AMES - 384 ISO™

384 Well Format

Users Guide

environmental bio-detection products inc.

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Kit Contents:

Reagent List

- A: Exposure Medium Concentrate 2.5 ml (1 unit)
- B: D-Glucose 1.5 ml (1 unit)
- C: Bromocresol Purple 1.5 ml (1 unit)
- D: D-Biotin 2 ml(1 unit)
- E: L-Histidine 50 µl (1 unit)
- F: Sterile Distilled Water 80 ml (1 unit)
- G: Growth Media 10 ml (1 unit)
- H: 10X Reversion Solution 4.5 ml (1 unit)
- I: **Reagent I** 50 µl (1 unit)
- N: 1X Exposure solution 3.5 ml (1 unit)

Basic Ames ISO strain: TA 100 *Salmonella typhimurium* (1 unit)
4NQO (5 ng/µl), 50 µl (1 unit)

Required Equipment

- Micropipette using disposable sterile tips in the range of 5 to 200 µl.
- An eight-channel multi-pipette (200 µl).
- Pipette Aid (Pipette gun) using 10 ml sterile pipettes tips.
- A 37°C Incubator.
- Parafilm piece (3 units)

Disposable list

- 24-well plate (2 units)
- 384 well plate (3 units)
- 5 ml tube (2 unit)
- 15 ml conical tube (1 unit)
- A biohazard disposable bag

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Applications:

- Testing of industrial effluents for presence of possible mutagenic compounds.
- Screening of municipal discharges for spill contamination, improper chemical disposal.
- Routine monitoring of waste water effluent for quality and mutagenicity.
- Screening of recycled potable water supplies for presence of priority pollutants and genotoxins.
- Screening air particulate mater (PM) for sub chronic human health effects.
- Evaluating water and soil samples for elevated levels of personal care product (PCP) residues.

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Standard Ames Strains

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Strain	Type of Reversion mutation	Standard mutagen	Comment
***Salmonella typhimurium*: Histidine dependent**			
TA97a	Frameshift	9-AA or ICR191	Derived from TA1537 and includes plasmid pKM 101 which induces error-prone DNA repair enzymes to increase sensitivity
TA98	Frameshift	2NF	Derived from TA1538 and includes plasmid pKM 101 which induces error-prone DNA repair enzymes to increase sensitivity
TA100	Base-pair substitution, oxidative	4NQO	Derived from TA1535 and includes plasmid pKM 101 which induces error-prone DNA repair enzymes to increase sensitivity
TA1535	Base-pair substitution, oxidative	4NQO	uvrB repair deficient, rfa mutation increases permeability to mutagens. Sensitive to 3 unique mutagens compared to TA100 (acetaldehyde oxime, 6-mercaptopurine and 1,3-butadiene)
***Escherichia coli*: Tryptophan dependent**			
E. coli WP2 uvrA (pKM101)	Base- pair substitution	4NQO	uvrA deletion mutation eliminates accurate excision repair mechanism, contains plasmid pKM 101 which induces error-prone DNA repair enzymes and increases sensitivity

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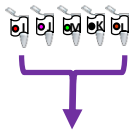
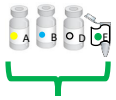


Filter Sterilize **Sample**

0.8 mL Filtered Sample + 0.8mL H_2O

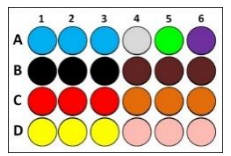
Incubate at 37°C for 12-16 hrs



Add **40% glucose**, **D-biotin**, **L-histidine** and **10X exposure solution** into a 15 ml tube to make a **master exposure mix**

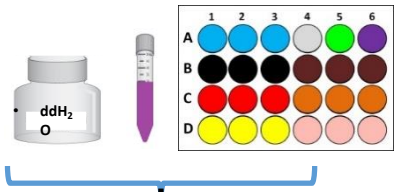
Transfer **0.8 ml of filtered sample**, **0.1 ml of bacteria** and **0.1 ml of master exposure mix** into each well

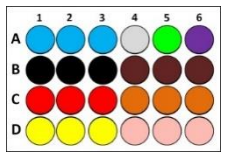
	1	2	3	4	5	6
A	Negative Control	Negative Control	Negative Control	Positive Control 1	Positive Control 2	Blank
B	No Dilution	No Dilution	No Dilution	Dilution 1	Dilution 1	Dilution 1
C	Dilution 2	Dilution 2	Dilution 2	Dilution 3	Dilution 3	Dilution 3
D	Dilution 4	Dilution 4	Dilution 4	Dilution 5	Dilution 5	Dilution 5



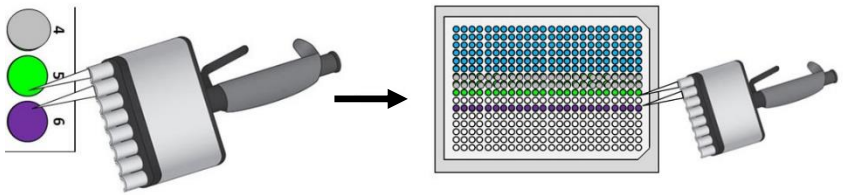
Incubate exposure plate for 120 min

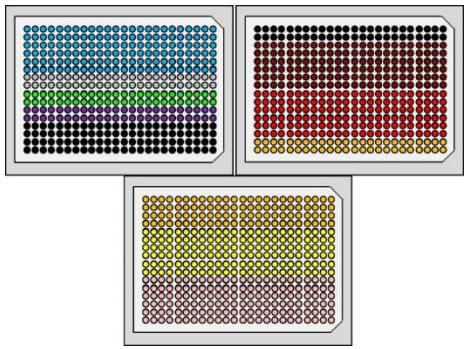
Add **40% glucose**, **bromocresol purple**, **D-biotin** and **10X reversion buffer** into a 15 ml tube to make a **master reversion mix**





After the 120 min incubation, transfer **0.5 ml of exposed bacteria from a single well**, **0.3 ml of master reversion mix**, **2.2 ml of H_2O** per well on a 24 well plate.





Parafilm seal and transfer plates into a sealing bag. Incubate at 37°C for 2-3 days

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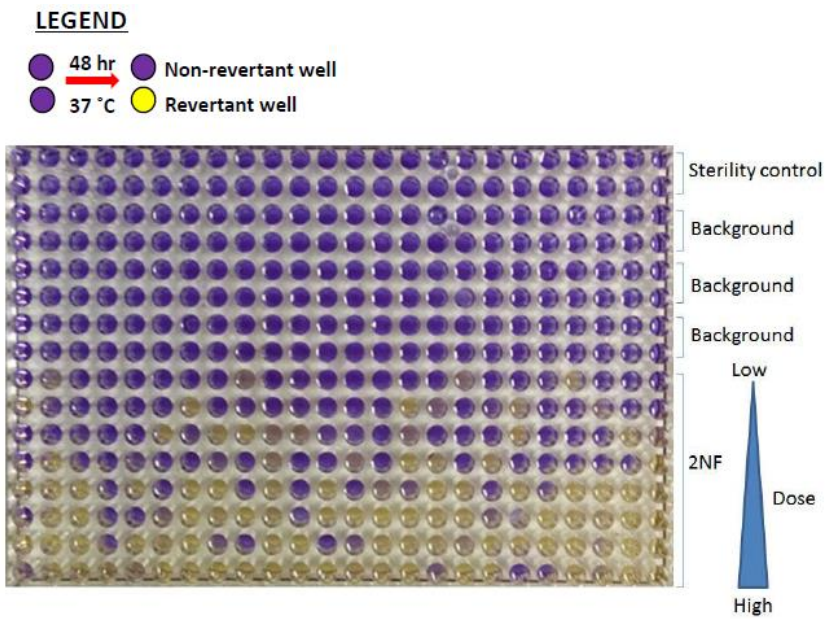
Ames-384 ISO™ Advantages

- Reagents, bacteria and other consumable components are supplied ready-to-use in a non-specialized laboratory
- Assay endpoints are easy to read colorimetric changes that require no specialized training
- Procedure uses less reagents, plastics and incubation space compared to traditional Ames tests. More samples can be run with less cleanup.
- Less sample is required for testing. Small sample volumes still produce great results.
- Pre-exposure and bacterial dilution steps ensure mutagen exposure occurs during bacterial log growth phase to improve results.



Sample 384 ISO result plate

Bioactivation (S9 kit add-on)

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- Many mutagens must first be metabolized into their reactive form by enzymes
- Depending on the compound under study, bioactivation may be required for detection
- EBPI offers traditional methods of pro mutagen activation through the addition of S9 liver fraction
- EBPI employs a commonly used metabolic activation system which includes post-mitochondrial liver fractions isolated from Sprague Dawley rats, supplemented with cofactors
- The rats are pre-treated with Aroclor 1254 to stimulate enzyme production prior to liver extraction
- This option is offered with all mutagenicity and genotoxicity testing kits for a supplemental cost

S9 Kit Contents

S9A: $MgCl_2$ + KCl solution 0.4 mL (1 unit)
S9B: Glucose-6-phosphate solution 80 µL (1 unit)
S9C: NADP solution 250 µL (1 unit)
S9D: Phosphate buffer 1.5 mL (1 unit)
S9F: Lyophilized S9 fraction

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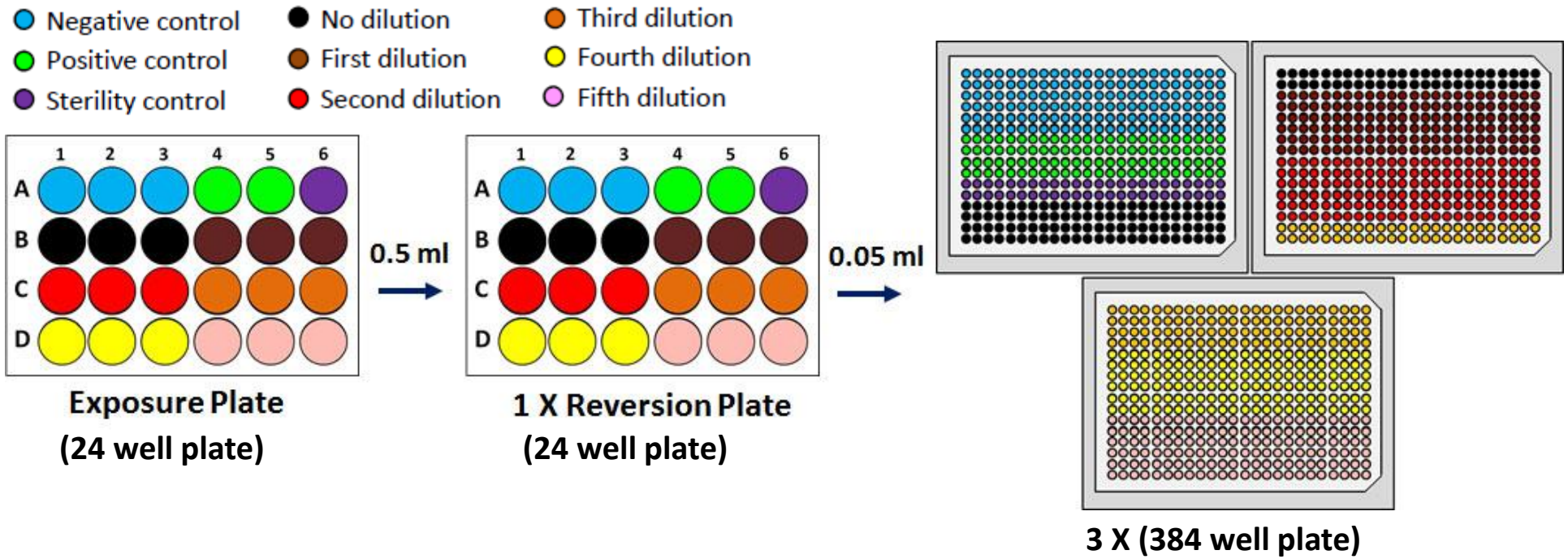
Ames-384 ISO™ Procedure

Experiment overview



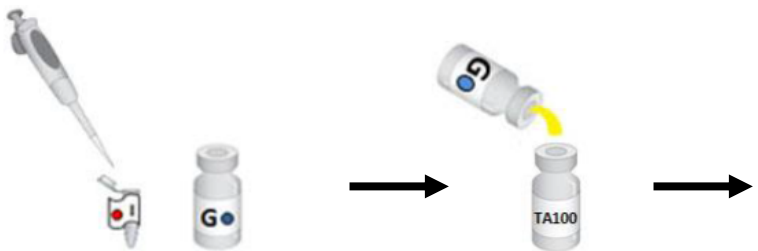
Note: Prior to using our test kits, we highly recommend the development of individual outlines that are representative of the respective experiment. This outline is only provided as a guideline for one possible method

The image above is an example of a test that utilizes one sample with five distinct dilutions in a triplicate manner.

Detailed Procedure

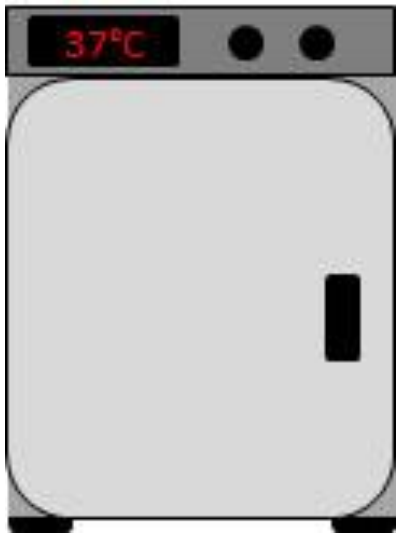
1. Overnight inoculation and initial bacterial growth

- Always use aseptic techniques for all steps in this procedure
- Add Reagent V to nutrient broth, mix and immediately transfer to bacterial bottle
- Shake to dissolve and place in incubator overnight at 37 °C with shaking (if possible)
    - aeration will aid bacterial growth.



Pipette 20 µl of **Reagent V** into the nutrient broth bottle

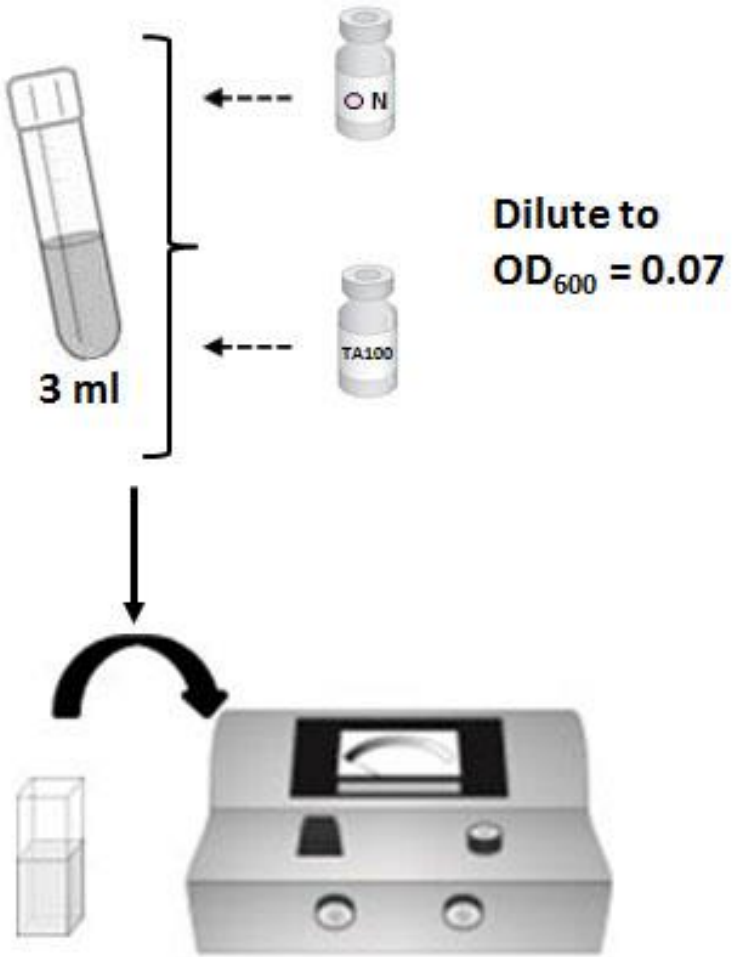
Pour media into lyophilized vial



Incubate at 37°C overnight (12-16 hrs)

2. Bacteria dilution

- The next morning observe bacterial vial for turbidity
- If turbidity is seen proceed with initial OD_{600} measurement
- If turbidity is not seen continue growth in incubator
- Perform bacterial dilution upon OD_{600} measurement to working dilution OD_{600} value given for each strain (eg. TA100 = 0.07)
    - Bacterial dilution encourages log growth phase and increases uptake of solution components



Dilution Calculation

X = Overnight Calculation,
Y = Working Concentration,
Final volume = 3 mL
Volume of overnight bacteria (mL) for dilution = (Y/X) X 3 mL

Example:
TA100 was grown overnight with an overnight **OD_{600}** = 1.6
Working dilution of TA100 is **OD_{600}** = 0.07
Volume of overnight TA100 for dilution = (0.07/1.6) X 3 mL **= 0.131 mL**

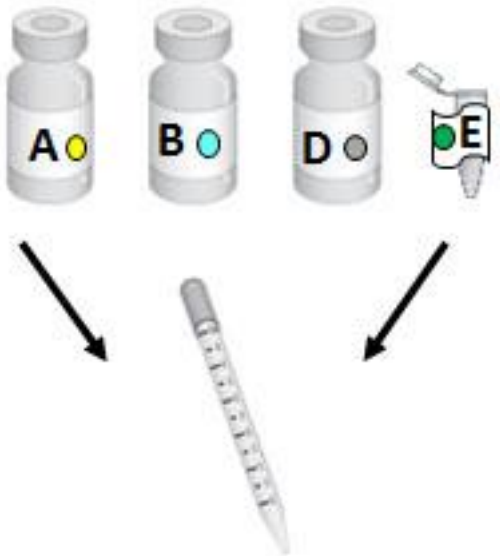
Therefore, you will add **0.131 mL of overnight TA100 bacteria** in 2.869 mL of 1X exposure buffer to give a **final volume of 3 mL**.

3. Exposure media preparation

- Prepare exposure media according to guidelines below
- Ensure final solution is well mixed before continuing to next step

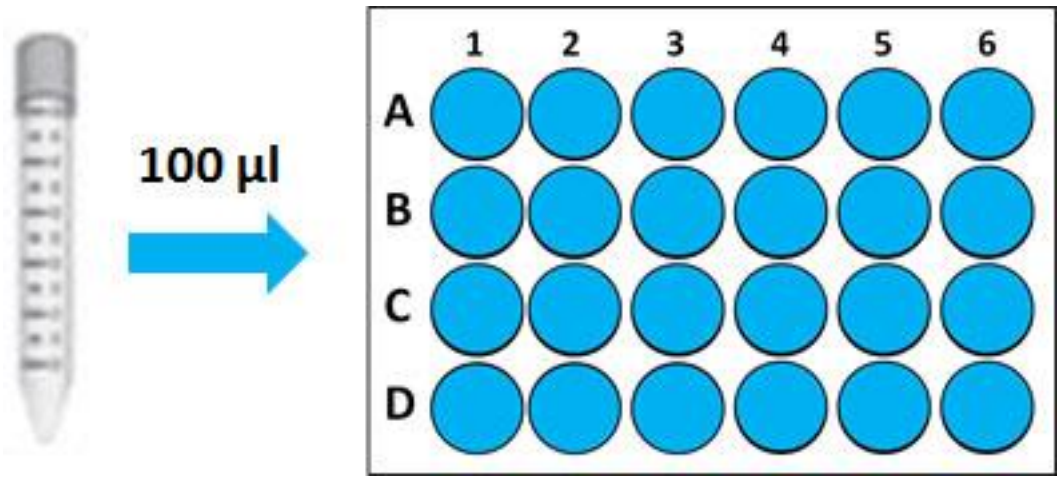
Master Exposure Mix (2.5 ml per experiment)

A) Exposure medium concentrate	2.075 ml
D) D- Biotin	0.150 ml
E) L-Histidine	0.025 ml
B) 40% D-Glucose	+ 0.250 ml
Total	2.50 ml



Master Exposure Mix

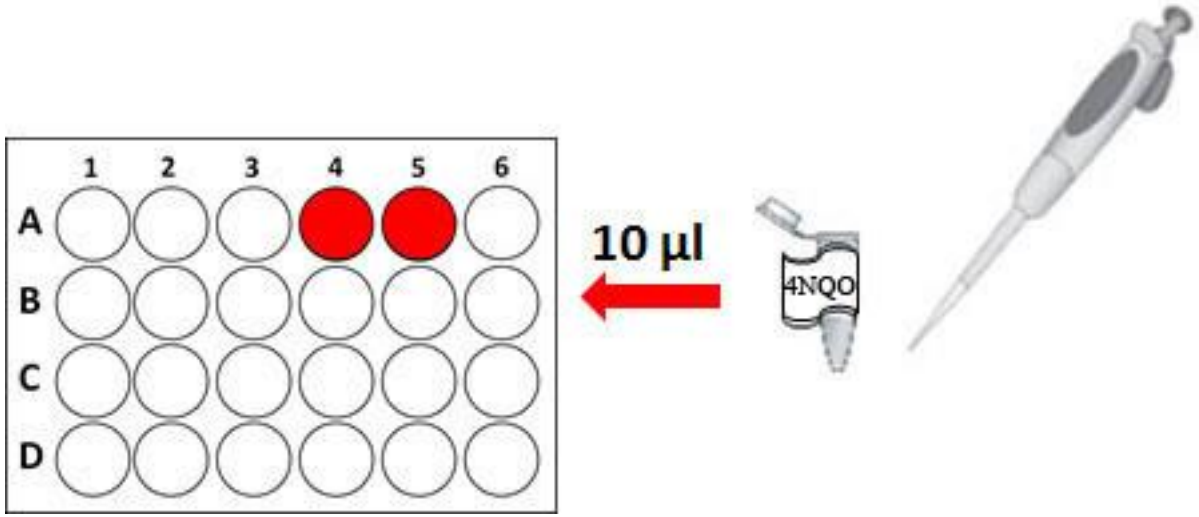
4. Transfer exposure media to 24 well plate



Master Exposure Mix

Exposure Plate

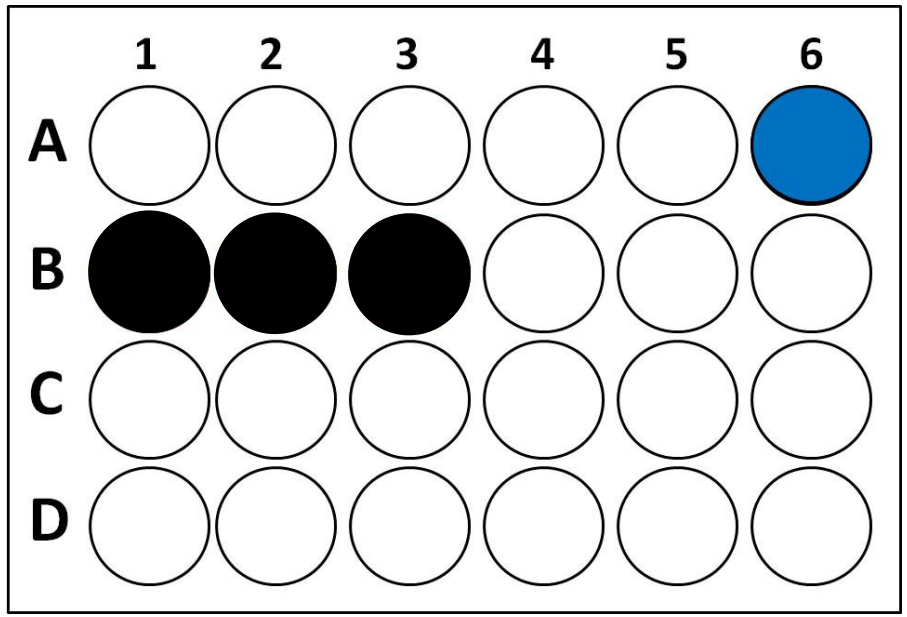
5. Transfer positive control mutagen to respective wells



- **Pipette 50 µl of 4NQO into assigned positive control wells**
- **Two positive control wells are used to increase statistical significance of response**
- **Mix wells by repeatedly reinjecting solution through pipette**

6. Transfer undiluted sample to respective wells

- Direct add 800 µL of undiluted sample to three wells. This will serve as the most concentrated sample of the series
- One well is left without sample or bacteria to check the sterility of the kit reagents (dark blue)

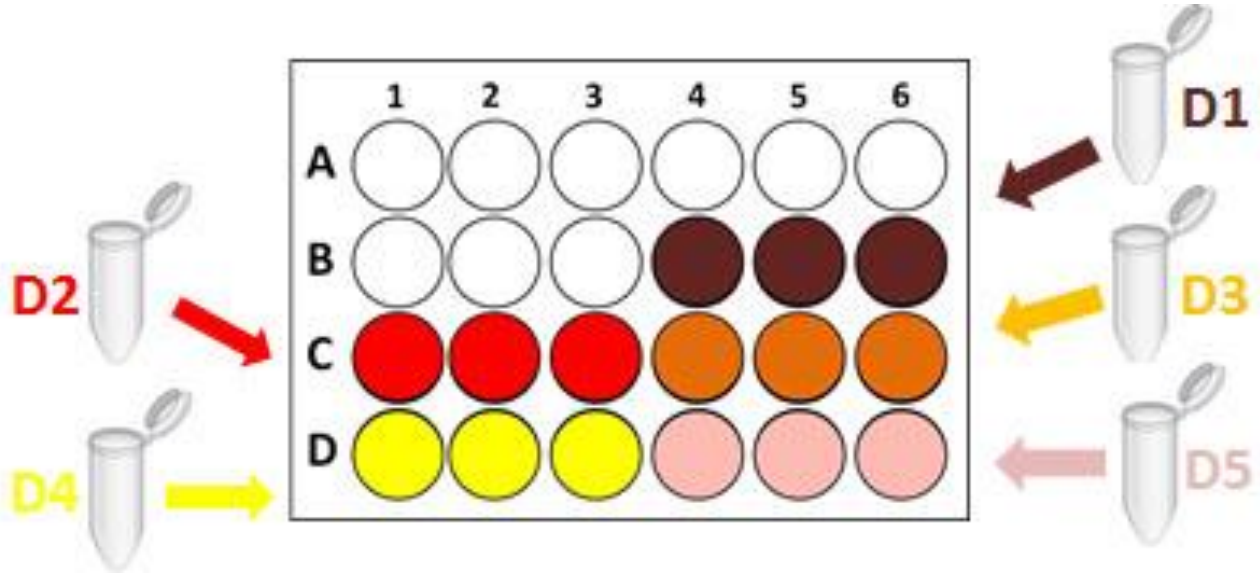


Reagent blank (no sample no bacteria)
Undiluted sample wells

Pipette800 µl of undiluted samples into assigned wells

7. Transfer diluted samples to respective wells

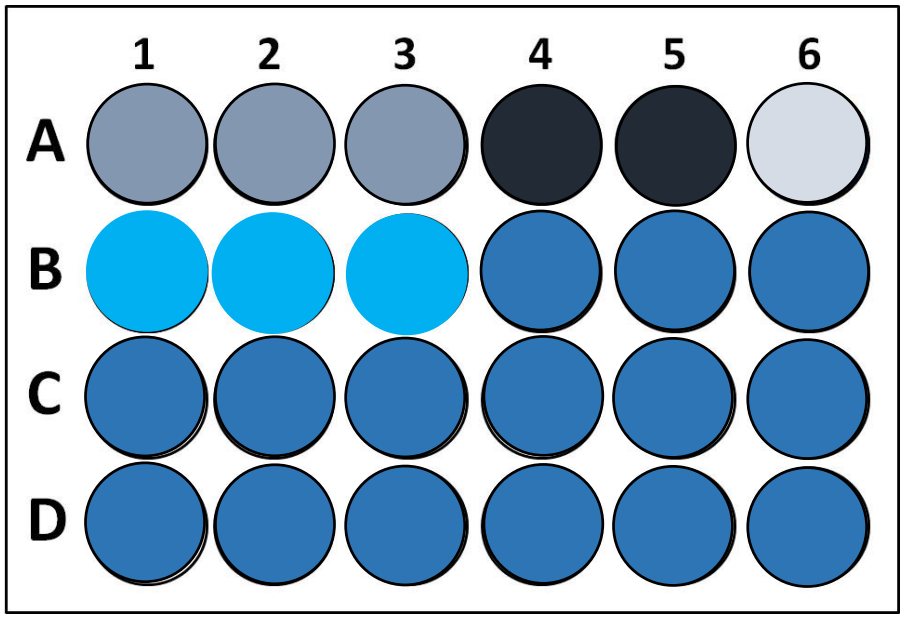
- There are enough wells in the exposure plate for 6 different concentrations. (1 undiluted + 5 dilutions)
- Each concentration is run in triplicate to enhance the statistical relevance of the results
- Dilution ratios can be changed depending on purpose of assay
  - Initial screening experiments may warrant wider ranges to find effective concentrations



Pipette diluted samples (X µl ≤ 800 µl) into assigned wells

8. Transfer sterile water to 24-well plate

- Sterile water is added to bring the **FINAL** volume of all wells to 900 µL
- Depending on sample concentrations amount of water added to the sample wells may require adjustment



800 µL of sterile water

750 µL of sterile water

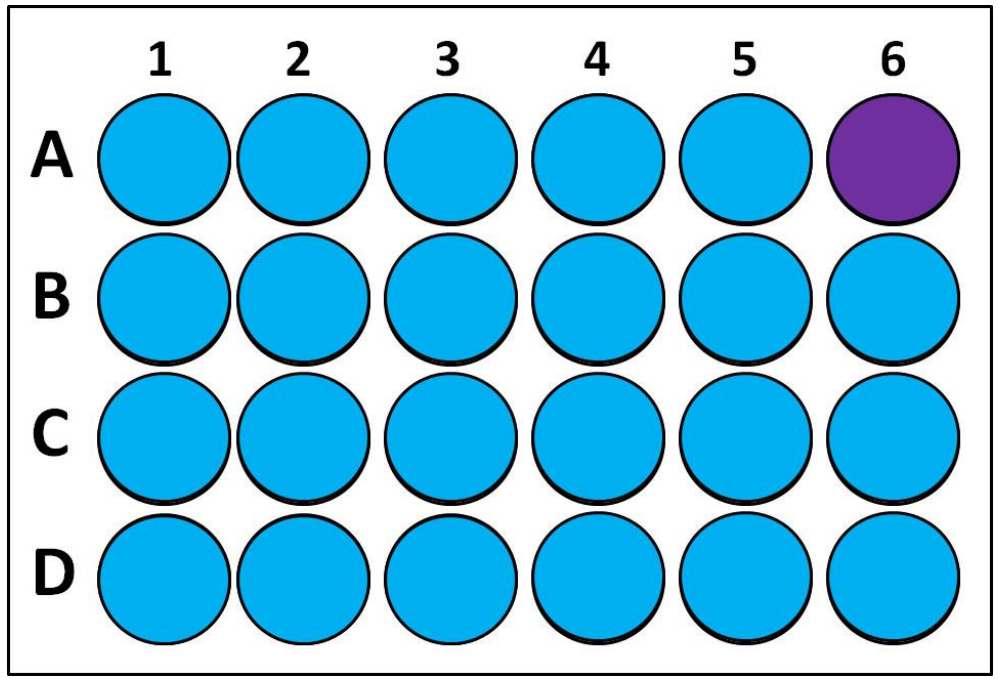
900 µL of sterile water

Volume of sterile water =
800 µL **– volume of samples**

0 µL of sterile water

9. Transfer bacteria to 24-well plate

- Bacteria is added to all wells except the reagent control (sterility control well, denoted by purple)
- **FINAL** volume of all wells after bacterial addition should be 1 mL

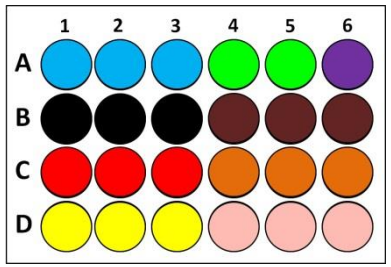


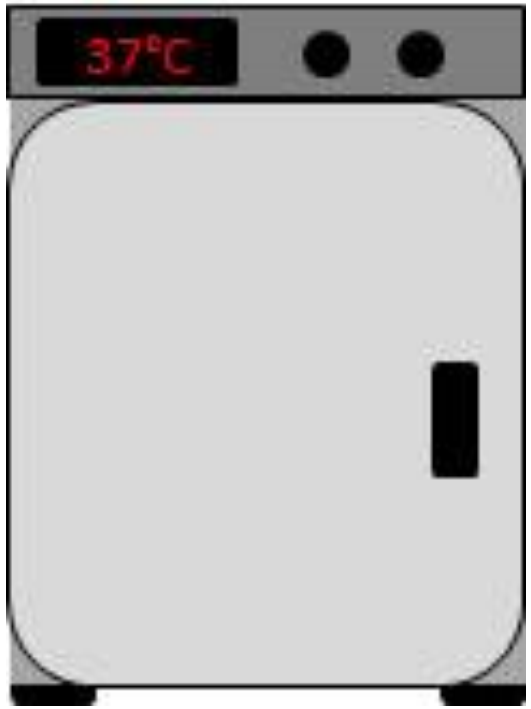
Pipette 100 µl of diluted bacteria into assigned wells

10. Exposure Incubation

- Mix exposure plate prior to incubation or during incubation (if possible)

- Negative control
- Positive control
- Sterility control
- No dilution
- First dilution
- Second dilution
- Third dilution
- Fourth dilution
- Fifth dilution





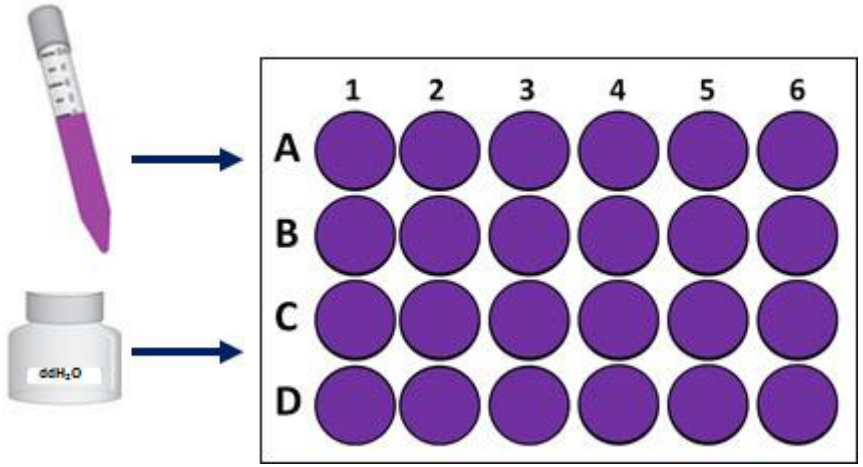
Incubate at 37°C
For 100 minute

11. Preparation of Reversion solution and Plate

- Near the end of the incubation period, prepare master reversion mix solution as outlined below
- Dispense 300 µL of reversion mix and 2.2 mL of sterile water to all wells of a second 24-well plate

Master Reversion Mix

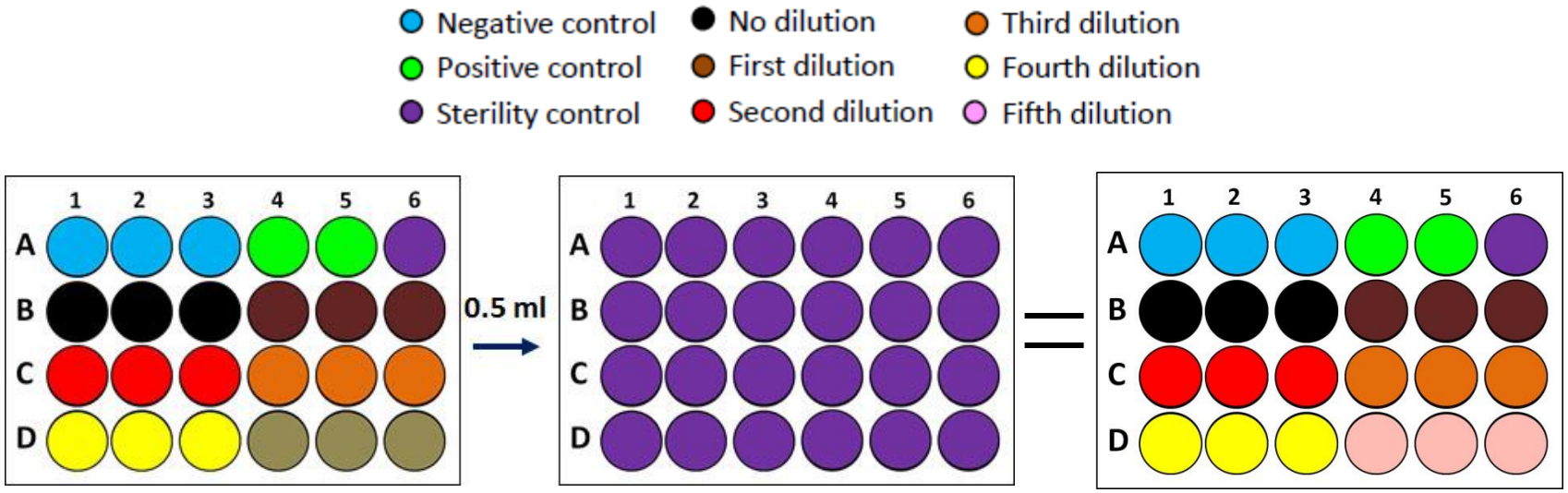
B) 40% D-Glucose	0.77 ml
C) Bromocresol purple	1.2 ml
D) D-Biotin	1.58 ml
H) 10X Reversion Solution I	3.95 ml
Total	7.5 ml



1X Reversion Plate

Add 0.3 mL of Master reversion mix and 2.2 mL of sterile water into each well of the reversion plate

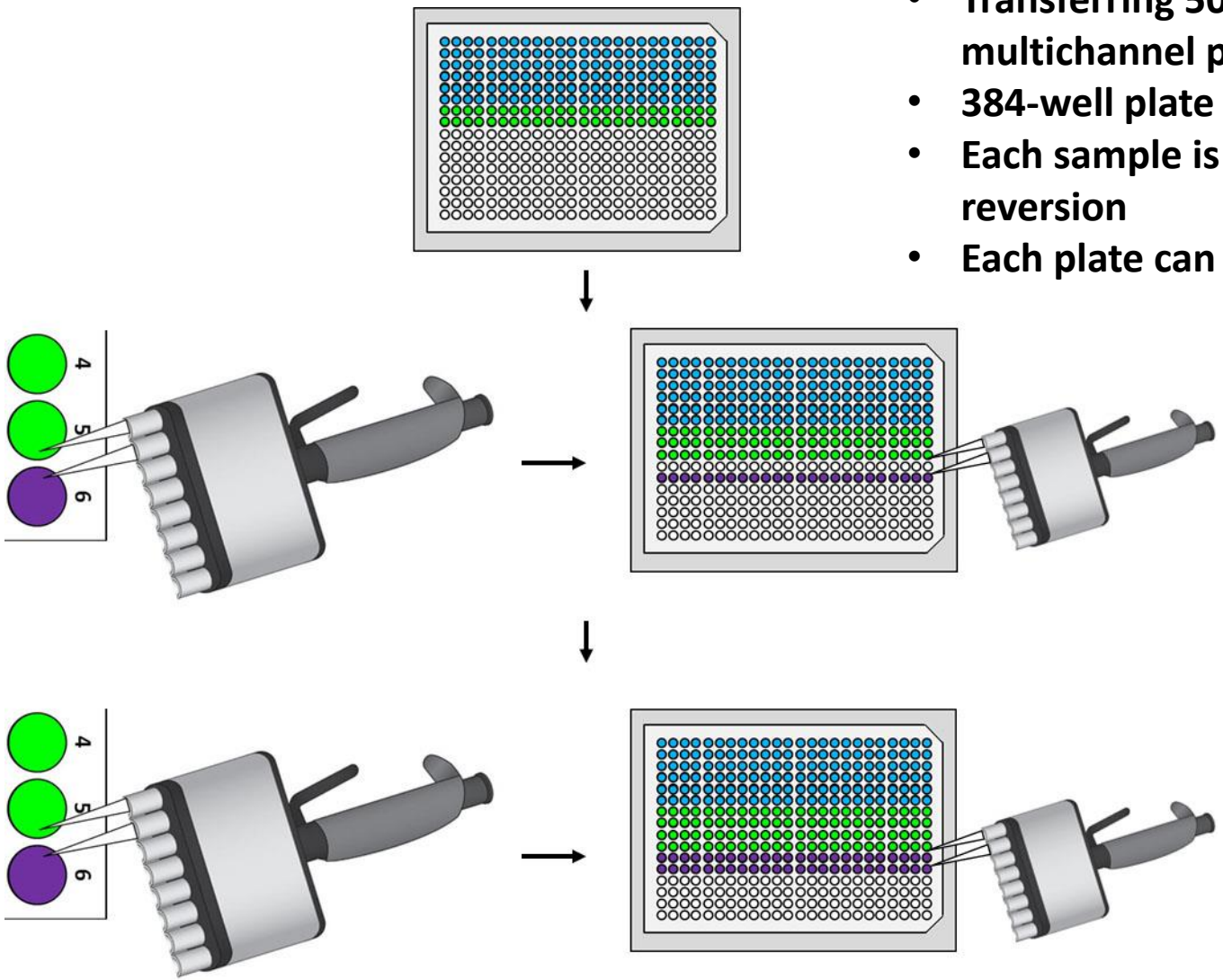
12. Transfer bacteria from exposure plate to reversion plate



Exposure Plate | **Reversion Plate** | **Reversion Plate with transferred bacteria**

After 100 min incubation, transfer 0.5 mL of exposed bacterial solution from exposure plate into respective wells in the Reversion Plate.

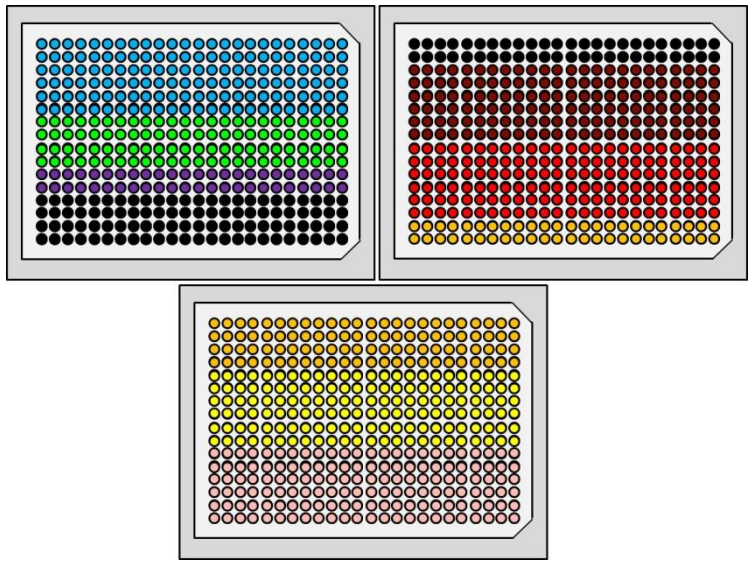
13. Transfer bacteria-reversion mix into 384-well plate

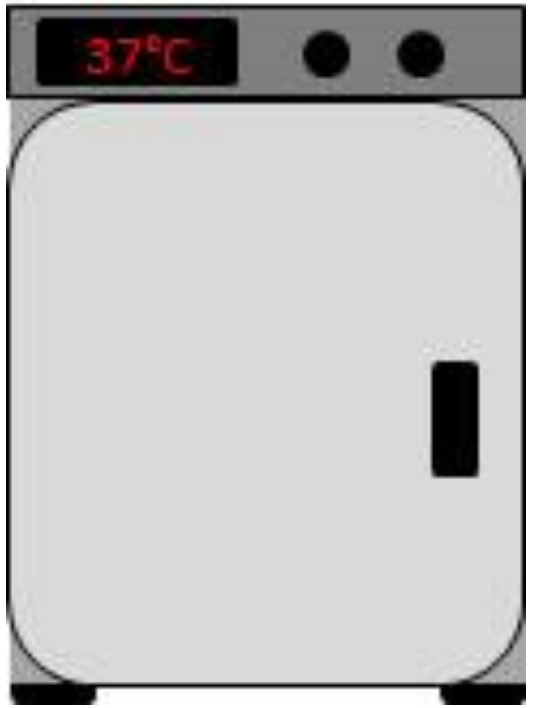


- **Transferring 50 µL of bacteria-reversion mix using a multichannel pipette**
- **384-well plate is divided into rows of 24 wells**
- **Each sample is pipetted into two rows (48 wells) to observe reversion**
- **Each plate can accommodate 8 samples**

14. Incubation of 384 well plate

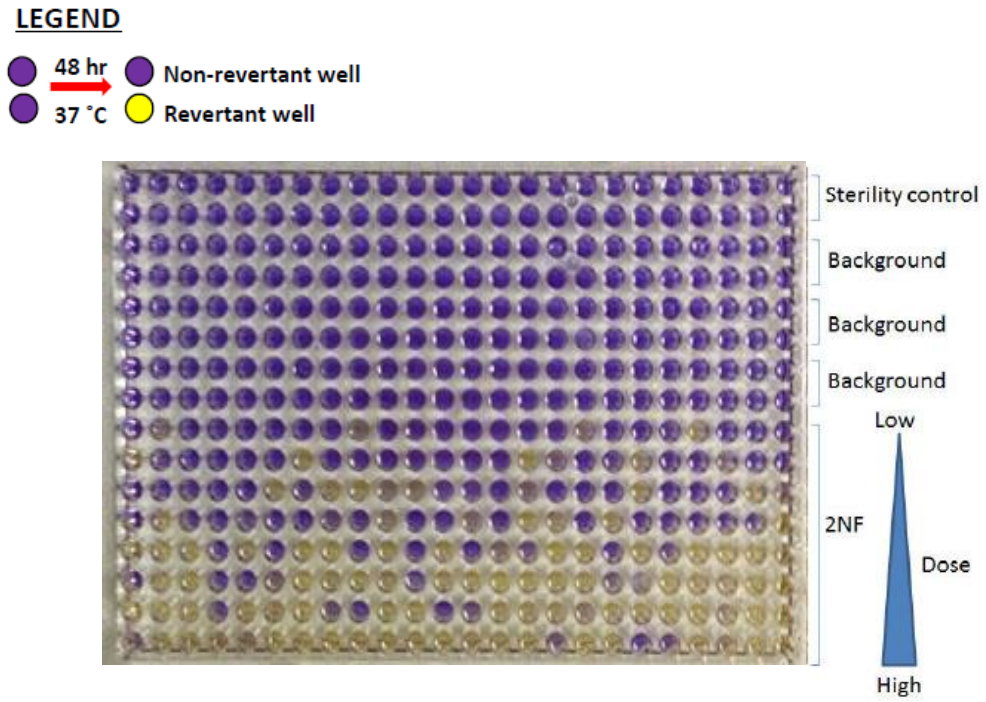
- Incubate 384-well plates at 37 °C for 2 days
- After two days, remove plates and observe the number of wells that have changed from purple to yellow
- Ensure that negative controls have small numbers of reversions, positive controls have many revertants and reagent sterility control does not have any revertants





Incubate at 37°C
For 2 days

Result Interpretation



Sample 384-well result plate

1. Plates were scored visually. Yellow and partial yellow wells are scored as positive. Purple wells are scored as negative.
2. The test is valid if the following criteria are met:
   a) Observe the `Blank' (sterility assessment) wells. Proceed only if the blank wells are sterile (purple). If the well is turbid or yellow, the assay may be contaminated or the sample is interfering with the reagents; results will be invalid.
   b) Average score for negative or background control is ≥ 0 and ≤ 15 revertant wells per 48-well section on day 2.
   c) Average score for positive (standard mutagen) controls is ≥ 25 revertant wells per 48-well section on day 2.

If any of these criteria are not met, the test is considered invalid!

Result Significance

No. Wells Positive in Background Plate	No. Wells Positive in Treatment Plate			No. Wells Positive in Background Plate	No. Wells Positive in Treatment Plate		
	0.05	0.01	0.001		0.05	0.01	0.001
0	3	6	9	24	33	36	40
1	5	8	12	25	34	37	40
2	7	10	14	26	35	38	41
3	9	12	15	27	36	39	42
4	10	13	17	28	36	40	43
5	12	15	19	29	37	40	43
6	13	16	20	30	38	41	44
7	14	18	21	31	39	42	45
8	16	19	23	32	40	43	46
9	17	20	24	33	41	44	46
10	18	21	25	34	42	44	47
11	19	23	27	35	43	45	47
12	20	24	28	36	43	46	48
13	21	25	29	37	44	46	49
14	22	26	30	38	45	47	49
15	24	27	31	39	46	48	50
16	25	28	32	40	46	48	50
17	26	29	33	41	47	49	50

- Depending of amounts of revertant wells in tested samples and revertant wells in negative controls, different levels of significance can be assigned
- Use the quick reference chart included with your procedure (left) or more advanced statistical methods to assign significance to mutagenicity results

In general, for mutagenicity to be suggested, there must be greater than double the amount of revertant wells compared to controls and evidence of a concentration-dependant dose-response relationship. (lower doses produce lower amounts of revertants)

AMES 384 ISO EXCEL Spreadsheet

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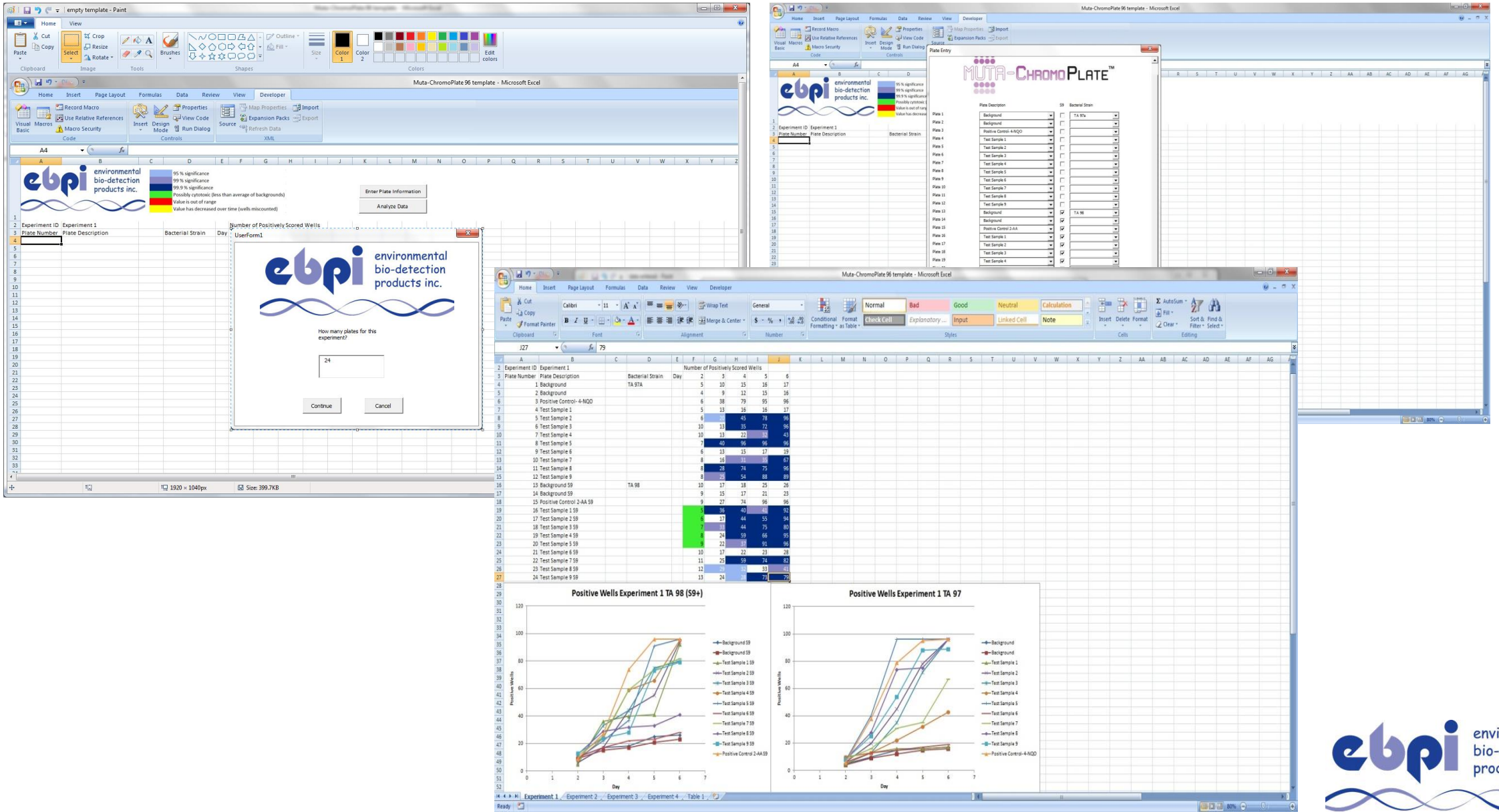

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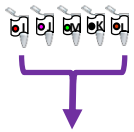
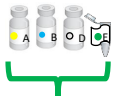


Filter Sterilize **Sample**

0.8 mL Filtered Sample + 0.8mL H_2O

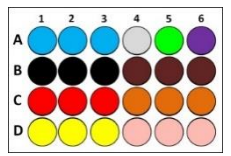
Incubate at 37°C for 12-16 hrs



Add **40% glucose**, **D-biotin**, **L-histidine** and **10X exposure solution** into a 15 ml tube to make a **master exposure mix**

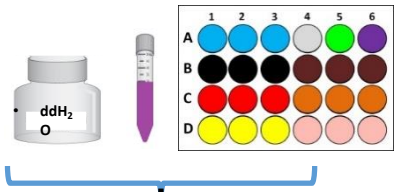
Transfer **0.8 ml of filtered sample**, **0.1 ml of bacteria** and **0.1 ml of master exposure mix** into each well

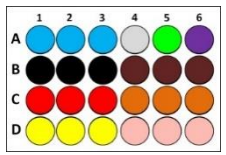
	1	2	3	4	5	6
A	Negative Control	Negative Control	Negative Control	Positive Control 1	Positive Control 2	Blank
B	No Dilution	No Dilution	No Dilution	Dilution 1	Dilution 1	Dilution 1
C	Dilution 2	Dilution 2	Dilution 2	Dilution 3	Dilution 3	Dilution 3
D	Dilution 4	Dilution 4	Dilution 4	Dilution 5	Dilution 5	Dilution 5



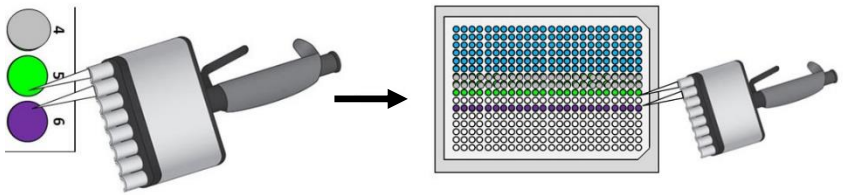
Incubate exposure plate for 120 min

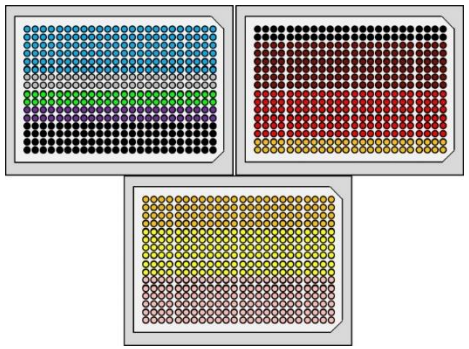
Add **40% glucose**, **bromocresol purple**, **D-biotin** and **10X reversion buffer** into a 15 ml tube to make a **master reversion mix**





After the 120 min incubation, transfer **0.5 ml of exposed bacteria from a single well**, **0.3 ml of master reversion mix**, **2.2 ml of H_2O** per well on a 24 well plate.





Parafilm seal and transfer plates into a sealing bag. Incubate at 37°C for 2-3 days

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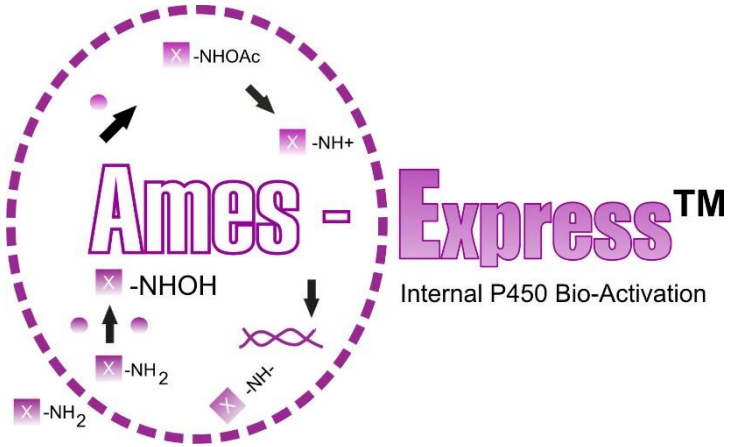
AMES - 384 ISO™
384 Well Format

Kit Options

- Basic Kits with and without or without S9 Activation
- TA100/TA98 Kits with or without S9 Activation
- OECD 471 Bacterial Based Kits
- 5, 10 Sample Kits
- Reagents Only Kits
- All kits/reagents can be modified to meet your requirements
- Ames Express Strains



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