MOLTOX®

Molecular Toxicology, Inc.

UMU Genotoxicity Test Kit Instruction Manual

31-400

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BEFORE YOU BEGIN

MOLTOX® UMU products are intended for research purposes only; they are not for use in humans or animals.

The Salmonella strain provided in the MOLTOX® UMU Water, Waste Water, & Chemical Test Kit is attenuated but is a potential etiologic agent and as such should be handled under Biosafety Level 2 conditions. Positive control chemicals are provided in very small quantities; nevertheless, they are mutagens/carcinogens and must be handled in strict accordance with your institutions chemical hygiene plan. Aseptic technique and standard microbiological and chemical safety precautions should be employed. Do not eat, drink, smoke, apply cosmetics, etc., while working in the laboratory area. Wear appropriate personal protection equipment (safety glasses, gloves, lab coat) when handling kit reagents and bacterial strains. Never pipette by mouth.

UMU Mutagenicity Assay Kit Components

31-400 UMU Water, Waste Water, & Chemical Test Kit		
26-714.1 *	TGA Culture Media, 100 mL *	2 each
26-715 *	10X TGA Culture Media, 10 mL *	1 each
26-716	B-Buffer, 35 mL	1 each
26-718	Stop Reagent, 30 mL	1 each
22-148L	ONPG, 1.1 mL (4.95 mg)	2 each
22-149	2-Mercaptoethanol, 100 µL	2 each
73-1535pSK	*S. typhimurium* TA1535/pSK1002, 1 mL	2 each
60-163	4-NQO, 12.5 µg/vial	1 each
60-164	2-AA, 50 µg/vial	1 each
22-147	Ampicillin, 55 mg/vial	1 each
11-401.3L	30 % Mutazyme, reconstitute to 3.25 mL	1 each

* NOTE - Media is supplied in "TG" form. Prior to use, rehydrate 22-147 using 1.1 mL cold, sterile purified water. Mix well. Complete the formulation to "TGA" by adding ampicillin to a final concentration of 50 µg/mL. [i.e. 100 µL rehydrated ampicillin (22-147) to 100 mL TG media]. This is applicable to TGA media (26-714) and 10X TGA media (26-715). Once ampicillin is added, store at 2-8°C for up to 2 months.

Additional items you will need to complete the assay:

Sterile Pipette Tips (20-200 and 200-1000 µL)

Micropipettes : Single channel (2-20 µL, 20-200 µL and 100-1000 µL) and
8-channel (30-300 µL – highly recommended)

Sterile Pipettes (1, 5, 10 & 25 mL)

37°C bacteriological incubator

Shaker, variable speed

Spectrophotometer/colorimeter (600 nm)

Multi-well plate reader (600 nm)

Sterile 15 and 50 mL centrifuge tubes

Sterile 125 mL culture flasks with closures

Sterile 96 well microplates

Solvents including sterile purified water and DMSO

Sterile 25 mL reagent reservoirs

INTRODUCTION

Salmonella typhimurium TA1535 (hisG46, *rfa*, uvrB) has been modified to contain the plasmid pSK1002. This plasmid contains the gene umuC fused to a lacZ reporter gene. If genetic lesions are formed when exposed to potentially genotoxic compounds, the umuC gene is induced as part of the bacterial SOS response. Due to the lacZ-umuC fusion and the accompanying lacZ-encoded β-galactosidase activity, genotoxic induction can be detected by the colorimetric change of ONPG substrate (colorless) to 2-nitrophenol (yellow).

This protocol was adapted from ISO 13829 "Water Quality- Determination of the genotoxicity of water and waste water using the UMU test". The MOLTOX® UMU Kit provides components to perform both aqueous and chemical tests based on the user's needs. Instructions diverge at the "Exposure to Test Samples" section. All other sections of this manual are appropriate for either type of test material.
If you desire assistance in any phase of the assay, please contact our Customer Service department at (828) 264-9099. We will be happy to work with you to help solve any problems that might arise.

Sample Preparation and Preservation

Test water and waste water samples as soon as possible after sampling. Samples may be stored at 4°C for up to 48 hours. If longer storage is required, store below -18°C in accordance with ISO 5667-16.

If sample is known/suspected to be highly acidic or alkaline, adjust the pH of the sample to 7.0 ± 0.2 before use.

TEST PERFORMANCE

1. Preparation of the Overnight Culture (See *NOTE under "Kit Components" section)

a. Aliquot 10 mL of TGA media into a sterile 50 mL conical tube. Aliquot 3 mL of TGA media into another sterile conical tube to serve as an overnight sterility control.

b. Thaw the TA1535/pSK1002 culture (73-1535pSK) carefully. Add 100 µL of TA1535/ pSK1002 culture to the tube containing the TGA media.

c. Cap both tubes loosely and incubate overnight (14 -16 hours) at 37 ± 1°C with shaking so as to assure adequate aeration without excessive foaming (often ~ 250 rpm).

2. Measure the Overnight Culture Value

a. Prepare 3 cuvettes by adding 900 µL of TGA media to each (for blank, sterility control, & to measure the density of the overnight culture).

b. Tighten the cap of the overnight culture and invert or swirl gently several times to ensure homogeneity.

c. Add 100 µL of the overnight culture to one cuvette containing 900 µL TGA media and invert 3X to mix well.

d. Blank the spectrophotometer at 600 nm using the cuvette with TGA media only.

e. Measure the optical density of the overnight culture and the sterility control at 600 nm. Record.

f. Determine the actual OD of the overnight culture by multiplying the OD_{600} of the overnight culture reading by 10.

g. The culture is acceptable for use if the value of the overnight culture at OD_{600} is 2.0 or greater and the sterility control at $OD_{600} \leq 0.005$.

NOTE: If the overnight culture has an $OD_{600} \leq 2.0$, there has not been sufficient growth. The culture tubes may be incubated for an additional period, but significant growth should be measurable in 2 hours. Ensure caps are loosened for adequate aeration.

If the OD_{600} of the sterility control is > 0.005, contamination may be present and the culture should not be used. Repeat STEPS 1 & 2 above using best possible aseptic technique.

3. Growth of Culture to Exponential Phase

Re-growth of the test organism is necessary to achieve the exponential growth phase required for the assay.

a. Place 13.5 mL of TGA media (26-714.1) into a sterile tube and warm to approximately 37°C.

b. In this tube, prepare a 1:10 dilution of acceptable overnight culture by adding 1.5 mL of overnight culture directly to the tube.

c. Cap loosely and incubate at 37°C with shaking for an additional 1.5 - 2 hours.

d. Measure the OD_{600} of the regrown culture as above using TGA media (26-714.1) as blank.

e. The OD_{600} of the regrown culture should be approximately 70 - 80% of the original OD_{600} for the overnight culture. Use TGA media to dilute if needed.

Note: During the culture reincubation step (STEP 3c) and referring to the next section, dilute the test samples and prepare the test microplates. After the 2nd incubation period, the test must be started within 10 minutes.

PROCEDURE DIVERGES AT THIS SECTION. FOLLOW APPROPRIATE SECTION FOR AQUEOUS (4.1) OR CONCENTRATED & SOLID (4.2) SAMPLE TESTING.

4.1 Aqueous Sample Testing

Perform the test in triplicate for each sample/dilution using the pipetting scheme shown in Table 1. (2) 96 well microplates should be prepared, one with S9, one without S9, as directed below.

Table 1- Pipetting Scheme, Plate A, Aqueous Sample Testing

		1:1.5			1:3			1:6			1:12		
		1	2	3	4	5	6	7	8	9	10	11	12
	A	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
	B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
	C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
	D	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	E	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5
	F	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6
	G	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	H	PC	PC	PC	SC	SC	SC	BL	BL	BL	BL	BL	BL
	S1= Sample 1, dilutions 1:1.5, 1:3, 1:6, 1:12 (3 of each); S2- sample 2, etc. NC = Negative control PC = Positive control SC = Solvent control BL = Blank												

Preparation of Plate A, Without S9:

a. Add 1 mL of DMSO to 60-163 vial. Cap and invert gently to mix. Resultant concentration is 12.5 µg/mL.

b. Aliquot 180 µL of sterile purified water to all the wells, except rows A to F, 1 - 3, and row H, 1 - 6 (Positive Controls/Solvent Controls).

c. For the Positive and Solvent Control wells, add 170 µL of sterile purified water to row H, 1 - 6 (Rows 1 - 3 are Positive Controls, Rows 4 - 6 are Solvent Controls).

d. Add 10 µL of the appropriate reconstituted Positive Control (4-NQO, 60-163) to columns 1 - 3 of row H. Mix well.

e. Add 10 µL of Solvent Control (DMSO) to columns 4 - 6 of row H. Mix well.

f. Add 360 µL of your test sample to columns 1 - 3 of rows A - F.

g. Perform 1:2 dilutions of samples by pipetting 180 µL from rows A - F, column 1, into rows A - F, column 4, 180 µL from the rows in column 2 to the rows in column 5, and 180 µL from rows in column 3 to the rows in column 6. Repeat for the 1:6 and 1:12 dilutions (column 4 into column 7, etc.), mixing each dilution thoroughly before removing next aliquot. Discard 180 µL from the last 3 columns (10 - 12).

h. Aliquot 20 µL of 10X TGA (26-715) media to all the wells on the microplate (Rows A - H, Columns 1 - 12).

i. Add an additional 70 µL of 10X TGA media to row H, columns 7 - 12 (Blank).

j. Add 70 µL of regrown inoculum to all wells of rows A - F and mix.

k. Add 70 µL of regrown inoculum to row G, columns 1 - 12 (negative control) and row H, columns1 - 6 (positive and solvent controls) and mix.

Mix the whole microplate from right to left, triturating each well to mix thoroughly.

Cover the microplate with the lid and incubate with shaking at 37 ± 1°C for 2 hours, 120 - 150 rpm.

Preparation of Plate A, With S9:

Follow above instructions for microplate set-up (Steps 4.1.a to 4.1.i) EXCEPT in step (a), add 1 mL of DMSO to 60-164. Cap and mix gently; resultant concentration is 50 µg/mL. Also, in Step (d) use 10 µL 2-AA, 60-164, as positive control. Continue as follows:

l. Reconstitute 30% S9 Mix by adding 3.25 mL of, ice cold sterile purified water to 30% S9 vial and mix. Hold on ice.

m. Mix 470 µL TGA media and 76 µL 30% S9 Mix. Add 50 µL of this mixture to wells H7 - H12 (Blank).

n. Mix 470 µL of the 30% S9 Mix and 2.9 mL of the regrown culture. Add 70 µL of bacteria/ S9 mix to all wells in rows A - H (except H7 to H12).

Mix the whole microplate from right to left, triturating each well to mix thoroughly.

Cover the 96 well microplate with the lid and incubate with shaking at 37 ± 1°C for 2 hours, 120 - 150 rpm.

4.2 Concentrated & Solid Sample Testing

Perform the test in triplicate for each sample/dilution using the pipetting scheme shown in Table 1. (2) 96-well microplates should be prepared, one with S9, one without S9, as directed below.

Table 2 -Pipetting Scheme, Plate A, Chemical Sample Testing

		25X			50X			100X			200X		
		1	2	3	4	5	6	7	8	9	10	11	12
	A	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
	B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
	C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
	D	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	E	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5
	F	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6
	G	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	H	PC	PC	PC	SC	SC	SC	BL	BL	BL	BL	BL	BL
	S1= Sample 1, dilutions 25X, 50X, 100X, 200X (3 of each); S2- sample 2, etc. NC = Negative control PC = Positive control (- S9/+S9) SC = Solvent control BL = Blank												

Preparation of Plate A, Without S9:

a. Add 1 mL of DMSO to 60-163 vial. Cap and invert gently to mix. Resultant concentration is 12.5 µg/mL.

b. In a 96 well microplate, add 190 µL of TGA media to positive control (PC) and solvent control (SC) wells in row H, columns 1 - 6.

c. Add 380 µL of TGA media (26-714.1) to columns 1 - 3 of rows A - F.

d. To all other wells, add 200 µL of TGA media. Add an additional 50 µL of TGA media to wells H7 - H12 (Blank).

e. Add 10 µL of reconstituted Positive Control to the wells in columns 1 - 3 of row H (Use 4-NQO, 60-163). Mix well.

f. Add 10 µL of Solvent Control (DMSO) to the wells in columns 4 - 6 of row H. Mix well.

g. Add 20 µL of test sample to the wells in columns 1 - 3, rows A - F.

h. Perform 1:2 dilutions of samples by pipetting 200 µL from column 1 rows A - F, into rows A - F column 4, 200 µL from the wells of column 2 to the wells of column 5, and 200 µL from wells of column 3 to the wells of column 6. Repeat for the 100X and 200X dilutions (column 4 into column 7, etc.), mixing each dilution thoroughly before removing next aliquot. Discard 200 µL from the last 3 columns (10 - 12).

i. Add 50 µL of regrown inoculum to all the wells excluding the Blank wells (except H7 - H12).

Mix the whole microplate from right to left, triturating to mix well.

Cover the 96 well microplate with lid and incubate with shaking at 37 ± 1°C for 2 hours, 120 - 150 rpm.

Preparation of Plate A, With S9:

Follow above instructions for plate set-up (Steps 4.2.a to 4.2.h) EXCEPT in step (a), add 1 mL of DMSO to 60-164. Cap and mix gently; resultant concentrations is 50 µg/mL. Also, in Step (f) use 10 µL 2-AA, 60-164, as positive control. Continue as follows:

j. Reconstitute 30% S9 Mix by adding 3.25 mL of, ice cold sterile purified water to 30% S9 vial and mix. Hold on ice.

k. Mix 344 µL TGA media and 76 µL 30% S9 Mix. Add 50 µL of this mixture to wells H7 - H12.

l. Mix 470 µL S9 Mix and 2.1 mL of the regrown culture. Add 50 µL of bacteria/S9 mix to all wells in rows A - H (except H7 to H12).

Mix the whole microplate from right to left, triturating each well to mix thoroughly.

Cover the 96 well microplate with the lid and incubate with shaking at 37 ± 1°C for 2 hours, 120 - 150 rpm.

PROCEDURE CONVERGES AT THIS SECTION. FOLLOW THE STEPS BELOW FOR BOTH AQUEOUS AND CHEMICAL TESTING.

5. Incubation

While Plate A is incubating, prepare the 2nd plate (Plate B) as such:

a. Add 270 µL of TGA media to every well. Replace the lid and incubate at 37°C to bring media to 37 ± 1°C.

b. After the 2 hour incubation of Plate A is complete, working from right to left, pipette 30 µL from each well of Plate A to the corresponding well on Plate B (a ten-fold dilution).

c. Remove the lid of the Plate B and read the OD_{600} value of each well (removing bubbles if needed).

d. Incubate Plate B for 2 hours at 37± 1°C with shaking (120-150 rpm).

Note: During the incubation of Plate B, continue with STEP 5e to prepare Plate C and appropriate reagents.

e. Hydrate the ONPG with room temperature sterile purified water. Mix well. Store in the amber vial and/or the dark.

f. Prepare a B-buffer/ONPG solution by mixing 15 mL of B-buffer (26-716), 41 µL of 2-Mercaptoethanol (22-149), & 1 mL of reconstituted ONPG (22-148).

g. Add 150 µL of B-buffer/ONPG solution to each well of Plate C.

h. Pre-warm plate to 28°C (this temperature is per ISO 13829 guideline. A different temperature for incubation may be used at this step, however, results may not compare to that found in the ISO 13829 guideline).

i. Following the 2 hour incubation of Plate B, mix each well and measure the OD_{600} values of each well in Plate B.

6. umuC Induction Assessment

a. Mix and pipette 30 µL from each well of Plate B into the corresponding well of Plate C, working from right to left (from lower to higher concentration).

b. Without delay, incubate Plate C for 30 minutes at 28 ± 1°C, 120 - 150 rpm.

c. When incubation is complete, pipette 120 µL of Stop Reagent to each well of Plate C.

d. Mix each well thoroughly and remove bubbles.

e. Immediately measure the OD_{420} of each well.

All microplates should be decontaminated and disposed of properly.

CALCULATIONS AND DEFINITIONS

Calculate the Growth Factor (G), the β-galactosidase activity (relative units, U_T), and the Induction Ratio (I_R) for each sample dilution as follows:

Growth factor (G)

$$G = \frac{A_{600,\,T} - A_{600,\,B}}{A_{600,\,N} - A_{600,\,B}}$$

Where: $A_{600,\,T}$ = sample absorbance at 600 nm
$A_{600,\,B}$ = blank absorbance at 600 nm
$A_{600,\,N}$ = negative control absorbance at 600 nm

β-galactosidase activity (U_T)

$$U_T = \frac{A_{420,\,T} - A_{420,\,B}}{A_{600,\,T} - A_{600,\,B}}$$

Where: $A_{600,\,T}$ = sample absorbance at 600 nm
$A_{600,\,B}$ = blank absorbance at 600 nm
$A_{420,\,S}$ = sample absorbance at 420 nm
$A_{420,\,B}$ = blank absorbance at 420 nm

(This activity, U_T, is expressed in relative units)

Induction Ratio (I_R)

$$I_R = \frac{1}{G} \times \frac{A_{420,\,T} - A_{420,\,B}}{A_{420,\,N} - A_{420,\,B}}$$

Where: $A_{420,\,T}$ = sample absorbance at 420 nm
$A_{420,\,B}$ = blank absorbance at 420 nm
$A_{420,\,N}$ = negative control absorbance at 420 nm

Lowest ineffective dilution (D_{LI}) = The lowest dilution of the dilution series with an $I_R < 1.5$

Highest ineffective concentration (C_{HI}) = The highest concentration of the dilution series with an $I_R < 1.5$

EVALUATING DATA AND VALIDITY

Validity

The test is valid if the following criteria are met:

⇒ The positive controls reach an I_R of ≥ 2

⇒ The average OD of the Negative Controls on Plate B increases by a factor of ≥ 2 during the 2 hour incubation period.

⇒ Growth factor (G) ≥0.5.

Genotoxicity

The sample is considered genotoxic if the following criteria are met:

⇒ If the I_R is ≥ 1.5 and the growth factor (G) is ≥ 0.5

⇒ A dose response is observed (recommended).

APPENDIX

UMU Testing - Aqueous Sample Short Protocol

Day Prior To Assay:

1. Aliquot 10 mL of TGA (Ampicillin supplemented) into a 50 mL conical tube. Aliquot 3mL TGA media into a separate tube to serve as sterility control (Section 1)

2. Thaw the culture carefully and add 100 µL to the tube containing 10 mL TGA.

3. Cap both tubes loosely and incubate with shaking overnight. Date and Time:_____

Day of the Assay:

Without S9

1. Measure the OD of the grown culture to ensure proper growth (> 2.0 @ OD_{600}; see Section 2). If the growth is acceptable, add 13.5 mL TGA media and 1.5 mL culture to a separate tube and incubate with shaking for an additional 1.5-2 hours (Section 3). Date and Time:_____

2. Prepare samples as necessary. Add 180 µL of sterile purified water to all wells except rows A - F, 1 - 3, and row H, 1 - 6 (Section 4.1.b). For the control wells, add 170 µL of sterile purified water to row H, 1 - 6 (Section 4.1.c); Plate A.

3. Add 10 µL of reconstituted Positive Control and Solvent Control to appropriate wells (Columns 1 - 3, Columns 4 - 6, Row H, respectively; (Section 4.1.d - e).

4. Aliquot 360 µL of the test sample(s) to columns 1-3, rows A - F (Section 4.1 f).

5. Perform 1:2 dilutions of test material across the plate, left to right. (Section 4.1 g).

6. Aliquot 10X TGA; 20 µL to all wells, additional 70 µL to row H, columns 7 - 12 (Section 4.1 h - i).

7. Aliquot 70 µL of regrown inoculum to all wells excluding the Blank wells (Section 4.1 j - k).

With S9

1. Follow the above steps through step 6, omitting the extra 10X TGA for row H, columns 7-12.

2. Reconstitute the 30% S9. Hold on ice. (Section 4.1.l)

3. Mix 470 µL TGA media and 76 µL 30% S9. Add 50 µL to wells H7-H12 (Blank). (Section 4.1.m)

4. Mix 470 µL of the 30% S9 mix and 2.9 mL of the regrown culture. Aliquot 70 µL of this mixture to all wells excluding the Blank wells (H7 – H12). (Section 4.1.n)

5. Mix plate right to left.

Procedure converges at this point. Use for Without and With S9

1. Incubate at 37°C for 2 hours with shaking.
   Date and Time:_____

2. Towards the end of the 2 hour incubation, prepare the second plate (Plate B) by aliquoting 270 µL TGA into each well (Section 5 a).

3. Once Plate A incubation is complete, aliquot 30 µL of each well into the corresponding well in Plate B (Section 5 b).

4. Read Plate B OD_{600}- and then incubate at 37°C with shaking for an additional 2 hours (Section 5 c - d).

5. Towards end of Plate B incubation period, hydrate ONPG. Mix 15 mL B-buffer, 1 mL ONPG, & 41 µL 2-mercaptoethanol. Aliquot 150 µL of the mix into each well of Plate C (Section 5 e - g).

6. Warm Plate C to 28°C (Section 5 h).

7. After the incubation period of Plate B is complete, mix and then read the OD_{600} of the plate (Section 5 i). Transfer 30 µL to the corresponding wells in Plate C (Section 6 a).

8. Incubate at 28°C with shaking for 30 minutes (Section 6 b).
   Date and Time:_____

9. Add 120 µL of Stop Reagent to each well and read the OD_{420} (Section 6 c - e).

Table 1: Suggested 96 Well Microplate Set Up

		1:1.5			1:3			1:6			1:12		
		1	2	3	4	5	6	7	8	9	10	11	12
	A	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
	B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
	C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
	D	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	E	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5
	F	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6
	G	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	H	PC	PC	PC	SC	SC	SC	BL	BL	BL	BL	BL	BL
	S1= Sample 1, dilutions 1:1.5, 1:3, 1:6, 1:12 (3 of each); S2- sample 2, etc. NC = Negative control PC = Positive control SC = Solvent control BL = Blank												

Day Prior To Assay:

1. Aliquot 10 mL of TGA (Ampicillin supplemented) into a 50 mL conical tube. Aliquot 3 mL TGA media into a separate tube to serve as sterility control (Section 1).

2. Thaw the culture carefully and add 100 µL to the tube containing 10 mL TGA.

3. Cap both tubes loosely and incubate with shaking overnight.
   Date and Time:_____

Day of the Assay:

Without S9

1. Measure the OD of the grown culture to ensure proper growth (> 2.0 @ OD_{600}; see Section 2). If the growth is acceptable, add 13.5 mL TGA media and 1.5 mL culture to a separate tube and incubate with shaking for an additional 1.5 - 2 hours (Section 3).
   Date and Time:_____

2. Prepare samples as necessary. Aliquot 190 µL TGA to positive and solvent control wells. Add 380 µL of TGA to rows A - F, columns 1 - 3 (Section 4.2 b - c). Add 200 µL TGA to all other wells and 50 µL extra to H7 - H12 (Section 4.2 d - e); Plate A.

3. Aliquot 20 µL of the test sample and 10 µL reconstituted control substances (as applicable) into appropriate wells (Section 4.2 f - h).

4. Perform dilutions (Section 4.2 i).

5. Aliquot 50 µL of regrown inoculum to all wells excluding the Blank wells (Section 4.2 j).

With S9

1. Follow the above steps through step 4.

2. Reconstitute the 30% S9 mix by adding 3.25 mL sterile, ice cold sterile purified water and mixing. Hold this mixture on ice (Section 4.2 k).

3. Mix 344 µL TGA media and 76 µL 30% S9. Add 50 µL of this mix to wells H7 - H12 (Section 4.2 l).

4. Mix 470 µL of the 30% S9 mix and 2.1 mL of the regrown culture. Aliquot 50 µL of this mixture to all wells excluding the Blank wells (Section 4.2 m).

Procedure converges at this point. Use for Without and With S9

1. Incubate at 37°C with shaking. Date and Time:_____

2. Near the end of the initial 2 hour incubation, prepare the second plate by aliquoting 270 µL TGA into each well (Section 5 a).

3. Once the initial incubation is complete, aliquot 30 µL of each well into the corresponding well in Plate B (Section 5 b).

4. Read the OD_{600} and then incubate at 37°C with shaking for an additional 2 hours (Section 5 c - d).

5. Near the end of the incubation period hydrate the ONPG. Prepare the 15 mL B-buffer, 41 µL 2-mercaptoethanol, 1 mL ONPG solution and aliquot 150 µL of the mixture into each well of Plate C (Section 5 e - g).

6. Warm plate to 28°C (Section 5 h).

7. After the incubation period is complete, mix and then read the OD_{600} of the plate (Section 5 i). Transfer 30 µL to the corresponding wells in Plate C (Section 6 a).

8. Incubate at 28°C with shaking for 30 minutes (Section 6 b).
   Date and Time:_____

9. After the incubation period is complete, add 120 µL of Stop Reagent and read the OD_{420} (Section 6 c - e).

Table 1 – Suggested 96-Well Plate Set Up, Chemical Sample Testing

		25X			50X			100X			200X		
		1	2	3	4	5	6	7	8	9	10	11	12
	A	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1
	B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2
	C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3
	D	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4	S4
	E	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5	S5
	F	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6	S6
	G	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
	H	PC	PC	PC	SC	SC	SC	BL	BL	BL	BL	BL	BL
	S1= Sample 1, dilutions 25X, 50X, 100X, 200X (3 of each); S2- sample 2, etc. NC = Negative control PC = Positive control (- S9/+S9) SC = Solvent control BL = Blank												

Version

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