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Spot Plating Assay for the Determination of Survival and Plating Efficiency of *Escherichia coli* in sub-MIC Levels of Antibiotics

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Spot plating is an alternative method to spread plating that generates more replicates in shorter periods of time. The spot plate assay is a method to determine the bacterial sensitivity to a sub-minimum inhibitory concentration of antibiotic by comparing the amount of cells plated to surviving cells that form colonies. This method is based on the principle that sensitive strains will form less colonies on a plate when challenged with antibiotics. Using this method, a greater amount of replicates can be performed in parallel to account for biological variation and obtain reliable and conclusive results.

INTRODUCTION

Spot plating is an alternative method to spread plating and turbidity-based methods used to quantify bacterial growth. These methods are also used for determining relative bacterial sensitivity to sub-Minimum Inhibitory Concentration (MIC) of antibiotics. Here, we discuss the methodology of a spot plating assay, adapted from Laubacher and Ades (1). Advantages of this method include speed, the ability to have multiple replicates per plate, and minimization of the number of plates required.

The assay utilizes the principle that a bacterial culture would have a decreasing percentage of viable cells with increasing concentrations of antibiotics. At a constant sub-MIC level of an antibiotic, the sensitivity of a bacterial strain could be correlated with the percentage of cells that survive and form a colony. This ratio of the surviving cells to total number of plated cells is the plating efficiency. The key to a successful spot plating assay is to spot plate the same number of cells onto each spot. By comparing the surviving cells on the antibiotic plates to the control, the sensitivity of the cells to the antibiotic concentration by plating efficiency percentage can be determined. This method can be used to obtain the plating efficiency for any combination of a bacterial strain and an antibiotic. Therefore, the plating efficiency allows us to compare the antibiotic sensitivity between different strains.

Key words: Antibiotics, *E. coli*, Spotting, Plating Efficiency, Survival, Assay, Serial Dilutions, Sub-MIC

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MATERIALS AND EQUIPMENT

- Overnight cultures of *E. coli* strains of interest
- Incubator
- Media plates, supplemented with and without antibiotics at desired concentrations
- Spectrophotometer and Cuvettes
- Micropipettes and Tips
- Laminar Flow Hood

PROTOCOL

A. Determination of the optimal sub-MIC level of antibiotics: The chosen sub-MIC level antibiotic should be chosen with care. The concentration should be high enough to inhibit growth, but not to inhibit all growth. This optimal sub-MIC concentration is usually just below the MIC of the antibiotic. For example, the MIC of penicillin and *E. coli* K12 is 30 µg/mL. The optimal sub-MIC of penicillin for the spot plate assay was found to be 25 µg/mL.

B. Preparation of media: Growth media with and without antibiotics should be made. See B1-B3 in the methods section.

C. Preparation of bacterial stock: The bacterial stocks should be grown overnight at their optimal growth conditions (such as in LB media at 37°C and shaking at 200 rpm). After overnight growth, the culture should be diluted to a concentration that would allow countable isolated colonies on the final spots of the LB plates. We recommend dilutions of $1x10^3$ CFU/mL. See C1 in the methods section.

D. Spot plating: A small volume (20 µL) of the working stock of bacterial culture should be plated up to 8 times on each plate. See D1 in the methods section.

E. **Calculate plating efficiency:** The number of colonies in each spot should be counted and tallied. The number of colonies on the antibiotic plates and the control plates are used to calculate plating efficiency. See E1 in the methods section.

METHODS

Lysogeny Broth Media Recipe

Media Plate Recipe (for 1 L)

➔ NaCl: 10 g, Yeast Extract: 5 g, Tryptone: 10 g.

Media Broth Recipe (for 1 L)

➔ NaCl: 10 g, Yeast Extract: 5 g, Tryptone: 10 g. Agar: 15 g.

Lysogeny Broth Media Preparation

1. Weigh out appropriate masses of reagents as seen in A1.
2. Dissolve and mix well.
3. Autoclave.
4. For antibiotic plates only: add antibiotics to warm media.
5. Pour plates into plastic petri dishes.

Antibiotic stock preparation

1. Weigh out the required amount of antibiotic on an analytical balance.
2. Dissolve in a falcon tube with required amount of sterile dH_2O.
3. Filter through a 0.22 µm filter into a sterile 15 mL falcon tube.

Dilution of bacterial culture to a working concentration

1. Determine the OD_{600} of the bacterial culture using a spectrophotometer.
2. Multiply the OD_{600} of the bacterial culture by the appropriate conversion ratio of OD_{600} to CFU/mL (for *E. coli* K12, $OD_{600} = 8x10^8$ CFU/mL)
3. Determine the dilution scheme required to obtain the working concentration.

Spot plating

1. Prepare media plates, supplemented with and without antibiotic(s) of interest at desired concentrations.
2. Inoculate an overnight culture (eg, a 5 mL culture tube) and incubate overnight.
3. The next day, determine optical density of diluted cultures and convert into CFU/mL using the conversion factor of the strain if known (example: 1.00 OD_{600} to $8x10^8$ cells/mL).
4. Serially dilute cultures (eg 100 µL of culture into 900 µL of fresh growth media) to obtain a $1x10^3$ CFU/mL culture. Mix or vortex well between dilutions.
5. Aseptically pipette 8 separated spots onto the plate using 20 µL of $1x10^3$ CFU/mL culture.
6. For the control, prepare a $1x10^2$ CFU/mL culture from the $1x10^3$ CFU/mL culture. Spot plate 2 spots to a new plate with no antibiotics by pipetting 200 µL of the $1x10^2$ CFU/mL culture.
7. Let the spots dry completely before incubating the plates overnight at 37°C.
8. After incubation, count the isolated colonies per spot.

Calculating plating efficiency

1. Count the number of colonies on each spot of the control plates.

2. Count the number of colonies on each spot of the antibiotic plates.
3. Plating efficiency is the number of isolated colonies divided by the number of plated cells and multiplied by 100 as seen in the following equation:

$$Plating\ Efficiency = \frac{Number\ of\ isolated\ colonies\ in\ spot}{Number\ of\ plated\ colonies} \times 100$$

For example: If 20 µL of $1x10^3$ CFU/mL is plated, the number of plated cells is 20.

ANTICIPATED RESULTS AND CONTROLS

Results can be presented as number of colonies per spot or as plating efficiency (Number of colonies on antibiotic/Number of colonies on no antibiotic). Controls include spot plating on plates without antibiotics to ensure the amount of plated cells is the expected number, and to determine if any decrease in survival or plating efficiency is attributable to the antibiotic. The number of colonies from any spots on any antibiotic-containing plates should be equal to or less than the number of colonies on the spots of the control plates.

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REFERENCES

1. **Laubacher, ME, Ades, SE.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J. Bacteriol. **190:**2065-2074

TROUBLESHOOTING

Issue/Observation	Possible Explanation	Solution
Too many colonies are present, colonies overlap.	Too many cells were plated, and/or cells grew too well.	• Use a higher concentration of antibiotics (but still sub-MIC) • Make more diluted cell cultures and repeat the experiment. • Incubate plates for less time. • Incubate plates at a lower temperature.
No colonies are present, but are present in the controls.	Antibiotic concentration may be lethal, no cells were spotted.	• Repeat experiment with lower levels of antibiotic. • Make less diluted cell cultures and repeat the experiment.
Spots on the plates connected together and are not separated.	Movement of plates before evaporation, too much volume was spotted.	• Do not move plates before total evaporation has occurred. • Reduce spotting volumes or space spots more generously.