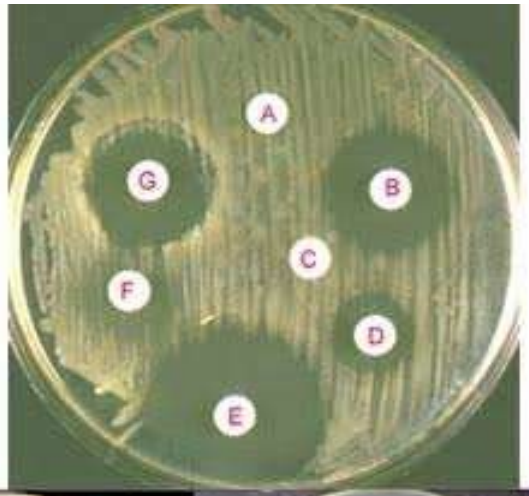
Microbiological Assay

Mueller Hinton Agar Plate



M173
E. coli
25922

Introduction

- **A microbiological assay defined as qualitative or quantitative determination of chemical compound from a simple or even complex material with the use of microorganisms**.
- Many anti-microbial agents, which inhibit the growth of microorganisms **(antibiotics)** or are essential for their growth **(vitamins** and amino acids).
- RELATIVE POTENCY: **defined as the term used to express the biological activity of a sample preparation compared to a standard preparation.**

Advantages:

- **Simple**
- **Specific**
- **Minimum requirements of space, labour, materials and time**
- **Inexpensive and**
- **Convenient method**

Disadvantages:

- **Required skilled technician**
- **Required proper calibration**
- **Less reproducible**
- **Chances of greater error**

Microbiological assay of an antibiotic

- The microbiological assay of an antibiotic is **based upon a comparison of the inhibition of growth of micro-organisms by measured concentrations of the antibiotics under examination with that produced by known concentrations of a standard preparation of the antibiotic having a known activity.**
- To **be utilised for demonstrating the therapeutic efficacy of antibiotics.**
- **Assay: it is a biological testing method to determine content or quality of substances.**

Two general methods are usually employed:

I) The cylinder-plate (or cup-plate) method (method A) and

II) The turbidimetric (or tube assay) method(method B)

- The cylinder-plate method depends upon diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish to an extent such that growth of the added micro-organism is prevented entirely in a zone around the cylinder containing a solution of the antibiotic.
- The turbidimetric method depends upon the inhibition of growth of a microbial culture in a uniform solution of the antibiotic in a fluid medium

Culture media used for antibiotic assay

- Dissolved all the required ingredients (table1) in sufficient water to produced 1000ml
- pH can be maintained by adding 1M solution of HCl or NaOH.
- Followed by the sterilization of the media

as required, so that after sterilization the pH is ...

Table 13.1: Composition of media: Quantities in gm per 1000 ml medium

Ingredient	A	B	C	D	E	F	G	H	I	J
Peptone	6.0	6.0	5.0	6.0	6.0	6.0	9.4	–	10.0	–
Yeast extract	3.0	3.0	1.5	3.0	3.0	3.0	4.7	–	–	–
Beef extract	1.5	1.5	1.5	1.5	1.5	1.5	2.4	–	10.0	–
Pancreatic digest of casein	4.0	–	–	4.0	–	–	–	17.0	–	15.0
Dextrose	1.0	–	1.0	1.0	–	–	10.0	2.5	–	–
Papaic digest of soyabean	–	–	–	–	–	–	–	3.0	–	5.0
Agar	15.0	15.0	–	15.0	15.0	15.0	23.5	12.0	17.0	15.0
Glycerin	–	–	–	–	–	–	–	–	10.0	–
Polysorbate 80	–	–	–	–	–	–	–	10.0	–	–
Sodium chloride	–	–	3.5	–	–	–	10.0	5.0	3.0	5.0
Dipotassium hydrogen phosphate	–	–	3.68	–	–	–	–	2.5	–	–
Potassium dihydrogen phosphate	–	–	1.32	–	–	–	–	–	–	–
Final pH	6.5	6.5	6.95 –	7.8 –	7.8 –	5.8	6.0	7.1	6.9	7.2
	– 6.6	– 6.6	7.05	8.0	8.0	– 6.0	– 6.2	– 7.3	– 7.1	– 7.4

Buffer solution

- Prepare by dissolving the following quantities given in Table2.
- sufficient water to produce 1000 ml after sterilisation, adjusting the pH with 8 M phosphoric acid or 10 M potassium hydroxide.

Buffer No.	Dipotassium Hydrogen Phosphate, K_2HPO_4(g)	Potassium Dihydrogen phosphate, KH_2PO_4(g)	pH adjusted after sterilization to
1	2.0	8.0	6.0±0.1
2	16.73	0.523	8.0±0.1
3	-	13.61	4.5±0.1
4	20.0	80.00	6.0±0.1
5	35.0	-	10.5±0.1*
6	13.6	4.0	7.0±0.2

Preparation of standard

- A Standard Preparation is an authentic sample of the appropriate antibiotic for which the potency has been precisely determined by reference to the appropriate international standard.
- The Potency of the standard preparation may be expressed in International Units or in µg per mg of the pure antibiotic.
- Ex: Dissolve a quantity of the standard preparation of a given antibiotics in the solvents (table3). Dilute the preparation to get the required concentration as stated and stored in a refrigerator.
- Usually prepared in the ratio of 1:1.5

Table 13.2: Stock solutions and test dilutions of standard preparation

Antibiotic	Assay method	Initial solvent for std. stock solution	Final std. stock conc^{n}/ml	Final diluent for test dilution	Median dose ug or Units/ml of test sol^{n}.
Amikacin	B	Water	1 mg	Water	10 µg
Amphotericin B	A	Dimethyl sulphoxide	1 mg	B5	1 µg
Bacitracin	A	0.01 M HCl	100 units	B1	1 unit
Bleomycin	A	B6	2 units	B6	0.04 unit
Capreomycin	B	Water	1 mg	Water	100 µg
Carbenicillin	A	B1	1 mg	B6	20 µg
Chlortetracycline	A[1]	0.1 M HCl	1 mg	Water	2.5 µg
	B[2]	0.1 M HCl	1 mg	Water	0.24 µg
Colistimethate sodium	A	Water	1 mg	B4	1 unit
	B	Water	1 mg	B6	1 unit
Colistin sulphate	A	Water	1 mg	B6	1 µg
Erythromycin	A	Methanol	1 mg	B2	1 µg
Framycetin	A	B2	1 mg	B2	1 µg
Gentamicin	A	B2	1 mg	B2	0.1 µg
Kanamycin sulphate	A[1]	B2	800 units	B2	0.8 unit
	B[3]	Water	1000 units	Water	10 unit
Neomycin	A	B2	1 mg	B2	1 µg
Novobiocin	A	Ethanol	1 mg	B4	0.5 µg
Nystatin	A	Dimethyl formamide	1000 units	B4	20 unit
Oxytetracycline	A[4]	0.1 M HCl	1 mg	B3	2.5 µg
	B[3]	0.1 M HCl	1 mg	Water	0.24 µg
Polymyxin B	A	Water	10,000 units	B4	10 unit
Spiramycin	A[5]	Methanol	1 mg	B2	12-50 units
Streptomycin	A[5]	Water	1 mg	Water	1 µg
	B[6]	Water	1 mg	Water	30 µg
Tetracycline	A[4]	0.1 M HCl	1 mg	Water	2.5 µg
	B[3]	0.1 M HCl	1 mg	Water	0.24 µg
Tobramycin	B	Water	1 mg	Water	2.5 µg
Vancomycin	A	Water	1 mg	B3	10 µg

P.T.O.

Preparation of the test sample

- prepare on the day of the assay a stock solution and test dilution as specified for each antibiotic in Table 4 but with the same final diluents as used for the Standard Preparation.
- The assay with 5 levels of the Standard requires only one level of the unknown at a concentration assumed equal to the median level of the standard.

Preparation of Test organism

- The test organism for each antibiotic is listed in Table, together withits identification number in the American Type Culture

Antibiotic	Test Organism	ATCC1 No.
Amikacin	*Staphylococcus aureus*	29737
Amphotericin B	*Saccharomyces cerevisiae*	9763
Bacitracin	*Micrococcus luteus*	10240
Bleomycin	*Mycobacterium smegmatis*	607
Carbenicillin	*Pseudomonas aeruginosa*	25619
Chlortetracycline	*Bacillus pumilus*	14884
Erythromycin	*Micrococcus luteus*	9341
Framycetin	*Bacillus pumilus*	14884
	Bacillus subtilis	6633
Gentamicin	*Staphylococcus epidermidis*	12228
Kanamycin sulphate	*Bacillus pumilus*	14884
	Staphylococcus aureus	29737
Neomycin	*Staphylococcus epidermidis*	12228
Novobiocin	*Staphylococcus epidermidis*	12228
Nystatin	*Saccharomyces cerevisiae*	2601
Oxytetracycline	*Bacillus cereus var; mycoides*	11778
	Staphylococcus aureus	29737
Polymyxin B	*Bordetella bronchiseptica*	4617
Spiramycin	*Bacillus pumilus*	6633
Streptomycin	*Bacillus subtilis*	6633
	Klebsiella pnumoniae	10031
Tetracycline	*Bacillus cereus*	11778
	Staphylococcus aureus	29737
Tobramycin	*Staphylococcus aureus*	29737
Tylosin	*Staphylococcus aureus*	9144

Preparation of inoculums

- **Inoculums is the mixture of microbes along with the culture media in which it is growing.**

Steps involved:

- Maintain the test microbes on slant of medium A and transfer to a fresh slant once a week.
- Incubate the slant at the specified temperature for 1day
- Using 3ml of slant solution, wash the microbes from agar slant on to a large surface of medium A such as a Roux bottle containing 250ml of agar media
- Incubate for 1day at the required temperature
- Wash the growth from the nutrient surface using 50ml of saline solution.
- Store the test microbes under refrigerator

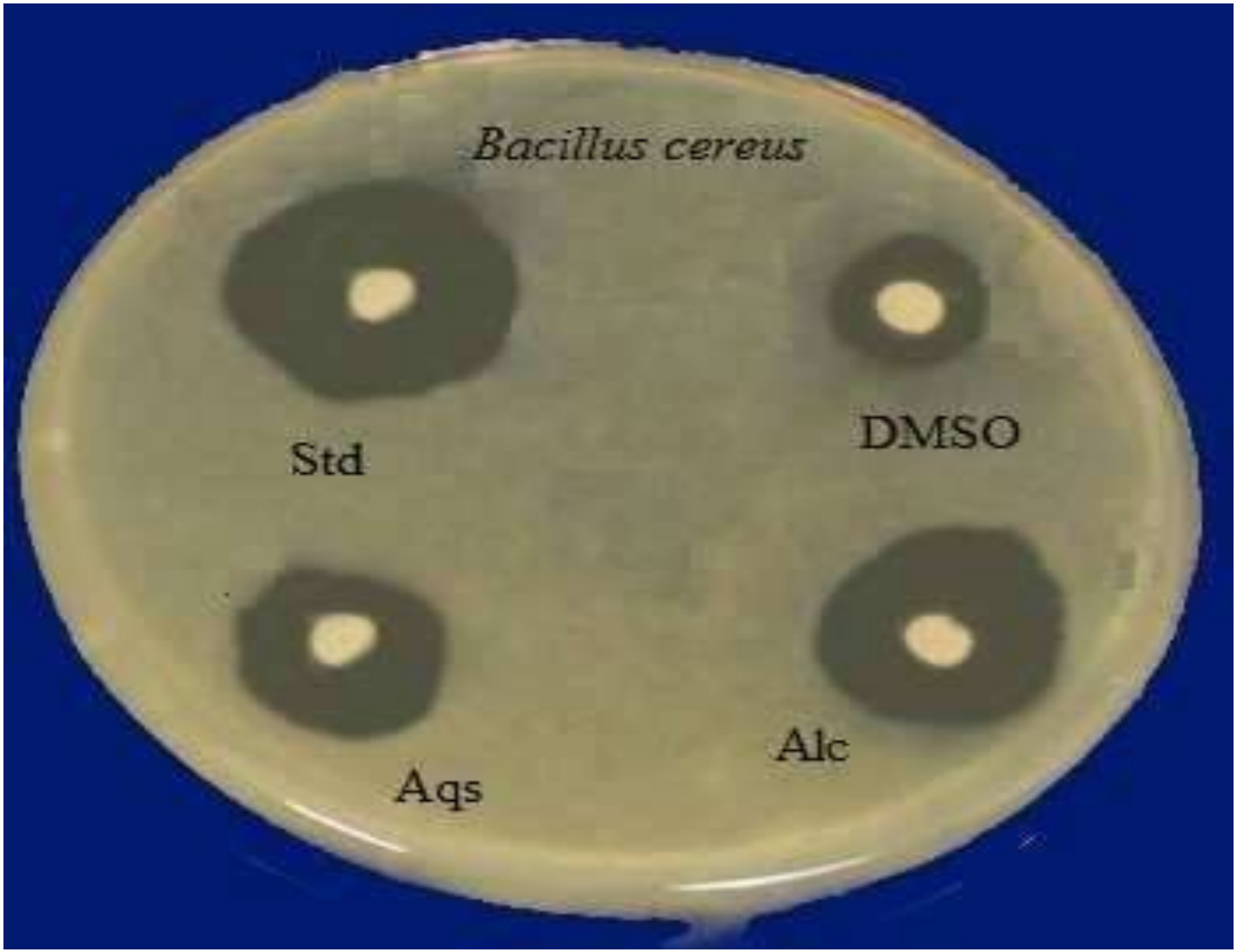
Methods of Microbiological Assay

- **A. Cylinder plate or Cup plate method**
- **B. Turbidimetric or Tube Assay method**

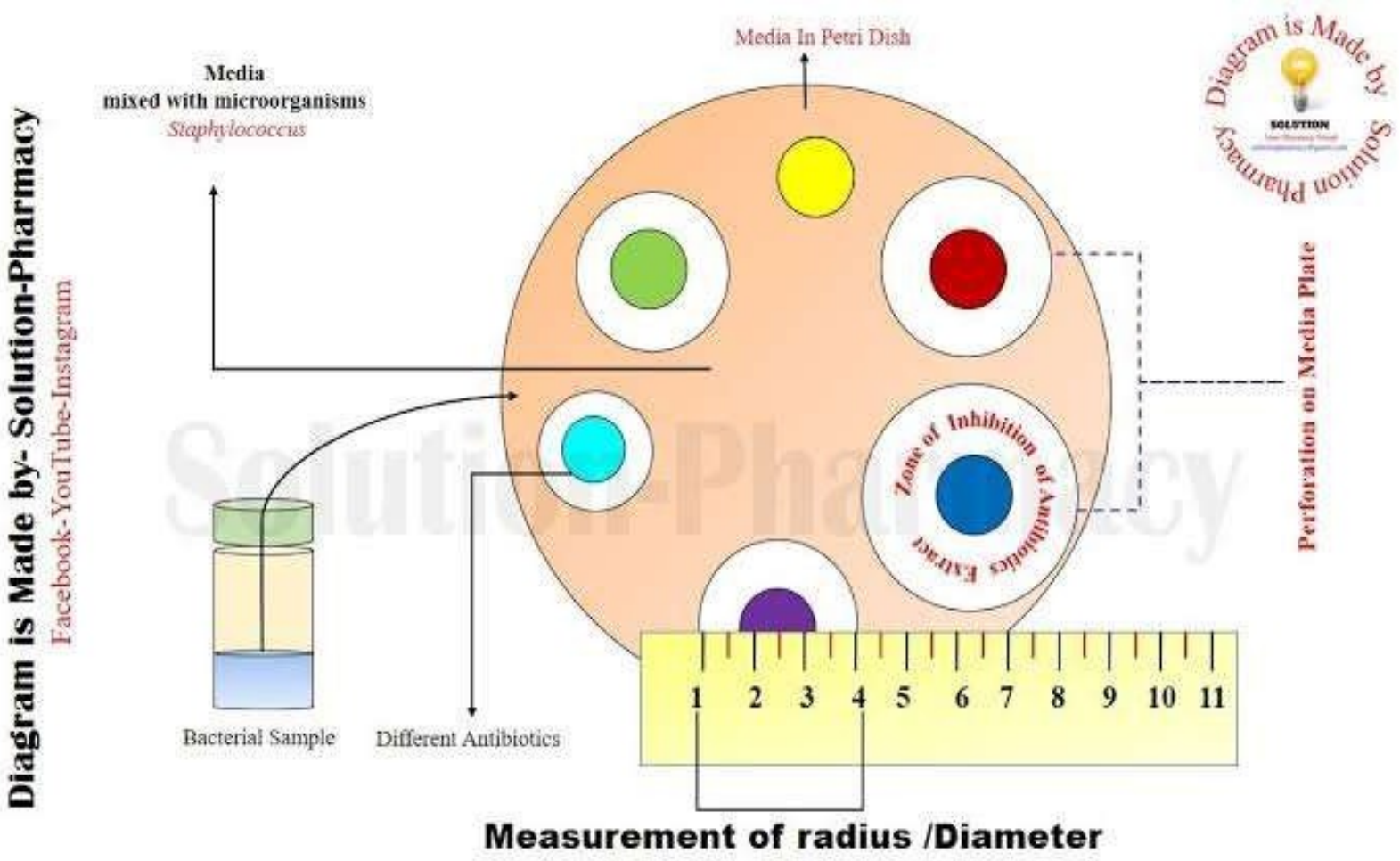
A. Cylinder plate or cup plate method

- A previously liquefied medium with the required quantity of microbial suspension is inoculated
- The suspension is added to the medium at a temperature between 40-50 degree and inoculated medium is immediately poured
- The solution are applied to the surface of the solider medium in sterile cylinder or in ager cavities
- They are incubated for about 18 hours at the temperature indicated
- The quantities estimation of antibiotic is done by accurately measuring the diameter of the circular inhibition zones .

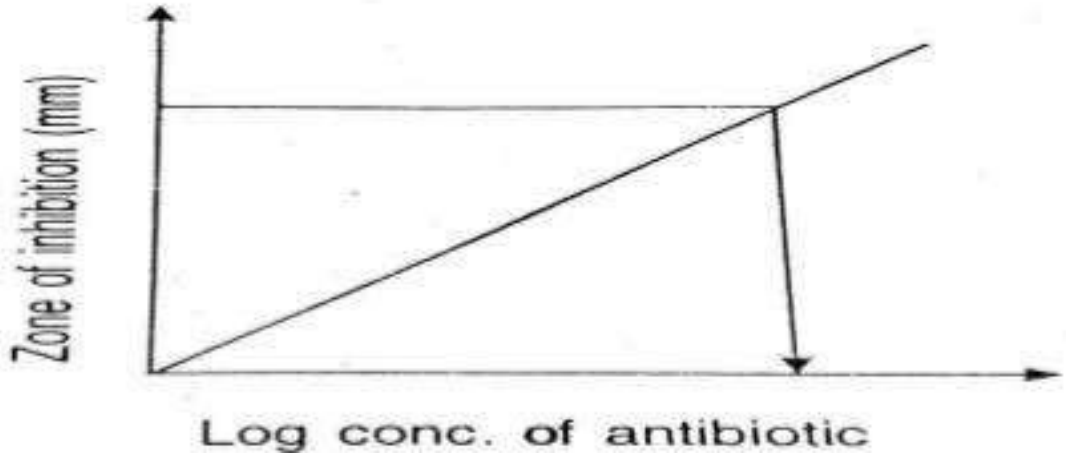
Simple image



Measurement of zone of inhibition

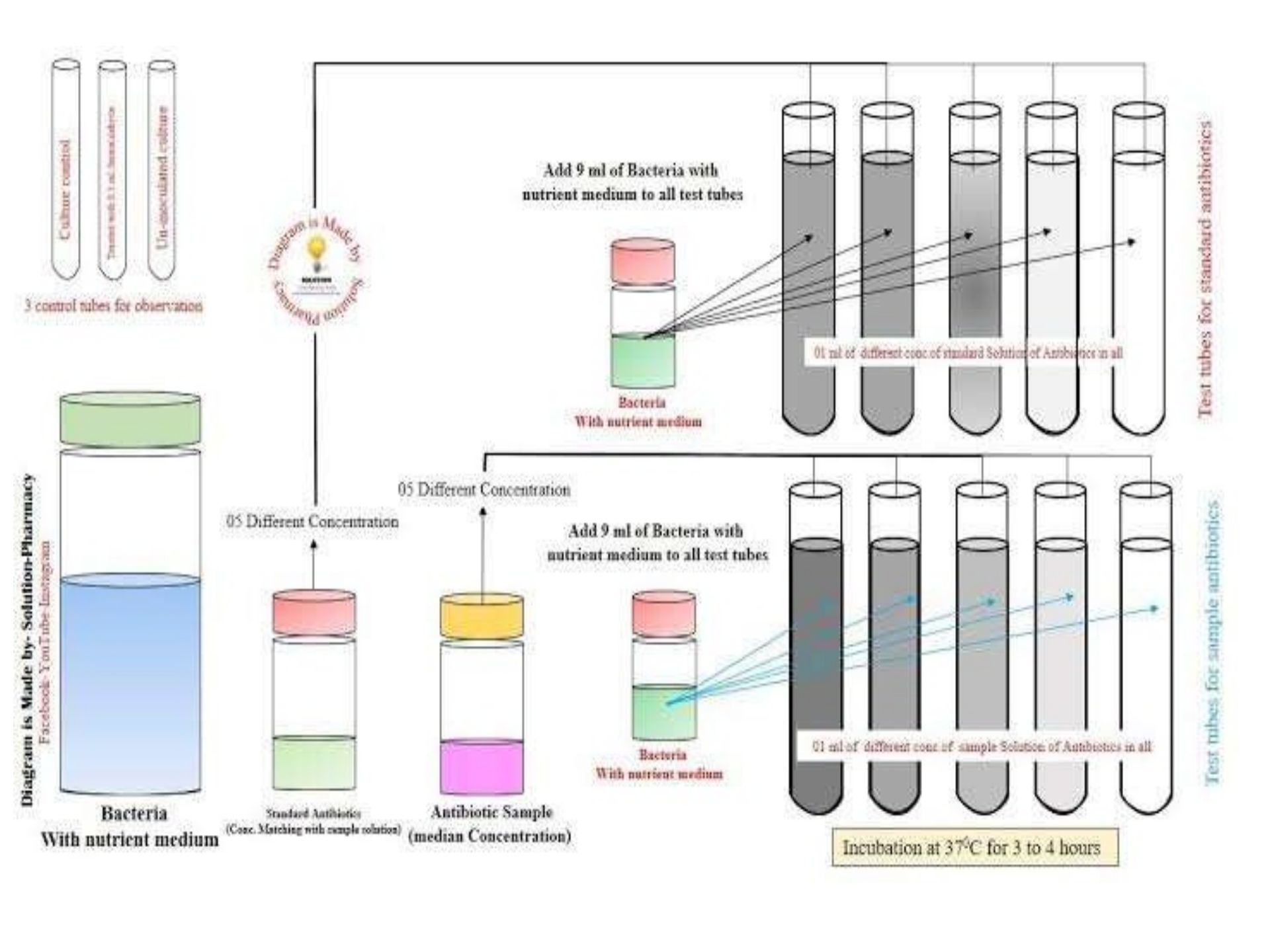


Standard curve of microbial assay of antibiotic (sample)



B. Turbidimetric or Tube Assay method

- **Advantage-** shorter incubation period for the growth of the test organism(usually 3 to 4 hrs)
- **Disadvantage-**The presence of solvent residues inhibitory substances affects more.
- Not recommended for cloudy or turbid preparation.
- Five different concentration of the standard solution are prepared for preparing the standard curve.
- 1ml of each concentration of the standard solution of the sample solution are placed in each of the tubes in duplicate at 9 ml of nutrients medium previously seeded with the appropriate test organism at to each other


Culture control
Un-inoculated culture
3 control tubes for observation
Diagram is Made by Solution Pharmacy
Add 9 ml of Bacteria with nutrient medium to all test tubes
Bacteria With nutrient medium
01 ml of different conc of standard Solution of Antibiotics in all
Test tubes for standard antibiotics
05 Different Concentration
05 Different Concentration
Add 9 ml of Bacteria with nutrient medium to all test tubes
Diagram is Made by- Solution-Pharmacy
Facebook-YouTube-Instagram
Bacteria With nutrient medium
Standard Antibiotic (Conc. Matching with sample solution)
Antibiotic Sample (median Concentration)
Bacteria With nutrient medium
01 ml of different conc of sample Solution of Antibiotics in all
Test tubes for sample antibiotics
Incubation at 37°C for 3 to 4 hours

- Five tubes containing the inoculated culture medium with standard drug with a specific dose and test organism.
- Five tubes containing culture medium with test organism and the test sample with different dosages.
- Another one treated immediately with **0.5 ml of dilute formaldehyde solution(blank)**
- All the tubes are placed in an incubator and maintain at the **specified temperature- 37°C for 3 to 4 hour.**
- The growth of the test organism is measured by determining the absorbance **at 530 nm of its against the blank.**
- The standard calibration curve is prepared and the absorbance obtained for the sample is plotted on it