



Manufactured By: Xenometrix AG

Ames MPF™ 1535 Microplate Format Mutagenicity Assay

S. typhimurium TA1535

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

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NOTE 1:

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

Article No.	Kit size*	Lyophilized liver S9	Positive Controls [#]
AA01-112	1	-	-
AA01-112-S1-P (Aroclor 1254-induced S9)	1	+	N ⁴ -ACT, 2-AA
AA01-112-S2-P (PBN- induced S9 [§])	1	+	N ⁴ -ACT, 2-AA
AA10-112	10	-	-
AA10-112-S1-P (Aroclor 1254-induced S9)	10	+	N ⁴ -ACT, 2-AA
AA10-112-S2-P (PBN-induced S9 [§])	10	+	N ⁴ -ACT, 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.

[#] N⁴-ACT: N⁴-Aminocytidine; 2-AA: 2-Aminoanthracene

[§] PBN-induced S9: Phenobarbital/ β -Naphthoflavone-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™ 98, Ames MPF™ 100, 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™ 1535 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.

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 strain provided in this kit is the *Salmonella typhimurium* strain TA1535. It detects mutagens that cause base-pair substitutions and contains the same *hisG46* mutation as TA100, but lacks the plasmid pKM101.

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™ 1535 assay.

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

Genotype of the TA1535 *Salmonella typhimurium* strain

Strain	Mutation	Type	Target	Cell Wall	Repair	pKM101
TA1535	<i>hisG46</i>	Base-pair subst.	GGG	<i>rfa</i>	<i>uvrB</i>	no

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™ 1535 Mutagenicity Assay kit contains the following components and should be stored as indicated:

-70° C to -80° C:

- Vials containing *Salmonella* strain (TA1535)

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:

- 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)

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

- Growth Medium
- Exposure 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)

- 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: N⁴-aminocytidine (for tests without S9) and 2-aminoanthracene (for tests with S9) and
- Liver S9 fraction (Aroclor 1254 or Phenobarbital/β-Naphtoflavone-induced)

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

A ready-to-use kit available separately from Xenometrix 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

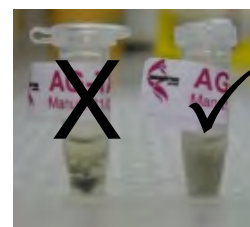
Using sterile technique, prepare an overnight culture of TA1535 by performing the following steps:

1. Remove the vial with the bacteria from the freezer. Let stand at ambient temperature for about 5 minutes. Add 200 µl of GM medium to the vial.
2. Prepare the overnight culture by performing the following steps:
 - Add 10 ml Growth Medium to a 50 ml culture tubes labeled 'TA1535'.
 - Add a few milliliter of Growth Medium to a tube labeled 'Negative Control'.

Note: Do not add ampicillin to the 'TA1535' culture tube, since this strain does not contain the R-factor plasmid pKM101 carrying ampicillin resistance.

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.

3. 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.



4. Pipette 25 µl of the dispersion into the 'TA1535' tube prepared under (3).
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 three cuvettes.
3. Swirl the overnight culture and transfer 100 µl of bacteria from the 'TA1535' 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 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 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*
TA1535		≥2.0
Negative Control		≤0.05

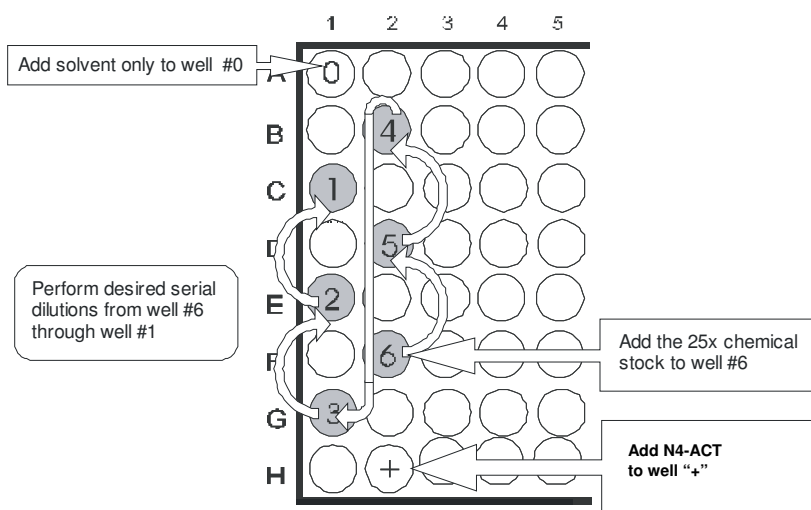
**This is actually a measurement of light scattering, which is dependent on the optical characteristics of a spectrophotometer. Therefore values can not be directly compared between different instruments. We suggest that you determine the maximum value for your instrument by measuring the growth of a 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 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 N4-ACT 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 μ l of each stock concentration will be required for dosing each strain (60 μ l 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 μ l.

When the calculated volume of the sample in well #6 is > 360 μ l 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** on the USB stick or which can be downloaded from the Aniara homepage www.aniara.com.

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 50 μ l of the positive control compound, N4-ACT (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 plate.

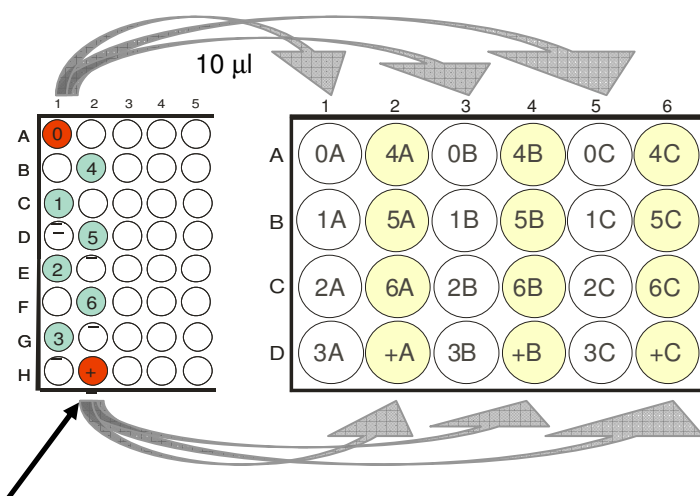
9. Fill in the chart below with the chemical and solvent used along with the dilutions performed. (An example in gray is given for a stock at 125 mg/ml, 2x dilutions, triplicates, for 1 strain, with and without S9).

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

Well	Add 25 X Stock	Add Solvent	Transfer to next well, mix	Exposure Conc. (1X)
0	-	80 μ l	-	Solvent control
1	-	80 μ l		0.16 mg/ml
2	-	80 μ l	80 μ l \uparrow	0.31 mg/ml
3	-	80 μ l	80 μ l \uparrow	0.63 mg/ml
4	-	80 μ l	80 μ l \uparrow	1.25 mg/ml
5	-	80 μ l	80 μ l \uparrow	2.5 mg/ml
6	160 μ l	-	80 μ l \uparrow	5 mg/ml

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 chemical N4-ACT 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 \pm S9 that are to be used (e.g. TA1535 - S9).
2. Using an 8-channel pipettor with 4 evenly spaced tips (every other channel), transfer 10 μ l 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 μ l 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 μ l 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 μ l 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.

Preparation of Exposure Cultures

TA1535 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!

TA1535 with S9 (see Appendix B for the preparation of 30% S9 mix):

1. Add **5.25 ml Exposure Medium** 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, mix.
4. **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.

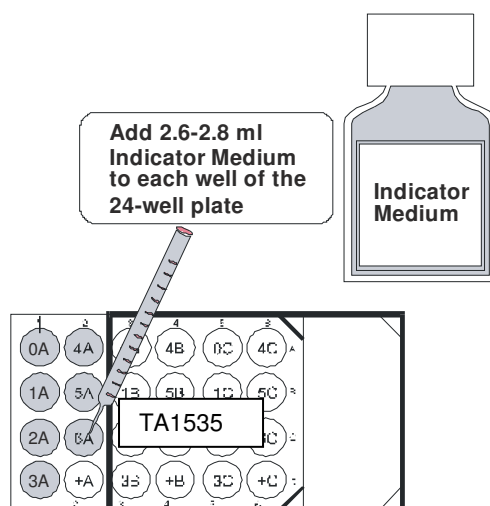
The final concentration of S9 in the culture is 4.5%.

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.

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.

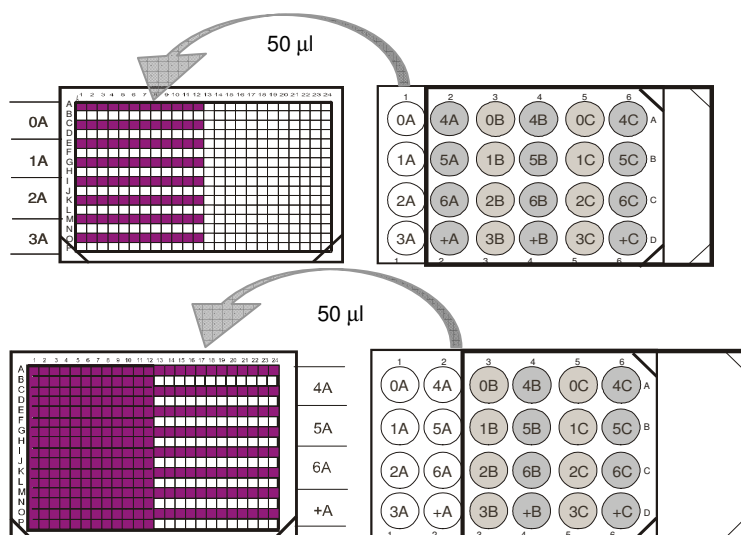
Time Incubation Started	Time Incubation Stopped	Date

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 10.
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. TA1535-1 -S9, TA1535-2 -S9, TA1535-3 -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

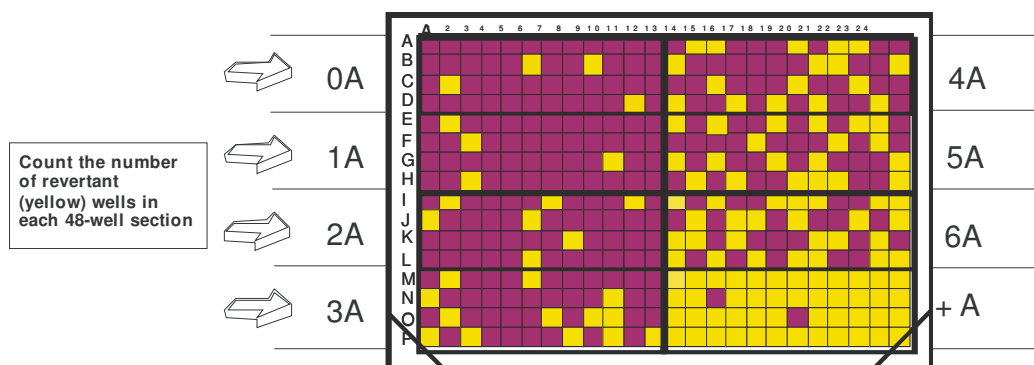
Incubation of Revertant Colony Selection Plates

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		

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.

TA1535 # Positive Wells -S9

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

TA1535 # 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 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. TA1535 -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 \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:** Compound 1 dropdown menu containing "Compound 1".
- Row 35-40:** Concentration selection table:

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:** Compound 1 dropdown menu containing "Compound 1".
- Row 45-46:** Concentration selection table:

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

Spontaneous
TA 100 -S9
3
4
6
2
1
2

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

Spontaneous
TA 100 +S9
7
1
0
4
2
2

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

Spontaneous
TA 100 -S9
3

Sheet 3: Summary

Definitions and Explanations

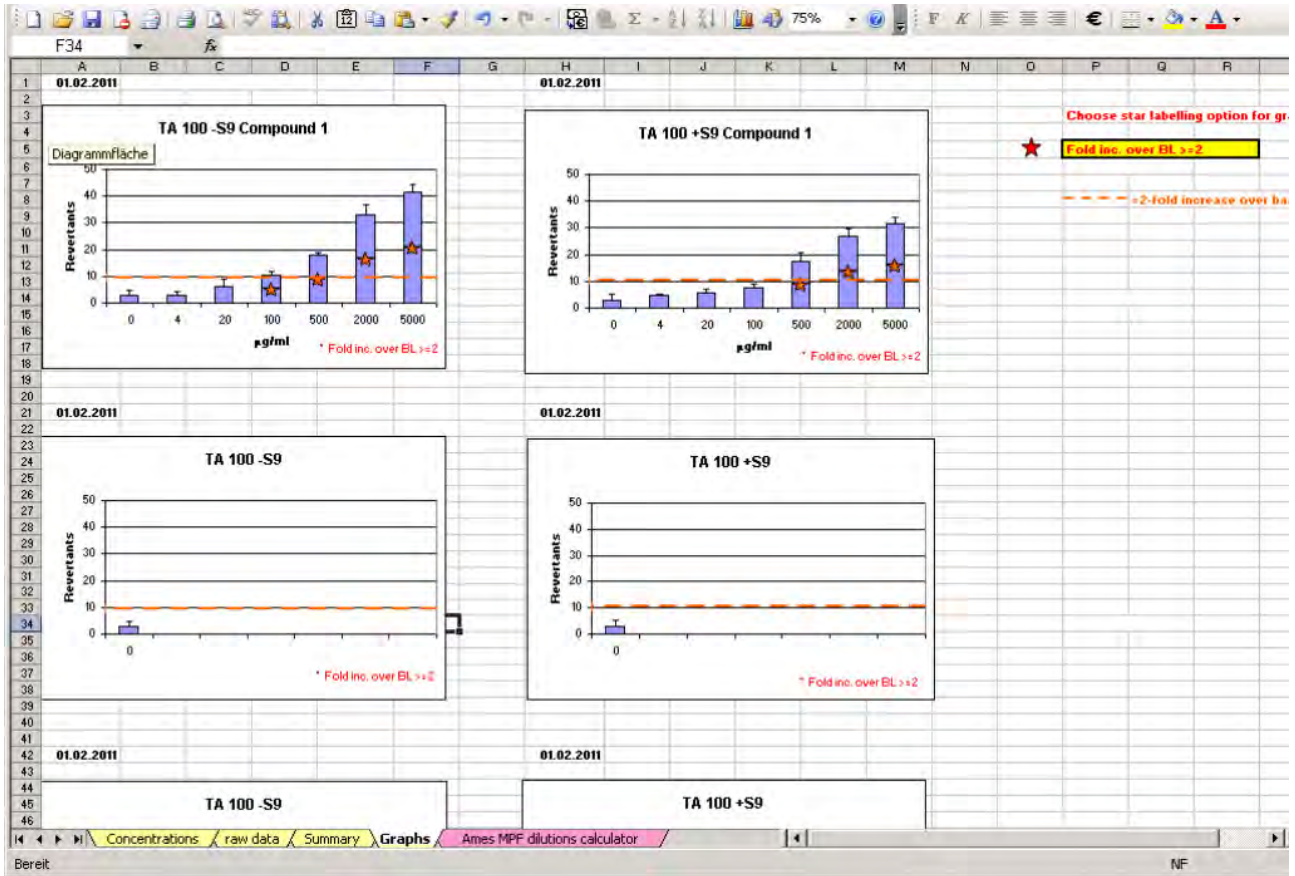
- 'n' = number of replicates
- 'Baseline' = Mean + 1 SD
- If 'mean # pos.wells' of zero dose control is less than 1, value will appear in gray and will be set to 1 in the column 'Corr. mean'. Fold increase (zero value) will then be calculated from the corrected mean.
- Baseline values < 1 will be set 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	0.56	0.3959
4	3	2.67	1.53		1.32	0.0263
20	3	6.33	2.52		2.16	0.0003
100	3	10.33	1.53		3.76	0.0000
500	3	18.00	1.00		6.89	0.0000
2000	3	33.00	3.61		8.63	0.0000
5000	3	41.33	3.21			
Pos. Control	3	47.67	0.58			

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

Sheet 4: Graphs



Appendix A Preparation of Positive Controls

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

	Chemical	25X Stock	Final Conc.
Without S9	N ⁴ ACT	2.5 mg/ml	100 µg/ml
With Aroclor 1254 S9	2-AA	125 µg/ml	5 µg/ml
With Phenobarbital/β-Naphtoflavone S9	2-AA	62.5 µg/ml	2.5 µg/ml

Preparation of positive controls for 1 test compound

A. Without S9:

1. Prepare a 2.5 mg/ml stock solution of N⁴-aminocytidine (N⁴-ACT) in DMSO.
2. Transfer 50 µl to the positive control (+) well in the 96-well chemical dilution plate. This gives in a final assay concentration of 100 µg/ml N⁴-ACT and should result in ≥ 25 positive wells.

Note: Na-azide is not recommended as positive control for TA1535

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)

1. Prepare a 125 µg/ml stock solution of 2-AA in DMSO.
2. Transfer 10 µl to the positive control triplicate wells (A+, B+ and C+) in the TA1535 24-well exposure plate.
2-aminoanthracene, at a final assay concentration of 5 µg/ml, should result in ≥ 25 positive wells.

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

1. Prepare a 62.5 $\mu\text{g/ml}$ stock solution of 2-AA in DMSO.
2. Transfer 10 μl to the positive control triplicate wells (A+, B+ and C+) in the TA1535 24-well exposure plate.
2-aminoanthracene, at a final assay concentration of 2.5 $\mu\text{g/ml}$, should result in ≥ 25 positive wells.

Appendix B

Preparation of 30% 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 Xenometrix 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 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	0.043 ml
0.25 M	MgCl ₂ ·6H ₂ O	0.042 ml
0.20 M	Glucose-6-phosphate	0.033 ml
0.04 M	NADP	0.130 ml
0.20 M	NaH ₂ PO ₄ buffer	0.663 ml
	S9 fraction	0.390 ml
Final Volume =		1.301 ml

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	0.748 ml
S9 - G-6-P	0.033 ml
S9 - NADP	0.130 ml
S9 fraction	0.390 ml
Final Volume =	1.301 ml

Appendix C Acceptable Values

	<u>TA1535</u>
<u>Without S9:</u>	
Solvent control	≤8
N4-ACT (100 µg/ml)	≥25
<u>With S9:</u>	
Solvent control	≤8
2-AA (5 - 2.5 µg/ml)	≥25

Mean of replicates

Appendix D

Pre-screen Determination of Dose Range (optional)

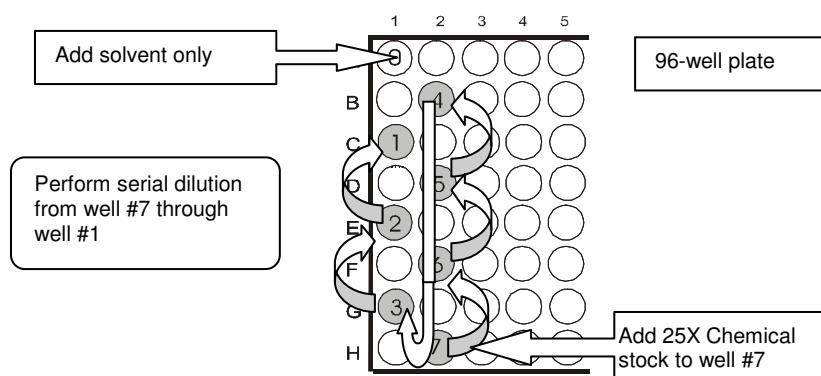
If you test a compound for the first time, you may like to determine its cytotoxicity and solubility in a pre-screening assay before the Ames MPF™ 1535 assay. After the pre-screening test you can choose the proper concentrations of compound to use. The top concentration of compound that can be used for 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™ 1535 assay, then both assays have to be performed on the same day.

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 μ l of this 25X stock to well #7 of the 96-well plate (see figure below).
4. Add 30 μ l 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 $\frac{1}{2}$ log serial dilution of the test chemical by transferring 14 μ l from well #7 to #6, and mix by pipetting up and down thoroughly.
6. Transfer 14 μ l 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.

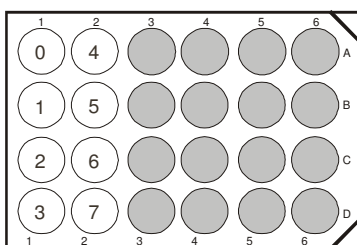


Chemical: _____ Solvent: _____

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

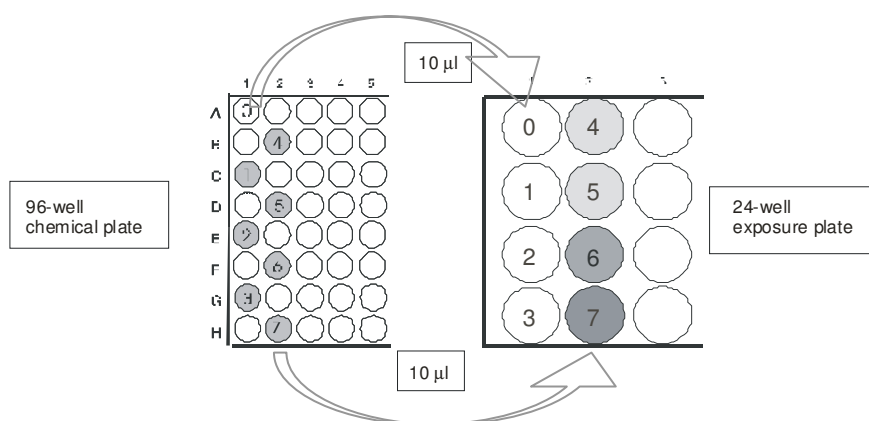
Transfer of Chemical to the Exposure Plate

Unwrap a 24-well plate for the pre-screen test using the dilutions indicated below. Label the plate (e.g. TA1535 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 μ l from the first column of wells #0 - 3 of the 96-well plate to column 1 of the 24-well plate.
2. Transfer 10 μ l from the second column of wells #4-7 of the 96-well plate to column 2 of the 24-well plate.



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 μ l 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 μ l will allow for the transfer of 240 μ l 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 Ames MPF™ 1535 mutagenicity assay.

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

384 WELL PLATE
