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APPENDIX

APPENDIX

1. Preparation of Stock Solution and Medium

1.1 Vogel-Bonner medium E stock salt solution (VB salt)

Use : Minimal agar

Ingredient	1 liter
Warm distilled water (45 $^{\circ}$C)	670 ml
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate, dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate tetrahydrate ($NaNH_4HPO_4.4H_2O$)	175 g

Salts were added to water in order that indicated above and allowed each salt to dissolve completely before adding the next. The solutions were filtered and then autoclaved at 121°C for 15 min.

1.2 Minimal glucose agar plate

Use : Mutagenicity assay

Ingredient	1 liter
Bacto agar	15 g
Distilled water	930 ml
VB salts	20 ml
40% glucose	50 ml

Agar was added to distilled water in a glass bottle and then autoclave at 121°C for 15 min. When the solution has cooled slightly, sterile VB salts and sterile 40% glucose were added, mixed and poured 30 ml into each sterile petri plate. Minimal glucose agar plates were kept in incubator at 37°C before using.

1.3 Oxoid nutrient broth NO.2

Use : Growing culture

2.5 g of nutrient broth No.2 was dissolved in 100 ml distilled H_2O and 12 ml of nutrient broth was transferred into each flask (covered with sterile gauze). They were autoclaved at 121°C for 15 min.

1.4 Top agar

Use : Mutagenic assay

Ingredient	100 ml
Bacto agar	0.6 g
Sodium chloride (NaCl)	0.5 g
Distilled water	100 ml

All ingredients were dissolved in water and stored in a glass bottle. The solution was autoclaved at 121°C for 15 min. 10 ml of 0.5 mM histidine HCl-0.5 mM biotin was added for 100 ml of top agar.

1.5 0.1 M L-histidine HCl stock

Use : Fortification of minimal agar plate

Ingredient	100 ml

L-histidine HCl	2.096 g
Distilled water	100 ml

2.096 g of L-histidine HCl (MW 209.63) was dissolved in 100 ml distilled water.

1.6 1 mM L-histidine HCl stock

Use : Fortification of minimal agar plate.

Ingredient	100 ml
0.1 M L-histidine HCl	1 ml
Distilled water	99 ml

1 ml of 0.1 M L-histidine HCl was diluted in 99 ml of distilled water

1.7 1 mM biotin stock

Use : Fortification of minimal agar plate.

Ingredient	100 ml
Biotin	24.43 mg
Distilled water	100 ml

Biotin (MW 244.3) was dissolved in distilled water, warmed and stirred untill dissolve completely.

1.8 0.5 mM L-histidine HCl / biotin solution.

Use : Mutagenicity assay (add 10 ml to 100 ml of Top agar)

Ingredient	200 ml
1 mM L-histidine HCl	100 ml
1 mM biotin	100 ml

The ingredients were mixed and autoclaved at 121°C for 15 min.

1.9 Na_3PO_4-KCl buffer

Use : Mutagenicity assay

Ingredient	330 ml
0.5 M Na_3PO_4 pH 7.4	100 ml
1 M KCl	16.5 ml
Distilled water	213.5 ml

The ingredient were mixed and autoclaved at 121°C for 15 min.

1.10 1 M Potassium chloride

Use : Na_3PO_4-KCl buffer for mutagenicity assay

Ingredient	1,000 ml
Potassium chloride	74.56 g
Distilled water	1,000 ml

Potassium chloride was dissolved into water.

1.11 0.5 M sodium phosphate pH 7.4

Use : Na_3PO_4-KCl buffer for mutagenicity assay

Ingredient

0.5 M Sodium dihydrogen phosphate (NaH_2PO_4) (MW120) (30 g / 500ml)

0.5 M Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (MW 177.99)

(44.5 g / 500 ml)

44.5 g of disodium hydrogen phosphate dihydrate was dissolved in 300 ml of distilled water and 0.5 M of disodium hydrogen phosphate dihydrate was added untill to pH 7.4 and then it was autoclaved at 121°C for 15 min.

2. Recipes for Some Reagents and Test Chemicals

2.1 2M sodium nitrite

Use : Nitrosation

Ingredient	10 ml
Sodium nitrite	1.38 g
Distilled water to	10 ml

The ingredient were mixed and autoclaved at 121°C for 15 min.

2.2 2M ammonium sulfamate

Use : reaction mixture

Ingredient	10 ml
Ammonium sulfamate	2.28 g
Distilled water to	10 ml

Ammonium sulfamate was dissolved in distilled water. Then, it was autoclaved for 15 min at 121°C.

2.3 0.5 N hydrochloric acid

Use : reaction mixture

Ingredient	100 ml

Conc. hydrochloric acid	5.37 ml
Sterile distilled water	94.63 ml

Conc.hydrochloric acid was dissolved in sterile distilled water and stored in sterile glass tubes or bottles with screw cap.

Note : Preparation of 0.5 N HCl must be used sterile technique because hydrochloric acid cannot be autoclaved.

2.4 3 mg/ml aminopyrene

Use : Standard solution for mutagenicity assay

Ingredient	1 ml
Aminopyrene	3 mg
Acetonitrile	1 ml

Aminopyrene was dissolved in acetonitrile. Then, it was stored in sterile vial with screw cap in the freezer. Preparation of this solution must be used sterile technique.

2.5 8 mg/ml ampicillin solution

Ingredient	10 ml
Ampicillin (sodium)	80 mg
Distilled H_2O	10 ml

Ampicillin was dissolved into water and stored in glass bottle with screw cap. Preparation of this solution must be used sterile technique.

2.6 0.1% crystal violet

Ingredient	10 ml
Distilled H_2O	10 ml
Crystal violet	10 mg

Crystal violet was dissolved into water and stored in glass bottle with screw cap. Preparation of this solution must be used sterile technique.

3. Procedure for Re-isolation and Growing Culture

Tester strains, TA98 and TA100 were grown in Oxoid nutrient broth No.2 and incubated overnight in 37°C in shaking water bath. The growth period should not exceed 16 hours. These cultures were re-isolated by streaking on minimal glucose agar plates which the surface were spreaded with 0.1 ml of 8 mg/ml ampicillin, 0.3 ml of 0.1 M histidine HCl and 0.1 ml of 1 mM biotin. These plates were incubated at 37°C for 48 hours. After incubation, the 4 single colonies per strain of TA98 and TA100 were picked up and grown in Oxoid nutrient broth No.2 and shaken overnight at 37°C in shaking water bath. Each culture was confirmed genotypes of the strains and kept the cultures as the source of bacteria for mutagenicity testing. For each 1 ml of culture, 0.09 ml of spectrophotometric grade DMSO was added. Combine the culture and DMSO in a sterile tube and distribute 200 µl of the culture aseptically into sterile cryotube (Nunc) and store in freezer at –80°C.

4. Confirming Genotype of Tester Strains

The broth cultures of TA98 and TA100 were used to confirm genotypes in the following ways.

4.1 Histidine requirement

The his⁻ character of the strains was confirmed by demonstrating the histidine requirement for growth on the minimal glucose agar plates enriched with histidine and biotin

Procedure:

plate a	no histidine and biotin
plate b	0.1 ml of 1 mM biotin
plate c	0.3 ml of 0.1 M his-HCl
plate d	0.3 ml of 0.1 M his-HCl + 0.1 ml of 1 mM biotin

Four minimal glucose agar plates were required for each tester strains. Each of the plates was applied on the surface with 0.1 ml of 1 mM biotin, 0.3 ml of 0.1 M his-HCl, 0.3 ml of 0.1 M his-HCl plus 0.1 ml of 1 mM biotin and no application (plate b, c, d, a respectively). A single streak of each strain was made across these plates. Four strains could be tested on the same plate. Incubated at 37°C for 24 hours. The growing of bacteria on histidine plus biotin plate was the result of histidine requirement.

4.2 R-factor

The R-factor strains (TA97, TA98, TA100 and TA102) should be tested routinely for the presence of the ampicillin resistance factor because the plasmid is somewhat unstable and can be lost from the bacteria.

Procedure: For each tester strain (TA98 and TA100), 0.3 ml of overnight culture was added to a tube containing 0.1 ml of 0.1 M histidine-HCl. And then 2 ml of moltentop agar containing 0.5 mM histidine-HCL and 0.5 mM biotin were added, mixed and poured on a minimal agar plate. The plate was rotated in order to distribute the mixtures and allowed several minutes for agar to become firm. R-factor and rfa mutation (see the next section) are performed in the same plate by dividing the plate into 2 parts, one for R-factor and the other for rfa mutation. For R-factor, filter paper disc containing 8 mg/ml ampicillin is applied on the surface of the agar by using sterile forceps. The disc was pressed lightly to embed in the overlay. The plates were incubated at 37°C for 24 hours. The absence of the clear zones of inhibition around the disc indicates resistance to ampicillin.

4.3 Rfa mutation

Strains having the deep rough (rfa) character should be tested for crystal violet sensitivity.

Procedure: 0.1% solution of crystal violet was pipetted to the sterile filter paper disc (1/4 inch) and transfer the disc to plates that seed with bacteria (the procedure was similar to R-factor). Incubated at 37°C for 48 hours. The clear zone appered around the

disc indicated the presence of the rfa mutation (crystal violet transferred into the cell and kill bacteria).

5. Spontaneous Reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a uniform background lawn of auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. Neverthrless, there is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, and it is advisable to include at least 2-3 spontaneous mutation control plates for each strain in a mutagenicity assay.

Procedure: 0.1 ml of DMSO was added to capped culture tube. 0.5 ml of $NaPO_4$-KCl buffer pH 7.4 and 0.1 ml of fresh overnight culture of TA98 or TA100 was added. The mixture was incubated in shaking water bath at 37°C in 20 min. After that 2.0 ml of molten top agar was added to the mixture, mixed and then poured on the incubated at 37°C for 48 hours. The his^+ revertants colonies that grown on the minimal glucose agar plate were counted.

6. The Response to Standard Mutagen

Standard mutagens or positive mutagens are used routinely in mutagenicity experiments to confirm the reversion property and specificity of each strain. The standard mutagen, which used in this experiment, was nitrosated-aminopyrene. Tester strains that highly response to positive mutagens will be chosen.

Procedure: The procedure is as described in spontaneous reversion except aminopyrene (0.06 and 0.12 µl per plate for TA98 and TA100, respectively) are used instead of sterile distilled water in absence S-9 mix, respectively. The characteristic of stock culture for TA98 and TA100 as the source of bacteria for mutagenicity is

a) contained R-factor (pKM 101) and rfa mutation

b) His^+ requirement

c) Low spontaneous reversion

d) Highly response to standard carcinogen

After the characteristic of the culture was tested, the mutagenicity test was Started.

VITA

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