P R O T O X K I T F

FRESHWATER TOXICITY TEST
WITH A CILIATE PROTOZOAN

BENCH PROTOCOL

Principle

The **Protoxkit F** contains all the materials, including the test species *Tetrahymena thermophila*, to perform six complete 24h growth inhibition assays.
The test is based on optical density (OD) measurement of the food substrate provided to the ciliates, in 1 cm disposable spectrophotometric cells. Ciliate growth inhibition is reflected by higher turbidity in the test cells containing the toxicant after 24h exposure, in comparison to the controls.
The stock culture vial containing the ciliates in a stationary growth phase can be stored at room temperature for several months.
The tests <u>are started</u> from the stock culture vial, without any intermediate time loss for hatching or reactivation of the test biota.

Preparation of the toxicant dilution series

Prepare the dilution series of the effluent or the chemical according to the detailed instructions given in the Standard Operational Procedure Manual.

Preparation of the ciliate inoculum

After gently shaking the stock culture vial, take 500 µl with a sterile syringe and transfer the ciliate suspension into a 1.5 ml stock-culture cell.

Add 1 ml distilled water, cap the cell and shake gently.
Measure the OD at 440 nm in a spectrophotometer.
Read the ciliate density (OD) and determine the dilution factor (F) and the dilution volume of distilled water (V), with the aid of the formulas :

$$F = ODvalue/0.040$$
$$V = 0.5 \times (F-1)$$

Transfer 500 µl of ciliate suspension in the stock-culture cell into a 5 ml ciliate inoculum tube and add V ml distilled water.
Stopper the tube and mix gently.

Preparation of the food suspension

Slowly defrost one vial of reconstitution medium and one vial of food substrate, and transfer the full contents of the former tube into the latter one.
Close the food substrate tube and mix thoroughly.

Inoculation of the test cells

Take 12 test cells and label them in pairs (C0 to C5).
Add 2 ml distilled water to the two control cells C0.
Transfer 2 ml from each toxicant dilution into each of the two parallel cells, from C1 to C5.
Subsequently add 40 µl food suspension to each of the 12 test cells.
Take the ciliate inoculum tube and mix the contents gently. Transfer 40 µl of ciliate inoculum into each of the 12 test cells.
Close the cells with their lids, shake gently, and put the cells in one of the cardboard holding trays.

N.B. Following this procedure, the ciliate density should be (close to) 100 organisms/ml in each test cell.

OD measurements and incubation of the test cells

Zero-calibrate the spectrophotometer at 440 nm with a test cell containing 2 ml distilled water.

Subsequently measure the OD of each test cell at the same wavelenght, after gentle shaking of the cell immediately prior to the measurement (T0 scoring).
Record the T0 data on the Result Sheet.
Put the holding tray with the cells in an incubator at 30°C for 24h.

IMPORTANT REMARK

In some cases (which are batch dependent) it is advised to extend the incubation period to 28 hours in order to obtain enough OD decrease in the controls (see also section Validity of the test - Important Remark in the Standard Operational Procedure Manual).

N.B. Since the growth of the ciliates is very temperature dependent, the outcome and the repeatability of the bioassays will also be highly dependent of the temperature precision and stability of the incubator !!

After 24h incubation, recalibrate the measuring equipment at 440 nm with a test cell containing 2 ml distilled water.
Subsequently re-measure the OD of each test cell after gentle shaking (T24h scoring).
Record the T24 data on the Result Sheet.

Testing of coloured samples

The Standard Operational Procedure describes a procedure to deal with OD interferences from coloured samples.

Data treatment

Calculate the mean for the two parallels for each toxicant dilution and the control.
Calculate the difference between the mean OD at T0 and T24 for each toxicant dilution (ΔOD_{C1-C5}) and for the control (ΔOD_{C0}).

Calculate the % inhibition for each toxicant dilution with the following equation :

$$\% \ inhibition_{(C1-C5)} = \left(1 - \frac{\Delta OD_{(C1 - C5)}}{\Delta OD_{C0}}\right) \times 100$$

Calculate the 24h EC_{50} using the specific Protoxkit programme that can be obtained - free of charge - from all Toxkit distributors.

Validity of the test

Besides all other prerequisites for toxicity bioassays, for the test to be acceptable the OD in the controls after 24h incubation must show a decrease of the T0 value by at least 60% (the OD-24h shall be 40% or less than the OD-T0).

Reference test

It is recommended that every 5-10 assays, a quality control test be carried out in order to check proper adherence to the test protocol, as well as the test sensitivity.

A reference test can be carried out with potassium dichromate ($K_2Cr_2O_7$). The 24h EC_{50} of the quality control test should be within the 95% confidence limits stipulated on the specification sheet.

The dilution series to be prepared for the reference test with potassium dichromate is : 5.6-10-18-32-56 mg/l.