Preparing Spread Plates Protocols

Created: Monday, 09 October 2006

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Information

One method of distributing bacteria evenly over the surface of an agar plate medium is commonly referred to as the spread plate method. Classically a small volume of a bacterial suspension is spread evenly over the agar surface using a sterile bent glass rod as the spreading device. The goal in evenly distributing the bacterial suspension is typically to permit the growth of colonies that can subsequently be enumerated (see Serial Dilution Protocols) and/or sampled following incubation. Each plate is spread with a single inoculum of the bacterial suspension.

An alternative approach to spreading a single inoculum volume with a smooth device is to apply a smaller volume and tip the plate, allowing gravity to distribute the inoculum in a band or track (track method) or to allow the inoculum to dry in place (drop method). With this alternative approach, several sample dilutions can be distributed on a single agar plate.

History

Since the development of the agar plate in Robert Koch's laboratory, several methods have been used to achieve an even distribution of bacterial growth on or in the agar. The most common methods used to achieve this type of distribution are: spread, pour, thin-layer, layered, and membrane filter (2).

Principles

Using the spread method a small volume of a bacterial suspension is distributed evenly over the surface of an agar plate using a smooth sterilized spreader (2). In the case of track plates, gravity is used to spread the inoculum down the agar in a column forming a track (1).

PROTOCOL: Spread Plates

Agar plates:

Select and prepare an agar medium based upon the type of bacteria to be enumerated or selected.

After autoclaving, cool the agar to between 45°C and 50°C prior to pouring the plates to minimize the amount of condensation that forms. The thickness of the agar should be roughly 0.3 cm, which can be

achieved by pouring 15 to 20 ml of media per 100 x 15 mm plate.

Freshly prepared plates do not work as well as dry plates as it takes longer for the inoculum to absorb into the agar. Plates may be dried by keeping them at room temperature for roughly 24 hours. Plates will dry faster in lower humidity so placing them in a laminar flow hood will speed the drying process. Once dried, plates may be used or refrigerated in closed bags or containers until required. Refrigerated plates should be warmed to room temperature prior to use.

Inoculations:

When enumerating colony-forming units (CFUs), plates with between 20 to 300 (or 25 to 250) CFUs can be used to calculate the number of CFUs/ml of the original sample. Typically a dilution series is prepared, often a ten-fold dilution series, using a suitable dilutent such as phosphate-buffered saline. Serial Dilution Protocols

A convenient inoculum volume, in terms of spreading, absorption, and calculations, is 0.1 ml (100 microliters).

Since some bacteria rapidly attach to the agar surface, the inoculum should be spread soon after it is applied.

Working from the most dilute suspension to the most concentrated is advised. Proceeding from most dilute to most concentrated makes it unnecessary to change pipette tips between the dilutions.

Spreading:

A reusable glass or metal spreader should be flame sterilized by dipping in alcohol (such as 70% isopropyl or ethanol), shaking off the excess alcohol, and igniting the residue (Fig. 2, Atlas page). The spreader is then allowed to cool.

The spreader is placed in contact with the inoculum on the surface of the plate and positioned to allow the inoculum to run evenly along the length of the spreader. Even pressure is applied to the spreader and the plate is spun, on a turntable or by hand, as illustrated in Fig. 3-7 on the Atlas page.

Alternatively the spreader may be rotated over the agar surface (Fig. 10, Atlas page).

Avoid spreading the inoculum all the way to the edge of the agar.

The goal is to evenly distribute the inoculum and to allow it to be absorbed into the agar. The plate, or spreader, should be rotated long enough to avoid pooling along the spreader once the rotation is stopped.

Avoid disturbing plates for 10 to 20 minutes after spreading. Drying time varies with the room temperature and humidity.

Incubation:

After the spread plates have been permitted to absorb the inocula for 10 to 20 minutes they may be inverted and incubated as desired.

Observe the plates before the colonies have had time to fully develop. Closely positioned colonies may be difficult to resolve as separate colonies later. Continue the incubation as necessary.

Incubation in closed humidified containers will help avoid problems with plates drying out when working with slow-growing colonies.

Counting and Selection:

After appropriate incubation, plates are inspected. When plating a dilution series, the growth on the plates should reflect the predictable drop in CFUs/plate as illustrated in this picture (Fig. 20, Atlas page) of a 10-fold dilution series prepared from an overnight broth culture of *Escherichia coli.*

FIG. 20. Picture of spread plates showing bacterial growth (*E. coli*, 40 hours, room temperature) on five plates prepared from a ten-fold dilution series. Care was taken to avoid spreading to the edges of the plates as it is more difficult to count colonies along the edge of the agar.

Duplicate or triplicate plates with 30 to 300 CFUs/plate are used to

calculate CFUs/ml.

Plates with well isolated colonies may be inspected and, if desired, colonies "picked" to establish new cultures.

PROTOCOL: Track Plate Method

Agar plates:

Select and prepare an agar medium based upon the type of bacteria to be enumerated or selected.

After autoclaving, cool the agar to between 45°C and 50°C prior to pouring the plates to minimize the amount of condensation forming. The thickness of the agar should be roughly 0.3 cm, which can be achieved by pouring 15 to 20 ml of media per plate. Square plates, 100 x 15 mm, with 13 mm grids or 100 mm round petri dishes may be used.

Freshly prepared plates do not work as well as dry plates as it takes longer for the inocula to absorb into the agar. Plates may be dried by keeping them at room temperature for roughly 24 hours. Plates will dry faster at lower humidity so placing them in a laminar flow hood will speed the drying process. Once dried, plates may be used or refrigerated in closed bags or containers until required. Refrigerated plates should be warmed to room temperature prior to use.

Inoculations:

When enumerating colony-forming units, tracks with 20 to 200 CFUs can be used to calculate the number of CFUs/ml of the original sample. Typically a dilution series is prepared, often a ten-fold dilution series, using a suitable dilutent such as phosphate-buffered saline. Serial Dilution Protocols

A convenient inoculate volume, in terms of spreading, absorption, and calculations, is 0.01 ml (10 microliters). Some bacteria rapidly attach to the agar surface so the plates should be tipped soon after delivery. Working from the most dilute suspension to the most concentrated is advised.

Six tracks will comfortably fit on a square agar plate and three or four tracks will fit on a round plate. Use a marker to establish the places to initially dispense inocula onto a round plate.

Spreading:

Inocula are placed in the appropriate squares or on the appropriate designated spots. To speed up the inoculation process and minimize the number of pipette tip changes, add the inocula to the plate in ascending order of bacterial concentration (most dilute to most concentrated).

Once the dilutions have been applied, the plate is tipped at roughly a 60° angle and then the angle is adjusted (45° to 90°) to control the flow of

the inocula forming the tracks as illustrated in Fig. 33 on the Atlas page.

When the "fastest" track comes to within 0.5 cm of the edge of the agar, the plate may be tipped backwards slightly (Fig. 37, Atlas page) to avoid pooling at the base of the tracks.

The plates should be left undisturbed for 5 to 10 minutes.

Incubation:

After the tracks have been permitted to absorb into the agar for 5 to 10 minutes, the plates should be inverted and incubated as desired.

Observe the plates before the colonies have had time to fully develop. Closely positioned colonies may be difficult to resolve as separate colonies later. Continue the incubation as necessary.

Incubation in closed humidified containers will help avoid problems with plates drying out when working with slow-growing colonies.

Counting and Selection:

After appropriate incubation, plates are inspected. When plating a dilution series, the growth on the plates should reflect the predictable drop in CFUs/plate as illustrated in this picture (Fig. 38, Atlas page).

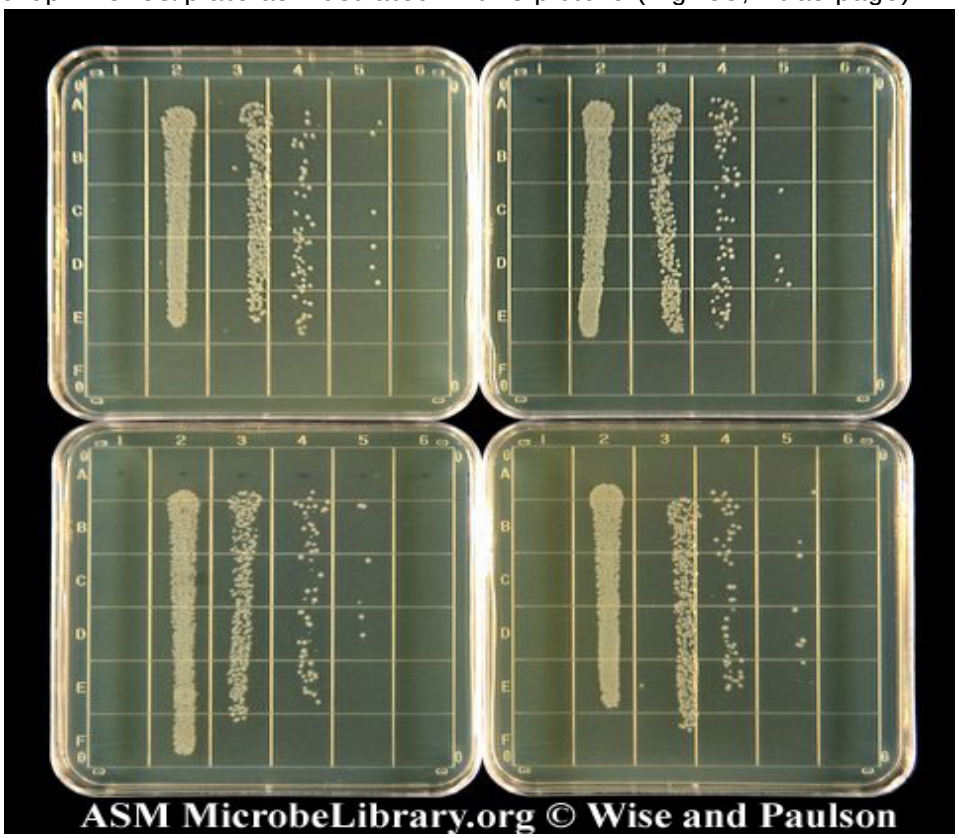

FIG. 38. Picture of four replica plates showing *E. coli* (room temperature, 40 hours) growing in four tracks.

Duplicate or triplicate tracks with 20 to 200 CFUs/plate are used to calculate CFUs/ml.

Plates with well isolated colonies may be inspected and colonies "picked" to establish new cultures.

SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and the Guidelines for Biosafety in Teaching Laboratories.

Make sure the container of alcohol is covered when not in use and that it is kept out of the vicinity of the spreader once the alcohol-dipped spreader is ignited.

COMMENTS AND TIPS

Spread Plates

Make sure plates are sufficiently dry prior to use.

McFarland standards are very useful in estimating cell densities so that an appropriate dilution series can be prepared and the dilutions to be plated determined. Prepare dilutions to deliver an estimated 30 to 300 colonies on at least one plate.

Plates should be prepared in duplicate or triplicate.

Do not delay in spreading the inoculum once it has been applied to the plate since some cells will rapidly attach to the agar, especially if the plate agar is nice and dry.

Avoid spreading the inoculum to the edge of the agar as it is more difficult to inspect and count colonies along the agar's edge.

Once the dilution series has been made, inoculate plates within 30 minutes to minimize changes in the number of cells in each dilution due to cell division or death.

Make sure even pressure is applied to the spreader so that fluid is evenly distributed along its length as the plate or spreader is rotated.

Once the dilutions are made, work backwards spreading the most dilute samples first.

When making your own spreaders do not make the spreading edge too long, it should conveniently fit into the alcohol container as well as the plate. Fire-polish the end of the spreader the student will hold. Bending the glass to form a triangle rather than an "L" will help assure only smooth even surfaces touch the agar and minimize pooling.

Distributing the organisms by rotating the spreader rather than the plate tends to cause more pooling of the inoculum.

Sterile glass or plastic beads are sometimes used to spread the inoculum over the surface of an agar plate when an even distribution of colonies is not an important outcome.

Track plates

Use 0.01 ml (10 microliters) or 0.005 ml (5 microliters) as the inoculum volume.

An ordinary 100 mm round petri dish may comfortably hold three to four tracks. If for your enumeration you plate triplicates, one plate per dilution works well.

Saves on plates and media as well as time.

Reduces labeling errors.

Gridded square plates are great but not necessary

Use 20 to 200 visible colonies per track as the countable range rather than the 30 to 300 or 25 to 250 ranges commonly used with classic spread plates.

Quickly apply the inocula onto a plate.

Tip the plate keeping one edge in contact with the bench top and sharpen or reduce the angle as needed to encourage the inocula to run down the plate forming tracks.

Do not let the tracks run to the edge of the agar.

Once the tracks have been formed, tip the plate back slightly so that the fluid does not collect or pool at the base of the track.

Let the plates sit 5 to 10 minutes before inverting and incubating them. Drying time varies with the room temperature and humidity.

ACKNOWLEDGMENTS

Minnesota State University—Moorhead undergraduates Nicole True and Rachel Aanenson made valuable suggestions and contributions throughout the development of the images for this Atlas and the preparation of the protocol.

REFERENCES

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