T H A M N O T O X K I T F

FRESHWATER TOXICITY SCREENING TEST

BENCH PROTOCOL

Principle :

The ***Thamnotoxkit*** contains all the materials to perform standardized, simple and low-cost bioassays for toxicity screening in freshwater. Using Instar II-III larvae of the fairy shrimp *Thamnocephalus platyurus* hatched from cysts, an acute toxicity test is executed in 24 hours in multiwall plates. Each Thamnotoxkit F provides for 6 complete tests (range-finding or definitive 24h-LC_{50}), or 5 bioassays and 1 quality control test with a reference toxicant.

1. Preparation of Standard Freshwater :

Fill a 1 liter volumetric flask with approximately 800 ml deionized water and add the contents of the five* vials of concentrated salt solutions, in the sequence 1 to 4 as indicated on the flasks. Add deionized water up to the 1000 ml mark and shake to homogenize the medium.
** Note that there are 2 vials with $CaSO_4.2H_2O$, both of which must be used!*

2. Storage of the Standard Freshwater :

The 1 liter solution of Standard Freshwater suffices for the 6 bioassays of each Toxkit. If all 6 tests are not carried out within a few days after preparation of the medium, store the Standard Freshwater in the refrigerator in darkness. Take care to bring the cooled medium (gradually) back to room temperature and aerate it prior to use.

3. Hatching of the cysts :

3.1. Preparation of the hatching medium

Hatching is performed in diluted Standard Freshwater (dilution 1:8 with deionized water). Transfer 2.5 ml Standard Freshwater into a vial and add 17.5 ml deionized water.

3.2. Hatching of the cysts

Hatching of the *Thamnocephalus* cyst should be initiated **24 hours** before the start of the toxicity test.

Cyst pre-hydration

Open a tube with cysts and fill it with diluted hatching medium (approx. 1 ml). Close the tube and shake it at regular intervals for approx. 30 minutes.

3.3. Transfer of pre-hydrated cysts into the hatching petri dish

Put 10 ml hatching medium into a small petri dish and empty the contents of the vial with pre-hydrated cysts into this petri dish; make sure most of the cysts are transferred by rinsing the tube with hatching medium. Swirl the petri dish gently to distribute the cysts evenly. Cover the hatching petri dish and incubate **at 25 °C for 20-22 hours**, under continuous illumination (light source of min. 3000-4000 lux).

4. Preparation of the Toxicant Dilution Series :

Prepare a dilution series of the test compound or effluent according to standard methods (e.g. USEPA, 1985).

5. Filling of the Test Plate :

The bioassays are conducted in disposable multiwell test plates with 24 (6 x 4) test wells. The wells are labelled as columns 1 to 6 across, and rows A to D down (see figure).
The distribution of the test solutions should always be carried out starting from the control (column 1, left) towards the highest concentration (column 6, right). To fill the control column, add 1 ml Standard Freshwater to the four wells of column 1. Repeat this procedure for the other columns with the respective toxicant concentrations, progressing from low to high concentrations in columns 2 to 6.

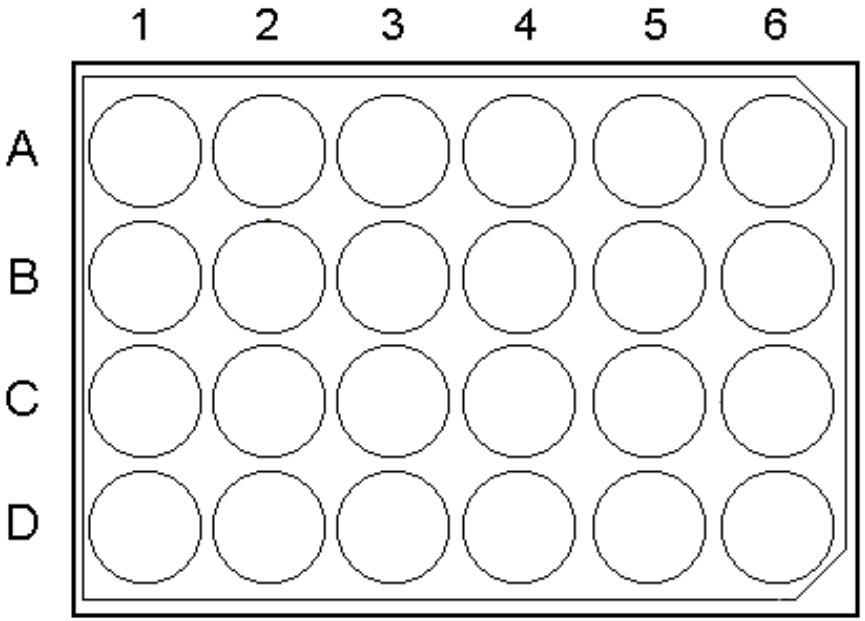

Figure: Multiwell test plate composed of 6 x 4 wells; the 6 wells of row D serve as rinsing wells.

6. Transfer of the larvae to the test wells :

Using a dissection microscope at magnification 10-12x, transfer approximately 50 instar II-III larvae with a micropipette from the transfer petri dish to each well in row D (rinsing wells*) of the multiwell plate. Subsequently transfer 10 larvae from the rinsing well of column 1 to the three wells of this column. Take care, during this operation, to minimize the transfer of medium along with the larvae. Repeat this operation for columns 2 to 6.

* *The intermediate passage of the fairy shrimp larvae from the petri dish to the definitive test wells via rinsing wells "washes" the larvae in the appropriate test solution before they enter the actual test well, thus minimizing dilution of the test solution during transfer.*

The test design of the THAMNOTOXKIT **is based on one control and five toxicant concentrations, each with 3 replicates of 10 animals. Each bioassay shall be performed in a new multiwell with a new micropipette.**

7. Incubation of the Test Plate and Scoring of the Results :

Put a strip of Parafilm on the test plate, cover it and incubate at 25°C in darkness. **After 24 hours**, count the dead* larvae in each test well and fill out the results sheet.
Calculate the % mortality** and, for the definitive tests, the 24h-LC_{50} using a standard statistical method (*A data treatment program to calculate the 24h EC_{50} for the Thamnotoxkit microbiotest is available on demand from MicroBioTests Inc.)*.

* *Larvae are considered dead if they do not exhibit any internal or external movement in 10 seconds of observation.*
** *For the THAMNOTOXKIT test to be valid, control mortality should not exceed 10%*

8. Reference test

In order to check the correct execution of the test procedure and the good physiological condition of the test animals, it is advisable to perform a reference test from time to time.

Such a reference test can e.g. be performed with the reference chemical potassium dichromate ($K_2Cr_2O_7$).

Preparation of the stock solution and the dilution series of the reference chemical :
Weigh 100 mg potassium dichromate on an analytical balance, transfer it to a 100 ml volumetric flask and fill to the mark with deionized water to make a 1000 ppm stock solution.
Make a dilution series 0.32 - 0.18 - 0.10 - 0.056 - 0.032 mg/l for the reference test and proceed further as prescribed for the toxicity test.

The value of the 24h LC_{50} should be within the 95% confidence limits stipulated in the specification sheet.