1. INTRODUCTION

EBPI Toxi-ChromoTest™ is a rapid bacterial-based colorimetric bioassay kit for the determination of toxicity. It is sensitive to a wide spectrum of toxic substances such as heavy metals, and organic and inorganic pollutants, and may be used to detect the presence of toxicants in water and soil/sediment extracts. The Toxi-ChromoPad™ assay may be used for direct testing of soils or sediments. The assay is based on the ability of substances (toxicants) to inhibit the *de novo* synthesis of an inducible enzyme - β-galactosidase in a highly permeable mutant of *E. coli*.

Exposing the bacteria to stressing conditions and then lyophilizing them enhance the sensitivity of the test. Upon being rehydrated in a cocktail containing a specific inducer of β-galactosidase, and essential factors required for the recovery of the bacteria from their stressed condition they are ready for testing. The activity of the induced enzyme released by actively growing recovered cells is detected by the hydrolysis of a chromogenic substrate. Toxic materials interfere with the recovery process and thus with the synthesis of the enzyme and the colour reaction.

WARRANTY

EBPI warrants that, at the time of shipment, the products sold by it are free of defects in material and workmanship, and confirm to company's specifications. Since actual experimental conditions prevailing at user's laboratory are beyond the control of EBPI or its representatives, EBPI makes no other warranty, express or implied, with respect to the products. Notification of any breach of warranty must be made within 120 days of delivery. The sole and exclusive remedy of the customer of any liability of EBPI of any kind, including liability based upon warranty (express or implied, whether contained herein or elsewhere) is limited to the replacement of the products or the refund of the invoice price of the products.

Complete results of the control reactions should accompany all replacement claims.

2. HANDLING THE TOXI-CHROMOTEST™ KIT

Handle the Toxi-ChromoTest™ Kit and your tested samples as you would any potentially hazardous material.

Although the bacterial strain is not a known pathogen, it is advisable and good laboratory practice to sterilize the remains of the Toxi-ChromoTest™ Kit before disposal (use the included biohazard bag).

Due to the short incubation time and chemical configuration of the kit, sterile handling is not imperative.

The Toxi-ChromoTest™ Kit should be stored under refrigeration (2 to 8°C), and should be protected from high temperatures and temperature changes. If being stored for long (months to years) periods of time it is advisable to keep the bacteria frozen

3. LIST OF TOXI-CHROMOTEST™ COMPONENTS

Each Toxi-ChromoTest™ package contains the components of three kits, including bacteria, media, micro-titration plates and some accessories. The contents of the bottles in each kit are:

A) **Reaction mixture** (3) - a cocktail containing an inducer for the enzyme β-galactosidase, and co-factors required for the recovery of the bacteria from their stressed condition.

10 ml per vial

B) **The Toxi-ChromoTest™ lyophilized bacteria** (3) - a highly permeable rough mutant of *E. coli*.

0.1 g per vial

C) **Rehydration solution** (3) - a solution to hydrate the bacteria.

10 ml per vial

D) **Standard toxic substance** (1) - 4 µg/ml mercury chloride in water.

2 ml per vial

F) **Chromogenic substrate** (3):
- Blue chromogen cocktail, ready for use

10 ml per vial

G) **Diluent** (3): for standard toxicant and samples.

10 ml per vial

* There is **no** bottle E included in this kit

Hardware – 3 96 well microtiter plates
3 biohazard bags

4. USING ONE TOXI-CHROMOTEST™ KIT

Each Toxi-ChromoTest™ package contains enough materials for three separate analytical sessions. The materials for each session can be used separately before expiry date if stored under refrigeration. During each analytical session you will use one bottle of each A, B, C, E and F. Discard remaining material from these bottles after the test session is complete. Bottle D contains enough standard toxicant to be used during all the test sessions.

Each kit also includes 3 - 96 well plates (one for each test session) and one biohazard bag.

5. TOXI-CHROMOTEST™ PROCEDURE

This chapter describes the steps of the Toxi-ChromoTest™ procedure. Figures 1 presents a suggested layout of the test microtiter plate for a typical test session. The layout can be changed to meet the individual needs of the test being carried out. The layout shown in figure 1 enables the testing of 5 materials at 14 different concentrations. Be sure to include a blank (column 1 fig 1) and a standard positive toxicant control (column 2 fig 1) with each test.

The first column of the microtiter plate is used for a reagent blank, and the second column for standard toxicant ($HgCl_2$). The remainder of the columns are used with the samples to be tested and their dilutions. Row H, last well in each column, is used as reference. The wells in this row will contain no toxic material.

5.1 **Dispense** 100 µl of the diluent from Bottle G to all wells of the micro-well modules in which tests are to be conducted except wells of Row A from A2, A3, A5, A7, A9, and A11 where the full strength material to be tested will be added (see Figure 1).

5.2 **Dispense** 200 µl of the standard toxicant (Bottle D) to the first well of Column 2, Well A2. Mix well and transfer 100 micro-litres of the solution to Well B2. Mix well and transfer 100 µl to Well C2. Repeat this procedure down the column as far as well G. From the last Well G2, discard 100 µl of solution. You have now completed the two fold dilutions of the positive known toxic material, mercury chloride starting at a concentration of 4ug/ml which should induce complete toxicity and ending with a 0.06 µg/ml concentration in row G. The last row in the column (H) is used as a zero concentration blank control where no toxicity should be present.

5.3 **Dispense** 200 µl of the first samples to be screened in the first wells of the 3rd, 5th, 7th, 9th and 11th wells (if following the suggested layout in fig 1) and dilute as you have done with the standard toxic material down column 3 and continue with the serial dilutions down column 4 until a series of 13 two fold dilutions have been completed. This is repeated in columns 5 and 6 for sample B. 7 and 8 for sample C, 9and 10 for sample D and 11 and 12 for sample E.

5.4 **Dispense** 100 µl of the reaction mixture from Bottle A to wells of Column 1 as shown in fig 1. These wells are used for a reagent blank.

5.5 **Rehydrate the bacteria** in Bottle B with the solution in Bottle C. Keep both bottles cold before mixing. Remove the seals and stoppers from the two bottles and immediately transfer the medium from Bottle C to Bottle B. Mix well by gently shaking. Leave at room temperature for 15 minutes, and then transfer 1 ml of the bacterial suspension (now in Bottle B) into the reaction mixture in Bottle A. Time: _____

5.6 **Dispense** 100 µl of the bacterial suspension from reaction mixture (Bottle A) which now includes the bacteria to each well of Columns 2 to 12 as shown in figure 1 (but not in column 1.)

5.7 **Incubate** the micro plate at 37°C for 90 minutes. During this time the bacteria attempt to induce the enzyme systems necessary to produce and excrete beta galactosidase. Bottle F (Blue Chromogen) should also be incubated at this time. (Note: during this incubation period a biological reaction is occurring. 37˚C is considered the optimal temperature for growth and should be incubated within +/- 1 degree). Time: _____

5.8 After removal from the incubator **Dispense** 100 µl (0.1 ml) of the chromogenic substrate in Bottle F to all the wells. The blue substrate in Bottle F is in a ready for use solution. It is advised to warm chromogenic cocktail to 37°C before dispensing. After dispensing, mix well by tapping the plate.

5.9 **Incubate** the micro plate at 37°C for 30 minutes for the blue substrate to develop. If colour is not well developed in the control wells (row H), incubate for a longer period. (Note: the colour development reaction can be accelerated if desired by incubation at a higher temperature up to 65^0C as a chemical reaction is occurring at this time). Time: _____

5.10 **Reading the colour reaction**: For blue colour, there is no need of stopping the reaction since hydrolysis of blue substrate is very slow. The colour can be read either visually against a colour scale (for reference the gradation developed in the reference toxicant can be used) or in a plate reader. Read results at 615 nm if a plate reader is being used.

As Toxi-ChromoTest™ is a biological test, to get more reliable results, it is recommended to replicate samples. One may also increase or decrease the number of dilutions according to previous information or to specific needs.

If a possible toxic effect of the tested material Solvent is suspected, it should be tested by running the solvent as a tested material in a special row or with the control as a reference.

The experimental procedure of the Toxi-ChromoTest™ is now completed. The next section will explain analysis of the results.

Note: The Toxi-ChromoTest™ procedure is suitable for samples completely soluble in water. If the sample tested is best dissolved in organic solution, you can make all your dilutions in water containing 5% DMSO.

6. ANALYSIS OF THE RESULTS

Analysis of toxic activity of a tested material can be carried out visually or quantitatively by using photometric instrumentation, such as an ELISA plate reader. Positive toxic result is a blue colour density below the colour density of the controls without the tested materials (wells of Row H).

6.1 Check the colour density appearing in the wells that contain the dilutions of the positive standard toxic solution of mercury chloride (Column 2 in figure 1). The colours should appear in different densities according to the concentration of the chemical. The control Well H2 (without toxicant) should give the highest colour density, which decreases with the increase of the toxicant concentration (up to Well A2 where no colour should have developed). There should be a sharp colour gradient in the reference toxicant at near the 0.25 and 0.5 mg/L $HgCl_2$ concentrations. (See figure 2 of data for the reference toxicant measured using a plate reader.)

If no colour appears in Column 2 in the lower rows (containing the lowest concentrations), testing the toxicant standard, it would mean that the Toxi-ChromoTest™ bacteria were not functioning properly and results obtained are invalid.

6.2 For visual analysis, check the colour density appearing in the wells of your tested materials. High toxicant concentrations may not have any colour due to toxicity. As the material is diluted out, toxicity is reduced and colour density will increase up to the maximal level of the controls. The colour densities can be assigned by ± signs (i.e., =, -, ±, +, ++, +++, etc.). Compare with values obtained with the standard toxicant as a reference for colour development.

For instrumental analysis of the results, read the absorption at about 615 nm for the blue chromogen. Blank the photometric reader automatically or manually on the blanking wells (wells of Column 1). Measure the optical density of the other micro-wells. Draw the obtained OD values versus concentration plot (see figure 2).

6.3 Toxicity of a tested material causes colour density below the OD values of the controls without the tested material. Toxicity can be calculated by the equation:

$$\% \text{ Toxicity} = [1 - \text{OD}^* \text{ treated cells}/\text{OD control cells}] \times 100$$

Minimal Inhibitory Concentration (MIC) is defined as the concentration of a chemical causing 20% toxicity.

* Treated cells are those incubated with a toxicant/tested material. Control wells are without tested material (wells of Row H).

TOXI-CHROMOTEST™ KIT SHORT PROTOCOL

Leave Bottles B and C cool at 4°C until ready to use

Warm Bottle F to 37°C or room temperature if 37^0C is not available.

SAMPLE PREPARATION

1. Dispense 200 µl of standard toxicant and 200 µl of your 100% concentration samples to the appropriate wells of Row A.

2. Dispense 100 µl of diluent from Bottle G to all other wells.

3. Now prepare the required serial two-fold dilutions of each sample (and the standard toxicant) by transferring 100 µl from Well A of each column into the next well (B) and continuing so by serial transferring 100 µl until Well G and if more dilutions required starting in row A of the next column and down the column again.

BLANK

To the blank column (1), dispense 100 µl of reaction mixture (A) to each well in the column.

REHYDRATION OF BACTERIA

Transfer rehydration solution in bottle C to bacteria in Bottle B. Mix and leave at room temperature for 15 minutes. Time: _____

TOXI-TEST

1. Transfer 1 ml of rehydrated bacteria from Bottle B to Bottle A.

2. Dispense 100 µl of reaction mixture including bacteria (from Bottle A) to all wells except the reagent blank (row 1).

3. Incubate at 37°C for 90 minutes. Time:_____

COLOUR DEVELOPMENT

1. Dispense 100 µl of chromogenic substrate (bottle F) to all wells.
2. Incubate at 37°C for 30 minutes or until a blue colour develops in the zero concentration wells (row H).

RESULTS

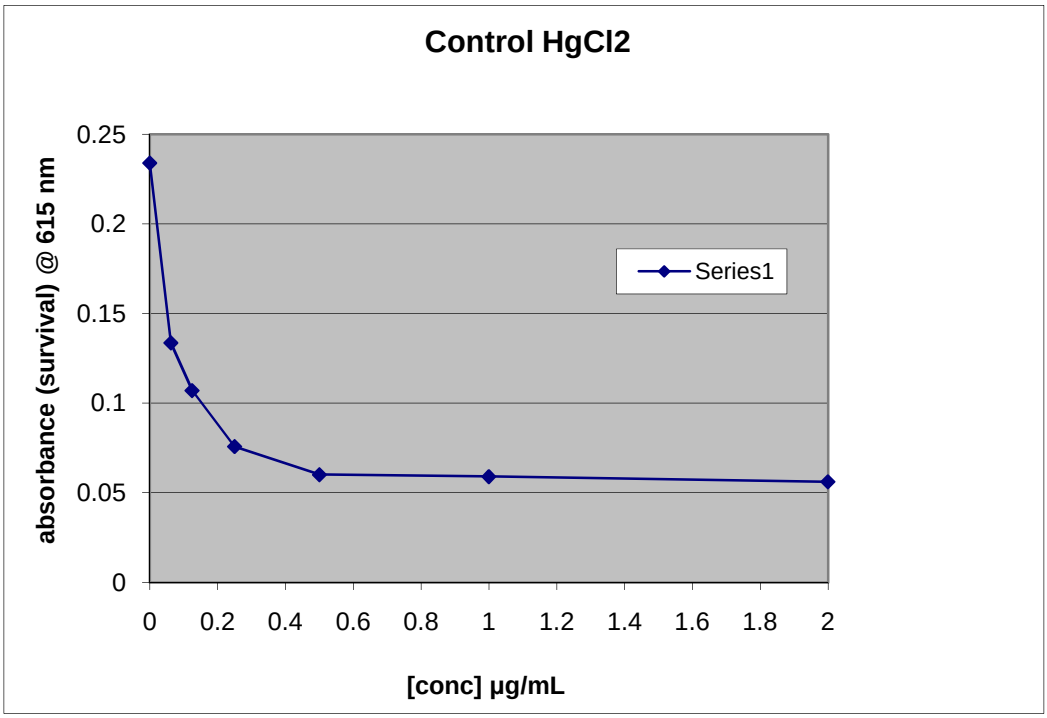
Read blue colour visually and score or
Measure optical density at 615 nm for blue colour.

FIGURE 1: SUGGESTED LAYOUT OF TOXI-CHROMOTEST™

Note if the approximate toxic concentration of the sample being tested is known the range of dilutions can be reduced such that the response if captured in the range of concentrations tested.

	COLUMN											
	Blank	$HgC1_2$	Sample A		Sample B		Sample C		Sample D		Sample E	
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	-	4 ug/mL	Undiluted	1:128	Same as columns 3 and 4 for sample B		Same as columns 3and 4 for sample C					
B	-	2	1:2	1:256								
C	-	1	1:4	1:512								
D	-	0.5	1:8	1: 1024								
E	-	0.25	1:16	1: 2048								
F	-	0.125	1:32	1: 4096								
G	-	0.06	1:64	1: 8192								
H	-	0	0		0		0					

Figure 2: Plot of absorbance at 615 nm for the reference toxicant. Full colour development produced an absorbance of 0.23 and no colour development (acutely toxic) an absorbance of 0.05



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