



Manufactured By: Xenometrix AG

umuC Easy AQ

1-Day Microplate Format Genotoxicity Assay for testing of water samples

using *S. typhimurium* TA1535/pSK1002
with media and reagents as described in ISO 13829

Instructions for use

6 - Sample Kit (2 x 96 determinations)
Art. No. AF06-118

Upon receipt of your umuC Easy AQ Genotoxicity Assay kit, **make sure that all reagents are stored appropriately (see pg. 4 for storage instructions)**. If components are damaged or if any problems occur, please contact Aniara by phone: (866) 783-3797 ; fax: (513) 573-9241, or e-mail: info@aniara.com

For Research use only

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umuC Easy AQ

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Principle of the Test

Salmonella typhimurium TA1535 [pSK1002] bacteria are exposed to potentially genotoxic water samples. If genotoxic lesions are produced by the samples, the umuC gene is induced as part of the bacterial SOS response. The plasmid pSK1002 contains the umuC gene fused to the lacZ reporter gene. The induction of lacZ is measured by the conversion of colorless ONPG substrate (*o*-nitrophenyl- β -D-galactopyranoside) to the yellow product *o*-nitrophenol by the lacZ-encoded β -galactosidase.

Since different kinds of genotoxic lesions lead to the induction of the SOS response, one strain of *S. typhimurium* with the appropriate reporter gene construct is sufficient to identify all classes of bacterial genotoxins. As with other bacterial genotoxicity and mutagenicity assays, compounds requiring metabolic activation for activity can be identified by the addition of S9 microsomal rat liver extract.

The protocol was adapted from ISO 13829 "Water quality - Determination of the genotoxicity of water and waste water using the umu-test". All media and reagents except for the S9 co-factor concentrations are as described in ISO 13829 and can be used with the original ISO protocol or with this optimized umuC- Easy AQ protocol.

For the testing of concentrated samples please use the umuC Easy CS kit!

Assay Description

S. typhimurium TA1535 [pSK1002] bacteria in the exponential growth phase are exposed for 120 minutes to 4 concentrations of an aqueous test sample, as well as to a positive and a negative control. After 2 hrs, the exposure cultures are diluted in fresh medium and allowed to grow for another 2 hrs. The induction and expression of the umuC - lacZ reporter gene is then assessed after lysis of the bacteria. Colorless ONPG is converted to the yellow product *o*-nitrophenol in the presence of induced β -galactosidase (lacZ). The intensity of the color correlates with the amount of β -galactosidase present and thus with the genotoxic potency of the test compound.

Measurement of the OD₆₀₀ before and after the 2 hr growth phase allows to calculate an Induction Ratio (IR) and to identify toxic growth inhibitory effects.

The genotoxic potential of samples can be assessed directly or in the presence of liver S9 fractions.

Genotype of *S. typhimurium* 1535 [pSK1002]

Strain	Cell Wall	Repair	pSK1002
TA1535	<i>rfa</i>	<i>uvrB</i>	yes

rfa: This mutation leads to a defective lipopolysaccharide (LPS) layer that coats the cell surface, making the bacteria more permeable to bulky chemicals.

uvrB: The *uvrB* deletion mutation eliminates the accurate excision repair mechanism, thereby allowing more DNA lesions to be repaired by error-prone DNA repair mechanisms.

pSK1002: The multicopy plasmid pSK1002, a fusion product of genes of the *umu* operon (which was derived from *E. coli* K12 AB1157) with the vector pMC1403 (a pBR322 derivative), carries a *umuC'*-*lacZ* gene fusion product. The *umu* operon is regulated by the SOS genes *recA* (protease) and *lexA* (repressor) and consists of a promoter, operator, *umuD* and *umuC* genes. The vector carries the *lacZ* and *lacY* genes, the ampicillin resistance gene and *ori*.

Safety Precautions

- Not for use in humans and animals. For research purposes only.
- Do not drink, eat, smoke, or apply cosmetics in designated work areas. Wear laboratory coats and gloves when handling specimens and kit reagents. Wash hands thoroughly afterwards. Do not pipette by mouth.
- Handle specimens as if capable of transmitting infectious agents. Thoroughly clean and disinfect all materials and surfaces that have been in contact with specimens. Discard all waste associated with specimens in a biohazard waste container

Kit Components

Note1:

Each Xenometrix umuC Easy AQ kit contains enough bacteria, media and reagents for 192 determinations. This allows e.g. to test a total of 6 compounds, 4 dilutions each, in triplicates, with positive and negative controls, in the absence and presence of S9. The assay can be performed as 1 or 2 separate experiments (6 or 3 compounds per experiment, respectively).

Alternative plate layouts, dilution schemes or replicate numbers are possible, but are not described in this manual and are not supported by the Excel calculation sheet provided by Anlara.

Note2:

The umuC Easy TG medium corresponds to the TGA medium described in the ISO 13829 protocol, except that ampicillin is added just prior to use.

Kit contents:

- 2 vials containing *S. typhimurium* TA1535 [pSK1002] in a semi-solid medium. Each vial contains 50 µl to which 200 µl TG medium has to be added just prior to use.
- 4 vials containing sterile ampicillin (4 x 50 µl at 50 mg/ml)
- 2 x 1.1 ml ONPG substrate solution
- 100 µl 2-mercaptoethanol
- 120 ml 1x TG medium (single strength medium)
- 5 ml 10x TG medium (10x concentrated medium)
- 35 ml B-buffer
- 30 ml Stop reagent

Storage Conditions

Each Xenometrix umuC Easy kit is shipped at ambient temperature. Please contact Aniara if you received the kit later than 10 days after the shipment date indicated on the delivery note (phone: (866) 783-3797 ; fax: (513) 573-9241 or email info@aniara.com)

The shipment contains the following components which should be stored **immediately upon arrival** as follows (*storage conditions for additional reagents which are not part of the kit are printed in italics*):

1. -70 °C to -80 °C:

- 2 vials containing *S. typhimurium* TA1535 [pSK1002] in a semi-solid medium. Each vial contains 50 µl to which 200 µl TGA medium has to be added just prior to use.

➡ The bacteria must be stored at least at -70 °C. Improper storage at -20 °C may compromise the viability and performance of the strain. The tubes are not suitable for liquid nitrogen storage.

(If no -70 °C storage is available at your institution please contact Aniara)

2. -20 °C:

- Ampicillin
- ONPG substrate solution. Protect from light!
- *S9 (available separately, see www.aniara.com)*
- *S9 buffer components (not provided, preparation see next page): Glucose-6-phosphate, NADP*
- *Dissolved positive controls*

3. 2 - 8 °C

- 2-mercaptoethanol
- *S9 buffer components (not provided, preparation see next page): KCl, MgCl₂*
- *Solid positive controls (available separately, see www.aniara.com)*

4. 20 - 25 °C (room temperature, protected from light):

- 1x TG culture medium (single strength medium)
- 10x TG medium (10x concentrated medium)
- B-buffer
- Stop reagent

Required Equipment and Consumables NOT Included with the Kit

Note: all plastic ware has to be sterile!

- Environmental shaker capable of 37°C and 28°C, 150 - 250 rpm incubations
- 37°C dry incubator
- Spectrophotometer for measuring optical density at 600 nm
- Microplate reader capable to read at 420 and 600 nm
- 20 µl and 200 µl adjustable pipettes and sterile tips
- 5-50 µl and 50-200 µl 8-channel pipettes

- 50 ml tubes with (filter) caps
- 96-well microtiter plates
- Reagent reservoirs
- 5 ml and 10 ml pipettes
- Spectrophotometer cuvettes
- Solvents for sample dilution and zero dose control
- S9 buffer components and cofactors

Cofactor Stock solution for S9:

Stock concentration	Reagent	MW	Total volume	Amount	Storage
1.00 M	KCl	74.55	50 ml	3.728 g	4 °C
0.25 M	MgCl ₂ x6 H ₂ O	203.31	50 ml	2.541 g	4 °C
0.20 M	Glucose-6-P ^a	282.10	10 ml	0.564 g	-20 °C
0.04 M	NADP ^b	765.40	10 ml	0.306 g	-20 °C

^a as Glucose-6-phosphate sodium salt

^b as NADP sodium salt hydrate, MW of anhydrous form

- filter-sterilize and store as indicated (KCl and MgCl₂ can also be autoclaved)

Available separately (see www.aniara.com)

- Positive control chemicals: 4-nitroquinoline N-oxide, (for tests without S9) or 2-aminoanthracene (for tests with S9)
- Liver S9 fraction

ASSAY PROCEDURE - DAY 1:

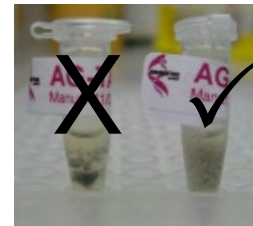
Overnight Culture Preparation

Using sterile technique, prepare an overnight culture of the strain by performing the following steps:

- Remove the vial of TA1535/pSK1002 from the freezer. Let stand at ambient temperature for about 5 minutes. Add 200 μ l of 1x TG medium to the vial.
- Remove the ampicillin vial from the -20°C storage and allow it to thaw at room temperature.
- Add 10 ml 1x TG medium to a 50 ml culture tube and 3 ml 1x TG medium to a sterility control tube
- Add 10 μ l ampicillin (50 mg/ml) to the tube with 10 ml 1x TG medium (=TGA medium)

For the following dispersion step we recommend to use tips with 300 μ l capacity and the volume of the pipette set to 200 μ l. If only 200 μ l capacity tips are available we recommend to set the pipette to 100 μ l. This helps to avoid contact of the liquid or bubbles with the non-sterile orifice of the pipette. As an additional precaution you may wipe the orifice with 70% alcohol prior to attaching the tip.

- Attach a sterile tip to the pipette and disrupt mechanically the dark semi-solid pellet with the tip. Pipette up and down until a uniform suspension is obtained which can be pipetted repeatedly without clogging the tip and which shows visually a homogeneous distribution of the dark fragments (see picture). If necessary, disperse large fragments mechanically with the tip until pipetting is possible.
- Pipette 50 μ l of the dispersion into the tube with 10 ml of TGA medium and label it "Culture".
- Place the caps loosely on the tubes, and secure with tape. After taping each cap, rock the cap back and forth. This disrupts the seal and insures that the cultures will receive sufficient aeration for complete overnight growth.
- Note: Using culture tubes with filter caps allows aeration even when the caps are firmly attached to the tubes. They do not have to be secured with tape.
- Incubate the tubes in an environmental shaker set at 37°C, 250 rpm for 14 -16 hrs.



ASSAY PROCEDURE - DAY 2:

Determination of the OD₆₀₀ Values of the Overnight Cultures

After the overnight incubation measure the OD₆₀₀ of the cultures:

1. Add 900 µl of 1x TG medium to 2 cuvettes.
2. Add 900 µl from the overnight sterility control tube to a third cuvette
3. Swirl the contents of the culture tube and transfer a 100 µl aliquot to one of the cuvettes containing 900 µl 1x TG Medium. Mix the contents of the cuvette.
4. Blank the spectrophotometer at 600 nm using the cuvette with 900 µl TG medium only.
5. Read the OD₆₀₀ of the cuvettes containing overnight culture and the sterility control.
6. Multiply the OD₆₀₀ reading of the culture cuvette by ten to obtain the actual optical density for the overnight culture.
7. Verify that the OD₆₀₀ values for the culture is at least 2.0*, and that the OD₆₀₀ value of the sterility control is ≤0.005. Use cultures for the next steps only if these criteria are met.

If the overnight culture has an OD₆₀₀ <2.0*, there was insufficient growth. Verify that the caps were loose to allow for aeration and that the bacteria were stored correctly upon receipt at ≤ -70 °C. The culture tubes may be incubated for additional time if necessary, but significant growth should be measurable within 2 hrs. If the OD₆₀₀ value of the sterility control is greater than 0.005, contamination has occurred and it is not recommended that the culture is used for the assay.

**This is actually a measurement of light scattering, which is dependent on the optical characteristics of a spectrophotometer. Therefore values can not be directly compared between different instruments. We suggest that you determine the maximum value for your instrument by measuring the growth of a bacterial overnight culture until it reaches a constant plateau value. The culture should be growing optimally, i.e. with agitation and sufficient aeration. Such a culture will be strongly turbid after an incubation time of 14 hrs at 37 °C. For an overnight culture to be considered acceptable it should have reached at least 70% of the maximal OD₆₀₀ value determined for your spectrophotometer. The value of 2.0 is a reasonable approximation for many instruments.*

Re-incubation of Bacteria

This step ensures exponential growth of the bacteria.

Dilute the overnight culture 1:4 (2 ml culture + 6 ml 1x TG +8 µl ampicillin) and incubate as before for about 1.5 -2 hrs to obtain an OD₆₀₀ corresponding to about 70-80% of the original OD of the overnight culture. Again, observe proper aeration of the tube. During this time, prepare the sample dilutions and the test plates.

Measure the OD₆₀₀ and dilute - if necessary - to the required OD₆₀₀ using 1x TG medium.

Exposure to Test Samples

The dilution scheme shown is for three 2-fold dilution steps starting with a 1.5-fold dilution of the aqueous sample. If you wish to use other dilutions or layouts you have to adjust the following procedure accordingly. The Excel calculation sheet available from Anlara (see page 10) only works with the suggested plate layout described here.

- Prepare the following Positive Controls:
Without S9: 4-Nitroquinolone (4-NQO), 12.5 µg/ml in DMSO. The final concentration in the assay will be 463 ng/ml.
With S9: 2-Aminoanthracene (2-AA), 50 µg/ml in DMSO. The final concentration in the assay will be 1.85 µg/ml.
- Add 180 µl of distilled sterile water to all wells in columns 4 - 12
- Add 360 µl of test sample to columns 1-3, rows A - C and E - G (This fills the wells almost completely. **Do not allow the lid to touch the liquid!** Place the lid in a tilted way to avoid contact with the liquid. Place the lid normally after the dilution steps)
- Add 170 µl of distilled sterile water to columns 1 - 3 of rows D and H (Pos. Control wells)

Rows A - D without S9 - Rows E - H with S9

	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	S1 - S3	Samples 1 - 3
B	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	PC	Positive control
C	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	SC	Solvent control
D	PC	PC	PC	SC	SC	SC	NC	NC	NC	BL	BL	BL	NC	Negative control
E	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	S1	BL	Blank
F	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2	S2		
G	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3	S3		
H	PC	PC	PC	SC	SC	SC	NC	NC	NC	BL	BL	BL		
	1.5x			3x			6x			12x			Final sample dilution	

- Add 10 µl of the appropriate Positive Control to columns 1 - 3 of row D (-S9) and row H (+S9)
- Remove and discard 10 µl from the Solvent Control wells in rows D and H, columns 4 - 6.
- Add 10 µl of Solvent Control (DMSO) to columns 4 - 6 of rows D and H
- Place tips on channels 1 - 3 and 5 - 7 of a multichannel pipette. Prepare the 3x dilutions of samples S1 - S3 and S1+ - S3+ by transferring and mixing 180 µl from rows A - C and E - G, column 1 into 4, 2 into 5, 3 into 6. Repeat for the 6x and 12x dilutions (column 4 into column 7 and so on). Discard 180 µl from the last 3 columns.
- For 1 plate: add 22 µl ampicillin (50 mg/ml) to 2.2 ml 10x TG medium (=10x TGA), mix
- Add 20 µl of 10x TGA to all wells

- Prepare a fresh co-factor solution from the stock solutions for rows E - H (+S9)

Volume	Reagent	Stock concentration	Concentration in mixture with bacteria and S9	Final concentration in assay
70 μ l	KCl	1.00 M	19 mM	5 mM
67 μ l	MgCl ₂	0.25 M	4.6 mM	1.2 mM
53 μ l	Glucose-6-P	0.20 M	2.89 mM	0.75 mM
211 μ l	NADP	0.04 M	2.3 mM	0.6 mM

- Mix, then remove and save 30 μ l for the Blank

For the wells without S9 (rows A - D):

- Add 70 μ l 1x TG medium to wells D10 - D12 (Blank)
- Add 3.3 ml re-incubated bacteria to a reservoir and add 70 μ l to all wells of rows A - D except D10 - D12 (Blank) (Use 4 tips, remove 1 tip after filling column 9)

For the S9 wells (rows E - H):

- Add 235 μ l 1x TG and 7.9 μ l S9 to the saved 30 μ l co-factor mix for the Blank and add 70 μ l thereof to the Blank wells H10-H12
- Add the freshly prepared co-factor solution (371 μ l) to a reservoir
- Add 2.93 ml of re-incubated bacteria
- Add 98 μ l S9 (prepared freshly and kept on ice), mix (= 2.88 %)
- Add 70 μ l of bacteria/S9 mix to all wells in rows E - H except H10-H12 (Blank) (Use 4 tips, remove 1 tip after filling column 9).
This gives a final S9 concentration of 0.75%
- Mix the whole plate with 8 tips from right to left
- Cover the microplate with a lid and incubate for 2 hrs at 37°C, 120 - 150 rpm

Dilution Step and Second Incubation

- During the 2 hr exposure prepare a second plate by pipetting 270 μ l of 1x TG medium with freshly added ampicillin to all wells (for 1 plate: 28 μ l ampicillin stock (50 mg/ml) to 28 ml 1x TG medium). Cover with a lid and place in an incubator to pre-warm the medium.
- After 2 hrs incubation, mix and transfer 30 μ l of the contents of the first plate to the second plate, column by column, from right to left.
- Read the OD₆₀₀ of the second plate (remove bubbles if necessary, read without lid).
- Incubate the second plate for 2 hrs at 37°C, with shaking (120 - 150 rpm)
- During this time: thaw the ONPG solution at RT in the dark
- Prepare a third plate with 150 μ l B-buffer/ONPG mixture (for 1 plate: 15 ml of B buffer, 40.5 μ l 2-mercaptoethanol, 1 ml ONPG solution) and pre-warm to 28°C. If no 28°C incubator is available put at 37°C for 15 minutes.*
- At the end of the 2 hrs incubation: mix and then read the OD₆₀₀ of the second plate.
- Set the temperature of the shaker to 28°C before reading the OD₆₀₀. *

Measurement of umuC Induction

- Remove the 3rd plate from the 28°C or 37°C incubator.
- Mix and transfer 30 μ l from each well of the second plate to the third plate (right to left)
- Incubate for 30 minutes at 28°C, with shaking (120 - 150 rpm)*
- After 30 min. add 120 μ l of Stop Reagent to each well.
- Mix, and remove bubbles
- Read the OD₄₂₀

* The incubation of the third plate at 28°C is as described in the ISO 13829 procedure. Incubations at different temperatures (e.g. room temperature or 37°C) may be used as well, but have different kinetic characteristics and may lead to results not directly comparable to results obtained with the ISO 13829 procedure.

Calculation and Definitions

For each sample dilution calculate the Growth Factor, the β -galactosidase activity (relative units), and the Induction Ratio I_R as follows:

$$\text{Growth factor } G: \quad G = \frac{A_{600, S} - A_{600, B}}{A_{600, N} - A_{600, B}}$$

where $A_{600, S}$ is the absorbance of the sample S at 600 nm
 $A_{600, B}$ is the absorbance of the blank at 600 nm
 $A_{600, N}$ is the absorbance of the negative control at 600 nm

$$\beta\text{-galactosidase activity (relative units) } U_S: \quad U_S = \frac{A_{420, S} - A_{420, B}}{A_{600, S} - A_{600, B}}$$

$$\text{Induction Ratio } I_R: \quad I_R = \frac{1}{G} \times \frac{A_{420, S} - A_{420, B}}{A_{420, N} - A_{420, B}}$$

Lowest ineffective dilution: D_{LI} = The lowest dilution of the dilution series with a $I_R < 1.5$

Highest ineffective concentration: C_{HI} = The highest concentration of the dilution series with a $I_R < 1.5$

For easier calculation you may download an **umuC Easy AQ Excel calculation sheet** from our Homepage at www.aniara.com and copy the output from the plate reader into this sheet.

The calculation sheet is based on the suggested plate layout and starting dilution (1.5x, see page 8), but allows to choose different dilution steps.

Data Evaluation, Validity Criteria

The whole test is considered valid if the positive controls reach an induction ratio I_R of ≥ 2

The average OD_{600} of the Negative Controls of the second plate should increase by a factor of ≥ 2 during the 2 hr incubation (growth control).

A sample dilution is considered genotoxic if the Induction ratio $I_R \geq 1.5$ and the Growth factor $G \geq 0.5$.

To call a test compound genotoxic in the umuC Easy AQ test we recommend that a dose response should be observed.