INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES

*Improving The Quality of Life*

PROTOCOL

# *In Vitro* Testing of Deodorants' Antibacterial Activity against Bacteria Isolated from Human Axilla


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## FOREWORD

This protocol is based on *in vitro* study conducted at Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The in vitro study is done to test and compare the antimicrobial activity of deodorants against Indonesian males' axillary microbiome. The study consists of bacterial growth curve and standard curve; Minimum Inhibitory Concentration (MIC); preliminary test of ideal bacteria-to-deodorant ratio; spot plating assay; time-kill assay; serial dilution; and, bacterial culture on agar media, observation and counting of colony forming unit (CFU).

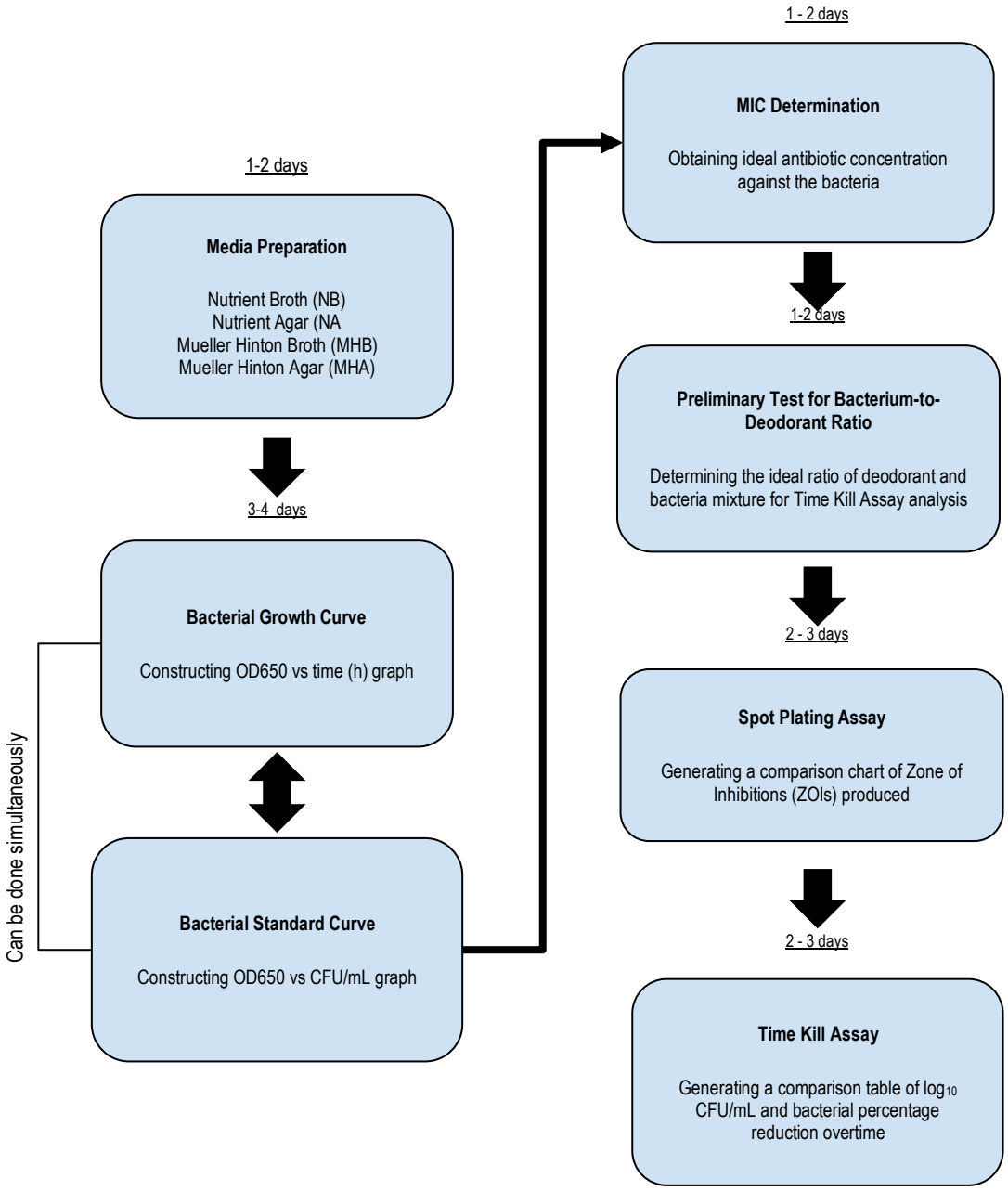
This protocol covers all steps except for the identification of bacteria using selective agar media and gram-staining and DNA sequencing, which will be covered in a separate protocol titled "Isolation and Identification of Bad Axillary Odors Producing Microorganism from Human Axillary Region". This protocol can be an example for those conducting similar studies testing antimicrobial activity in cosmetic products through *in vitro* studies, as adjustments can be made according to the objectives of the study.

## INTRODUCTION

Deodorant is a cosmetic product that is applied on axillary skin to reduce sweat production (i.e. antiperspirant); to prevent malodor generation by skin microbiota; to reduce bacterial growth by its antibacterial properties; and/or, to reduce body odor by absorbing or masking it, by fragrances for instance (Darlenski and Fluhr, 2011). To precisely identify the effectiveness of the antibacterial agents in the deodorant product, clinical trials are commonly conducted using human subjects. Such processes are long, tedious, risky, and costly. In the hope of providing solutions to those challenges, some *in vitro* procedures are adapted to shorten the period of trials and eliminate the need for ethical reviews, pay incentives for the volunteers, achieve large sample sizes, and ensure safety, to name a few. Spot-Plating Assay and Time-Kill Assay are some *in vitro* procedures that can be adapted to mimic clinical trials on humans in treating bacteria with deodorant on agar or in test tubes/well plates (Chojnacki et al., 2021). Spot-Plating Assay tests the deodorant's antimicrobial performance through bacterial Zone of Inhibition, whereas, Time-Kill Assay measures bacterial reduction over time for samples treated with deodorant.

This study aims to test and compare the antimicrobial activity of deodorants by the adopted in vitro testing methods against Indonesian males' axillary microbiome. This protocol is suitable for deodorants in liquid consistency, which are mostly available in roll-on products. This protocol utilizes *Corynebacterium tuberculostearicum*, *Micrococcus luteus*, *Staphylococcus hominis*, and *Staphylococcus epidermidis* as test microorganisms as they have been found to be dominant microbes present in Indonesian male subjects' axilla involved in the previous study (Rudden et al., 2020; Callewaert et al., 2014; Zinn, Singer, & Bockmühl, 2021). Before undergoing these tests, a bacterial growth curve and standard curve need to be generated to ensure consistency and optimacy of growth conditions throughout the study. Additionally, preliminary tests are also required to determine the minimum inhibitory concentration of positive control (antibiotic) and bacteria-to-deodorant ratio.

## Flowchart


1-2 days
Media Preparation
Nutrient Broth (NB)
Nutrient Agar (NA
Mueller Hinton Broth (MHB)
Mueller Hinton Agar (MHA)
3-4 days
Bacterial Growth Curve
Constructing OD650 vs time (h) graph
Can be done simultaneously
Bacterial Standard Curve
Constructing OD650 vs CFU/mL graph
1 - 2 days
MIC Determination
Obtaining ideal antibiotic concentration against the bacteria
1-2 days
Preliminary Test for Bacterium-to-Deodorant Ratio
Determining the ideal ratio of deodorant and bacteria mixture for Time Kill Assay analysis
2 - 3 days
Spot Plating Assay
Generating a comparison chart of Zone of Inhibitions (ZOIs) produced
2 - 3 days
Time Kill Assay
Generating a comparison table of log10 CFU/mL and bacterial percentage reduction overtime

## METHODOLOGY

### Bacterial Growth Curve

**Background**

One of the most popular techniques in microbiology for tracking the expansion and proliferation of microorganisms over time is growth curve assessment based on optical density (OD). This technique offers a straightforward and dependable means to comprehend numerous elements of microbes. For instance, in this study, a growth curve was used to monitor the development of bacteria. In general, bacteria go through four phases of growth: the lag phase, during which the bacteria must first adapt to their new environment; the logarithmic or exponential phase, where cell doubling is at its maximum rate; the stationary phase, during which bacterial growth flattens due to depleting nutrients and equaling number of living and dead cells; and finally, the death phase, during which the bacteria lose their capacity to divide and the number of dead cells outnumbers the number of living cells. To have the greatest bactericidal impact, antimicrobial agents are typically tested against bacteria during their log phase of proliferation where cells reproduce actively. Therefore, to establish the log phase of the bacteria that would be used in this experiment, bacterial growth curves were constructed.

**Goal**

To identify the log phase of bacteria (in OD) and discover how long it takes for the bacteria to reach the log phase.

**Materials**

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], Nutrient broth (NB), Micropipette tips.

**Equipment**

96 well-plate, 10 mL glass test tubes, Orbital shaker, Micropipette, Plate reader (Infinite®M200 NanoQuant).

**Procedure**

1. Inoculate a few colonies of 3-day culture into 3 mL of fresh NB, then incubate the subcultures overnight in the 37oC orbital shaker incubator at 120 rpm.
2. Inoculate 10% of the subcultures into 3 mL of fresh NB for activation; incubate the activated cultures in the 37oC orbital shaker incubator at 120 rpm.
3. On the next day, transfer 200 µL of activated cultures into a 96 well-plate according to the scheme below (Figure 1; Blank: NB only).

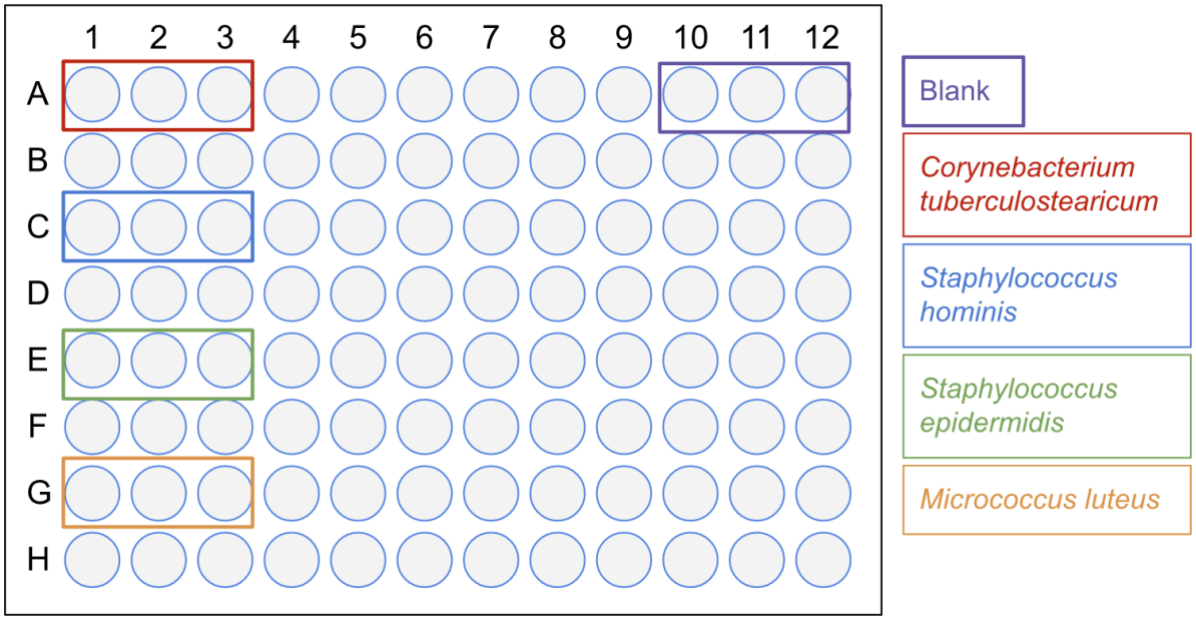



**Figure 1.** 96 well-plate scheme of the bacterial growth curve

4. Measure the OD of the activated cultures at 650 nm using a Plate Reader (Infinite®M200 NanoQuant) with the setting: [COS96ft] - Corning 96 Flat Transparent, open cover; record the data as T0*.
5. Continue the incubation and OD measurement of the well plate every hour for 10-12 hours.
6. Record all the OD measurements and construct the OD650 vs. hour graph.

*If it reached 0.1, the culture could serve as T0 for the growth curve. Otherwise, culture reactivation is required, i.e Step 2.

## METHODOLOGY

### Bacterial Standard Curve

**Background**

A standard curve standardizes bacterial density or concentration in CFU/mL, a unit commonly used in antimicrobial susceptibility tests. By establishing a standard curve, bacterial density/concentration can be assumed just by measuring the Optical Density (OD). A standard curve also makes it easier to compare and reproduce the findings of various investigations.

**Goal**

To translate bacterial OD measurement into CFU/mL, which will be used to standardize the initial bacterial concentration to $10^8$ CFU/mL.

**Materials**

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], Nutrient broth (NB), nutrient agar (NA), 0.9% sodium chloride (NaCl) solution, micropipette tips.

**Equipment**

10 mL glass test tubes, sterile inoculation loop, 96-well plate, 37℃ incubator, 37℃ orbital shaker, plate reader (Infinite®M200 NanoQuant), micropipettes, plastic petri dish, colony counter, marker.

**Procedure**

1. Inoculate a glass tube of 3 mL of NB with a loopful of bacterial colonies aseptically. Mix by vortexing.
2. Prepare another tube to act as a negative control with 3 mL of NB only.
3. Incubate the culture overnight in a 37℃ orbital shaker at 120 rpm.
4. The next day, transfer 200 µL of the culture into a well in a 96-well-plate. Do this step in triplicates (see Figure 2).
5. Measure the OD of the bacterial culture in a plate reader (Setting: [COS96ft] - Corning 96 Flat Transparent, well-plate lid removed, and in 650 nm; leave the rest as default) to ensure the bacteria has reached the sufficient OD of the log phase, referring to the growth curve previously.
6. Record the results. Make sure to use the results that are subtracted with the blank and averaged among the triplicates.
7. Dilute the bacterial culture to a range of OD (e.g. 0.1, 0.2, 0.3, ..., 1.0) in the 96-well-plate with NB (Figure 2).
8. Measure the OD of the dilutions to validate.
9. To enumerate bacterial cells in the sample, serial dilution is performed by taking 5 µL of the bacterial sample into 195 µL of 0.9% NaCl solution as a diluent (40-fold dilution); repeat the dilution 12 times ($10^{-1}$ to $10^{-12}$). Refer to Figure 2 below.

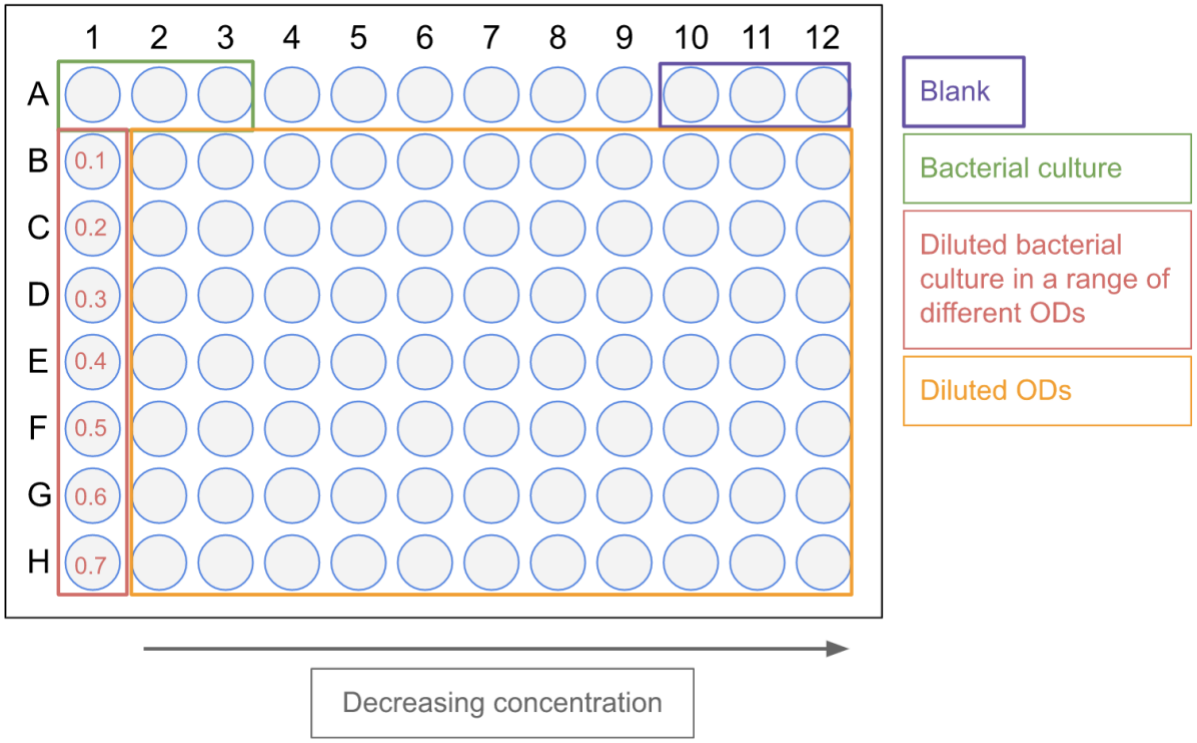



**Figure 2.** The scheme of bacterial standard curve dilution in a 96-well plate.

10. Plate all bacterial samples (and the negative control) onto NA by the Miles and Misra technique (10 µL/drop; 3 drops per quadrant; 6 quadrants per agar plate) (Figure 3).

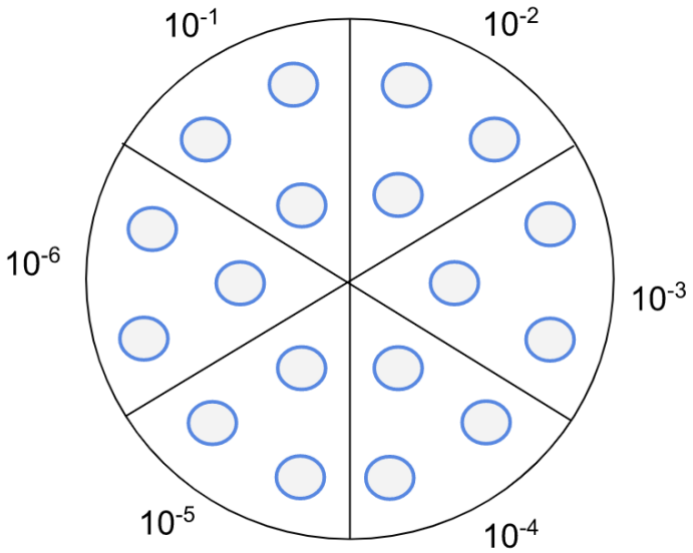



**Figure 3.** The layout out of bacterial samples' plating on nutrient agar by Miles and Misra technique for generating a bacterial standard curve.

11. Incubate the culture overnight at 37°C.
12. The next day, count the Colony Forming Units (CFU) of the quadrants on all agar plates (Range of countable colony: 2-20 per drop).

13. Calculate the CFU/mL of each quadrant using **Equation [1]** below.

$$\text{Original Cell Density} = \frac{Average\ of\ n \times (DF)^d}{10^{-2}} \text{CFU/mL}$$

Where:

n : Number of colonies in a quadrant (CFU)

DF : Dilution factor

d : Number of dilution performed to the sample plated

$10^{-2}$ : To convert µL to mL

14. Construct the OD650 vs. CFU/mL graph.

## METHODOLOGY

### Vancomycin (Positive Control) Minimum Inhibitory Concentration (MIC) Determination

**Background**

A minimum Inhibitory Concentration (MIC) test is conducted to determine the lowest concentration of the tested antimicrobial agent that prevents the observable growth of the bacterium of interest, under specific test conditions. MIC values are used to assess the efficacy of new antimicrobial medications as well as the drug susceptibilities of bacteria. In this study, the test is performed through broth dilution where bacteria is transferred into a liquid growth medium while being exposed to various concentrations of an antimicrobial agent. This is frequently done using 96-well microtiter plates. After incubation for a predetermined amount of time, growth is evaluated, and the MIC value is recorded. This procedure can be completed in about 3 days, and it is only applicable to aerobic bacteria.

**Goal**

To determine the MIC of vancomycin, an antibiotic that is effective against the bacteria of interest, as the positive control in this study.

**Materials**

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], Generik vancomycin hydrochloride injection powder (in vial; 0.5 g powder in 10 mL of sterile water), Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), Antibiotic, Micropipette tips.

**Equipment**

10 mL glass test tubes, sterile inoculation loop, 96-well plate, 37°C incubator, 37°C orbital shaker, plate reader (Infinite®M200 NanoQuant), micropipettes, plastic petri dish, colony counter, marker.

**Procedure**

1. Inoculate a glass tube of 3 mL of MHB with a loopful of bacterial colonies from the stock culture aseptically. Mix by vortexing.
2. Prepare another tube to act as a negative control with 3 mL of NB only.
3. Incubate the culture at 37°C in an orbital shaker incubator at 120 rpm for 6 hours or until 0.8 - 1 OD (refer to growth curve).
4. Adjust the bacterial culture to $10^8$ CFU/mL (refer to standard curve).
5. Prepare a sterile 96-well plate.
6. Pipette 200 µL of MHB in the sterility control well (column 12) and 100 µL in columns 2 to 11 (refer to Figure 4).
7. Transfer 100 µL of bacterial culture to column 11 and resuspend with the MHB (growth control)
8. Pipette 200 µL of vancomycin (128 mg/L) in column 1.
9. Dilute vancomycin serially (from columns 1 to 2, 2 to 3, 3 to 4, and so on up to column 10) by 2-fold (1:1).
10. Mix the bacterial suspension adjusted by vortexing.

11. Pipette 100 µL of the bacteria to columns 1 to 10 and column 11 (in duplicates [e.g. Row A is duplicated in Row B]; see Figure 4). Mix well.

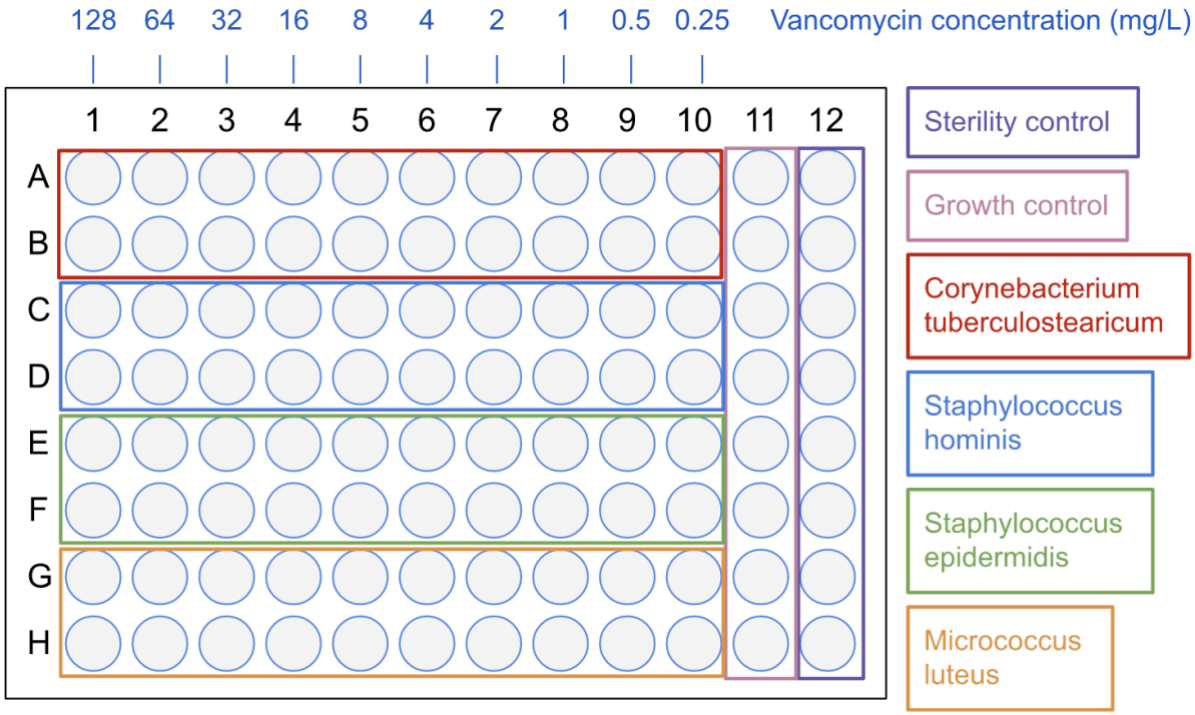



**Figure 4.** The scheme for determining the minimum inhibitory concentration of vancomycin against 4 different bacteria in a 96-well plate.

12. Incubate the well plate at 37°C in a shaker at 120 rpm overnight.
13. The next day, measure the OD of the bacterial culture in a plate reader (Setting: [COS96ft] - Corning 96 Flat Transparent, well-plate lid removed, and in 650 nm; leave the rest as default).
14. Determine wells with no apparent bacterial growth as the MIC candidates.
15. To validate MIC, enumerate bacterial cells in each well by serial dilution. Dilute each by removing 20 µL into a new 96-well plate filled with 180 µL of 0.9% NaCl (10-fold dilution) 12 times ($10^{-1}$ to $10^{-12}$). See Figure 2.
16. Plate all bacterial suspensions (and the negative control) onto NA by the Miles and Misra technique (10 µL/drop; 3 drops per quadrant; 6 quadrants per agar plate) (Figure 3).
17. Incubate the culture overnight at 37°C.
18. The next day, count the Colony Forming Units (CFU) on all agar plates (See Equation [1]) and determine the MIC of vancomycin for each bacteria.

## METHODOLOGY

### Preliminary Test for Bacterium-to-Deodorant Ratio

#### Background

In the Time Kill Assay, deodorant's efficacy is tested by incubating it with bacteria over time. To determine the ideal bacterium-to-deodorant volume ratio, pilot tests are performed. The ideal ratio is established by comparing the bacterial count of each ratio with the bacterial count of the positive and negative control. The ratio that gives the furthest bacterial count to the controls (i.e. greatest anti-bactericidal effects) is selected.

#### Goal

To determine the bacterium-to-deodorant ratios (0.25:1, 0.5:1, and 1:1 [vol/vol]) with the greatest dynamic range between the negative control (MHB) and the positive control (vancomycin antibiotic).

#### Materials

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], deodorants, Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), micropipette tips.

#### Equipment

10 mL glass test tubes, sterile inoculation loop, 96-well plate, 37°C incubator, 37°C orbital shaker, plate reader (Infinite®M200 NanoQuant), micropipettes, plastic petri dish, colony counter, marker, ruler.

#### Procedure

1. Inoculate a glass tube of 3 mL of MHB with a loopful of bacterial colonies aseptically. Mix by vortexing.
2. Prepare another tube to act as a negative control with 3 mL of NB only.
3. Incubate the culture at 37°C in an orbital shaker incubator at 120 rpm for 6 hours or until 0.8 - 1 OD (refer to growth curve).
4. Adjust the bacterial culture to $10^8$ CFU/mL (refer to standard curve). Mix well.
5. Prepare a sterile 96-well plate.
6. Pipette 1:1 volume of MHB and bacteria in the negative control well (column 11; total volume: 200 µL).
7. Pipette 1:1 volume of vancomycin (in MIC) and bacteria in the positive control well (column 12; total volume: 200 µL).
8. Pipette 0.25:1, 0.5:1 and 1:1 bacteria-to-deodorant volume ratios in duplicates (see Figure 5). Mix well.

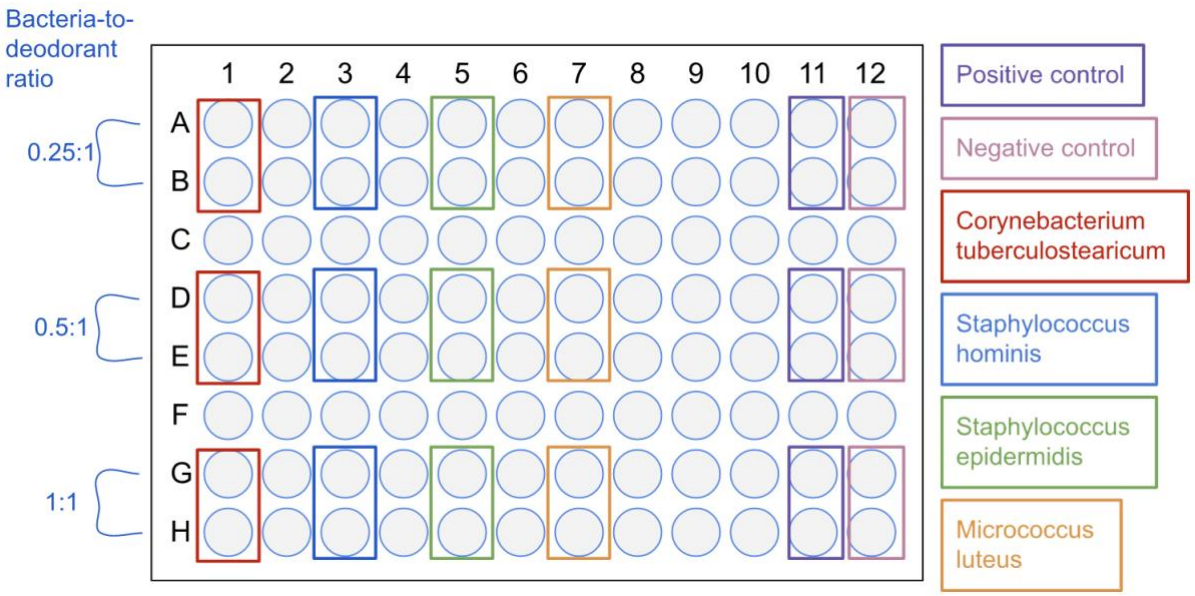



**Figure 5.** The scheme for determining the best bacteria-to-deodorant ratio against 4 different bacteria in a 96-well plate (in duplicates).

9. Incubate the well plate at 37°C in a shaker at 120 rpm overnight.
10. The next day, measure the OD of the bacterial culture in a plate reader (Setting: [COS96ft] - Corning 96 Flat Transparent, well-plate lid removed, and in 650 nm; leave the rest as default) to observe turbidity (i.e. Bacterial growth).
11. To validate the bacteria-to-deodorant ratio, enumerate bacterial cells in each well by serial dilution. Dilute each by removing 20 µL into a new 96-well plate filled with 180 µL of 0.9% NaCl (10-fold dilution) 12 times ($10^{-1}$ to $10^{-12}$). See Figure 2.
12. Plate all bacterial suspensions (and the negative control) onto NA by the Miles and Misra technique (10 µL/drop; 3 drops per quadrant; 6 quadrants per agar plate) (Figure 3).
13. Incubate the culture overnight at 37°C.
14. The next day, count the Colony Forming Units (CFU) on all agar plates and determine the bacteria-to-deodorant ratio with the greatest dynamic range between negative and positive control.

# METHODOLOGY

## Spot Plating Assay

### Background

In general, the Spot Plating Assay is known as a method for determining a bacterium's susceptibility to sub-minimum antibiotic concentration inhibition by comparing the number of live cells forming colonies with the amount of plated cells. With some modifications to the methodology, Spot Plating Assay could examine the antimicrobial activity of deodorants by presenting the zone of inhibitions (ZOI). The larger the zone's diameter, the more effective the deodorant is against bacteria.

### Goal

To determine the efficacy of the tested deodorant based on the average zone of growth inhibition (ZOI); the larger ZOI indicates more antibacterial efficacy of the deodorant.

### Materials

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], Nutrient Broth (NB), Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), NaCl, Micropipette tips, Microcentrifuge tubes, Type III water, Antibiotic, Deodorant, Parafilm.

### Equipment

Glass spreader, Micropipette, Caliper, Incubator, Marker, Lighter, Bunsen burner.

### Procedure

1. Grow the bacteria samples overnight in NB at 37°C and 120 rpm, then dilute to $10^8$ CFU/mL at the mid-log phase referring to the generated bacterial growth curve and standard curve.
2. Wash 5 mL of the bacterial samples by centrifuging them at 3500 rpm for 15 minutes, discarding the supernatant, and washing the pellet with NaCl twice.
3. Add 5 mL of MHB to the tube. Vortex until homogenous.
4. Spread 100 µL of $10^8$ CFU/mL of each bacteria onto the surface of the MHA plate aseptically.
5. Dry the plate for 5 minutes.
6. Apply 25 µL of deodorant/antibiotic/type III water* onto the center of the plate surface.
7. Incubate the plates overnight at 37°C.
8. Measure the diameter of bacterial growth inhibition in millimeters (Zone of Inhibition/ZOI).
9. Record the average of ZOI and standard deviation (SD).
10. Count and record the colonies within the ZOI.

**Positive control: Antibiotic; Negative control: Type III Water; Treatment: Deodorant*

## METHODOLOGY

### Time Kill Assay

**Background**

One of the most common methodologies for monitoring and evaluating the antimicrobial effectiveness of deodorants over time is the Time Kill Assay. The methodology is the most similar to the clinical trial, as the incubation period in this study was adjusted based on the incubation period of the clinical trial in one day of product testing (1, 5, and 9 hours incubation), making the results of this methodology the most likely to be compared with the clinical trial's results by comparing the bacterial reduction percentages.

**Goal**

To monitor and evaluates the antimicrobial effectiveness of deodorants over time and further determine the efficacy of the tested deodorant based on bacterial reduction percentage.

**Materials**

Isolated bacterial colonies from axilla on Tryptic Soy Agar stored in i3L's Culture Collection [*Corynebacterium tuberculostrearicum* (15.7.HMAR.T SP-CT), *Staphylococcus hominis* (15.7.HMAR.T SP-SHOM), *Staphylococcus epidermidis* (15.7.HMAR.T SP-SEPI), *Micrococcus luteus* (15.7.HMAR.T SP-ML)], Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), 0.9% NaCl, Micropipette tips, Type III water, Antibiotic, Deodorant, Parafilm.

**Equipment**

96 well-plate, Incubator, Micropipette, Colony counter.

**Procedure**

1. Grow the bacteria samples overnight in NB at 37$^{o}$C and 120 rpm, then dilute to $10^{8}$ CFU/mL in the mid-log phase referring to Standard Curve.
2. Wash 5 mL of the bacterial samples by centrifuging them at 3500 rpm for 15 minutes, discarding the supernatant, and washing the pellet with NaCl twice.
3. Add 5 mL of MHB to the tube. Vortex until homogenous.

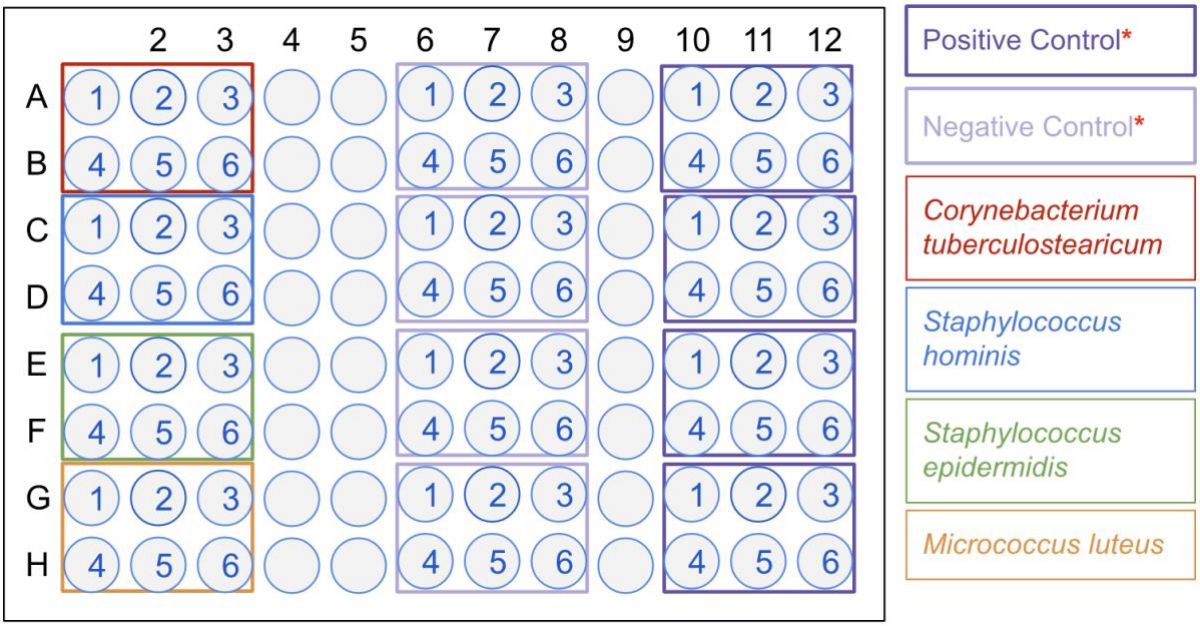



**Figure 6.** 96 well-plate scheme for Time Kill Assay.

4. Mix $10^8$ CFU/mL of bacteria with the deodorant/antibiotic/MHB* at a 1:1 ratio (determined from the preliminary test) in a 96 well-plate according to Figure 6 (the controls are mixed with the corresponding bacteria).
5. Remove the 20 µL aliquot after 1, 2, 3, 4, 5, and 9 hours of treatment. Transfer to 180 µL NaCl in a 96 well-plate.
6. Serially dilute the aliquot with 10-fold dilution up to $10^{-6}$ using sterile NaCl.
7. Plate the samples into MHA using the Miles and Misra technique to enumerate the number of bacteria $ml^{-1}$ remaining.
8. Test the samples in triplicates and record the average $\log_{10}$ of the remaining bacteria.
9. Calculate the bacterial reduction percentage using the **Equation [2]** below.

$$Percent\ Reduction = \frac{(A - B)\ x\ 100}{A}$$

Where:

A = number of viable bacteria before treatment (log10 CFU)
B = number of viable bacteria after treatment (log10 CFU)

* *Positive control: Antibiotic; Negative control: MHB; Treatment: Deodorant*

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