ANIARA

Manufactured By: Xenometrix AG

Ames MPF™ 98/100
Microplate Format Mutagenicity Assay

S. typhimurium TA98 and TA100

Instructions for use
Version for semi-solid strain stocks

Please note: Items are shipped at ambient temperature with cooling elements. Kit contents will be fully active **if shipment is received within 10 days from dispatch and stored immediately as indicated on the individual items and as described on page 3 of this manual**. If components are damaged or if any problems occur, please contact Aniara by phone: 866-783-3797; fax: 513-573-9241, or Email: info@aniara.com

For Research use only **Version 4.5_S January 2012**

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NOTE 1:

This manual applies to the following versions of the assay with <u>semi-solid</u> strain stocks:

Article No.	Kit size*	Lyophilized liver S9	Positive Controls[#]
AA01-210	1	-	-
AA01-210-S1-P (Aroclor 1254-induced S9)	1	+	2-NF, 4-NQO, 2-AA
AA01-210-S2-P (PBN- induced S9[§])	1	+	2-NF, 4-NQO, 2-AA
AA10-210	10	-	-
AA10-210-S1-P (Aroclor 1254-induced S9)	10	+	2-NF, 4-NQO, 2-AA
AA10-210-S2-P (PBN-induced S9[§])	10	+	2-NF, 4-NQO, 2-AA

* Sufficient for 1 or 10 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 480 measurements (10 sample kit) per strain.

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

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

For the kit versions with liquid strain stocks please refer to the manual shipped with those kits.

NOTE 2:

This kit can be used in combination with any other Ames kit available from Aniara (Ames II, Ames MPF™ 1535, Ames MPF™ 1537, Ames MPF™ E.coli Combo, Ames MPF™ E.coli pKM and Ames MPF™ E.coli uvrA)

NOTE 3:

Please read carefully the entire manual before starting the experiments!

Ames MPF™ 98/100 Mutagenicity Assay
Table of Contents

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.

A chemical's mutagenic potential is assessed by exposing these *his-* organisms to varying concentrations of chemical 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* strains TA98 and TA100. TA100 is for the detection of base substitution mutations and TA98 is for the detection of frameshift mutations.

Assay Description

Approximately 10^7 *his-* bacteria are exposed to 6 concentrations of a test agent, as well as a positive and a negative control, for 90 minutes in medium containing sufficient histidine to support approximately two cell divisions. After 90 minutes, the exposure cultures are diluted in 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 solvent (negative) 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 test chemical relative to the solvent controls indicates that the chemical is mutagenic in the Ames MPF™ 98/100 assay.

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

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Genotypes of the TA98 and TA100 *Salmonella typhimurium* strains

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
TA98	*hisD3052*	Frameshifts	GCGCGCGC	*rfa*	*uvrB*	yes
TA100	*hisG46*	Base-pair subst.	GGG	*rfa*	*uvrB*	yes

rfa: This mutation leads to a defective lipopolysaccharide (LPS) layer that coats the cell surface, making the bacteria more permeable to bulky chemicals and non-pathogenic (Mortelsmans and Zeiger (2000), Mutat. Res. 455, 29-60).

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 MPF™ 98/100 Mutagenicity Assay kit contains the following components and should be stored as indicated:

-70° C to -80° C:

- Vials containing *Salmonella* strains (TA98, TA100)

Note: The strains are shipped with cool packs, but not frozen. Upon arrival they must be immediately 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 included, see Note 1 at beginning of document for available kit configurations)
- Dissolved positive controls

4° C:

- Positive controls before reconstitution (if provided, see Note 1 at beginning of document)
- S9 100/1537 Booster solution (provided only in kits with S9)

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

- Growth Medium
- Exposure Medium
- Indicator Medium

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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)

- 50 ml tubes with (filter) caps
- 24-well plates
- 384-well microtiter plates
- 96-well microtiter plate
- Reagent reservoirs
- 5 ml and 10 ml pipettes
- Spectrophotometer cuvettes
- Solvents for sample dilution and solvent control
- S9 buffer components*

Included in some kit versions only (see Note 1 at beginning of this manual):
- Positive control chemicals: 2-nitrofluorene and 4-nitroquinoline N-oxide (for tests without S9) and 2-aminoanthracene (for tests with S9) and
- Liver S9 fraction (Aroclor 1254 or Phenobarbital/β-Naphtoflavone-induced), including S9 100/1537 Booster solution

***S9 Cofactor kit (Art. No. APCO-0800)**
A ready-to-use kit available separately from Aniara containing phosphate buffer pH 7.4, $MgCl_2$, 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.

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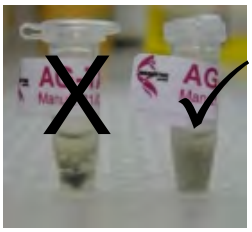
ASSAY PROCEDURE - DAY 1:
Overnight Culture Preparation

Using sterile technique, prepare overnight cultures of TA98 and TA100 by performing the following steps:

1. Remove the vials with the bacteria from the freezer. Let stand at ambient temperature for about 5 minutes. Add 200 µl of GM medium to each vial.
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 'TA100'.
   - Add a few milliliter of Growth Medium to a tube labeled 'Negative Control'.
   - Add 10 µl ampicillin (50 mg/ml) to tubes 'TA98' and 'TA100'.

Note: For the following dispersion step we recommend to use tips with 300 µl capacity and the volume of the pipette set to 200 µl. If only 200 µl capacity tips are available we recommend to set the pipette to 100 µl. 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.

4. 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.



5. Pipette 25 µl of the dispersion into the corresponding tubes prepared under (3).
6. 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.
7. 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!

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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.

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ASSAY PROCEDURE - DAY 2:
Determination of the OD_{600} Values of the Overnight Cultures

1. After the overnight incubation measure the OD_{600} 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 OD_{600} reading of each cuvette containing overnight culture and the negative control.
6. Multiply each OD_{600} reading by ten to obtain the actual optical density and enter the values into the chart below.
7. Verify that the OD_{600} values for the cultures is **at least 2.0***, and that the OD_{600} 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 OD_{600} <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 upon receipt at ≤ -70° 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 OD_{600} 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
TA100		≥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 OD_{600} value determined for your spectrophotometer. The value of 2.0 is a reasonable approximation for many instruments.*

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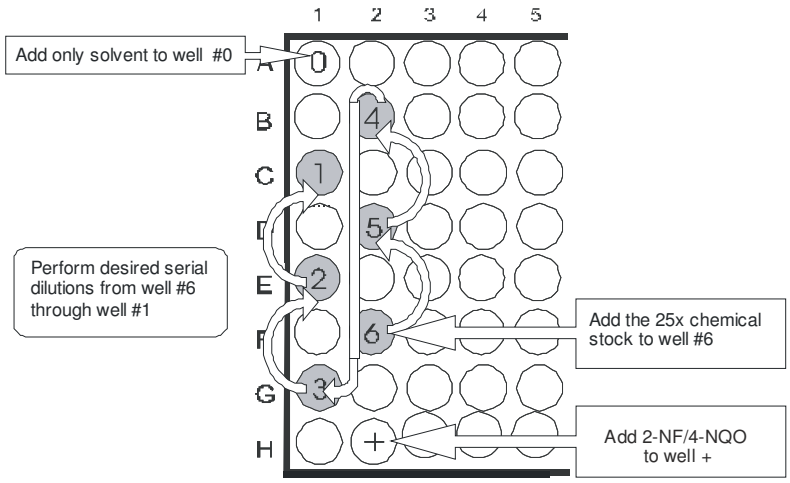
Preparation and Dilution of the Chemical Stock

Note: To determine the optimal dose range of chemical to use in the Ames MPF assay, refer to Appendix D for the *optional* protocol for the 'Pre-screen Determination of Optimal Dose Range'.

Note: The following procedure describes an assay for 1 test compound at 6 concentrations in triplicate with negative (solvent) and positive control.
If S9 fraction is to be used, refer to Appendix B for the preparation of the 30% S9-mix.

Prepare the dilutions of the chemical stock to be used in the assay by performing the following steps:



1. Prepare the positive control chemical stock solutions of 4-NQO and 2-NF (for the use without S9) and 2-AA (for the use with S9) as described in Appendix A.
2. Prepare a chemical stock which is 25 times more concentrated than the highest concentration to be used in the assay. 25X stock concentrations are necessary to achieve the desired 1X assay concentration due to the dilution into the exposure cultures.
3. Unwrap the 96-well chemical dilution plate and place it on the paper template for the 96-well plate layout. Be sure that the plate is oriented correctly.
4. Transfer the 25X stock to well #6 of the chemical dilution plate.

Note: Volumes added at this and the next steps are dependent on the dilution factor to be used. 30 µl of each stock concentration will be required for dosing each strain (60 µl 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 20 µl.

When the calculated volume of the sample in well #6 is > 360 µl we recommend to use a 24-well plate as the chemical dilution plate.

You can use the **"Ames MPF dilutions calculator"** which is included in the **Ames calculation sheet** which can be downloaded from the Aniara homepage www.aniara.com.

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5. Add the appropriate amount of solvent to wells #0-5 of the plate.
6. Perform the first dilution step by transferring the test chemical from well #6 to #5, and mix by pipetting up and down thoroughly.
7. Complete the serial dilutions from well #5 to #4, #4 to #3, #3 to #2, and #2 to #1. DO NOT transfer chemical to the 0 well. This is the solvent control for the assay and should contain solvent only.
8. Add 80 µl of of the positive control compounds, 2-NF/4-NQO (prepared in step 1) to the well labeled '+'

Note: The positive control 2-AA for tests in the presence of S9 should be added directly to the corresponding wells of the 24-well exposure plates. Please refer to the table in Appendix A.

9. Fill in the chart below with the chemical and solvent used along with the dilutions performed. (An example in gray is given for a stock at 125 mg/ml, 2x dilutions, triplicates, for 2 strains, with and without S9).

Chemical: _____ **25X Stock Conc.:** _____ **Solvent**: _____

Well	Add 25 X Stock	Add Solvent	Transfer to next well, mix	Exposure Conc. (1X)
0	-	140 µl	-	Solvent control
1	-	140 µl		0.16 mg/ml
2	-	140 µl	140 µl ↑	0.31 mg/ml
3	-	140 µl	140 µl ↑	0.63 mg/ml
4	-	140 µl	140 µl ↑	1.25 mg/ml
5	-	140 µl	140 µl ↑	2.5 mg/ml
6	280 µl	-	140 µl ↑	5 mg/ml

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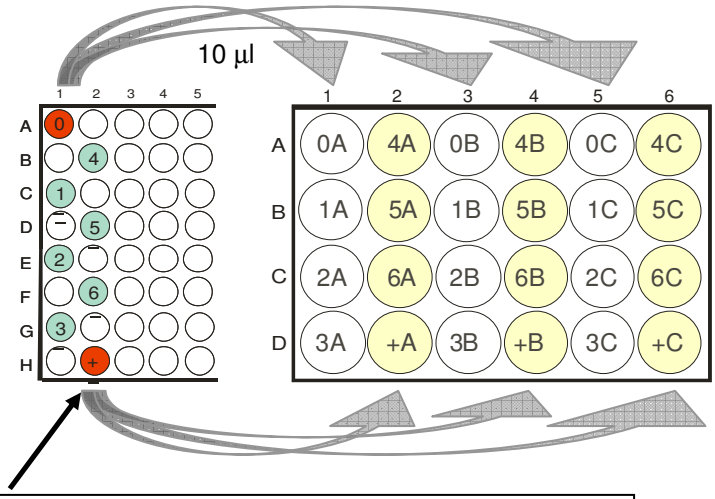
Transfer of Chemical to the Exposure Plates

Unwrap the 24-well exposure plates (2 plates per strain -/+ S9). Place the first plate to be used on the paper template for the 24-well plate layout. Be sure that the plate is oriented correctly.



Add the positive control chemicals 2-NF/4-NQO to well +. The positive control 2-AA for tests with S9 is added directly to the corresponding wells of the 24-well exposure plates.

Transfer the chemical dilutions by performing the following steps:

1. Label four 24-well plates with the strains ± S9 that are to be used (e.g. TA98 - S9).
2. Using an 8-channel pipettor with 4 evenly spaced tips (every other channel), transfer 10 µl from the first column of the chemical dilution plate (wells #0-3) to the bottom of columns 1, 3, and 5 of all 24-well plates.
3. Transfer 10 µl from the second column of the chemical dilution plate (wells #4-6 and +) to the bottom of columns 2, 4, and 6 of the 24-well plates without S9.
4. Remove the lowest tip and transfer 10 µl from the second column of the chemical dilution plate (wells #4-6 without +) to the bottom of columns 2, 4, and 6 of the 24-well plates with S9.
5. Add 10 µl of the positive control 2-AA directly into the corresponding wells of the 24-well plates with S9.

Caution: Different concentrations of volatile compounds should not be dispensed in a single 24-well plate. Use 1 tube or one plate per concentration.

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Preparation of Exposure Cultures

Note: For tests with *Salmonella* strains in the presence of S9 the Salmonella Exposure Medium should be supplemented with the "S9 100/1537 Booster Solution". Refer to Appendix B for its preparation and for more information.

TA98 without S9:

1. Add **6.3 ml Exposure Medium** to a reservoir.
2. Swirl the contents of the culture tube and add **0.7 ml** of the overnight culture to the Exposure Medium (1 : 10 dilution).
3. Using an 8-channel pipettor, mix the contents of the reservoir by pipetting up and down thoroughly and transfer 240 µl to all wells of the 24-well exposure plate. DO NOT touch the chemical on the bottom of the wells with tips!

TA98 with S9 (see Appendix B for the preparation of 30% S9 mix):

1. Add **5.25 ml Exposure Medium/Booster Solution** to a pipetting reservoir.
2. Add **0.7 ml** of bacterial culture to the reservoir. Swirl the contents of the culture tube before removing the sample in case any settling has occurred.
3. Transfer **1.05** ml of the 30% S9 mix to the reservoir containing overnight culture and Exposure Medium/Booster Solution, mix.
4. **Immediately** transfer 240 µl to each well of the 24 well exposure plate.

The final concentration of S9 in the culture is 4.5%.

Note: Two tips of the 8-channel pipettor dispense into each well of the 24-well exposure plate. Therefore, setting the pipettor to 120 µl will allow for the transfer of 240 µl to each well.

TA100 without S9:

1. Add **6.65 ml Exposure Medium** to a reservoir.
2. Swirl the contents of the culture tube and add **0.35 ml** of the TA100 culture to the Exposure Medium (1 : 20 dilution).
3. Using an 8-channel pipettor, mix the contents of the reservoir by pipetting up and down thoroughly and transfer 240 µl to all wells of the 24-well exposure plate. DO NOT touch the chemical on the bottom of the wells with tips!

TA100 with S9 (see Appendix B for the preparation of 30% S9 mix):

1. Add **5.6 ml Exposure Medium/Booster Solution** to a pipetting reservoir.
2. Add **0.35 ml** of the TA100 culture to the reservoir. Swirl the contents of the culture tube before removing the sample in case any settling has occurred.
3. Transfer **1.05** ml of the 30% S9 mix to the reservoir containing bacterial culture and Exposure Medium/Booster Solution, mix.
4. **Immediately** transfer 240 µl to each well of the 24 well exposure plate.

The final concentration of S9 in the culture is 4.5%.

Note: Two tips of the 8-channel pipettor dispense into each well of the 24-well exposure plate. Therefore, setting the pipettor to 120 µl will allow for the transfer of 240 µl to each well.

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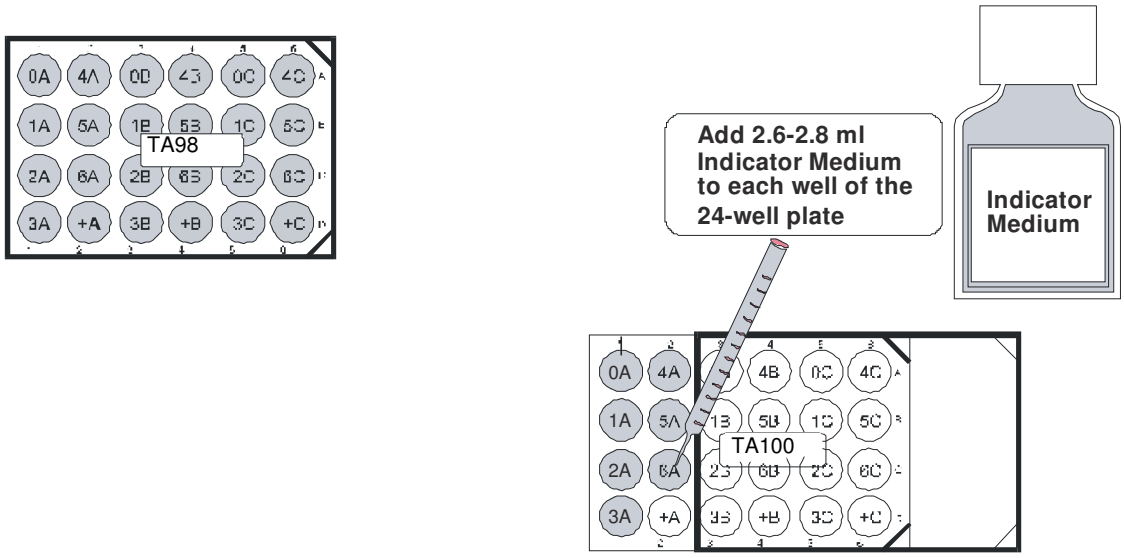
Secure the 24-well plates to the base of a 37° C environmental shaker. Incubate the plates for 90 minutes at 37° C, 250 rpm. Record the time the incubation was started in the chart below.

Time Incubation Started	Time Incubation Stopped	Date

Addition of Indicator Medium



1. After the 90 minute incubation, remove the exposure plates from the environmental shaker. Record the time the incubations were stopped in the chart on page 11.
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.

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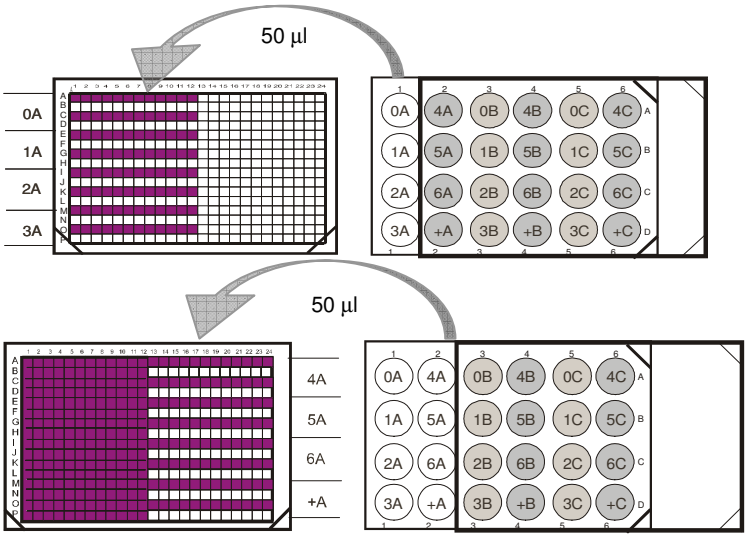
Transfer of Exposed Cultures from 24-well Plate to 384-well Plates



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. TA98-1 -S9, TA98-2 -S9, TA98-3 -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

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Incubation of Revertant Colony Selection Plates

1. 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.
2. 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		

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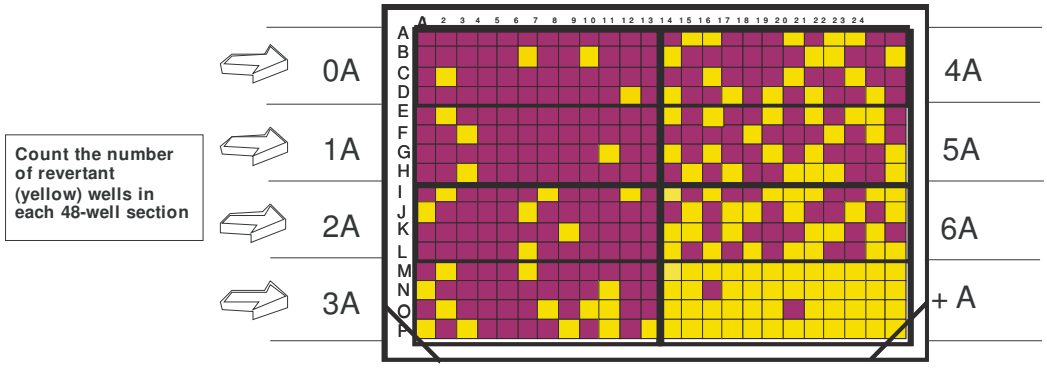
ASSAY PROCEDURE - DAY 4

Plate Scoring



1. 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 on the previous page.
2. 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 chart below. 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			
+			

TA100 # Positive Wells -S9

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

TA100 # Positive Wells +S9

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

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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 Ames MPF 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 (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.

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 solvent control.

Example: Mean ± 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 may be downloaded from the Aniara homepage www.aniara.com.

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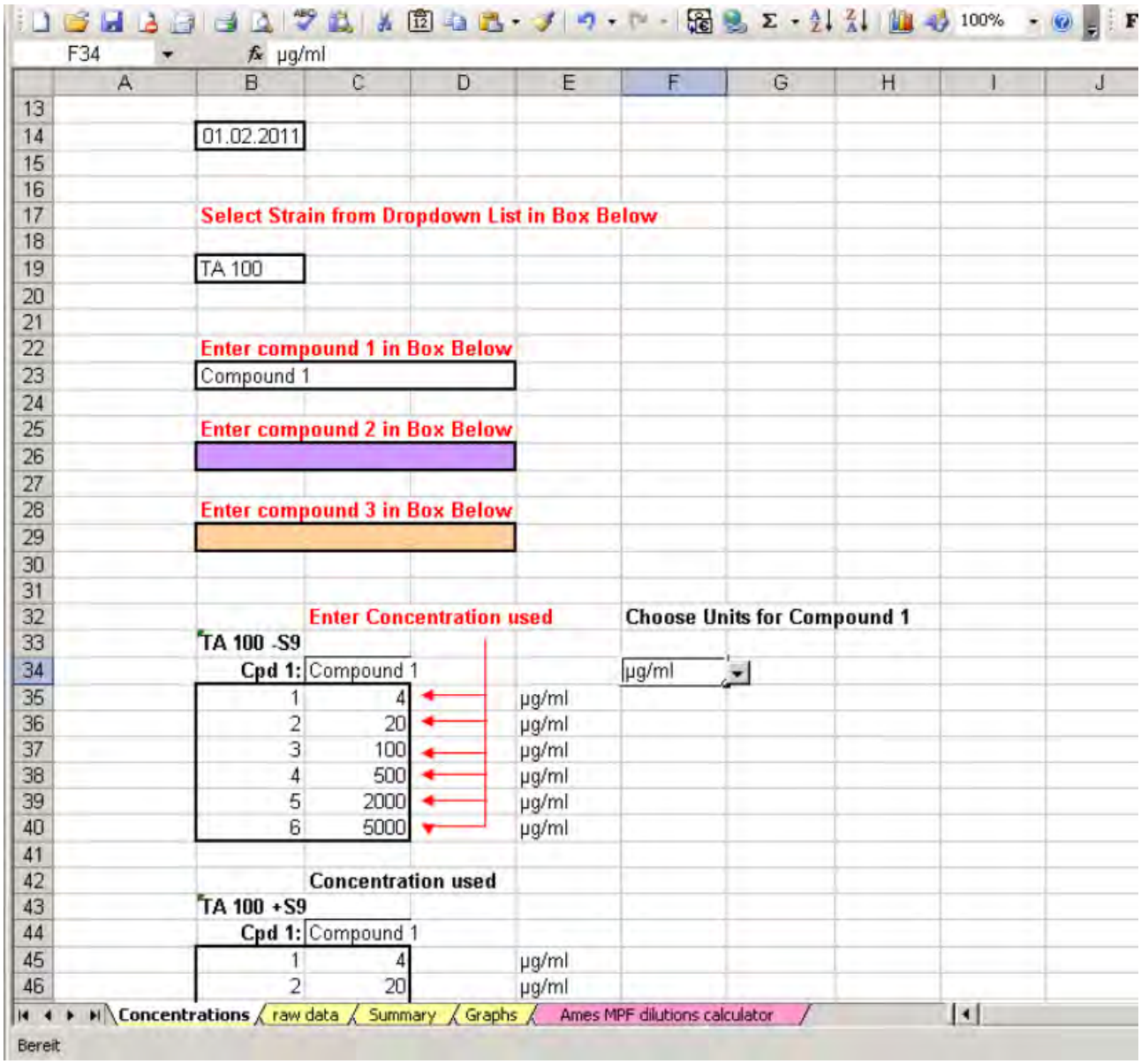
Layout of the Xenometrix Excel Calculation Sheet

Sheet 1: Concentrations



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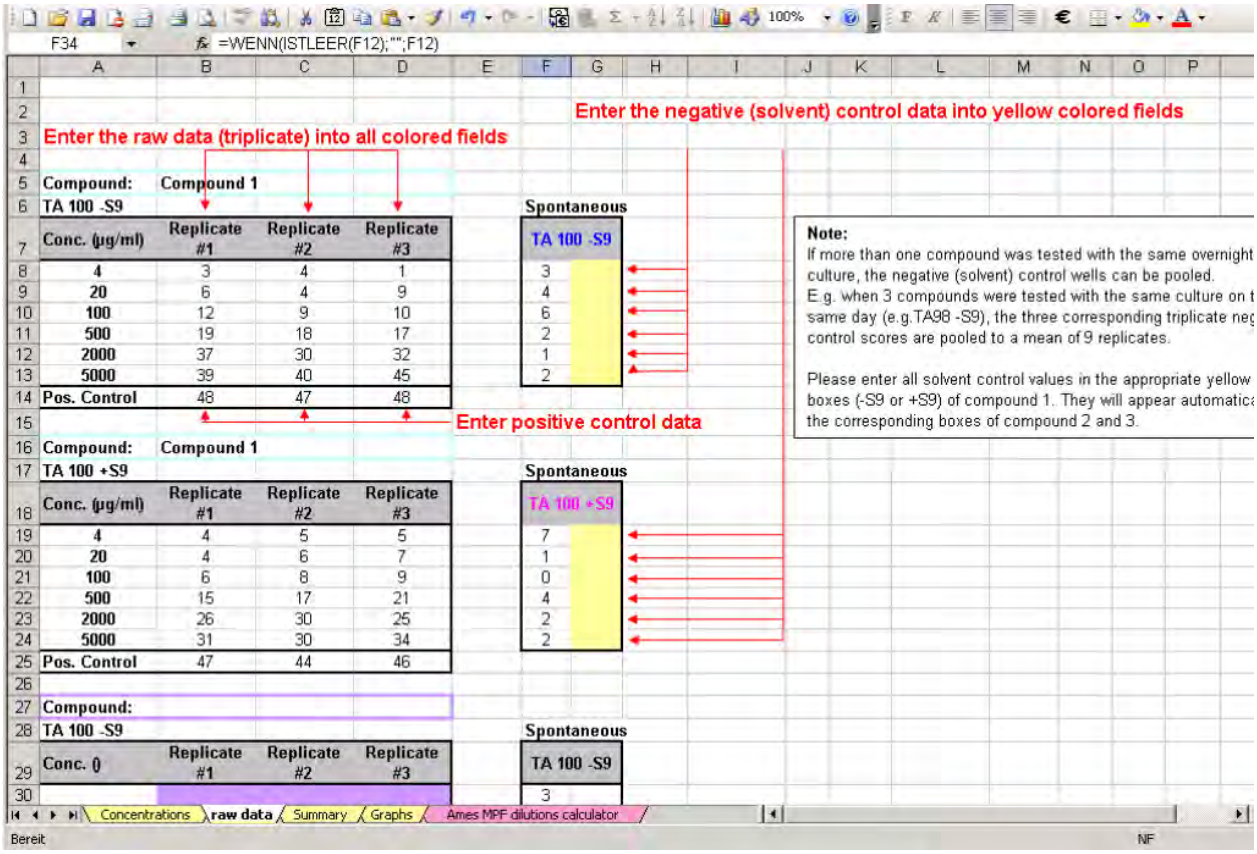
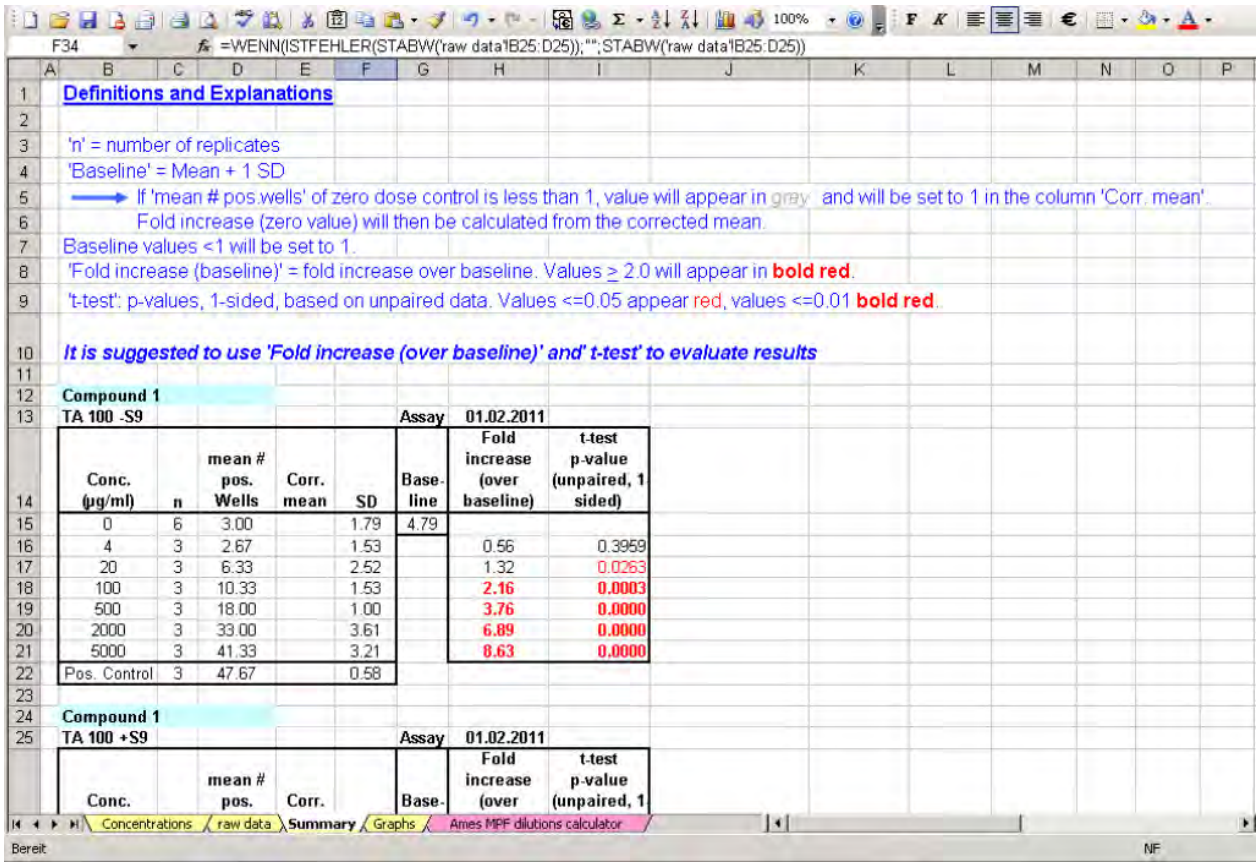
Sheet 2: Raw Data



Sheet 3: Summary



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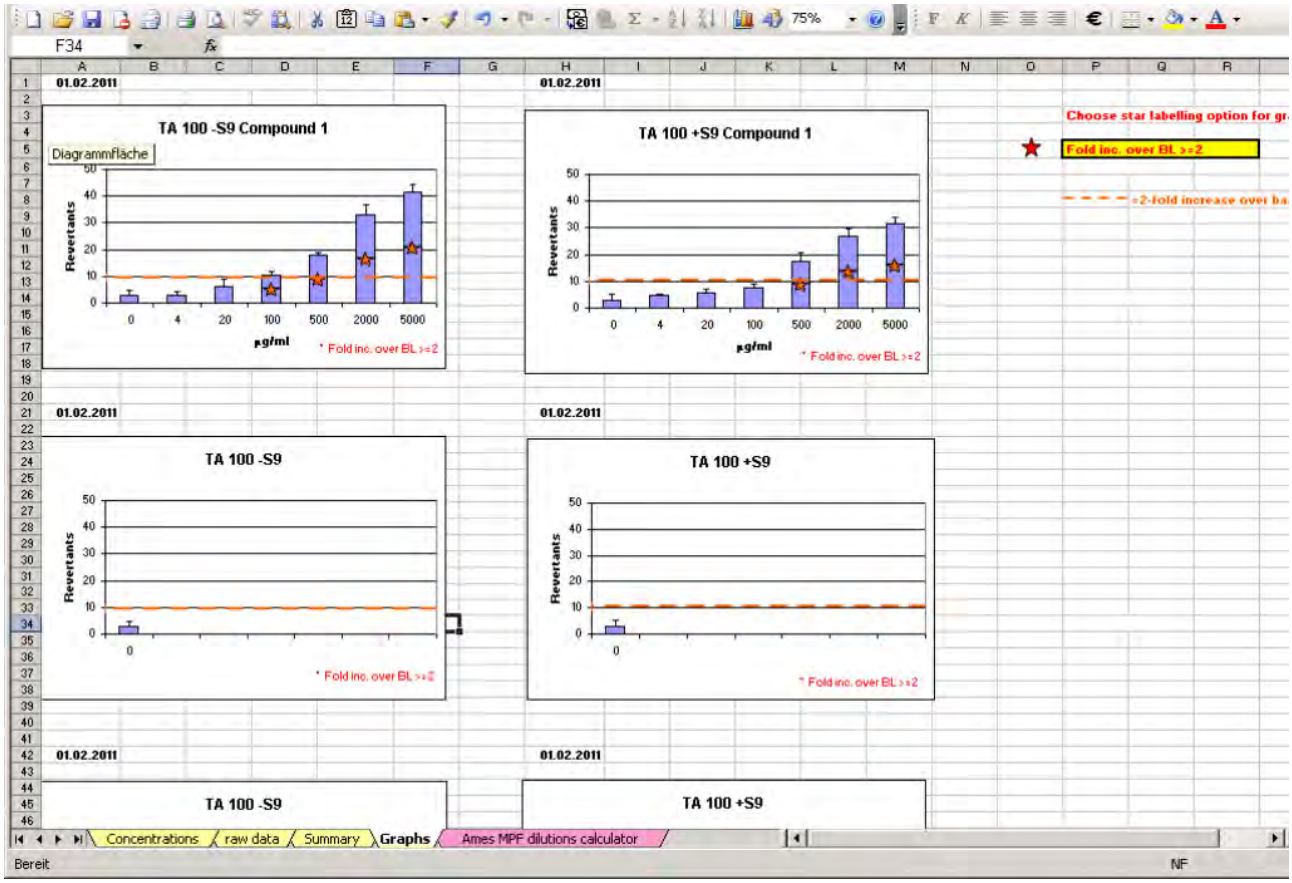
Sheet 4: Graphs



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Appendix A
Preparation of Positive Controls

Positive Controls without S9

Strain	Chemical	25X Stock	Final Conc.
TA98 and TA100	2-NF/4-NQO	50 µg/ml/2.5 µg/ml	2 µg/ml/0.1 µg/ml

Positive Control with S9

	With Aroclor 1254 S9			With Phenobarbital/β-Naphtoflavone S9		
	Chemical	25X Stock	Final Conc.	Chemical	25X Stock	Final Conc.
TA98	2-AA	25 µg/ml	1 µg/ml	2-AA	12.5 µg/ml	0.5µg/ml
TA100	2-AA	62.5 µg/ml	2.5 µg/ml	2-AA	31.25 µg/ml	1.25 µg/ml

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 5 µg/ml stock solution of 4-nitroquinoline N-oxide (4-NQO) in DMSO.
3. Combine 40 µl of the 2-NF stock solution (from step 1) with 40 µl of the 4-NQO stock solution (from step 2) directly in the positive control well of the 96-well chemical dilution plate and use this mixture as the positive control for both the TA100 and TA98 cultures. The positive control chemical well now contains 2.5 µg/ml of 4-NQO and 50 µg/ml 2-NF. This results in a final assay concentration of 0.1 µg/ml for 4-NQO and 2 µg/ml for 2-NF.

The positive control should result in ≥ 25 positive wells both cultures.

Note: Na-azide is not recommended as positive control for TA100!

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B. With S9:

Note:	Differently induced S9 preparations vary in their mutagenic potencies for the positive control 2-AA. Therefore, the 2-AA concentrations differ when using either Aroclor 1254- or Phenobarbital/β-Naphtoflavone-induced S9.

B1. With Aroclor 1254-induced S9 (Refer to Appendix B for the preparation of S9 Mix)

Note:	In previous versions of the instruction manuals we recommended to use a final 2-AA concentration of 5 µg/ml although the strains show different sensitivities to 2-AA (refer to the S9 certificate of analysis). The reason was to simplify the handling of the transfer to the 24-well exposure plates. When using **Aroclor** induced S9, a 2-AA concentration of 5 µg/ml may be used if the preparation of different concentrations seems to be too laborious. Since TA98 is more sensitive to 2-AA than TA100, the use of strain-specific 2-AA concentrations will better indicate eventual problems with the condition of a culture.

Prepare a 62.5 µg/ml stock solution of 2-aminoanthracene (2-AA) in DMSO.

TA100:

Transfer 10 µl of the 62.5 µg/ml stock to the positive control triplicate wells (A+, B+ and C+) in the TA100 24-well exposure plate.
2-aminoanthracene, at a final assay concentration of 2.5 µg/ml, should result in ≥ 25 positive wells.

TA98:

1. Dilute the 62.5 µg/ml 2-AA stock solution 1 : 2.5 to 25 µg/ml in DMSO.
2. Transfer 10 µl to the positive control triplicate wells (A+, B+ and C+) in the TA98 24-well exposure plate.
   2-aminoanthracene, at a final assay concentration of 1.0 µg/ml, should result in ≥ 25 positive wells.

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

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B2. With Phenobarbital/β-Naphtoflavone-induced S9 (Refer to Appendix B for the preparation of S9 Mix)

Note: In previous versions of the instruction manuals we recommended to use a final 2-AA concentration of 5 µg/ml although the strains show different sensitivities to 2-AA (refer to the S9 certificate of analysis). The reason was to simplify the handling of the transfer to the 24-well exposure plates.
Please use the 2-AA concentrations indicated below when using **Phenobarbital/β-Naphtoflavone** induced S9, since high concentrations of 2-AA (5 µg/ml) can be toxic to TA100.

Prepare a 31.25 µg/ml stock solution of 2-aminoanthracene (2-AA) in DMSO.

TA100:

Transfer 10 µl of the 31.25 µg/ml stock to the positive control triplicate wells (A+, B+ and C+) in the corresponding 24-well exposure plate.
2-aminoanthracene, at a final assay concentration of 1.25 µg/ml, should result in ≥ 25 positive wells

TA98:

1. Dilute the 31.25 µg/ml 2-AA stock solution 1 : 2.5 to 12.5 µg/ml in DMSO.
2. Transfer 10 µl to the positive control triplicate wells (A+, B+ and C+) in the TA98 24-well exposure plate.
   2-aminoanthracene, at a final assay concentration of 0.5 µg/ml, should result in ≥ 25 positive.

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Appendix B
Preparation of 30% S9-Mix

Note: Some batches of S9 can lead to signs of toxicity under certain conditions. We have observed that strains TA100 and TA1537 when tested with the positive control chemical 2-AA may not always reach the required acceptable value of ≥25 positive wells in the presence of the suggested final S9 concentration of 4.5%. A reduction of the S9 concentration may lead to higher values with 2-AA, but could also lead to a reduction of positive wells with other compounds which require high S9 concentrations.

We therefore ship our S9 fraction together with a "S9 100/1537 Booster Solution" to protect strains TA100 and TA1537 from possible toxic S9 effects. This solution will be mixed with the Exposure Medium when using S9 in the Ames MPF assay. Although TA98 and TA1535 are not or only marginally affected, the booster solution can be used for all *Salmonella* strains to simplify Exposure Medium handling.

Adding the "Booster Solution" to batches of S9 that do not actually need it has no negative effects on the performance of S9 with any of the 4 Ames MPF *Salmonella* strains.

The S9 100/1537 Booster Solution should not be used with *E.coli* strains which are tested in a different Exposure Medium.

Procedure for assays with S9:

Mix the S9 100/1537 Booster Solution with the Ames MPF Exposure Medium at a ratio 1 : 667 (e.g. 10 ml Exposure Medium + 15 µl Booster Solution). Prepare the required volume of Exposure Medium / S9 100/1537 Booster mixture.

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	$MgCl_2x6H_2O$[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_2PO_4 buffer[a*]	1 liter	31.200 g	4°C

[a] autoclave
[b] filter sterilize
[*] Add 31.2 g $NaH_2PO_4x2H_2O$ 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).

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

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	0.076 ml
0.25 M	$MgCl_2 \bullet 6H_2O$	0.074 ml
0.20 M	Glucose-6-phosphate	0.058 ml
0.04 M	NADP	0.230 ml
0.20 M	NaH_2PO_4 buffer	1.173 ml
	S9 fraction	0.690 ml
	Final Volume =	2.301 ml

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

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	1.323 ml
S9 - G-6-P	0.058 ml
S9 - NADP	0.230 ml
S9 fraction	0.690 ml
Final Volume =	2.301 ml

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Appendix C
Acceptable Values

	TA98	TA100
Without S9:		
Solvent control	≤8	≤8
2-NF (2.0 µg/ml)+4-NQO (0.1 µg/ml)	≥25	≥25
With S9:		
Solvent control	≤8	≤8
2-AA (0.5 - 2.5 µg/ml)	≥25	≥25

Mean of replicates

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Appendix D
Pre-screen Determination of Dose Range (optional)

If you test a compound for the first time, you may like to determine its cytotoxicity and solubility in a pre-screening assay before the Ames MPF™ 98/100 assay. After the pre-screening test you can choose the proper concentrations of compound to use. The top concentration of compound that can be used for the pre-screening assay can be based either on the highest concentration of compound that is soluble in the desired solvent or by individual requirements. The following pre-screening assay can then be used to evaluate the cytotoxic effects of the soluble concentrations.

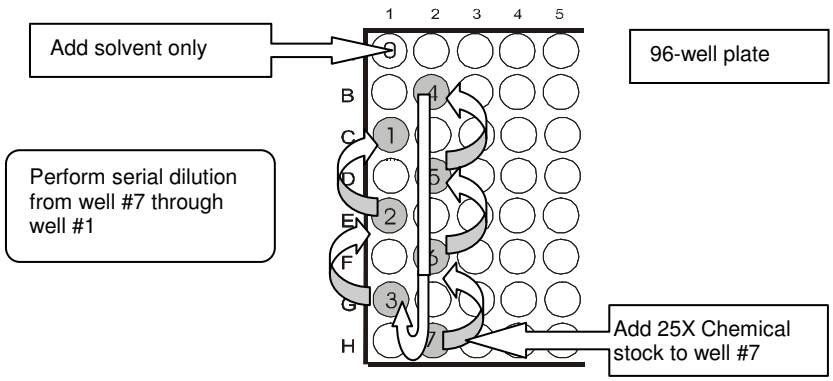
If the same overnight culture is used for the pre-screening assay and the Ames MPF™ 98/100 assay, then both assays have to be performed on the same day.

Either strain may be used for pre-screening but not all strains respond identically to toxic compounds. We suggest to use TA98 for this pre-screening.

Dilutions of the Chemical Stock

Prepare the chemical stock concentrations to be used in the assay by performing the following steps:

1. Prepare a chemical stock, which is 25 times more concentrated than the highest concentration to be used in the assay. 25X stock concentrations are necessary to achieve the desired 1X assay concentration due to the dilution into the exposure culture.
2. Unwrap a 96-well plate.
3. Transfer 44 µl of this 25X stock to well #7 of the 96-well plate (see figure below).
4. Add 30 µl of solvent to wells #0-6 of the chemical dilution plate. Solvent concentrations should be the same in all wells #0-7.
5. Perform a ½ log serial dilution of the test chemical by transferring 14 µl from well #7 to #6, and mix by pipetting up and down thoroughly.
6. Transfer 14 µl of the uniformly mixed solution from well #6 to chemical well #5, and mix by pipetting up and down thoroughly.
7. Complete this serial dilution from well #5 to #4, #4 to #3, #3 to #2 and #2 to #1.
   Do not transfer chemical to well 0. The 0 well is the solvent control and should contain solvent only.
8. Fill in the chart below with the chemicals and solvent used along with the dilutions performed.



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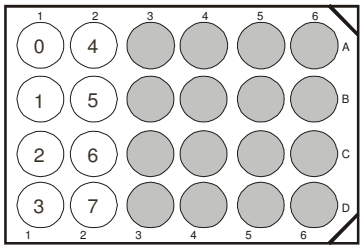
Chemical:_____ **Solvent**:_____

Well #	25X Conc.	Exposure Conc. (1X)
1		
2		
3		
4		
5		
6		
7		

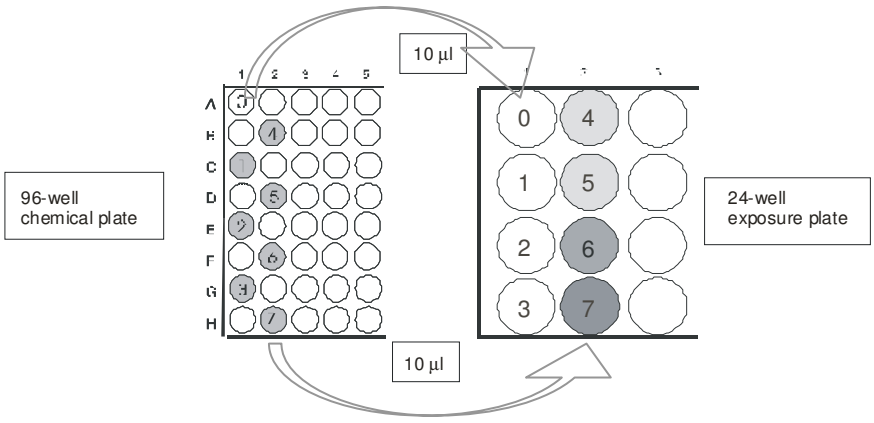
Transfer of Chemical to the Exposure Plate

Unwrap a 24-well plate for the pre-screen test using the dilutions indicated below. Label the plate (e.g. TA98 pre-screen)



Dose the culture with chemical by performing the following steps:

1. Using an 8-channel pipettor with 4 evenly spaced tips (every other channel), transfer 10 µl from the first column of wells #0 - 3 of the 96-well plate to column 1 of the 24-well plate.
2. Transfer 10 µl from the second column of wells #4-7 of the 96-well plate to column 2 of the 24-well plate.



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Preparation of Pre-Screen Exposure Culture

1. Dispense 2.7 ml Exposure Medium to a pipetting reservoir and add 0.3 ml of the overnight culture to the Exposure Medium. Swirl the contents of the culture tube before removing the sample in case any settling has occurred.
2. Using an 8-channel pipettor, mix the contents of the reservoir by pipetting up and down and transfer 240 µl to all wells of columns 1 and 2 of the 24-well exposure plate.

Note: Two tips of the 8-channel pipettor dispense into each well of the 24-well exposure plate. Therefore, setting the pipettor to 120 µl will allow for the transfer of 240 µl to each well.

3. Secure the 24-well plate to the base of 37° C environmental shaker. Incubate the plate for 90 minutes at 37° C, 250 rpm.

Determination of Dose Range

1. After 90 minutes, remove the 24-well plate from the shaker. Visually compare the turbidity of each well. Non-cytotoxic doses of the chemical should be turbid, like the solvent control (well 1). Highly cytotoxic doses will be clear due to bacterial lysis. If differences in turbidity are difficult to distinguish visibly, a 24-well plate reader set at 600 nm can be used to identify wells with reduced growth or with lysis. If no 24-well plate reader is available, the content of the wells of the 24-well plate can be transferred to a 96-well plate.
2. Select the lowest chemical concentration which showed cytotoxicity, or the highest soluble concentration as your highest concentration for the assay.

Note: This pre-screen procedure can only identify compounds that lyse bacteria within 90 minutes. Compounds which take longer to kill bacteria may go unnoticed and may lead to a drop of the number of positive wells to zero at high compound concentrations in the in the Ames MPF™ 98/100 mutagenicity assay.

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Appendix E
Optional Strain Handling to Reduce the Risk of Occasional Elevated Baseline Values

Salmonella TA100 and *E.coli* wp2 [pKM101] have a higher spontaneous reversion rate than the other strains available in the Ames MPF™ format. This leads to a higher frequency of overnight cultures with elevated baseline values making it difficult to evaluate mutagenic agents, especially weak ones. While Xenometrix makes every effort to sell bacterial strains with optimal sensitivities and low spontaneous reversion frequencies, emergence of unsuitable overnight cultures can still occur. Depending on the lot, storage, handling and growth conditions such unfavorable TA100 and wp2 [pKM101] overnight cultures typically occur with frequencies in the range of 5 - 10%. While this can be just inconvenient with readily available samples, it can be very frustrating and annoying with valuable samples or under conditions where results must become available in a very short time.

Xenometrix has therefore developed a method to minimize the risk of performing an experiment with a unsuitable TA100 or [pKM101] overnight culture. This risk reduction needs more media (not included in the basic kit), an additional day of experimentation and consumption of somewhat more plasticware.

Note: The spontaneous revertants of TA100 may already be scored after approximately 24 hrs, producing representative values for starting the actual experiment after 1 day. However, the spontaneous revertants of wp2 [pKM101] can not be reliably scored after 24 hrs. We suggest the following procedure schedule for testing *E.coli* wp2 [pkM101] or both strains concurrently. When only TA100 is tested the schedule indicated in *italics* may be used as an alternative.

Procedure:

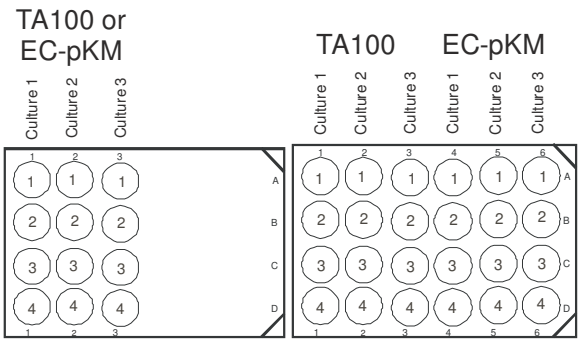
Overnight culture (Thursday evening*)(Monday evening)*:

Start 3 instead of 1 overnight cultures using stock from 1 vial.

Pre-experiment (Friday morning) *(Tuesday morning)*:

Record the OD_{600} of each overnight culture. Pipet 1 ml of each culture into fresh sterile 50 ml tubes. Add 1 ml of fresh growth medium to each tube and mix the contents by gently swirling. Store the diluted cultures in a refrigerator. Ensure sufficient aeration by using loose caps or tubes with filter caps (highly recommended to avoid airborne contaminations).

Perform a small Ames MPF assay with solvent only for the determination of the spontaneous reversion rate with each of the 3 undiluted overnight cultures. We recommend 4 replicates for each culture:



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Experimental day (Monday morning) *(Wednesday morning)*:

Score the 384-well plates obtained with the 3 cultures from Friday *(Tuesday)* for spontaneous revertants. Retrieve the culture with the best (lowest) spontaneous reversion rate from the refrigerator. Swirl the content of the tube. Add 2 ml of Growth Medium and 3 µl of ampicillin to the refrigerated culture and incubate at 37° C and 250 rpm for approximately 2 hrs until the OD_{600} reaches about 75% of the density of the original overnight culture.

Proceed to "Preparation and Dilution of the Chemical Stock" as described on page 7 to perform the actual Ames MPF assay.

Note: The same procedure can be used for the other Ames MPF™ strains. Due to their low spontaneous reversion rate the risk of obtaining an unfavorable overnight culture with the standard procedure is very small and the benefit of this new procedure is accordingly smaller.

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96 WELL CHEMICAL DILUTION PLATE

0											
	4										
1											
	5										
2											
	6										
3											
	+										

24 WELL 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

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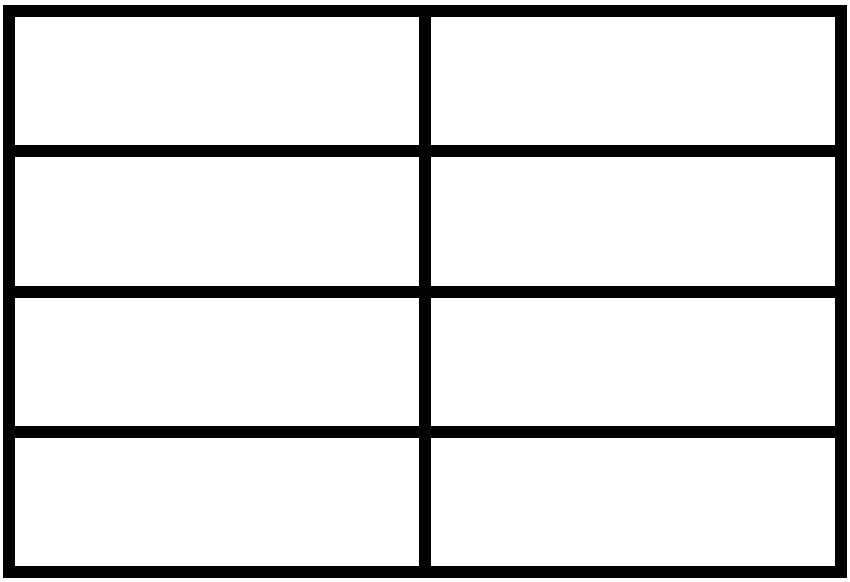
384 WELL PLATE



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