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Microbial Mutagenicity Assay: Ames Test

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[Abstract] The Microbial mutagenicity Ames test is a bacterial bioassay accomplished *in vitro* to evaluate the mutagenicity of various environmental carcinogens and toxins. While Ames test is used to identify the revert mutations which are present in strains, it can also be used to detect the mutagenicity of environmental samples such as drugs, dyes, reagents, cosmetics, waste water, pesticides and other substances which are easily solubilized in a liquid suspension. We present the protocol for conducting Ames test in the laboratory.

Keywords: Mutagenicity, Carcinogenicity, *Salmonella* strains, Gene mutation, Revertants

[Background] The Microbial Ames test is a simple, rapid and robust bacterial assay consisting of different strains and applications of *Salmonella typhimurium/E. coli,* used for ascertaining the mutagenic potential (Levin *et al*., 1982; Gupta *et al.*, 2009). In 1975, Ames and his followers standardized the traditional Ames assay protocol and reappraised in 1980's (Maron and Ames, 1983). Induction of new mutations replacing existing mutations allows restoring of gene function. The newly formed mutant cells are allowed to grow in the absence of histidine and form colonies, hence this test is also called as 'Reversion assay' (Ames, 1971). While traditional Ames test is quite laborious and time consuming for initial monitoring of mutagenic compounds, miniaturization of liquid suspension significantly impacted the usability by making it more convenient. The standard doses (2 µl, 5 µl, 10 µl, 50 µl and 100 µl) were set to evaluate the mutagenicity from lower to higher concentration (Hayes, 1982). Mice liver has been used as a tissue for preparing homogenate 9,000 *x g* (S9 hepatic fraction) whereas in S9 mix, hepatocytes are used to minimize the mammalian metabolic activation formed in the mice liver. In Ames bioassay, the sensitivity of a compound for mutagenicity is based on the knowledge that a substance which is mutagenic in the presence of liver enzymes metabolizing compound might be a carcinogen (Mathur *et al*., 2005).

Genetic Approach: The *Salmonella/E. coli* tester strains: Several strains of *Salmonella typhimurium* have been used in Ames assay which requires histidine synthesis to assess the mutagenicity. In the histidine operon, each tester strain contains a different mutation. In addition to the histidine mutation, the standard tester strain of *Salmonella typhimurium* contains other mutations that greatly enhance their ability to detect the mutations (Figure 1). One of the mutations (*rfa*) causes partial loss of the lipopolysaccharides barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo[*a*]pyrene allowing not to penetrate in the normal cell wall (Mortelman and Zeiger, 2000). The mutagens present in the tested samples give rise to induced

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revertants on a minimal medium (absence of histidine). They are further used to observe revertants in previously mutated strains (that are not able to grow in a medium without histidine). The other mutation (*uvrB)* is a deletion mutation in which deletion of a gene, coding for the DNA excision repair system, causing gradually increased sensitivity in detecting many mutagens (Ames *et al*., 1973a). The reason behind this mutation is the deletion excising the *uvrB* gene emulsifying these bacteria requiring biotin for growth. The standard strains such as TA 97, TA 98, TA 100 and TA 102 contain the R-factor plasmid, pKM101. These R-factor strains are reverted by a number of mutagens that are detected weakly or not at all with the non R-factor parent strains (Ames *et al*., 1975a).

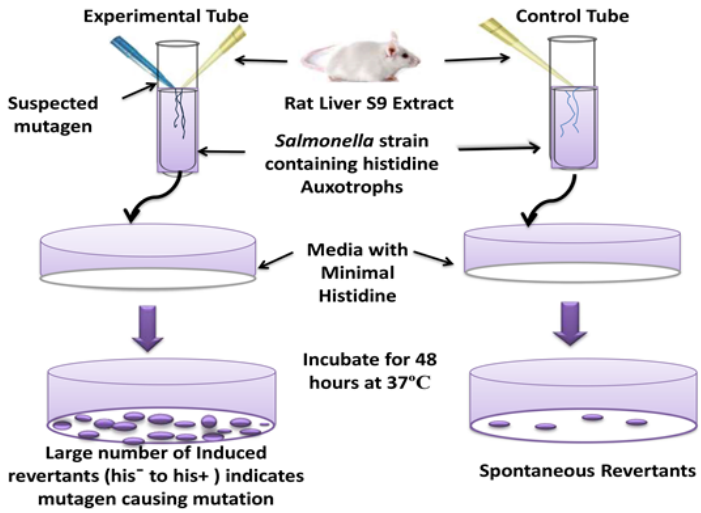

Figure 1. Genetic approach for assessing the mutagenicity in *Salmonella* strains (modified from https://en.wikipedia.org/wiki/Ames_test)

Many studies (Ames *et al.*, 1975b; Levin *et al.*, 1982) revealed that development of plasmid pKM101 in TA 1535 and TA 1538 strains leads to complement other isogenic strains such as TA 98, TA 100, TA 104 and TA 102. The *his* G46 mutation in TA 100 and TA 1535 codes for the first enzyme of histidine biosynthesis (*hisG)* (Ames *et al.*, 1975b). This mutation, determined by DNA sequence analysis, substitutes proline (-GGG-) for leucine (-GAG-) in the wild type organism (Barnes *et al.*, 1982). The tester strains TA 1535 and its R-factor derivative present in TA 100, detect mutagens which causes base-pair substitutions generally at one of these G-C pairs. The hisD3052 mutation in TA 1538 and TA 98 is in the *hisD* gene coding for histodinol dehydrogenase. TA 1538 and its R-factor derivative TA 98 detect various frameshift mutagens in repetitive sequences as ‘hot spots’ resulting in a frame shift mutation (Walker and Dobson, 1979; Shanabruch and Walker, 1980) (Table 1).

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Table 1. Genotype of the *Salmonella* strain used for mutagenesis testing

Strain	Reversion event	Histidine mutation	LPS defect, markers	R-factor	Target DNA	Plasmid	References
TA 98	Frame shift	D3052	*rfa, uvrB*	+R	CGCGCGCG	pKM101	Simmon *et al.*, 1977
TA 1538	Frame shift	D3052	*rfa, uvrB*	-R	CGCGCGCG	-	Ames *et al.*, 1975b
TA 100	Base pair substitution	G46	*rfa, uvrB*	+R	GGG	pKM101	Maron and Ames, 1981 and 1983
TA 1535	Base pair substitution	G46	*rfa, uvrB*	-R	GGG	-	Maron and Ames, 1983
TA 1537	Frame shift	C3076	*rfa, uvrB*	-	CCC	-	Zeiger *et al.*, 1985
TA 102	Transition/Transversion	G428	*rfa, uvrB*	+R	TAA	pKM101, pAQ1	Venitt and Boswoth, 1983
***E. coli* WP2 uvrA**	Base pair substitution	-	*uvrA*	-	-	-	Brusick *et al.*, 1980

Levin *et al*. (1982) described a standard strain *Salmonella typhimurium* bacterium called TA 102 which was used to evaluate the effect of some compounds reacting with nucleotides AT. Tester strain TA102 containing nucleotides AT, present in *hisG* gene carrying plasmid pAQ1. There are certain mutagenic agents which are detected by TA 102 but not by TA 1535, TA 1537, TA 1538, TA 98 and TA 100 (Wilcox *et al*., 1990). Before performing experiment, a new set of fresh strains are prepared; and the genotypes are assessed (R-factor, His, *rfa* and *uvrB* mutations). For these, we refer readers to many excellent reviews (Walker, 1979; Czyz *et al.,* 2002; Fluckiger-Isler *et al.,* 2004).

Certain carcinogens present in active forms in biological reaction are easily catalyzed by cytochrome-P450. Metabolic activation system is absent in *Salmonella*, and in order to improve the potentiality of bacterial test systems, liver extracts of Swiss albino mice are used. This serves as a rich source in converting carcinogens to electrophilic chemicals that are incorporated to detect *in vivo* mutagens and carcinogens (Garner *et al*., 1972; Ames *et al*., 1973a). The crude liver homogenate as 9,000 *x g* S9 fraction contains free endoplasmic reticulum, microsomes, soluble enzymes and some cofactors set with S9 concentration to 10% (Franz and Malling, 1975). The oxygenase requires the reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADP) which is generally *in situ* by the action of glucose-6-phosphate dehydrogenase and reducing NADP both work as cofactors in assay (Prival *et al*., 1984; Henderson *et al*., 2000). While water is considered as a negative control, sodium azide, 2-nitrofluorine and mitomycin for TA 98, TA 100 and TA 102 without S9 metabolic activation and 2-anthramine with S9 hepatic fraction are used as positive controls for conducting the test (Table 2). Before performing the experiment, fresh solutions must be prepared.

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Table 2. Positive controls with and without S9 metabolic activation (DeFlora *et al*., 1984)

Positive controls	
With S9 metabolic activation	**Without S9 metabolic activation**
2-Aminofluorene	2-Nitrofluorene
1,8-Dihydroxyanthraquinone	Sodium azide
2-Aminoanthracene	Mitomycin C
Cyclophosphamide	Methyl methanesulfonate
2-Anthramine	9-Aminoacridine

Spontaneous Reversion Control: Each strain of *Salmonella* contains a specific mutant range. Selection of solvents shows the effect on the frequency range of spontaneous mutant (Maron and Ames, 1983) (Table 3). The range of revertants varies in research laboratories. The spontaneous revertants are visible through unaided eyes (Figure 2).

Table 3. Spontaneous revertants control values for various strain types and number of revertants (Mortelmans and Stocker, 1979)

Strain	**Spontaneous**	**Revertants**
	With S9	Without S9
TA 98	20-50	20-50
TA 100	75-200	75-200
TA 102	100-300	200-400
TA 104	200-300	300-400
TA 1535	5-20	5-20
TA1537	5-20	5-20

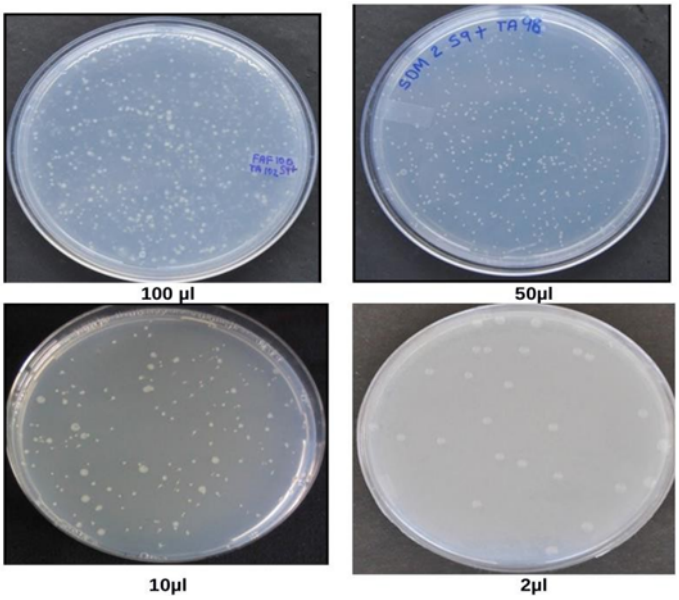

Figure 2. Spontaneous revertants colonies obtained after addition of waste water from health center in *Salmonella* mutagenicity assay at different concentrations, viz. 2 µl, 10 µl, 50 µl, 100 µl (Vijay, 2014)

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Materials and Reagents

A. Materials
1. Tips (1,000 µl, 200 µl, 10 µl) (Tarsons)
2. Sterile Petri plates (HiMedia Laboratories, catalog number: PW001)
3. Erlenmeyer flask and beaker (SchottDuran,10 ml, 250 ml, 500 ml)
4. Eppendorf tubes (Tarsons,1.5 ml, 2.0 ml)
5. Metal loop holder (metal loop Ch-2, HiMedia Laboratories, catalog number: LA012)
6. L shaped spreader(HiMedia Laboratories, catalog number: PW1085)

B. Mutagens
1. Sodium azide (HiMedia Laboratories, catalog number: GRM1038)
2. 4-Nitroquinoline *N*-oxide (Sigma-Aldrich, catalog number: N8141)
3. 2-Aminofluorene (Sigma-Aldrich, catalog number: A55500)
4. Benzo(*a*)pyrene (Sigma-Aldrich, catalog number: B1760)
5. Mitomycin C (Roche Diagnostics, catalog number: 10107409001)
6. 2,4,7-Trinitro-9-fluorenone (Accustandard, catalog number: R-033S)
7. 4-Nitro-*o*-phenylenediamine (Sigma-Aldrich, catalog number: 108898)

C. Reagents
1. Oxoid nutrient broth No. 2 (Sigma-Aldrich, catalog number: 70123)
   Note: This product has been discontinued.
2. 70% ethanol
3. Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) (HiMedia Laboratories, catalog number: RM683)
4. Citric acid monohydrate (HiMedia Laboratories, catalog number: GRM1008)
5. Potassium phosphate, dibasic (K_2HPO_4) (anhydrous) (Merck, catalog number: 61788005001730)
6. Sodium ammonium phosphate tetrahydrate ($NaNH_4HPO_4 \cdot 4H_2O$) (Sigma-Aldrich, catalog number: S9506)
7. D-biotin (HiMedia Laboratories, catalog number: TC096)
8. L-histidine (HiMedia Laboratories, catalog number: TC076)
9. Hydrochloric acid (HCl) (HiMedia Laboratories, catalog number: AS003)
10. Potassium chloride (KCl) (Merck, catalog number: 61753305001730)
11. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (HiMedia Laboratories, catalog number: MB040)
12. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (Merck, catalog number: 1063700050)
13. Disodium hydrogen phosphate (Na_2HPO_4) (HiMedia Laboratories, catalog number: TC051)
14. NADP (sodium salt) (HiMedia Laboratories, catalog number: RM392)

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15. D-glucose-6-phosphate (monosodium salt) (Sigma-Aldrich, catalog number: G7879)
16. Ampicillin trihydrate (Sigma-Aldrich, catalog number: A6140)
17. Sodium hydroxide (NaOH) (Merck, catalog number: 106462)
18. Crystal violet (Sigma-Aldrich, catalog number: C6158)
19. Agar-Agar (Himedia Laboratories, catalog number: RM026)
20. Nutrient broth (HiMedia Laboratories, catalog number: M002)
21. Tetracycline (Sigma-Aldrich, catalog number: 87128)
22. Dimethylsulfoxide (HiMedia Laboratories, catalog number: TC185)
23. Vogel-Bonner medium E (50x) (see Recipes)
24. 0.5 mM histidine/biotin solution (see Recipes)
25. Salt solution (1.65 M KCl + 0.4 M $MgCl_2$) (see Recipes)
26. 0.2 M sodium phosphate buffer, pH 7.4 (see Recipes)
27. 1 M Nicotinamide Adenine Dinucleotide Phosphate (NADP) solution (see Recipes)
28. 1 M glucose-6-phosphate (see Recipes)
29. Ampicillin solution (4 mg/ml) (see Recipes)
30. Crystal violet solution (0.1%) (see Recipes)
31. Minimal glucose plates (see Recipes)
32. Histidine/Biotin plates (see Recipes)
33. Ampicillin and tetracycline* plates (see Recipes)
34. Nutrient agar plates (see Recipes)
35. S9 mix (Rat Liver Microsomal Enzymes + Cofactors) (see Recipes)
36. Sodium azide (see Recipes)
37. Mitomycin (see Recipes)
38. 2-Anthramine (see Recipes)

Equipment

1. Orbital shaking incubator (Remi, model: RIS-24(BL))
2. Laminar Flow hood (Bio safety cabinet) (Deepak Meditech Pvt Ltd., Steri clean)
3. Pipettes (Eppendorf, model: Research® plus, catalog number: 3120000062, 1,000 µl; catalog number: 3120000046, 200 µl; catalog number: 3120000020, 10 µl)
4. Vortex mixer (Labnet International, catalog number: S0100)
5. Hot water bath (Daiki Sciences, catalog number: KBLee2001)
6. Autoclave (TSC)
7. Automatic Colony counter (Sonar)
8. Refrigerator centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus Biofuge Primo R)
9. pH meter (Labindia Analytical Instruments, model: PICO pH Meter, catalog number: PC13330101)

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10. Tissue tearor (Bio Spec Products, catalog number: 985370-04)

Procedure

1. Before performing the experiment, inoculate a single fresh colony of standard strains of *S. typhimurium* TA 98, 100 and 102, in oxoid nutrient broth-2 and incubate for 10-12 h at 37 °C in an incubator shaker at 120 rpm to ensure sufficient aeration for 1 x 10^9 bacterial cells. Each strain of *S. typhimurium* is grown separately in Erlenmeyer flasks (10 ml).
2. Prepare fresh mutagen for each experiment (see Recipes).
   Negative control: Autoclaved distilled water
   Positive controls for TA 98, TA 100 and TA 102 without S9 metabolic activation (S9 mix): sodium azide (1 µg/ml) 2-nitrofluorine (1 µg/ml) and mitomycin (0.125 µg/ml)
   For TA 98, TA 100 and TA 102 with S9 metabolic activation (S9 mix): 2-Anthramine (2 µg/ml)
3. Preparation of minimal glucose agar (MGA) plates: Mix the medium of minimal glucose agar plates (Recipe 9) and pour 25 ml into each Petri dish. Prepare the plates freshly before use.
4. Label all minimal glucose agar plates and Eppendorf tubes prior to experiment.
5. To the 2 ml sterile Eppendorf tubes, add the following each:
   a. 0.1 ml fresh culture of *Salmonella* strains
   b. 0.2 ml of His/Bio solution
   c. 0.5 ml sodium phosphate buffer (absence of S9 mix) or 0.5 ml S9 (presence of S9 mix)
   d. 0.1 ml of test sample or 0.1 ml of positive or negative control
   e. Make up to 1 ml with autoclaved distilled water.
6. Mix the contents of Eppendorf tubes and pour onto Petri plates and spread using L-shaped spreader on the surface of MGA plates. Cover the Petri plates with sterile aluminum foil to protect the testing sample from photo reactive substances.
7. After incubation of 48 h at 37 °C, spontaneous revertants colonies appear and are clearly visible with unaided eyes. All plates are run in triplicates.
8. Revertants form a uniform lawn of auxotrophic bacteria on the surface the background of medium.

Data analysis

Non-statistical analysis

The most widely used method for non-statistical analysis of result in Ames test is ‘two-fold rule’ described by Mortelmans and Zeiger (2000) and Morino-Caniello and Piegorsch (1996). On the basis that the increase in the number of revertant colonies, the concentration of the tested sample goes up (dose-dependent manner), mutagenicity ratio (MR) is calculated first by counting the number of revertant colonies per plate and then calculating the MR as described by Maron and Ames (1983) using the formula below (see Sample data below for results):

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$$M.R = \frac{S.R + I.R}{S.R(Negative\ control)}$$

M.R = Mutagenicity Ratio

S.R = Spontaneous Revertants

I.R = Induced Revertants

Sample data

Medical liquid waste was collected from different health care premises of Jaipur city. *Salmonella* mutagenicity test was performed on all the samples in their crude natural state using the plate incorporation procedure described by Maron and Ames, 1983. The results of *Salmonella* mutagenicity assay was analyzed through Mutagenicity Ratio method and shown in Table 4.

Table 4. Mutagenicity ratios of *S. typhimurium* strains TA98, TA100 and TA102 treated with waste water from different health premises (Vijay, 2014)

Health centers	Concentrations (µl)	Mutagenicity Ratio TA 98		Mutagenicity Ratio TA 100		Mutagenicity Ratio TA 102	
		-S9	+S9	-S9	+S9	-S9	+S9
Government Hospital (Untreated) (GH)	2	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	50	+	+	+	+	+	+
	100	+	+	+	+	+	+
Private Hospital (Untreated) (PH)	2	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	50	+	+	+	+	+	+
	100	+	+	+	+	+	+
Private Hospital (Treated) (PH)	2	-	-	-	-	-	-
	5	-	-	-	-	-	-
	10	-	-	-	-	-	-
	50	-	-	+	-	-	-
	100	-	-	-	-	-	-

+Mutagenicity Ratio > 2.0 imply mutagenic, -Ratio < 2.0 imply non-mutagenic

Conclusion

The Ames test is a widely accepted bacterial assay to detect the mutagenicity in pathogenic bacteria. In this protocol, although we have shown the step wise methodology to perform Ames assay applicable for three strains, this method can be used for studying all compounds to infer mutagenicity. Whereas the Ames assay experiments involve sterile measures, care must be taken in ensuring the sample/plasmid is not contaminated. The improved methods to detect the genotoxicity of compounds help us troubleshoot methods for studying the compounds tested in clinical trials.

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Notes

Sterilization (safety considerations while working with *Salmonella*)

1. As *S. typhimurium* is a pathogenic bacterium, it is prudent to use precautionary measures every time and apply standard biosafety guidelines such as using plugged pipettes, proper sterilization by 70% ethanol and autoclaving all contaminated material.
2. Handling of chemicals and strains should be done in biosafety cabinet. Before and after the use, cabinet must be sterilized using 70% ethanol and exposed to 15 min UV.
3. Care must be taken to protect from chemical exposure by wearing gowns, eye glasses and gloves.
4. Before discarding, all contaminated material (*e.g.*, test tubes, pipettes and pipette tips, gowns and gloves) should be properly autoclaved.

Limitations

Ames assay consists of *Salmonella typhimurium* strains and so it is not a perfect model for human. Mice liver S9 hepatic fraction is used to minimize the mammalian metabolic activations formed in the hepatic system so that the mutagenicity of metabolites can be assessed. There are several differences between human and mice metabolism which can affect the mutagenicity of testing substances. Major disadvantages of fluctuation test is slower and slightly more laborious than Ames protocol. The test is primarily used for testing aqueous samples containing low levels of mutagen and therefore, this test is well adapted for evaluating the mutagenicity of wastewater samples.

Recipes

1. Vogel-Bonner medium E (50x)
   For Minimal agar (Recipe 9)

Ingredients	Per 500 ml
Warm distilled H_2O (45 °C)	335 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	5 g
Citric acid monohydrate	50 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	250 g
Sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	87.5 g

   a. Salts are added to the warm water in a flask. Place the flask on a hot plate
   b. After each salt dissolves entirely, transfer the solution into glass bottles and autoclave for 20 min at 121 °C
   c. When the solution gets cool, cap the bottle tightly
   d. Store the solution at 4 °C

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2. 0.5 mM histidine/biotin solution

   For mutagenic bioassay

Ingredients	Per 125 ml
D-Biotin (F.W. 247.3)	15.45 mg
L-Histidine·HCl (F.W. 191.7)	12.0 mg
Distilled H_2O	125 ml

   Dissolve the biotin in hot distilled water. The solution is autoclaved for 20 min, at 121 °C and then stored at 4 °C

3. Salt solution (1.65 M KCl + 0.4 M $MgCl_2$)

   For S9 hepatic fraction

Ingredients	Per 250 ml
Potassium chloride (KCl)	30.75 g
Magnesium chloride ($MgCl_2·6H_2O$)	20.35 g
Distilled H_2O to final concentration of	250 ml

   All the components are dissolved in water. The solution is autoclaved for 20 min, at 121 °C and then stored at 4 °C

4. 0.2 M sodium phosphate buffer, pH 7.4

   For S9 hepatic fraction

Ingredients	Per 250 ml
0.2 M sodium dihydrogen phosphate ($NaH_2PO_4·H_2O$)	30 ml (6.9 g/250 ml)
0.2 M disodium hydrogen phosphate (Na_2HPO_4)	220 ml (7.1 g/250 ml)

   Adjust pH to 7.4. Sterilize the buffer by autoclaving for 20 min at 121 °C

5. 1 M nicotinamide adenine dinucleotide phosphate (NADP) solution

   For S9 hepatic fraction

Ingredients	Per 2.5 ml
NADP	191.5 mg
Sterile distilled H_2O	2.5 ml

   NADP is dissolved in the distilled water and mixed by vortexing. Tubes are placed in an ice bath. The solution can be used for up to six months

6. 1 M glucose-6-phosphate

   For S9 hepatic fraction

Ingredients	Per 5 ml
Glucose-6-phosphate (G-6-P)	1.41 g
Sterile distilled H_2O	5 ml

   Glucose-6-phosphate is dissolved in the 5 ml distilled water and mixed by vortexing. Tubes are placed in an ice bath. The solution can be used for up to six months

7. Ampicillin solution (4 mg/ml)

   Used in tests of ampicillin resistance

   Master plates for R-factor strains

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Ingredients	Per 500 ml
Ampicillin trihydrate	0.4 g
Sodium hydroxide (0.02 N)	50 ml

Ampicillin trihydrate is dissolved in the 50 ml of NaOH (0.02 N) and mixed by vortexing. Tubes are placed in an ice bath

8. Crystal violet solution (0.1%)

   Used in tests for crystal violet sensitivity (to confirm rfa mutation)

Ingredients	Per 500 ml
Crystal violet	0.05 g
Distilled H_2O	50 ml

9. Minimal glucose plates

   Used in Mutagenic bioassay

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	465 ml
50x VB salts (Recipe 1)	10 ml
40% glucose	25 ml

Add agar in 465 ml of distilled water and autoclave for 20 min, at 121 °C. After cooling, add the salts and glucose gently

10. Histidine/Biotin plates (Master plates for non R-factor strains)

    Used in tests for histidine requirement

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	457 ml
50x VB salts	10 ml
40% glucose	25 ml
Sterile histidine (2 g per 400 ml H_2O)	5 ml
Sterile 0.5 mM biotin	3 ml

Dissolve agar in the given concentration in distilled water. Autoclave each solution separately for 20 min. After cooling of solution, add each salt gently

11. Ampicillin and tetracycline* plates

    *Master plates for the cultivation of strains containing the plasmids pKM101 and pAQ1**

Ingredients	Per 500 ml
Agar	7.5 g
Distilled H_2O	405 ml
50x VB salts	10 ml
40% glucose	25 ml
Sterile histidine (2 g per 400 ml H_2O)	5 ml
Sterile 0.5 mM biotin	3 ml

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Sterile ampicillin solution (8 mg/ml 0.02 N NaOH)	1.58 ml
*Sterile tetracycline solution (8 mg/ml 0.02 N HCl)	0.125 ml

Dissolve agar in the given concentration in distilled water. Autoclave each solution separately for 20 min. After cooling of solution, add each salt gently

**Note: TA 102 is resistant to tetracycline. The shelf life of the plates is two weeks at 4 °C.*

12. Nutrient agar plates

Used in tests for genotypes [Crystal violet sensitivity (rfa) and UV sensitivity (AuvrB)] and viability of bacteria

Ingredients	Per 500 ml
Nutrient agar	7.5 g
Distilled H_2O	500 ml

Dissolve agar in the given concentration in distilled water. Autoclave separately for 20 min. Pour the cooled solution into the Petri plates

13. S9 mix (Rat Liver Microsomal Enzymes + Cofactors)

Ingredients	Standard S9 mix Per 25 ml
Mice liver	1.0 ml (2%)
$MgCl_2$-KCl salts	0.5 ml
1 M glucose-6-phosphate	0.125 ml
0.1 M NADP	1.0 ml
0.2 M phosphate buffer, pH 7.4	12.5 ml
Sterile distilled H_2O	9.86 ml

Note: Add each ingredient in the reverse order listed above (First water, and then phosphate buffer…). Avoid refreezing the S9 mix.

14. Sodium azide

Used in Mutagenicity assay

Ingredients	Per ml
Sodium azide	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

15. 2-Nitrofluorine

Used in Mutagenicity assay

Ingredients	Per ml
2-Nitrofluroine	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

16. Mitomycin

Used in Mutagenicity assay

Ingredients	Per ml
Mitomycin	10 µg

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Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

17. 2-Anthramine

Used in Mutagenicity assay

Ingredients	Per ml
2-Anthramine	10 µg
Autoclave distilled H_2O	990 µl (to make a total volume of 1 ml)

Working concentrations are prepared by taking 1, 2, 4 µl of 10 mg/ml

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