



Manufactured By: Xenometrix AG

Ames II AQUA

Microplate Format Mutagenicity Assay for testing of water samples

using *S. typhimurium* TA98 and TAMix

Instructions for use

Upon receipt of your Ames II AQUA Mutagenicity Assay kit, **make sure that all reagents are stored appropriately (see pg. 2 for storage instructions)**. If components are damaged or if any problems occur, please contact Aniara by phone: 866-783-3797; fax: 513-5739241 or e-mail: info@aniara.com.

For Research use only

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7768 Service Center Drive • West Chester OH 45069

Phone: 513.770.1991

Toll Free: 866.783.3797

Fax: 513.573.9241

Email: info@aniara.com

www.aniara.com

NOTE 1:

This manual applies to the following versions of the assay:

Article No.	Kit size*	lyophilized liver S9	Positive Controls [#]
AL01-213	1	-	-
AL01-213-S1-P (Aroclor 1254-induced S9)	1	+	2-NF, 4-NQO, 2-AA
AL01-213-S2-P (PBN- induced S9 [§])	1	+	2-NF, 4-NQO, 2-AA
AL05-213	5	-	-
AL05-213-S1-P (Aroclor 1254-induced S9)	5	+	2-NF, 4-NQO, 2-AA
AL05-213-S2-P (PBN-induced S9 [§])	5	+	2-NF, 4-NQO, 2-AA

* Sufficient for 1 or 5 samples when tested with and without S9, in triplicates, 6 concentrations, with negative and positive controls. This equals a total of 48 (1 sample kit) or 240 measurements (5 sample kit) per strain.

[#] 2-NF: 2-Nitrofluorene; 4-NQO: 4-Nitroquinoline-N-oxide; 2-AA: 2-Aminoanthracene

[§] PBN-induced S9: Phenobarbital / β -naphthoflavone-induced S9

NOTE 2:

The Ames II AQUA procedure can be used with any other Ames MPF™ *Salmonella* strain available from Aniara (TA100, TA1535, TA1537). Please note that TA1535 and TA1537 are NOT ampicillin - resistant!

NOTE 3:

Please read carefully the entire manual before starting the experiments!

NOTE 4:

For the testing of concentrated samples please use the standard Ames II and Ames MPF™ kits and Instructions for Use from Aniara.

Ames II AQUA Mutagenicity Assay

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Principle of the Test

Point mutations were made in the histidine (*His*) operon in *Salmonella typhimurium*, rendering the bacteria incapable of producing histidine. These mutations result in *his*- organisms that cannot grow unless histidine is supplied. When a mutagenic event occurs, base substitutions or frameshifts within the *His* gene may cause a reversion to histidine prototrophy. These reverted bacteria will then grow in histidine-deficient media.

The mutagenic potential of a test sample is assessed by exposing these *his*- organisms to varying concentrations of sample and selecting for the reversion event. Medium lacking histidine is used for this selection which allows only those cells that have undergone the reversion to histidine prototrophy to survive and grow.

The strains provided in this kit are the *Salmonella typhimurium* TAMix and TA98. The Ames II TAMix contains a mixture of equal proportions of the Ames II TA7001-TA7006 strains. Individually, these strains are designed to revert by only one specific base-pair substitution out of six possible changes. Thus, when mixed, all six base substitution mutations can be represented in one culture. This kit also contains TA98 for the detection of frameshift mutations.

Assay Description

Approximately 10^7 *his*- bacteria are exposed to 6 concentrations of an aqueous test sample, as well as to a positive and a negative (sterile water) control, for 90 minutes in a medium containing sufficient histidine to support approximately two cell divisions. After 90 minutes, the exposure cultures are diluted in a pH indicator medium lacking histidine, and aliquoted into 48 wells of a 384-well plate. Within two days, cells that have undergone the reversion to histidine prototrophy will grow into colonies. Bacterial metabolism reduces the pH of the medium, changing the color of that well. The number of wells containing revertant colonies are counted for each dose and compared to a negative (water) control. Each dose is done in triplicate to allow for statistical analysis of the data.

An increase in the number of revertant colonies upon exposure to a test sample relative to the negative controls indicates that the sample is mutagenic in the Ames II AQUA Assay.

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

Genotypes of the TA98 & TAMix *Salmonella typhimurium* strains

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
TA98	<i>hisD3052</i>	Frameshifts	GCGCGCGC	<i>rfa</i>	<i>uvrB</i>	yes
TAMix contains:						
TA7001	<i>hisG1775</i>	b.p. sub.	A:T>G:C	<i>rfa</i>	<i>uvrB</i>	yes
TA7002	<i>hisC9138</i>	b.p. sub.	T:A>A:T	<i>rfa</i>	<i>uvrB</i>	yes
TA7003	<i>hisG9074</i>	b.p. sub.	T:A>G:C	<i>rfa</i>	<i>uvrB</i>	yes
TA7004	<i>hisG9133</i>	b.p. sub.	G:C>A:T	<i>rfa</i>	<i>uvrB</i>	yes
TA7005	<i>hisG9130</i>	b.p. sub.	C:G>A:T	<i>rfa</i>	<i>uvrB</i>	yes
TA7006	<i>hisC9070</i>	b.p. sub.	C:G>G:C	<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. The deletion through the biotin gene makes the bacteria biotin dependent.

pKM101: This R factor plasmid enhances chemical and UV-induced mutagenesis via an error-prone recombinational DNA repair pathway. The plasmid also confers ampicillin resistance.

Kit Components and Storage Conditions

Each Xenometrix Ames II AQUA Mutagenicity Assay kit contains the following components and should be stored as indicated:

-70°C to -80°C:

- Vials containing frozen *Salmonella* strains (TAMix, TA98)

Note: The strains are shipped on dry ice and must be stored at least at -70°C. Improper storage at -20°C may compromise the viability of the strains. The tubes are not suitable for liquid nitrogen storage.

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

-20°C:

- Vial(s) containing sterile ampicillin (50 mg/ml)
- S9 (if provided, see Note 1 at beginning of document for available kit configurations)
- Dissolved positive controls

4°C:

- 10X Exposure Medium Solution A
- 10X Exposure Medium Solution B
- Positive controls before reconstitution (if provided, see Note 1 at beginning of document)

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

- Growth Medium
- Indicator Medium

Required Equipment and Consumables NOT Included with the Kit

Note: all plastic ware has to be sterile!

- Environmental shaker capable of 37°C, 250 rpm incubations with approx. 2.5 - 3 cm amplitude
- 37°C dry incubator
- Light table for scoring results (recommended)
- Spectrophotometer for measuring optical density at 600 nm
- 20 µl, 200 µl, and 1000 µl adjustable pipettes and sterile tips
- 5-50 µl and 50-200 µl 8-channel pipettes
- 8-Channel repeating pipettor and sterile tips (recommended)
- Optional: Plate reader capable of reading 96-well plates at OD₆₀₀ (for cytotoxicity measurement)

- 50 ml tubes with (filter) caps
- 24-well plates
- 384-well microtiter plates
- 96-well microtiter plate (optional, for cytotoxicity measurement)
- Reagent reservoirs
- 5 ml and 10 ml pipettes
- Spectrophotometer cuvettes
- Sterile water for sample dilution and negative control, DMSO for preparation of the positive controls
- S9 buffer components*

Included in some kit versions only (see Note 1 at beginning of this manual):

- Positive control chemicals: 2-nitrofluorene, 4-nitroquinoline N-oxide (for tests without S9) and 2-aminoanthracene (for tests with S9) and
- Liver S9 fraction (Aroclor 1254 or phenobarbital / β-naphthoflavone-induced)

*S9 Cofactor kit (Art. No. APCO-0800)

A ready-to-use kit available separately from Aniara containing phosphate buffer pH 7.4, MgCl₂, KCl, G-6-P and NADP for preparing the S9 mix. This kit replaces the self-made S9 buffer components (Appendix B).

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.

ASSAY PROCEDURE - DAY 1: Overnight Culture Preparation

Using sterile technique, prepare overnight cultures of TA98 and TAMix by performing the following steps:

1. Remove the vials with the bacteria from the freezer and allow them to thaw at room temperature. Use bacteria within 5 minutes after thawing.
2. Remove the ampicillin vial from the -20°C storage and allow it to thaw at room temperature.
3. Prepare the overnight cultures by performing the following steps:
 - Add 10 ml Growth Medium to two 50 ml culture tubes labeled 'TA98', and 'TAMix'.
 - Add a few milliliter of Growth Medium to a tube labeled 'Negative Control'.
 - Add 10 µl ampicillin (50 mg/ml) to tubes 'TA98' and 'TAMix'.
4. Re-suspend the thawed bacterial stock cultures and add 10 µl into the appropriately labeled tube.
5. 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.
6. Incubate the tubes in an environmental shaker set at 37°C, 250 rpm for 14 -16 hrs. Record the time the incubation is started in the chart below.

	Start Incubation	Stop Incubation	Incubation time
Date			
Time			

Note: Sufficient aeration of the overnight culture is crucial for optimal growth resulting in a dense culture. Your culture may grow insufficiently if you do not have an orbital environmental shaker with an amplitude of ~2.6 cm and a shaking frequency of 250 rpm. If your shaker does not fulfill these specifications, you may use sterile Erlenmeyers instead of 50 ml centrifuge tubes (larger liquid-air interface) or increase the shaking frequency to 350-400 rpm. Irrespective of using tubes or Erlenmeyer flasks it is critical not to cap them tightly in order to allow for sufficient aeration!

Note: As a culture increases in age, the number of spontaneous revertants may increase. Best results are obtained when cultures are started late on Day 1 and processed immediately after the overnight incubation (14 -16 hours).

Note: Xenometrix strongly suggests to discard remaining thawed bacteria after starting the overnight cultures. We DO NOT recommend to re-freeze bacteria for later use. The growth and performance of re-frozen cultures may be compromised.

ASSAY PROCEDURE - DAY 2:

Determination of the OD₆₀₀ Values of the Overnight Cultures

1. After the overnight incubation measure the OD₆₀₀ of the cultures:
2. Add 900 µl of Growth Medium to four cuvettes.
3. Swirl the overnight cultures and transfer 100 µl of bacteria from each tube as well as from the negative control tube to cuvettes containing 900 µl Growth Medium. Mix the contents of the cuvettes.
4. Blank the spectrophotometer at 600 nm using the cuvette with 900 µl Growth Medium only.
5. Take the OD₆₀₀ reading of each cuvette containing overnight culture and the negative control.
6. Multiply each OD₆₀₀ reading by ten to obtain the actual optical density and enter the values into the chart below.
7. Verify that the OD₆₀₀ values for the cultures is **at least 2.0***, and that the OD₆₀₀ value of the negative control is ≤ 0.05 . Use cultures for the next steps only if these criteria are met.
8. If the overnight cultures have an OD₆₀₀ $< 2.0^*$, there was insufficient growth. Verify that the caps were loose to allow for aeration, that the shaking was sufficient, that the temperature was 37°C, and that the strains were stored correctly up on receipt at $\leq -70^{\circ}\text{C}$. The culture tubes may be incubated for additional time if necessary, but an incubation time of 24 hours should not be exceeded. If the OD₆₀₀ value of the negative control is greater than 0.05, contamination has occurred and it is not recommended that the cultures be used for the assay.

Overnight Culture	OD ₆₀₀ (x10)	Acceptable Range*
TA98		≥ 2.0
TAMix		≥ 2.0
Negative Control		≤ 0.05

**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.*

Preparation and Dilution of the Test Sample Stock Concentrations

Note: The following procedure describes an assay for 1 test sample at 6 concentrations in triplicate with negative (sterile water) and positive controls. Refer to Appendix B for the preparation of S9 mix.

Prepare the dilutions of the test sample to be used in the assay by performing the following steps:

Samples containing solids should be centrifuged. In this case, only the supernatant is processed further and mutagenic activity associated with the pellet will be lost for the assay. Filter sterilize test samples (0.2 μm).

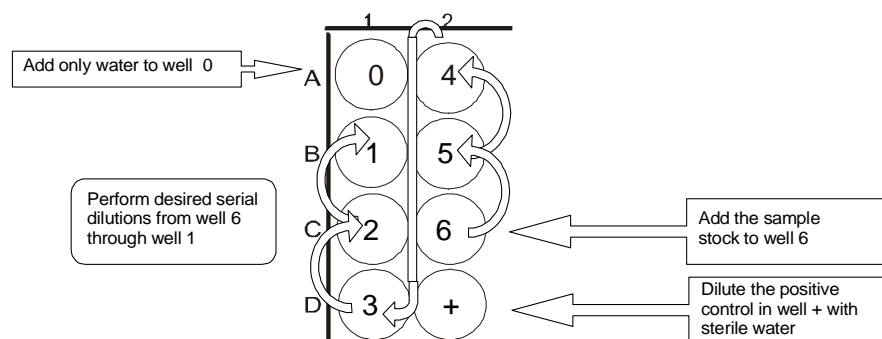
Note: The pH of strongly acidic or basic samples should be adjusted to ~7 before sterilization.

1. Prepare the positive control chemical stocks as described in Appendix A.
2. Unwrap a 24-well plate and place it on the paper template for the 24-well dilution plate layout. Be sure that the plate is oriented correctly.
3. Transfer the test sample to well #6 of the dilution plate (see picture below).

Note: Volumes added at this and the next steps are dependent on the dilution factor to be used. 1.110 ml of each sample concentration will be required for dosing both strains (2x 1.110 ml for testing with and without S9). Therefore, plan the volumes added in these steps such that after the serial dilutions are performed, there will be sufficient volumes for dosing. We recommend to calculate also a dead volume (pipetting reserve) of approximately 50 μl .

4. Add the appropriate volumes of sterile water to wells #0-5 of the plate.
5. Perform the desired dilution of test sample by transferring it from well #6 to #5, and mix by pipetting up and down thoroughly.
6. Complete the serial dilutions from well #5 to #4, #4 to #3, #3 to #2, and #2 to #1. DO NOT transfer test sample to the 0 well. This is the zero-dose control for the assay and should contain water only.
7. Add 1.10 ml of sterile water and 63 μl of the positive control stock (prepared in step 1) to the well labeled '+'. Mix well.

Note: The positive controls used for assays without and with S9 are different. Therefore prepare two dilution series using columns 1 plus 2 (no S9) and columns 3 plus 4 (with S9) of the 24-well dilution plate, each of them with the appropriate positive control in well '+'.



8. Fill in the chart below with the sample used along with the dilutions performed.
(An example in gray is given for 2x dilutions, triplicates, for 2 strains, with OR without S9).

Test sample: _____

Well	Add sample	Add water	Transfer to next well, mix	Final concentration
0	-	1.2 ml	-	Solv. Contr.
1	-	1.2 ml		3.13%
2	-	1.2 ml	1.2 ml ↑	6.25%
3	-	1.2 ml	1.2 ml ↑	12.5%
4	-	1.2 ml	1.2 ml ↑	25%
5	-	1.2 ml	1.2 ml ↑	50%
6	2.4 ml	-	1.2 ml ↑	100%

Preparation of the 10x Exposure Medium Working Solution

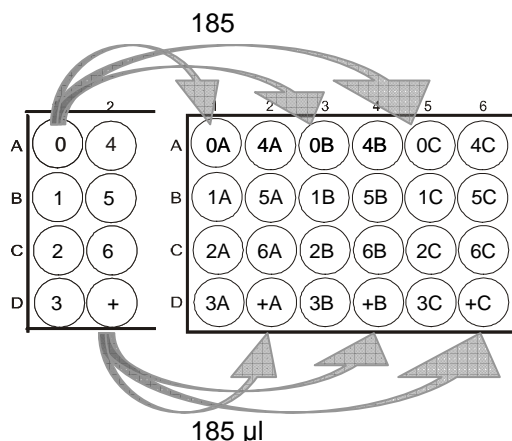
Prepare the 10X Exposure Medium working solution by adding 100 µl of Exposure Medium Solution B to 4 ml of Exposure Medium Solution A (ratio of 1:40). Mix well.

Preparation of Exposure Cultures

Unwrap the 24-well exposure plates (2 plates per strain +/- S9).

For the following steps use an 8-channel pipettor with 4 evenly spaced tips (every other channel)

1. Add ~1ml of sterile water to a reagent reservoir and transfer 15 µl to all wells of the plates without S9 (TA98 -S9, TAMix -S9).
2. Add ~2.7 ml of 10X Exposure Medium Working Solution to a reagent reservoir and transfer 25 µl to all wells of all plates (TA98 +/-S9, TAMix +/-S9).
3. Transfer 185 µl from the first column of the 24-well dilution plate (wells #0-3) to columns 1, 3, and 5 of all 24-well exposure plates (see picture below).
4. Transfer 185 µl from the second column of the 24-well dilution plate (wells #4-6 and +) to columns 2, 4, and 6 of all 24-well exposure plates (see picture below).

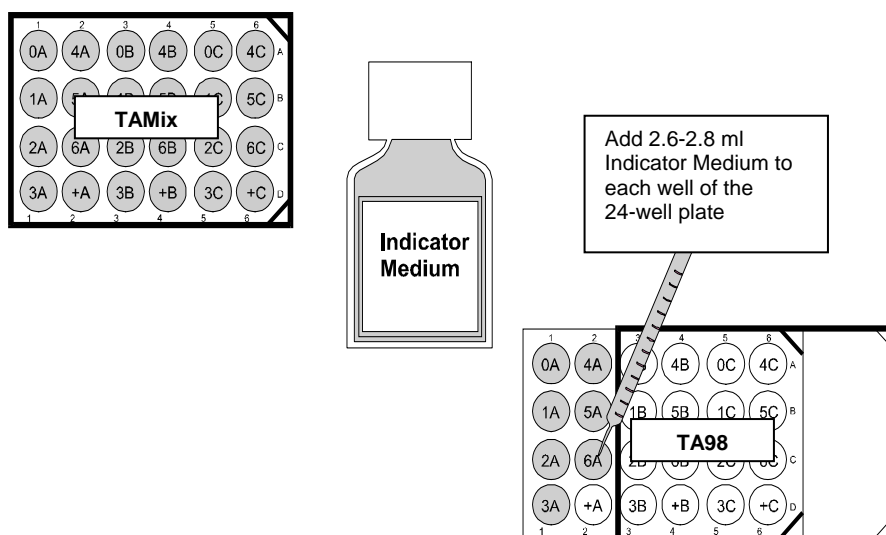


- 5.a Add ~1.6 ml of the **TA98 culture** to a reagent reservoir, swirl gently the contents and transfer 25 μ l to of all wells of the TA98 exposure plates. Pipet to the wall of the wells without touching the liquid to avoid cross-contamination.
- 5.b Add ~1.6 ml of the **TAMix culture** to a reagent reservoir, swirl gently the contents and transfer 25 μ l of to all wells of the TAMix exposure plates. Pipet to the wall of the wells without touching the liquid to avoid cross-contamination.
6. Add ~1 ml of S9 mix prepared as described in Appendix B to a reagent reservoir and transfer 15 μ l to all wells of the S9 plates. Pipet to the wall of the wells without touching the liquid to avoid cross-contamination.
7. Secure the 24-well plates to the base of 37°C environmental shaker. Incubate the plate for 90 minutes at 37°C, 250 rpm. Record the time the incubation was started.

Pipetting Scheme:

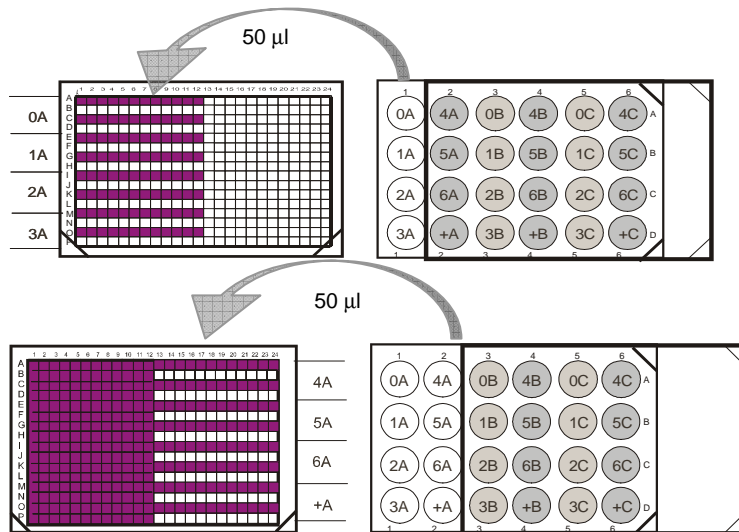
	TA98		TAMix	
	no S9	with S9	no S9	with S9
Sterile water	15 μ l	-	15 μ l	-
10X exposure medium working solution	25 μ l	25 μ l	25 μ l	25 μ l
Sample (or positive control)	185 μ l	185 μ l	185 μ l	185 μ l
Bacteria	25 μ l	25 μ l	25 μ l	25 μ l
S9 mix	-	15 μ l	-	15 μ l

Addition of Indicator Medium



1. After the 90 minute incubation, remove the exposure plates from the environmental shaker. Record the time the incubation was stopped.
2. Add 2.6 - 2.8 ml Indicator Medium (the minimal volume needed may depend on the characteristics of your 8-channel repeating pipettor used in the following step) to each well of the 24-well plates. Take care not to cross-contaminate wells or the bottle of Indicator Medium.

Transfer of Exposed Cultures from 24-well Plate to 384-well Plates



1. Unwrap three 384-well plates for each 24-well plate. Label each 384-well plate with the strain that it will contain and the plate replicate number (e.g. TAMix-1 -S9, TAMix-2 -S9, TAMix-3 -S9). Be sure that the plate is oriented correctly.
2. Place the appropriate 24-well exposure culture plate to the side of the related 384 well plates.
3. Slide the cover of the 24-well exposure culture plate to the right so that column 1 is uncovered.
4. Using an 8-channel pipettor (repeating type strongly suggested), mix the solution in the wells of column 1 of the 24-well plate by pipetting up and down gently.
Note: Two tips of the 8-channel pipettor fit into each well of the 24-well exposure plate.
5. Slide the cover of the first 384-well plate to the right so that the left half of the plate is uncovered.
6. Dispense 50 µl aliquots into columns 1-12 of the first 384-well plate. Each tip lines up with every other well of the plate, so a complete transfer will require two horizontal passes from column 1 to column 12. Place the cover back on the plate
7. Slide the cover of the 24-well exposure culture plate to the right one more column so that columns 1 and 2 are now uncovered.
8. Mix the solution in the wells of column 2 by pipetting up and down gently.
9. Slide the cover of the first 384-well plate to the left so that the second half of the plate is uncovered.
10. Dispense 50 µl aliquots into columns 13-24 of the first 384-well plate. Again, the transfer requires two horizontal passes from column 13 to column 24. Place the cover back on the plate
11. Change tips on the pipettor and repeat this procedure for the remaining columns of the 24-well plates. Columns 3 and 4 of the 24-well plate are aliquoted into the second 384-well plate and columns 5 and 6 - after changing tips - are aliquoted into the third 384-well plate.
12. Repeat above procedure for each 24-well plate

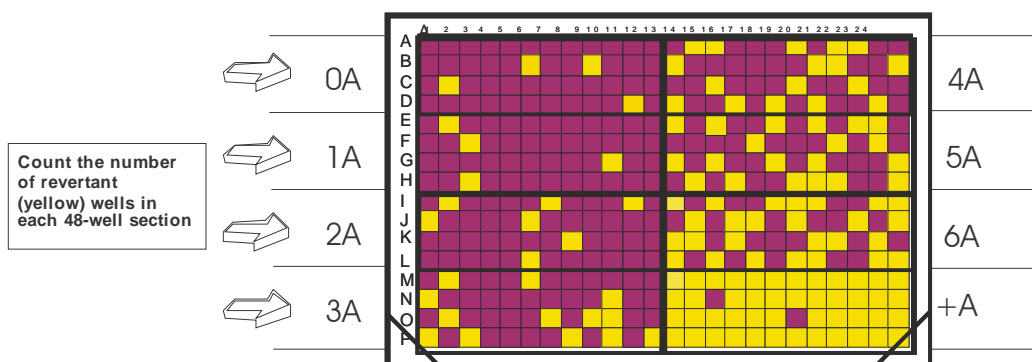
Incubation of Revertant Colony Selection Plates

- Carefully place the 384-well plates into the sealable plastic incubation bag provided and engage the seal. This will prevent evaporation during the 2 day selection incubation.
- Place the plastic bag into a 37°C dry incubator for 2 days. Record the time the incubation was started below.

	Date	Time
Incubation Started		
Incubation Stopped		

ASSAY PROCEDURE - DAY 4

Plate Scoring



- Remove the plastic bag containing the 384-well plates from the 37°C dry incubator. Record the time the incubation was stopped in the chart above.
- Score the three replicate 384-well plates per strain by using the transparent 384-well plate scoring template, e.g. by placing the template and the 384-well plate on top of a light table. Count the number of positive wells in each 48-well section, and enter the data in the charts on p. 11. Positive wells are those that have turned yellow or have a bacterial colony visible on the bottom of the well.

Note: Any indication of a color change from purple to yellow should be included in the positive count.

Note: In sections with many revertants it is easier to count the number of purple wells and to subtract this number from 48.

TA98 # Positive Wells -S9

Concentration	Plate 1	Plate 2	Plate 3
0			
1			
2			
3			
4			
5			
6			
+			

TA98 # Positive Wells +S9

Concentration	Plate 1	Plate 2	Plate 3
0			
1			
2			
3			
4			
5			
6			
+			

TAMix # Positive Wells -S9

Concentration	Plate 1	Plate 2	Plate 3
0			
1			
2			
3			
4			
5			
6			
+			

TAMix # Positive Wells +S9

Concentration	Plate 1	Plate 2	Plate 3
0			
1			
2			
3			
4			
5			
6			
+			

Data Entry

Prepare an Excel sheet with the necessary information (compound name, dose concentrations, units, strain used, metabolic activation, etc.) for the calculation of the raw data.

Enter the number of positive wells from the charts above for the appropriate replicate plate.

Note: If more than one compound was tested with the same overnight culture, the negative control wells (containing water only) can be pooled. E.g. when 3 samples were tested with the same culture on the same day (e.g. TA98 -S9), the three corresponding triplicate negative control scores are pooled to a mean of 9 replicates.

Calculate the '**Mean Number of Positive Wells per Concentration**' which is the average of the positive wells for the three replicates for each dose.

Calculate the '**Standard Deviation of Positive Wells per Concentration**' which are the standard deviation values for the Mean Number of Positive Wells.

Suggested Calculation, Data Interpretation

- A. Calculate the '**Fold Induction over the Baseline**' which is the ratio of the mean number of positive wells for the dose concentration divided by the baseline. The baseline is obtained by adding one standard deviation to the mean number of positive wells of the negative control.

Example: Mean \pm SD of negative control = 1.2 ± 0.7
Baseline ($1.2 + 0.7$) = 1.9

Note: If the baseline is less than 1.0, the value should be set to 1.0.

Fold inductions in revertant numbers over the baseline are generally not considered as positive if less than 2.0. Below this fold increase value, the data are unreliable with respect to determining mutagenicity. A compound that shows a clear dose response and/or yields multiple fold inductions greater than 2.0, is classified as a mutagen.

- B. **Student's t-test** (1-sided, unpaired) may be used to determine significance at the $\alpha = 0.05$ level.

Xenometrix suggests to use the free Excel calculation sheet which simplifies data entry and automates all necessary calculations. It is included on the USB stick or may be downloaded from the Aniara homepage www.aniara.com.

Layout of the Xenometrix Excel Calculation Sheet

Sheet 1: Concentrations

The screenshot shows an Excel spreadsheet with the following layout:

- Row 14:** Date input field containing "01.02.2011".
- Row 17:** Instruction: "Select Strain from Dropdown List in Box Below".
- Row 19:** Strain name input field containing "TA 100".
- Row 22:** Instruction: "Enter compound 1 in Box Below".
- Row 23:** Compound 1 input field containing "Compound 1".
- Row 25:** Instruction: "Enter compound 2 in Box Below".
- Row 26:** Compound 2 input field (highlighted in purple).
- Row 28:** Instruction: "Enter compound 3 in Box Below".
- Row 29:** Compound 3 input field (highlighted in orange).
- Row 32:** Section header "Enter Concentration used" and "Choose Units for Compound 1".
- Row 33:** Strain identifier "TA 100 -S9".
- Row 34:** "Cpd 1:" dropdown menu set to "Compound 1" and a units dropdown menu set to "µg/ml".
- Rows 35-40:** A table of concentration values with red arrows pointing to the right:

1	4	µg/ml
2	20	µg/ml
3	100	µg/ml
4	500	µg/ml
5	2000	µg/ml
6	5000	µg/ml
- Row 42:** Section header "Concentration used".
- Row 43:** Strain identifier "TA 100 +S9".
- Row 44:** "Cpd 1:" dropdown menu set to "Compound 1".
- Rows 45-46:** A table of concentration values:

1	4	µg/ml
2	20	µg/ml

The spreadsheet interface includes a toolbar at the top, a status bar at the bottom showing "Bereit", and a navigation pane at the bottom with tabs for "Concentrations", "raw data", "Summary", "Graphs", and "Ames MPF dilutions calculator".

Sheet 2: Raw Data

Enter the raw data (triplicate) into all colored fields

Enter the negative (solvent) control data into yellow colored fields

Enter positive control data

Note:
If more than one compound was tested with the same overnight culture, the negative (solvent) control wells can be pooled.
E.g. when 3 compounds were tested with the same culture on the same day (e.g. TA98 -S9), the three corresponding triplicate negative control scores are pooled to a mean of 9 replicates.

Please enter all solvent control values in the appropriate yellow boxes (-S9 or +S9) of compound 1. They will appear automatically in the corresponding boxes of compound 2 and 3.

Compound:	Compound 1			Spontaneous
TA 100 -S9	Replicate #1	Replicate #2	Replicate #3	TA 100 -S9
Conc. (µg/ml)				
4	3	4	1	3
20	6	4	9	4
100	12	9	10	6
500	19	18	17	2
2000	37	30	32	1
5000	39	40	45	2
Pos. Control	48	47	48	

Compound:	Compound 1			Spontaneous
TA 100 +S9	Replicate #1	Replicate #2	Replicate #3	TA 100 +S9
Conc. (µg/ml)				
4	4	5	5	7
20	4	6	7	1
100	6	8	9	0
500	15	17	21	4
2000	26	30	25	2
5000	31	30	34	2
Pos. Control	47	44	46	

Compound:	Compound 1			Spontaneous
TA 100 -S9	Replicate #1	Replicate #2	Replicate #3	TA 100 -S9
Conc. 0				
				3

Sheet 3: Summary

Definitions and Explanations

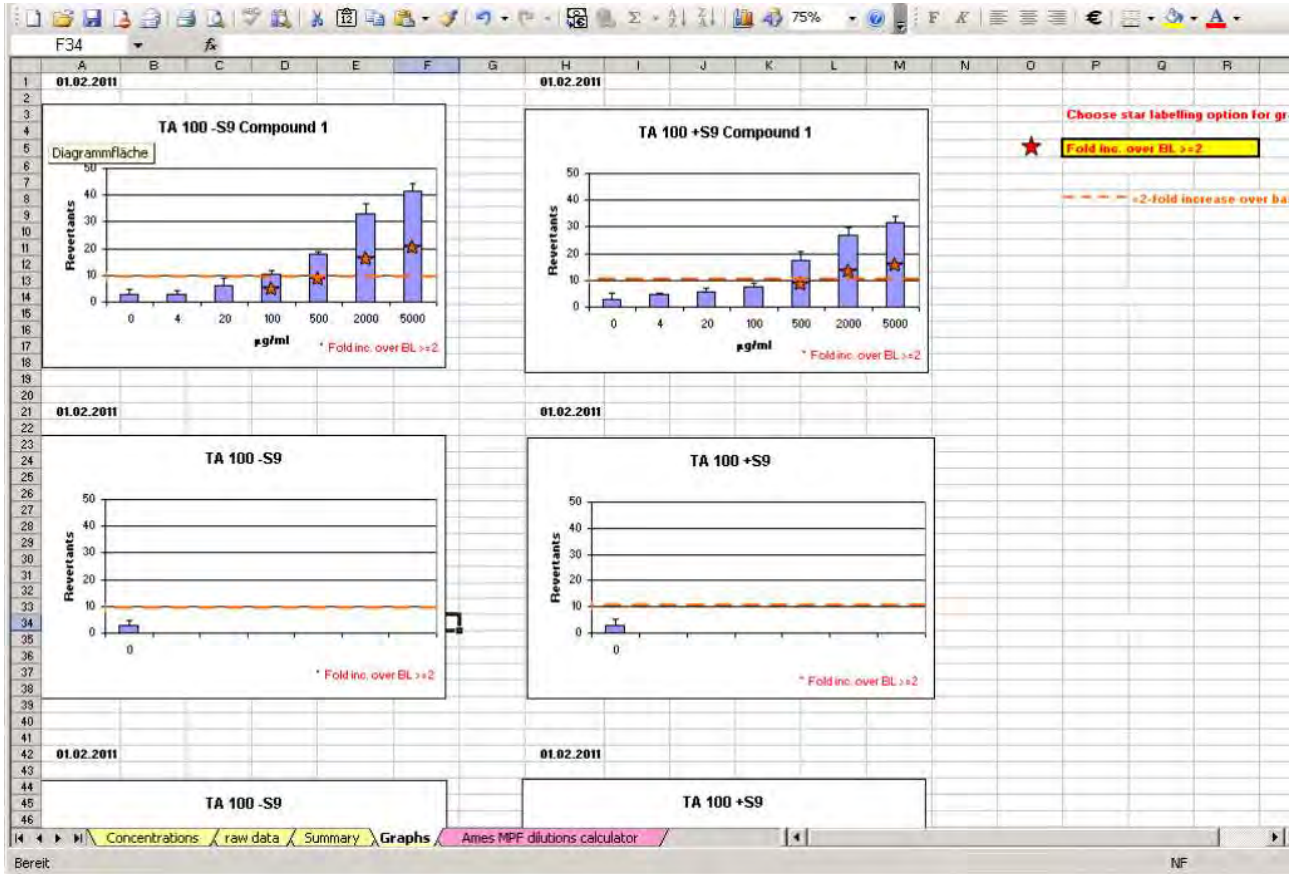
- 'n' = number of replicates
- 'Baseline' = Mean + 1 SD
- If 'mean # pos.wells' of zero dose control is less than 1, value will appear in grey and will be set to 1 in the column 'Corr. mean'. Fold increase (zero value) will then be calculated from the corrected mean.
- Baseline values <1 will be set to 1.
- 'Fold increase (baseline)' = fold increase over baseline. Values ≥ 2.0 will appear in bold red.
- 't-test': p-values, 1-sided, based on unpaired data. Values ≤0.05 appear red, values ≤0.01 bold red.

It is suggested to use 'Fold increase (over baseline)' and 't-test' to evaluate results

Compound 1		Assay		01.02.2011		
Conc. (µg/ml)	n	mean # pos. Wells	Corr. mean	Base-line	Fold increase (over baseline)	t-test p-value (unpaired, 1-sided)
0	6	3.00	1.79	4.79		
4	3	2.67	1.53		0.56	0.3959
20	3	6.33	2.52		1.32	0.0263
100	3	10.33	1.53		2.16	0.0003
500	3	18.00	1.00		3.76	0.0000
2000	3	33.00	3.61		6.89	0.0000
5000	3	41.33	3.21		8.63	0.0000
Pos. Control	3	47.67	0.58			

Compound 1		Assay		01.02.2011	
Conc.	mean # pos.	Corr.	Base-	Fold increase (over)	t-test p-value (unpaired, 1

Sheet 4: Graphs



Appendix A Preparation of Positive Control Stocks

Positive Controls

Strain	Chemical	Final Conc.
TA98 and TAMix	2-NF/4-NQO	2 µg/ml/0.5 µg/ml
TA98 and TAMix	2-AA	5 µg/ml

Note: Dissolved positive control chemicals should be stored at -20°C

Preparation of positive controls for 1 test compound

A. Without S9:

1. Prepare a 100 µg/ml stock solution of 2-nitrofluorene (2-NF) in DMSO.
2. Prepare a 25 µg/ml stock solution of 4-nitroquinoline N-oxide (4-NQO) in DMSO.
3. Add 31.5 µl of the 2-NF stock solution (from step 1) and 31.5 µl of the 4-NQO stock solution (from step 2) directly into the positive control well of the 24-well dilution plate containing 1.1 ml sterile water (page 6), mix well and use this mixture as the positive control for both the TA98 and TAMix cultures. The positive control chemical well now contains 2.7 µg/ml 2-NF and 0.67 µg/ml 4-NQO.

This results in a final assay concentration of 2 µg/ml for 2-NF and 0.5 µg/ml for 4-NQO. Combined, the chemicals should result in ≥ 25 positive wells for both the TA98 and TAMix cultures.

B. With S9: (Refer to Appendix B for the preparation of S9 Mix)

1. Prepare a 125 µg/ml stock solution of 2-aminoanthracene (2-AA) in DMSO.
2. Transfer 63 µl to the positive control (+) well of the 24-well dilution plate containing 1.1 ml sterile water (page 6). The positive control chemical well now contains 6.76 µg/ml of 2-AA.

2-aminoanthracene at a final assay concentration of 5 µg/ml, should result in ≥ 25 positive wells for both the TA98 and TAMix cultures.

Appendix B Preparation of S9 Mix

Stock Solutions for Preparing 30% S9 mix

Prepare the following reagents using the amount of compound given below and bring the volume up to the value shown in the 'Total Volume' column using de-ionized or distilled water. Sterilize each reagent as noted. Store each reagent at the temperature given.

Stock	Reagent	Total Volume	Amount	Storage
1.00 M	KCl ^a	50 ml	3.728 g	4°C
0.25 M	MgCl ₂ ·6H ₂ O ^a	50 ml	2.541 g	4°C
0.20 M	Glucose-6-phosphate Na salt ^b	10 ml	0.564 g	-20°C
0.04 M	NADP Na salt ^b	10 ml	0.306 g	-20°C
0.20 M	NaH ₂ PO ₄ buffer ^{a*}	1 liter	31.200 g	4°C

^a autoclave

^b filter sterilize

* Add 31.2 g NaH₂PO₄·2H₂O to 750 ml de-ionized or distilled water. Adjust pH to 7.4 with NaOH. Add water to 1 liter.

Note: The S9 cofactor kit (Art. No. APCO-0800) available separately from Aniara contains all the above reagents in 3 individual ready-to-use solutions (Buffer-salts, G-6-P and NADP).

Preparation of 30% S9 mix using self-made reagents (for 1 test sample)

Keep all (thawed) reagents on ice.

Immediately before use, prepare a 30% S9 mix by combining the volumes of reagents listed below in a sterile tube:

Stock	Reagent	Volumes
1.00 M	KCl	0.033 ml
0.25 M	MgCl ₂ ·6H ₂ O	0.032 ml
0.20 M	Glucose-6-phosphate	0.025 ml
0.04 M	NADP	0.100 ml
0.20 M	NaH ₂ PO ₄ buffer	0.510 ml
	S9 fraction	0.300 ml
Final Volume =		1.000 ml

The final concentration of S9 in the assay is 1.8%.

Preparation of 30% S9 mix when using the S9 Cofactor Kit PCO-0800 (for 1 test sample)

Keep all (thawed) reagents on ice.

Immediately before use, prepare a 30% S9 mix by combining the volumes of reagents listed below in a sterile tube:

Solution	Volumes
S9 - Buffer-Salts	0.575 ml
S9 - G-6-P	0.025 ml
S9 - NADP	0.100 ml
S9 fraction	0.300 ml
Final Volume =	1.000 ml

Appendix C Acceptable Values

	TA98	TAMix
Without S9:		
Solvent control	≤8	≤8
2-NF (2.0 µg/ml)+4-NQO (0.5 µg/ml)	≥25	≥25
With S9:		
Solvent control	≤8	≤8
2-AA (5.0 µg/ml)	≥25	≥25

Mean of replicates

Appendix D Measurement of Cytotoxicity (optional)

If you want to test your sample dilutions for cytotoxicity, please proceed as follows:

Prepare twice the amount of exposure cultures (500 µl) with TA98 as shown below:

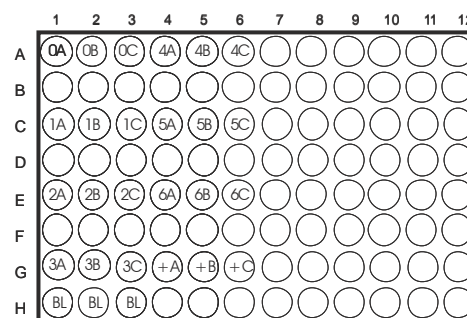
	TA98 no S9
Sterile water	30 µl
Exposure Medium working solution	50 µl
Sample	370 µl
Bacteria	50 µl
S9 mix	-

Note : Cytotoxicity of a sample may be different in different strains and in the presence and absence of S9. If only one strain is used for cytotoxicity (cell growth) measurement, we suggest to use TA98 as the TAMix consists of several strains.

1. Before incubation of the 24-well exposure plate for 90 minutes, transfer 250 µl to a 96-well plate by using an 8-channel pipettor with 4 evenly spaced tips (beginning with the low sample concentrations = columns 1, 3 and 5)
2. Add water to 3 wells as blank
3. Measure the OD₆₀₀ of the wells of the 96-well plate using a plate reader
4. Incubate the 96-well plate together with the exposure plates for 90 minutes at 37°C and 250 rpm
5. After the 90 minute incubation, remove the plates from the environmental shaker.
6. Proceed with the 24-well plates as described under 'Addition of Indicator Medium' on page 8.
7. Mix the wells of the 96-well plate, rows 1, 3, 5, then 2, 4, 6
8. Measure the OD₆₀₀ of the wells in the 96-well plates using a plate reader
9. Calculate cytotoxicity (CT) according to the equation below:

$$CT = 100 - 100 \left(\frac{S_{t=90} - S_{t=0}}{NC_{t=90} - NC_{t=0}} \right)$$

- CT Cytotoxicity in %
 S Mean OD₆₀₀ of sample, corrected for Blank
 NC Mean OD₆₀₀ of negative control, corrected for Blank
 t = 0 Initial value at t = 0
 t = 90 Final value after exposure



Note: This pre-screen procedure can only identify substances that arrest growth or lyse bacteria within 90 minutes. Substances which take longer to kill bacteria may go unnoticed and may lead to a drop of the number of positive wells to zero at cytotoxic concentrations.

Appendix E

References

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S. Flückiger-Isler, M. Baumeister, K. Braun, V. Gervais, N. Hasler-Nguyen, R. Reimann J. van Gompel, H.-G. Wunderlich, G. Engelhardt, Assessment of the performance of the Ames II™ assay: A collaborative study with 19 coded compounds. *Mutation Res.* (2004) 558, 181-197.

M. Kamber, S. Flückiger-Isler, G. Engelhardt, R. Jaechh, E. Zeiger; Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity; *Mutagenesis* (2009) 24, 359-366.

24 WELL DILUTION PLATE

0	4				
1	5				
2	6				
3	+				

24 WELL EXPOSURE PLATE

0A	4A	0B	4B	0C	4C
1A	5A	1B	5B	1C	5C
2A	6A	2B	6B	2C	6C
3A	+A	3B	+B	3C	+C

384 WELL PLATE
