

Antibiotic Technology Laboratory

Lab 7: Antimicrobial Synergy Testing using the Checkerboard Method

The rise of Multidrug-Resistant (MDR) pathogens has rendered many monotherapies ineffective. Synergy testing is a specialized technique used to evaluate the interaction between two antimicrobial agents (e.g., two antibiotics, or an antibiotic combined with a nanomaterial or plant extract or any other material).

The checkerboard assay: is an in vitro method to test antimicrobial synergy between two agents, like antibiotics.

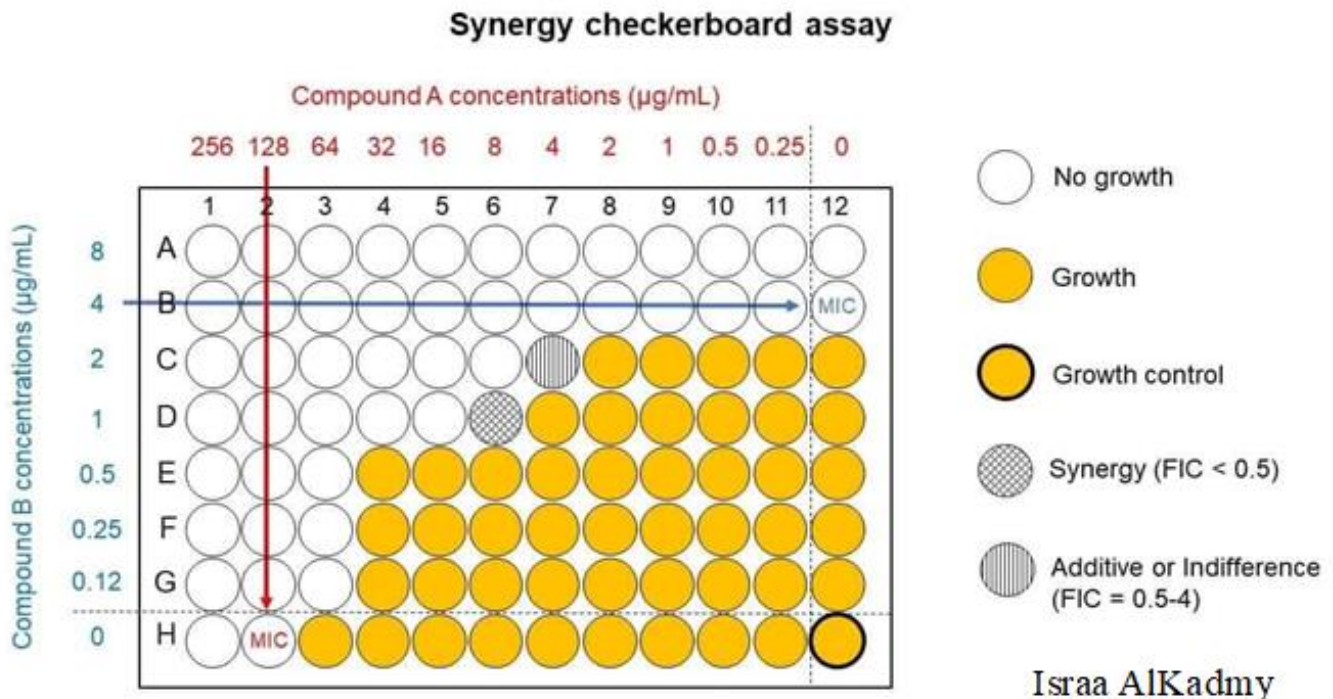
The goal is to determine if the combined effect is greater than the sum of their individual activities.

Purpose: It evaluates if drugs work better together (synergy), additively, or antagonistically against bacteria. Researchers use it for multidrug-resistant strains, such as *Pseudomonas aeruginosa*.

This assay helps identify combination therapies by comparing minimum inhibitory concentrations (MICs).

How It Works

Serial dilutions of each agent form a grid in a 96-well plate—one across columns, one down rows. Wells are inoculated with bacteria, incubated, and growth is assessed visually or via turbidity. The fractional inhibitory concentration index (FICI) quantifies interaction: $FICI \leq 0.5$ indicates synergy.



Objectives

1. To understand the concepts of Synergy, Additivity, and Antagonism.
2. To perform the Checkerboard Titration Method using 96-well microtiter plates.
3. To calculate and interpret the Fractional Inhibitory Concentration Index (FICI).

Feature of Microdilution Checkerboard:

1. Setup Time is Fast pipetting
2. Low Cost and small volume of broth media.
3. Contamination Risk is Minimal.
4. Synergy Readout by FICI grid.

Materials & Reagents

Microorganisms: Standardized bacterial suspension (e.g., *A. baumannii* or *S. aureus*) adjusted to 0.5 McFarland (1×10^8 CFU/mL).

Antimicrobials: Agent A (Standard Antibiotic).

Agent B (Second Antibiotic, Nanomaterial, or Plant Extract).

Media: Cation-Adjusted Mueller-Hinton Broth (CAMHB).

Equipment: 96-well microtiter plates, multichannel pipettes, incubator (37°C), and a microplate reader (optional).

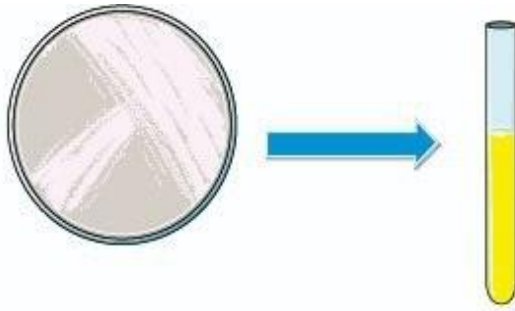
Experimental Procedure (The Checkerboard Layout)

The "Checkerboard" refers to the arrangement of two agents in a grid where the concentration of Agent A decreases along the columns and Agent B decreases along the rows. prepare a 96-well plate with serial 2-fold dilutions: drug A horizontally (columns), drug B vertically (rows), starting above expected MICs. Add bacterial inoculum ($\sim 5 \times 10^5$ CFU/mL), incubate (e.g., 18-24h at 37°C), and score growth (clear vs. turbid wells). **Serial Dilutions:** Dispense Agent A in horizontal rows (decreasing concentrations from left to right). Dispense Agent B in vertical columns (decreasing concentrations from top to bottom).

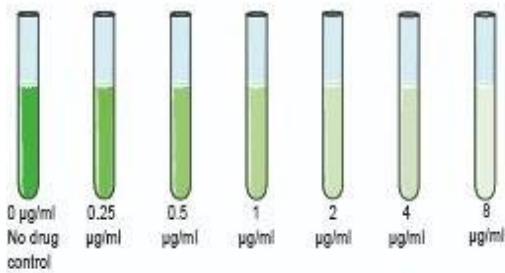
1. Prepare Plate for Drug A (Horizontal): Add 100 μ L 2x MHB to column 1; 50 μ L to columns 2-11; 100 μ L to column 12 (growth control). Serially dilute drug A 2-fold across columns 1-11 using 50 μ L transfers.
2. Add Drug B (Vertical): Pipette 50 μ L 2x drug B dilutions down rows A-H into columns 1-11 (leave column 12).
3. Mix Thoroughly: Use multichannel pipette for mixing in each column to ensure even concentrations.
4. Inoculate: Add 50 μ L standardized bacteria to all wells (final $\sim 5 \times 10^5$ CFU/mL); column 12 gets broth only.
5. Incubate: 37°C, 18-24h, static or shaking.
6. Read Results: Visually score turbidity (growth) vs. clear (inhibition); determine MICs alone/combo; calculate FICI.

Perform ≥ 3 replicates; automate, if possible, for precision.

After incubation, identify the MIC for each agent alone and in combination. The MIC is the lowest concentration that shows no visible growth.

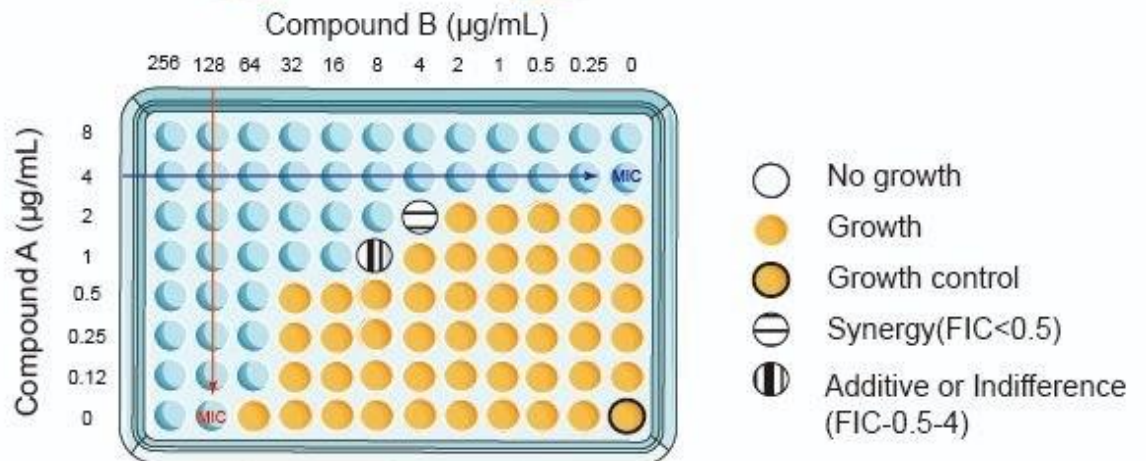
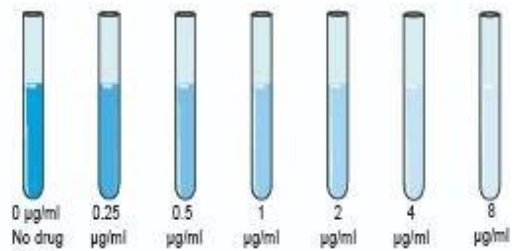


Compound A



0.5 McFarland turbidity
Pseudomonas aeruginosa

Compound B



96-Well Checkerboard Layout for Synergy Testing

0.5	512 / 64	256 / 64	128 / 64	64 / 64	32 / 64	16 / 64	8 / 64	4 / 64	2 / 64	1 / 64	0.5 / 64	0.25 / 64
1	512 / 32	256 / 32	128 / 32	64 / 32	32 / 32	16 / 32	8 / 32	4 / 32	2 / 32	1 / 32	0.5 / 32	0.25 / 32
2	512 / 16	256 / 16	128 / 16	64 / 16	32 / 16	16 / 16	8 / 16	4 / 16	2 / 16	1 / 16	0.5 / 16	0.25 / 16
4	512 / 8	256 / 8	128 / 8	64 / 8	32 / 8	16 / 8	8 / 8	4 / 8	2 / 8	1 / 8	0.5 / 8	0.25 / 8
8	512 / 4	256 / 4	128 / 4	64 / 4	32 / 4	16 / 4	8 / 4	4 / 4	2 / 4	1 / 4	0.5 / 4	0.25 / 4
16	512 / 2	256 / 2	128 / 2	64 / 2	32 / 2	16 / 2	8 / 2	4 / 2	2 / 2	1 / 2	0.5 / 2	0.25 / 2
32	512 / 1	256 / 1	128 / 1	64 / 1	32 / 1	16 / 1	8 / 1	4 / 1	2 / 1	1 / 1	0.5 / 1	0.25 / 1
64	512 / 0.5	256 / 0.5	128 / 0.5	64 / 0.5	32 / 0.5	16 / 0.5	8 / 0.5	4 / 0.5	2 / 0.5	1 / 0.5	0.5 / 0.5	0.25 / 0.5
	512	256	128	64	32	16	8	4	2	1	0.5	0.25

Caffeic Acid (µg/mL)

The fractional inhibitory concentration index (FICI) Formula:

The FICI formula clearly shows antimicrobial synergy in checkerboard assays for student understanding.

How to calculate FICI in checkerboard assay and why use these constrictions?

The fractional inhibitory concentration index quantifies antimicrobial interactions in checkerboard assays. The interaction is evaluated using the following equations:

Determine MICs for drugs A and B alone (MIC-A, MIC-B). In the checkerboard grid, identify the lowest concentrations inhibiting growth together (MIC_A_comb, MIC_B_comb). Compute FICI as:

$$FICI = \frac{MIC_A_comb}{MIC_A} + \frac{MIC_B_comb}{MIC_B}$$

Interpret: ≤0.5 (synergy), 0.5–1 (additive), 1–4 (indifferent), >4 (antagonism)

Example: MIC-A=16 µg/mL, MIC-B=32 µg/mL alone; comb=4+8 µg/mL →

$$FICI = 4/16 + 8/32 = 0.5 \text{ (synergy)}$$

According to international standards (e.g., CLSI/EUCAST), the FICI values are interpreted as follows:

FICI Value	Interpretation	Clinical Significance
$FICI \leq 0.5$	Synergy	The combination is significantly more effective individual drugs. drugs work better together
$0.5 < FICI \leq 1.0$	Additivity	The combined effect is a simple summation of individual effects. combined effect equals sum alone
$1.0 < FICI \leq 4.0$	Indifference	No significant interaction between the two agents. no clear interaction
$FICI > 4.0$	Antagonism	The drugs interfere with each other, reducing effectiveness. one reduces other's effectiveness