MPF™ YG

Microplate Format Mutagenicity Assay

S. typhimurium YG1021 or YG1024 or YG1041

Instructions for use

adapted from the Ames MPF™ Protocol

For Research use only **Version 1.1 April 2010**

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MPF™ YG Mutagenicity Assay
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NOTE:

Please read carefully the entire manual before starting the experiments!

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 assay described in this instruction manual is can be used with the *Salmonella typhimurium* strains YG1021, YG1024 or YG1041. It has not been tested with other YG strains. YG are plasmid-modified TA98 derivatives and detect mutagenic activity with different chemical sensitivity and specificity than TA98. YG1021 is a nitroreductase (NR) overproducing strain, YG1024 an O-acetyltranferase (OAT) overproducing strain, and YG1041 is a NR and OAT overproducing strain. The strains may be obtained from Dr. T. Nohmi of the National Institute of Hygienic Sciences,. Tokyo, Japan

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 three days, cells that have undergone the reversion to histidine prototrophy will grow into colonies. Metabolism by the bacterial colony 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 zero dose (solvent) 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 zero-dose controls indicates that the chemical is mutagenic in the assay.

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

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Storage Conditions

All media should be stored at room temperature.

Required equipment and consumables NOT included

- Tester strains YG1021, YG1024 and/or YG1041 (the strains may be obtained from Dr. T. Nohmi of the National Institute of Hygienic Sciences,. Tokyo, Japan)
- Na-Ampicillin, 50 mg/ml (YG1021, YG1024, YG1041)
- Tetracycline-HCl, 6.25 mg/ml for YG1021 and YG1024
- Kanamycin, 25 mg/ml for YG1041
- Solvents for sample dilution and zero dose control
- S9 and S9 buffer components
- Positive control chemicals: 2-nitroflourene (YG1021 and YG1024 without S9), 1-nitropyrene (YG1041 without S9), 2-aminoanthacene (all strains with S9)

- Environmental shaker capable of 37°C, 250 rpm incub ations
- 37°C dry incubator
- Light box 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 and sterile tips
- 8-Channel repeating pipettor and sterile tips

Note: all plasticware has to be sterile!

- 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

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 an overnight culture of a YG strain by performing the following steps:

1. Remove the vial of the frozen strain to be used from the -70°C storage and allow it to thaw at room temperature. Use bacteria within 5 minutes after thawing.
2. Prepare the overnight culture by performing the following steps:
   - Add 10 ml Growth Medium to one 50 ml culture tube labeled with the appropriate strain.
   - Add a few milliliter of Growth Medium to a tube labeled ‘Negative Control’.
   - For YG1021 and YG1024 cultures: Add 10 µl ampicillin (50 mg/ml) and 10 µl tetracycline (6.25 mg/ml) to the tube ‘YG’.
     For YG1041 culture: 10 µl ampicillin (50 mg/ml) and 10 µl kanamycin (25 mg/ml) to the tube ‘YG’.
   - Re-suspend the thawed bacterial stock culture and add 10 µl directly into the medium of the culture tube labeled ‘YG’.
3. Place the caps **loosely** on the tubes, and secure lightly with tape. This insures that the cultures are sufficiently aerated during 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.
4. Incubate the tubes in an environmental shaker set at 37°C, 250 rpm for 14 -16 hrs.

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).

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ASSAY PROCEDURE - DAY 2:

Determination of the OD_{600} Values of the Overnight Culture

1. After the overnight incubation measure the OD_{600} of the cultures:
2. Add 900 µl of Growth Medium to three cuvettes.
3. Swirl the contents of the culture tube and remove a 100 µl aliquot of cells from the overnight culture tube as well as from the negative control tube, and add it to a cuvette containing 900 µl Growth Medium. Mix the contents of the cuvette.
4. Blank the spectrophotometer at 600 nm using the cuvette with 900 µl Growth Medium only.
5. Take the OD_{600} reading of the cuvette containing overnight culture and the negative control.
6. Multiply each OD_{600} reading by ten to obtain the actual optical density for the overnight cultures and enter the values into the chart below.
7. Verify that the OD_{600} value for the culture 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 culture does have an OD_{600} <2.0*, there was insufficient growth. Verify that the cap was loose to allow for aeration and that the strain was stored correctly upon receipt at ≤ -70℃. The culture tube may be incubated for addit ional 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 culture be used for the assay.

Optical Density Results

Culture	OD_{600} (x10)	Acceptable range*
YG strain		>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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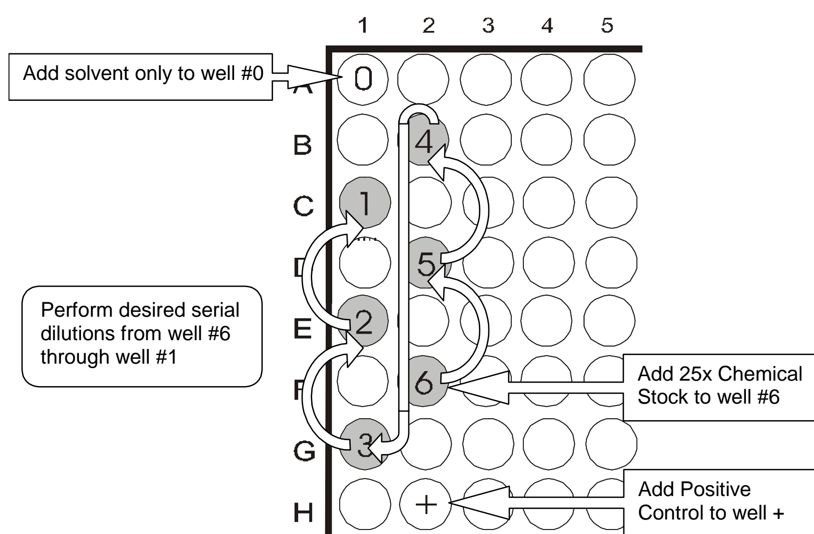
Preparation and Dilution of the Chemical Stock

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

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

1. Prepare the positive control chemical stock 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 (see below).
   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 (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.
5. Add the appropriate volumes of solvent to wells #0-5 of the plate. Solvent concentrations should be the same in all wells #0-6.
6. Perform the desired dilution of test chemical by transferring it from well #6 to #5, and mix by pipetting up and down thoroughly.
7. Perform the desired dilution of test chemical from well #5 to #4, and mix by pipetting up and down thoroughly.
8. Complete the serial dilutions from well #4 to #3, #3 to #2, and #2 to #1.
   DO NOT transfer chemical to the 0 well. This is the zero-dose control for the assay and should contain solvent only.
9. Add 50 µl of the positive control compound (prepared in step 1) to the well labeled '+'.



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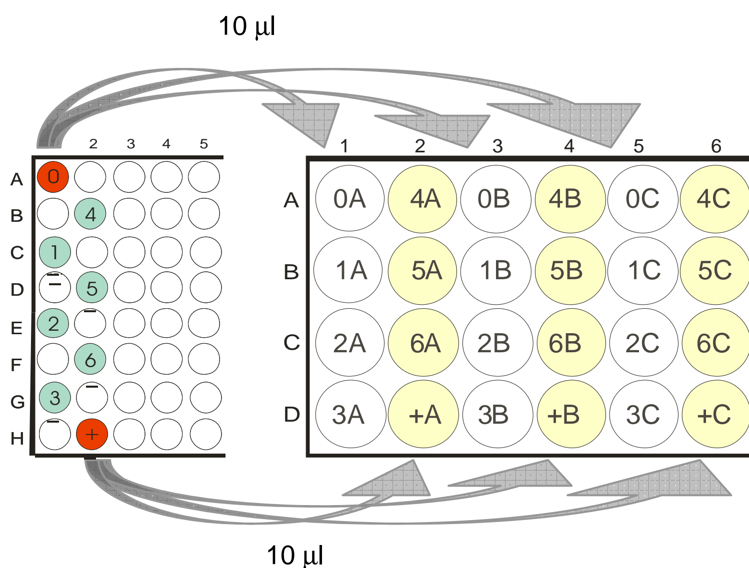
10. Fill in the chart below with the chemical and solvent used along with the dilutions performed.

Chemical: ________________________ **Solvent**: ________________________

Well	Volume of Solvent	Vol. of chem. transferred	25X Stock Conc.	Exposure Conc. (1X)
6				
5				
4				
3				
2				
1				
0				

Transfer of Chemical to the Exposure Plate

Unwrap a 24-well exposure plate. Place the plate on the paper template for the 24-well plate layout. Be sure that the plate is oriented correctly.



Transfer the chemical dilutions by performing the following steps:

1. Label the 24-well plate (e.g. YG1021 without 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 the 24-well plate.
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 plate.
4. Repeat steps 1.-3. with a second plate but with 2-AA as positive control chemical if S9 is added.

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 Culture

1. Add 7.2 ml Exposure Medium to a pipetting reservoir. If S9 fraction is to be used, refer to Appendix B for the procedure.
2. Swirl the contents of the culture tube and add 0.8 ml of the YG culture to the Exposure Medium (1 : 10 dilution).
3. Using an 8-channel pipettor, mix the contents of the pipetting reservoir by pipetting up and down thoroughly and transfer 240 µl of the Exposure Medium-strain mixture to all wells of the 24-well exposure plate. DO NOT touch the chemical on the bottom of the wells with tips!

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.

4. Secure the 24-well plate to the base of 37°C env ironmental shaker. Incubate the plate for exactly 90 minutes at 37°C, 250 rpm.

Addition of Indicator Medium

1. After the 90 minute incubation, remove the exposure plate(s) from the environmental shaker.
2. Add 2.8 ml Indicator Medium to each well of the 24-well plates. Take care not to cross-contaminate wells or the bottle of Indicator Medium.

Transfer of Exposed Culture 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 plate replicate number (i.e. YG1021-1, YG1021-2, YG1021-3). Be sure that the plate is oriented correctly.
2. Place the 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 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, a complete 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.

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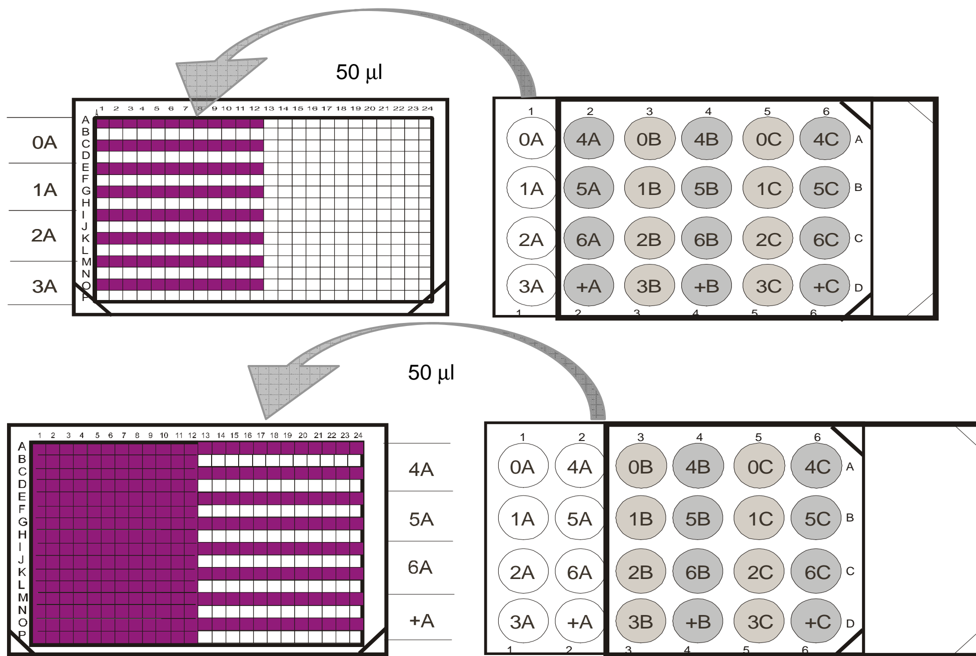

Incubation of Revertant Colony Selection Plates

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 **56 - 72 h**.

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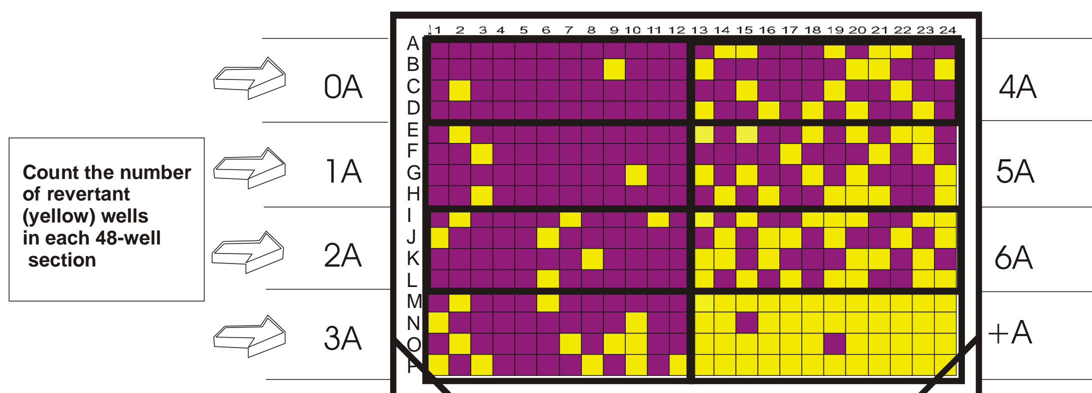
ASSAY PROCEDURE - DAY 4

Plate Scoring



Remove the plastic bag containing the 384-well plates from the 37°C dry incubator.

- Score the three replicate 384-well plates for the YG 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 box. 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.

 # Positive Wells -S9

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

 # 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 98 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, 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 zero dose baseline. The baseline is obtained by adding one standard deviation to the mean number of positive wells of the zero dose control.

Example: Mean ± S.D 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 is 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 offers a free Excel calculation template which simplifies data entry and automates all necessary calculations. Please download it from the Aniara homepage: www.aniara.com

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Layout of the Xenometrix Excel Calculation Sheet

Sheet 1: Concentrations

B19 TA 98

	B	C	D	E	F
14	01.01.2007				
17	Select Strain from Dropdown List in Box Below				
19	TA 98				
22	Enter compound 1 in Box Below				
23	Compound 1				
25	Enter compound 2 in Box Below				
28	Enter compound 3 in Box Below				
32		Enter Concentration used			Choose Units for Compound 1
33	TA 98 -S9				
34	Cpd 1:	Compound 1			µg/ml
35	1	4		µg/ml	
36	2	20		µg/ml	
37	3	100		µg/ml	
38	4	500		µg/ml	
39	5	2000		µg/ml	
40	6	5000		µg/ml	
42		Concentration used			
43	TA 98 +S9				

Concentrations / raw data / Summary / Graphs

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Sheet 2: Raw Data

Arial 10

D25 | 48

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

Enter the raw data (triplicate) into all colored fields

Compound: Compound 1

TA 98 -S9

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
4	0	1	1
20	2	3	5
100	6	9	10
500	12	18	17
2000	27	30	25
5000	39	36	35
Pos. Control	48	47	48

Spontaneous TA 98 -S9
3
1
0
2
1
1

Enter positive control data

Compound: Compound 1

TA 98 +S9

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
4	1	1	2
20	4	1	2
100	3	2	3
500	4	7	5
2000	10	12	13
5000	15	14	19
Pos. Control	48	47	48

Spontaneous TA 98 +S9
1
1
0
0
2
2

Compound:

TA 98 -S9 Spontaneous

Concentrations / raw data / Summary / Graphs

Sheet 3: Summary

Arial 10

D25 | 48

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

Enter the raw data (triplicate) into all colored fields

Compound: Compound 1

TA 98 -S9

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
4	0	1	1
20	2	3	5
100	6	9	10
500	12	18	17
2000	27	30	25
5000	39	36	35
Pos. Control	48	47	48

Spontaneous TA 98 -S9
3
1
0
2
1
1

Enter positive control data

Compound: Compound 1

TA 98 +S9

Conc. (µg/ml)	Replicate #1	Replicate #2	Replicate #3
4	1	1	2
20	4	1	2
100	3	2	3
500	4	7	5
2000	10	12	13
5000	15	14	19
Pos. Control	48	47	48

Spontaneous TA 98 +S9
1
1
0
0
2
2

Compound:

TA 98 -S9 Spontaneous

Concentrations / raw data / Summary / Graphs

Sheet 4: Graphs

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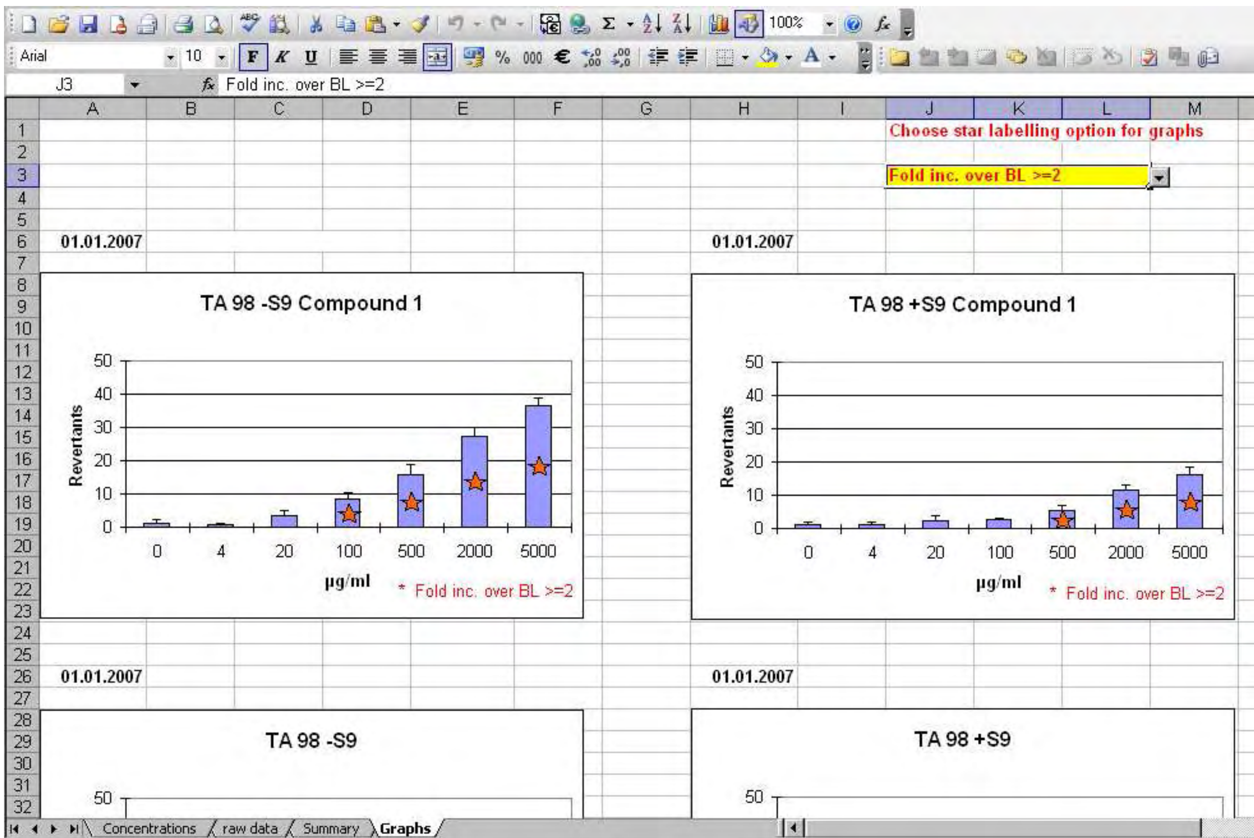

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Appendix A
Preparation of Positive Controls

A. Without S9:

1. For YG1021 and YG1024 cultures: Prepare a 10 µg/ml stock solution of 2-nitrofluorene (2-NF) in DMSO. This stock solution may be aliquoted and stored at -20°C.
2. Transfer 50 µl to the positive control (+) well in the 96-well chemical dilution plate. This results in a final assay concentration of 0.4 µg/ml 2-NF and should result in ≥ 25 positive wells.

1. For YG1041 culture: Prepare a 200 µg/ml stock solution of 1-nitropyrene (1-NP) in DMSO. This stock solution may be aliquoted and stored at -20°C. Before each experiment, prepare a fresh 500 ng/ml stock solution from the 200 µg/ml stock 1-NP.
2. Transfer 50 µl of the 500 ng/ml solution to the positive control (+) well in the 96-well chemical dilution plate. This results in a final assay concentration of 0.02 µg/ml 1-NP and should result in ≥ 25 positive wells.
   Note: The 500 ng/ml 1-NP solution should not be frozen and re-used. Discard the rest of the solution after use.

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

1. For YG1021 culture: Prepare a 25 µg/ml stock solution of 2-aminoanthracene (2-AA) in DMSO. This stock solution may be aliquoted and stored at -20°C.
2. Transfer 50 µl to the positive control (+) well in the 96-well chemical dilution plate. 2-aminoanthracene, at a final assay concentration of 1 µg/ml, should result in ≥ 25 positive wells.

1. For YG1024 and YG1041 cultures: Prepare a 1.25 µg/ml stock solution of 2-aminoanthracene (2-AA) in DMSO. This stock solution may be aliquoted and stored at -20°C.
2. Transfer 50 µl to the positive control (+) well in the 96-well chemical dilution plate. 2-aminoanthracene, at a final assay concentration of 0.05 µg/ml, should result in ≥ 25 positive wells.

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Appendix B
Modifications of the MPF™ YG Assay Protocol when Using S9

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 deionized 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 deionized or distilled water. Adjust pH to 7.4 with NaOH. Add water to 1 liter.

Preparation of 30% S9 Mix (for 1 test compound with TA98)

Keep all (thawed) reagents on ice.
Immediately before use, prepare 1.5 ml of 30% S9 mix by combining the volumes of reagents listed below in a sterile tube:

Stock	Reagent	Volume
1.00 M	KCl	0.050 ml
0.25 M	$MgCl_2 \bullet 6H_2O$	0.048 ml
0.20 M	Glucose-6-phosphate	0.038 ml
0.04 M	NADP	0.150 ml
0.20 M	NaH_2PO_4 buffer	0.765 ml
	S9 fraction (Aroclor 1254 induced rat liver)	0.450 ml
	Final Volume =	1.501 ml

Protocol Changes to Accommodate S9: Exposure Culture Preparation

1. Transfer the chemical to the exposure plate as described on page 8.
2. Add **6.0 ml** Exposure Medium to a pipetting reservoir.
3. Add **0.8 ml** of the YG overnight culture to the reservoir. Swirl the contents of the culture tube before removing the sample in case any settling has occurred.

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4. Transfer **1.2 ml** of the 30% S9 mix to the reservoir containing overnight culture and Exposure Medium, mix.
5. **Immediately** after the S9 mix is added to the reservoir, transfer 240 µl of the S9/bacterial culture/Exposure Medium mixture to each well 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.

The final concentration of S9 in the culture is 4.5%

Appendix C
Acceptable Values

Strain	Controls	Acceptable Mean Values
YG1021	solvent control -S9 solvent control +S9	≤8 ≤12
	2-NF (0.4 µg/ml) -S9	≥25
	2-AA (1 µg/ml) + S9	≥25
YG1024	solvent control -S9 solvent control +S9	≤8 ≤12
	2-NF (0.4 µg/ml) -S9	≥25
	2-AA (0.05 µg/ml) + S9	≥25
YG1021	solvent control -S9 solvent control +S9	≤8 ≤12
	1-NP (0.02 µg/ml) -S9	≥25
	2-AA (.05 µg/ml) + S9	≥25

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Appendix D
Pipetting and Scoring Templates