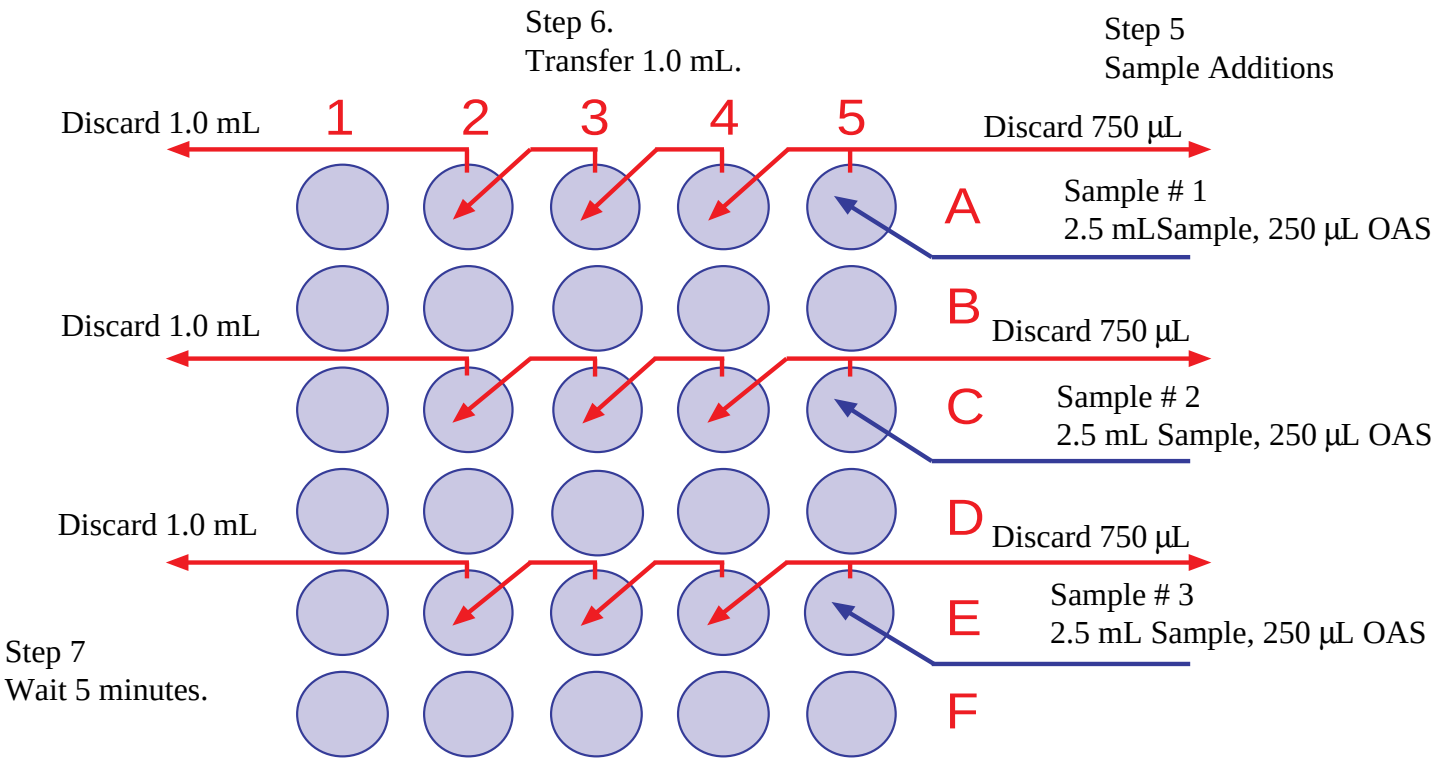
Step 4
Add 1.5 mL Diluent.



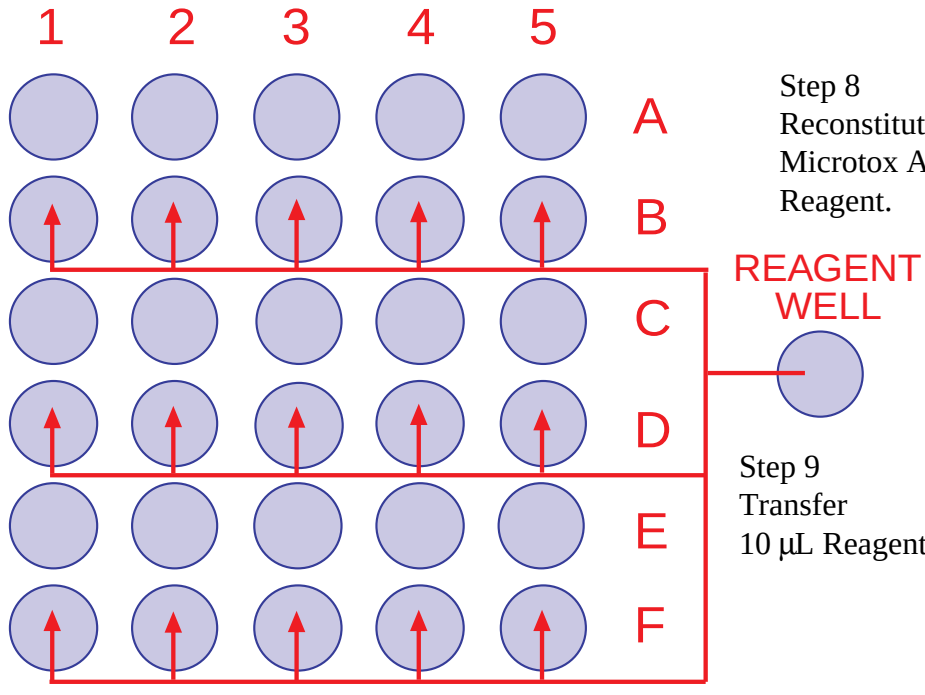
REAGENT WELL

Step 2
Add 1.0 mL Reconstitution Solution.

3 Samples Simultaneously template 2



3 Samples Simultaneously template 3



Step 8
Reconstitute a vial of Microtox Acute Test Reagent.

Step 9
Transfer
10 µL Reagent.

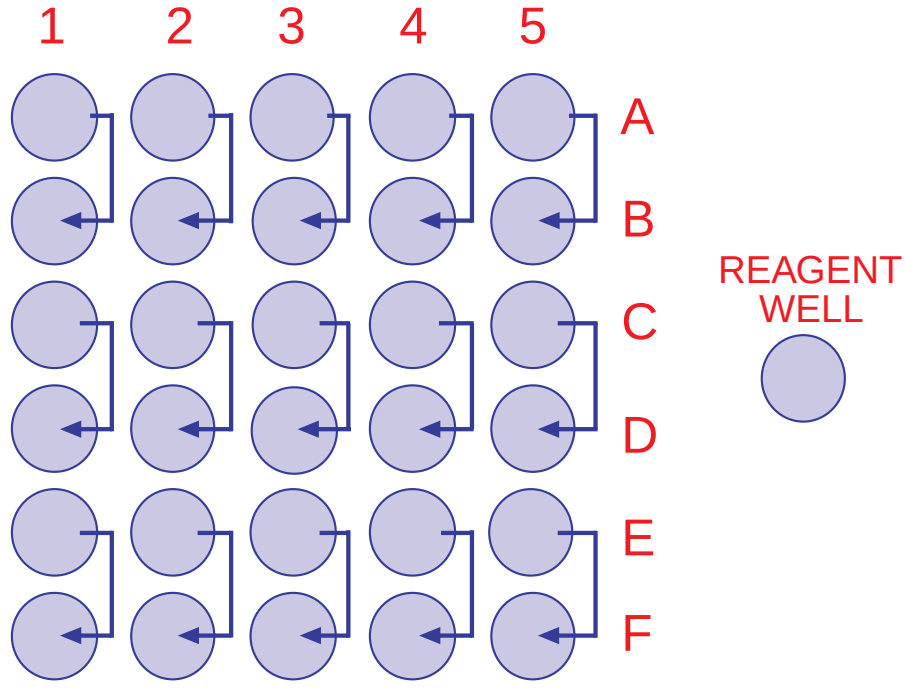
Step 10
Wait 15 minutes.

3 Samples Simultaneously template 4

Step 11
Read Io Light Levels

Step 12
Make 0.5 mL Transfers and mix.

Step 13
Read It Light Levels



90% SAMPLE CONCENTRATION BASIC TEST

Two controls, eight sample dilutions in duplicate.

This procedure tests a sample at a higher sample concentration than the Basic Test protocol. Recommended for samples that have EC50's between 27-100 % sample concentration.

ANALYZER PREPARATION

1. Place cuvettes in all incubator wells, READ Well and REAGENT Well.
2. Add 1.0 mL Reconstitution Solution to REAGENT Well.
3. Add 2.0 mL Diluent to A1 & A2.
4. Add 2.7 mL Diluent to the cuvette in the READ Well.
   Use 0.25-2.5 mL adjustable pipettor or a new disposable 5 mL glass or plastic pipette.
5. Label test tubes as:
   A3, A4, A5, D1, D2, D3, D4
6. To a large test tube labeled D5 add 25.0 mL of sample.
   Use a new disposable glass or plastic pipette.

OSMOTIC ADJUSTMENT

Osmotic adjustment is required when the salt content is below 2%. The amount of NaCl or OAS used is for when the sample salt content is 0.0%.

Osmotic Adjustment With Solid NaCl

1. Add 500.0 mg (0.500 g) solid NaCl to test tube D5, mix.

Optional Osmotic Adjustment With OAS

2. Add 2.5 mL OAS to test tube D5, mix.

SAMPLE DILUTION

1. Add the following amounts of sample from tube D5 to tube:
   - 1.5 mL to A3
   - 2.0 mL to A4
   - 2.5 mL to A5
   - 3.0 mL to D1
   - 3.5 mL to D2
   - 4.0 mL to D3
   - 4.5 mL to D4
2. Add the following amounts of Diluent to tube:
   - 3.5 mL to A3
   - 3.0 mL to A4
   - 2.5 mL to A5
   - 2.0 mL to D1
   - 1.5 mL to D2
   - 1.0 mL to D3
   - 0.5 mL to D4

   Do not touch the pipettor tip with the sample.
3. Mix contents of each test tube one at a time, then transfer 2.0 mL to its corresponding cuvette.
4. Wait 5 minutes.

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.
3. Add 0.3 mL reagent to READ Well.
4. Mix the cuvette in the READ Well.
5. Transfer 100 µL reagent/diluent from cuvette in the READ Well to:
   - B1 through B5
   - C1 through C5
   - E1 through E5
   - F1 through F5

   Use the 10-100 µL pipettor or a repeat pipettor.
6. Discard the cuvette in the READ Well.
7. Wait 15 minutes.

COMPUTER PREPARATION

1. Call up the Basic Test program.
2. Number of tests: 1
   Enter a file name for test
3. Set Current Test Parameters:

   Layout

   Number of controls (2)
   Number of dilutions (8)
   Replications (duplicate)
   Test Time (5, 15, 30)

Sample information

Initial Concentration (not used)
Dilution Factor (not used)
Concentration table (used)

Osmotic Adjustment	
NaCl	OAS
27.0	18.2
36.0	27.3
45.0	36.4
54.0	45.5
63.0	54.6
72.0	63.9
81.0	72.8
90.0	81.9

Units (%)
Osmotic adjustment (NaCl or OAS or none)

TEST PROTOCOL

1. Place B1 cuvette in READ well. Press the SET button.
2. Touch the computer <space bar> key.
3. READ zero time light levels as prompted by the computer monitor. Then immediately...
4. ... make the following 0.9 mL transfers:
   A1 to B1 & C1
   A2 to B2 & C2
   A3 to B3 & C3
   A4 to B4 & C4
   A5 to B5 & C5
   D1 to E1 & F1
   D2 to E2 & F2
   D3 to E3 & F3
   D4 to E4 & F4
   D5 to E5 & F5
5. Touch the computer <space bar> key.
6. When timer sounds, READ light levels as prompted by the computer monitor.

DATA REPORT

1. Reduce the data calculating EC20 or EC50 value.

ORGANIC SOLVENT SOLUBILIZATION OF SAMPLE

Two controls, eight sample dilutions in duplicate.

Many organic chemicals are insoluble in water. These require testing in the presence of an organic solvent.

Organic solvent solubilization (or extraction) will make available the nonvolatile organic compounds soluble in that organic solvent system.

The Reagent is sensitive to organic solvent toxicity.

This protocol uses an organic solvent concentration below the Reagent detectable toxicity level.

Keep the sample concentration as high as possible.
If no bioreactivity is found, the sample is either nontoxic, or is not soluble enough in the solvent used. The "90% Sample Concentration Basic Test" is very useful for low toxicity samples.

WHICH ORGANIC SOLVENTS

The three recommended organic solvents are Ethanol (Do Not use Denatured Ethanol), Methanol, and DMSO.

SOLVENT/DILUENT

It is recommended that the organic solvent concentration be No Higher than 1%, and that the organic solvent concentration be kept constant by adding 1% organic solvent to Diluent (solvent/diluent). This keeps the organic solvent concentration below its toxic effect, minimizing the sample masking effect that can be caused by the solvent system toxicity.

It is possible that both the organic solvent and samples can affect the same target site on the reagent. This test procedure will keep this to a minimum.

SAMPLE EXTRACTION

This procedure is used to dissolve organic solvent-soluble material from samples such as soils and sediment samples for testing.

Extract the sample with a pure organic solvent, using one of the published procedures. Microbics does not recommend a particular organic solvent extraction procedure. They all have some similarities.

Some procedures extract the sample in one organic solvent, then transfer the sample to a second organic solvent. The most common solvent used is 100% Ethanol (NON-DENATURED) for the final organic solvent system. Use this solution as your test sample.

SAMPLE SOLUBILIZATION

Dissolve non-aqueous samples in an organic solvent prior to testing.

Determine which of the three organic solvents (Ethanol, Methanol or DMSO) your sample will dissolve in. Then use that pure organic solvent in as high a concentration as possible. Use this as your test sample.

SOLVENT/DILUENT PREPARATION

Add 500 uL (0.5 mL) 100% solvent (Ethanol (Non-denatured, Methanol, or DMSO) to 49.5 mL Diluent.

ANALYZER PREPARATION

1. Place cuvettes in all incubator wells, READ Well and REAGENT Well.
2. Add 1.0 mL Reconstitution Solution (No Organic Solvent) to Reagent Well.
3. Add 2.0 mL Solvent/Diluent to A1 & A2.
4. Add 2.7 mL Solvent/Diluent to the cuvette in the READ Well.
   Use 0.25-2.5 mL adjustable pipettor or a new disposable 5 mL glass or plastic pipette.
5. Label test tubes as:
   A3, A4, A5, D1, D2, D3, D4
6. Add 2.5 mL Solvent/Diluent to test tubes:
   A3, A4, A5, D1, D2, D3, D4
7. To a large test tube labeled D5 add 5.94 mL of Diluent (No Organic Solvent).
   Use the 0.25 -2.5 mL adjustable pipettor

   If you do not have the 0.25 - 2.5 mL adjustable pipettor, add 24.75 mL.

SAMPLE DILUTION

1. Add 60 µL sample (in 100% organic solvent) to test tube D5, and mix.
   Use the 10 -100 µL adjustable pipettor

   If you do not have the 10 - 100 µL adjustable pipettor, add 250 µL to the 24.75 mL in D5.

   If a precipitate forms repeat step making a lower concentration of the sample.

   The sample concentration in A5 is 1 % of the original sample concentration and 1 % organic solvent.

   Example: 100 mg/L stock sample concentration
   100 mg/L × 0.01 (1%) = 1.0 mg/L conc. in A5

2. Make 1:2 serial dilutions by transferring 2.5 mL, mixing after each transfer:

   test tube to test tube

   D5 to D4
   D4 to D3
   D3 to D2
   D2 to D1
   D1 to A5
   A5 to A4
   A4 to A3

3. Transfer 2.5 mL from each test tube to its corresponding cuvette.
4. Wait 5 minutes.

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.
3. Add 0.3 mL reagent to READ Well.
4. Mix the cuvette in the READ Well.
5. Transfer 100 µL reagent/diluent from cuvette in the READ Well to:

   B1 through B5
   C1 through C5
   E1 through E5
   F1 through F5

   Use the 10-100 µL pipettor or a repeat pipettor.

6. Discard the cuvette in the READ Well.
7. Wait 15 minutes.

COMPUTER PREPARATION

1. Call up the Basic Test program.
2. Number of tests: 1
   Enter a file name for test
3. Set Current Test Parameters:

   Layout

   Number of Controls (2)
   Number of dilutions (8)
   Replications (duplicate)
   Test Time (5, 15, X)

   Sample information

   Initial concentration (0.90% of the original sample concentration)
   Dilution Factor (2)
   Concentration Table (not used)
   Units (%)
   Osmotic adjustment (none)

TEST PROTOCOL

1. Place B1 cuvette in READ well. Press the SET button.
2. Touch the computer <space bar> key.
3. READ zero time I_o light levels as prompted by the computer monitor. Then immediately...
4. ... make the following 0.9 mL transfers:
   A1 to B1 & C1
   A2 to B2 & C2
   A3 to B3 & C3
   A4 to B4 & C4
   A5 to B5 & C5
   D1 to E1 & F1
   D2 to E2 & F2
   D3 to E3 & F3
   D4 to E4 & F4
   D5 to E5 & F5
5. Touch the computer <space bar> key.

6. When timer sounds, READ light levels as prompted by the computer monitor.

DATA REPORT

1. Reduce the data.

SCREENING

One control, one sample dilution.

PURPOSE OF THE SPECIAL PROCEDURE

This procedure tests a sample at a single concentration. Therefore, a dose response curve is not generated. Since a dose response curve is not generated, the relative toxicity can not be determined. This procedure uses 90% sample concentration, though any concentration can be used. The Screening procedure is most effective when the highest possible sample (90%) concentration can be employed.

The following procedure screens 4 samples. The number of samples can be increased by adding samples to additional wells.

ANALYZER PREPARATION

1. Place cuvettes in incubator row A1 through A5 and REAGENT Well.
2. Add 1.0 mL Recon Solution to REAGENT Well.
3. Add 1.0 mL Diluent to A1.

SAMPLE PREPARATION

1. Add 1.0 mL sample 1 to A2.
2. Add 1.0 mL sample 2 to A3.
3. Add 1.0 mL sample 3 to A4.
4. Add 1.0 mL sample 4 to A5.

   ### Primary Dilution of Sample

   When all the sample dilution have a gamma greater than 10.0, a primary dilution of the sample is required.

   See Primary Dilution table on the next page.
5. If osmotic adjustment is required add 100 µL OAS to A2 through A5, mix.

   Osmotic adjustment is required when the salt content is below 2%. The amount of OAS used above is for when the sample salt content is 0.0%.

Primary Dilution Table

Primary Dilution	Sample / Diluent	Initial Concentration
none		90.0
1:10	sample / Diluent 250 µL / 2.25 mL	9.0
1:100	(250 µL / 2.25 mL) + (250 µL / 2.25 mL)	0.9
1:1000	(250 µL / 2.25 mL) + (250 µL / 2.25 mL) + (250 µL / 2.25 mL)	0.09

REAGENT PREPARATION

1. Reconstitute a vial of Microtox Acute Toxicity Test Reagent.
2. Mix reagent 10 times with pipettor set to 0.5 mL.

COMPUTER PREPARATION

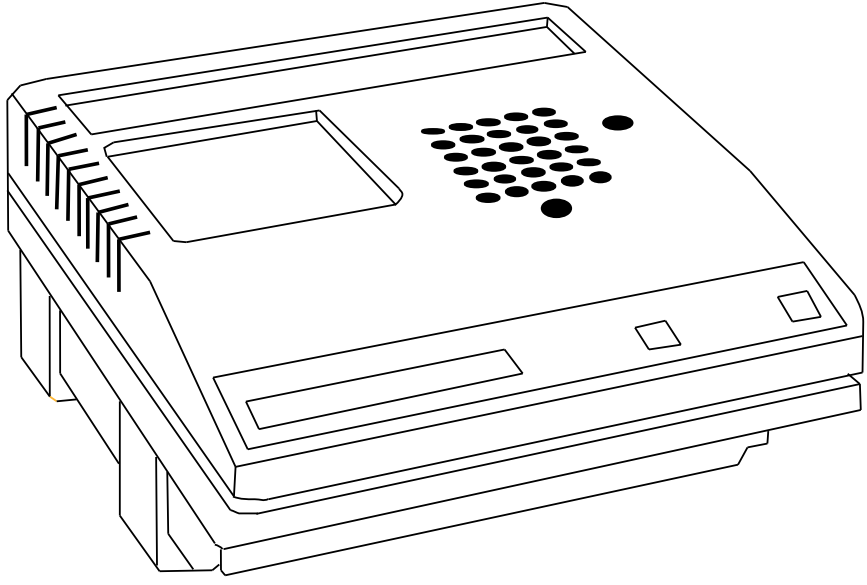
1. Call up the Screen Test program.
2. Select: Start Testing
3. Set Test Parameters:
   - Number of controls (1)
   - Number of samples (4)
   - Enter sample descriptions
   - Initial Concentration
     - (see Primary Dilution Table)
   - Units (%)
   - Osmotic adjustment (X)
   - Test Time (5, 15, X)

TEST PROTOCOL

1. Touch the computer <space bar> key.
2. Transfer 10 µL reagent to each cuvette: A1 through A5.
   Use a new pipettor tip for each sample.
   Use the 10-100 µL pipettor or a repeat pipettor.
3. Touch the computer <space bar> key.
4. Mix each cuvette by shaking: A1 through A5
5. When timer sounds, Place A1 cuvette in READ well. Press the SET button.
6. READ light levels as prompted by the computer monitor.
   There are NO zero time light readings for the Screen Protocol.

DATA REPORT

1. Reduce the data.



COLOR CORRECTION

When the sample has color, perform one of the Acute Test Protocols, and determine the sample's ECXX value. Check the sample concentration at the ECXX value for visible color. If this sample concentration contains distinct color perform the Color Correction Protocol.

ANALYZER PREPARATION

1. Place cuvettes in incubator wells A1, A3, A5.
2. Place an empty Color Correction Cuvette in the READ WELL.
   This is a special, cuvette.
3. Using a marker, place a mark on the lip of the Color Correction Cuvette. Keep this mark facing the same direction in the READ WELL when obtaining Color Correction light readings.
4. Add 1.0 mL diluent to A1, A3 and outer chamber of the Color Correction Cuvette in READ WELL.

SAMPLE PREPARATION

Do not use sample that contains Test Reagent.

1. If sample is turbid, it should have been centrifuged before the Basic Test Protocol was performed.
2. Make sample dilution close to the EC50 value (or the ECXX value of interest, e.g. EC20).
   Example, EC50 = 7.5%, make a 5% or 10% sample.
3. Add 1.0 mL of diluted sample to cuvette A5.
4. Wait 5 minutes.

REAGENT PREPARATION

Use the residual reagent from the Basic Test Protocol. For Color Correction the residual reagent can be used for up to eight hours after reconstitution.

1. Transfer 50 µL reagent to A3, and mix.
2. Using cuvette aspirator (glass Pasteur pipette, 9 inch) transfer diluted reagent (A3) to center chamber of the Color Correction Cuvette (READ WELL) until reagent is at the diluent level in outer chamber.
3. Wait 15 minutes.

COMPUTER PREPARATION

1. Call up the data file to be color corrected. Go to the Calculations Options, call up Color Correction.
2. Enter sample concentration used for Color Correction, when prompted by the computer.

TEST PROTOCOL

1. When the first diluent control has stabilized for 5 min., press the SET button.
2. Touch the computer <space bar> key.
3. Read the light level. Immediately...
4. ...remove and discard all of the Diluent in the outer chamber of the Color Correction Cuvette. Immediately...
5. ...transfer all of the sample (A5) to outside chamber of the Color Correction cuvette. Immediately...
   Use a clean cuvette aspirator.
6. ...touch the computer <space bar> key.
7. READ light level when prompted by the computer screen. Immediately.....
8. ...remove and discard all of the sample in the outer chamber of the Color Correction Cuvette. Immediately...
9. ...transfer all of the Diluent (A1) to outside chamber of the Color Correction cuvette. Immediately...
   Use a clean cuvette aspirator.
10. ...touch the computer <space bar> key.
11. READ light level when prompted by the computer screen.
12. Enter sample concentration.

DATA REPORT

The data file will now contain the Color Corrected information and a new Data Report will contain the results after Color Correction.