COMMONLY USED METHODS FOR CELL CULTURE

Common Bacterial Culture Techniques and Media

Bacterial species differ from each other in their metabolic capabilities, requiring researchers to use specific media and culture conditions. As those requirements often vary widely between species, readers should refer to the units in this manual focusing on their particular organism for details on cultivation and media. However, a number of common techniques are used in the study of a wide variety of bacteria, and these are presented in this appendix. In addition, since many strains of *Escherichia coli* serve as important tools for almost all modern biologists, commonly used techniques and media for that species are also included.

ASEPTIC TECHNIQUE

This phrase refers to all of the measures that are taken to prevent the introduction of sepsis (contamination) into cultures. Aseptic technique is sometimes mistakenly called "sterile technique," but this phrase is inappropriate since the goal of bacterial culture is to grow a microorganism rather than maintain a sterile environment devoid of bacteria. It is imperative that the microbiologist practice aseptic technique to ensure that cultures remain uncontaminated; otherwise, resulting studies will be meaningless. Examples of aseptic technique include cleaning and disinfecting laboratory surfaces prior to use, limiting the amount of time during which cultures or media are left uncapped and thus exposed to the air, keeping petri dishes closed whenever possible, effectively sterilizing inoculating loops and other equipment that come into contact with cultures or media, and avoiding breathing on cultures or sterile instruments.

COMMONLY USED TOOLS

Inoculating Loop

Many varieties of loops, made of nickel-chromium or platinum wire, are available from many suppliers. The wire may be permanently attached to a handle or replaceable, and may be obtained as either a straight wire or a twisted loop. Each type has its own advantages and disadvantages, and choice is usually made on the basis of availability or personal preference. The loop shape allows efficient transfer of liquids, which form a film over the loop. Standardized volumetric loops are available for reproducible transfers of specific volumes of liquid.

A loop can be easily made on the end of a straight wire by bending it around the tip of a sharp pencil. The loop itself should be 2 to 3 mm in diameter and must be complete, with the tip of the wire just touching the opposite side of the loop to allow formation of a liquid film for transfer.

The inoculating loop must be sterilized immediately before and immediately after use. To sterilize, first place the end of the wire closest to the handle in the blue (hottest) part of a burner flame, until the wire glows red. Next, slowly draw the wire through the flame, ensuring that the entire length of the wire glows red. The loop can be cooled quickly by

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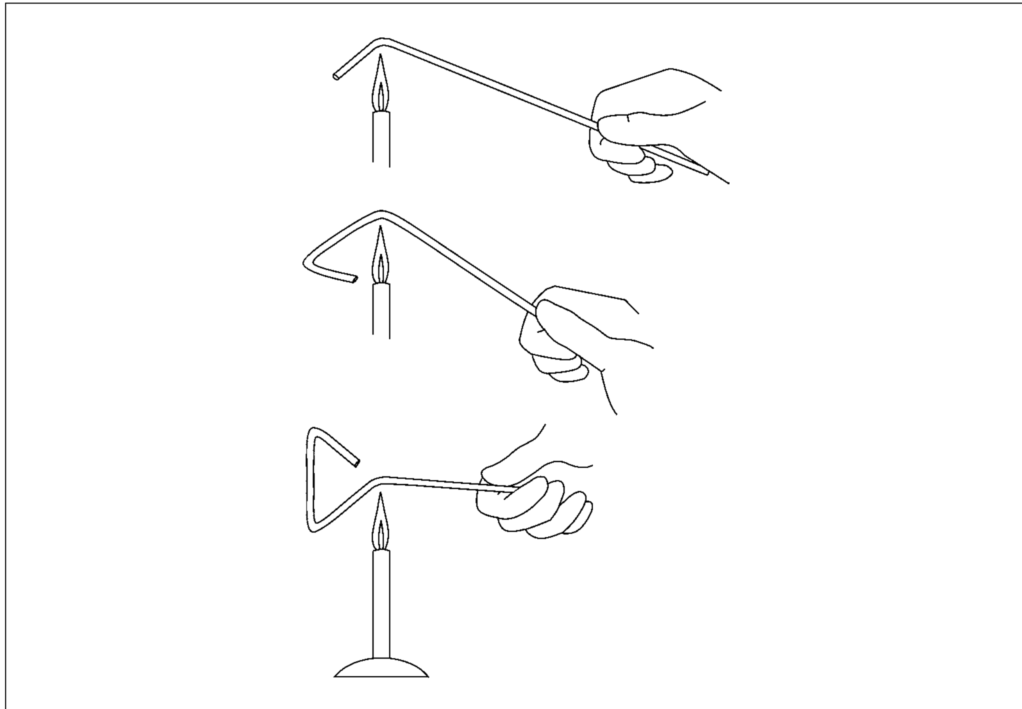

Figure A.4A.1 Making a spreader.

gently touching it to an unused, sterile part of an agar plate or by dipping it in sterile liquid medium. Do *not* blow on a hot loop to cool it off.

Disposable, plastic loops are also available, although they are expensive over the long term and create a large amount of waste. However, use of disposable loops is advisable with certain organisms in order to avoid generation of hazardous aerosols during flaming, or when working in oxygen-deficient environments that preclude use of a burner. See appropriate units of this manual to determine which type of loop is best for each organism being studied.

Culture Spreader

A spreader is used to evenly distribute bacterial cells over the surface of a plate. This will yield either a lawn on a nonselective medium or isolated colonies on a selective medium. A spreader can be easily made by heating and bending a piece of 4- to 5-mm diameter glass rod, as illustrated in Figure A.4A.1. In a pinch, a much less durable spreader can be quickly made by substituting a Pasteur pipet for the glass rod. For safety in handling, melt away sharp ends of the rod as a first step, i.e., prior to bending. A pair of needle-nose pliers or similar tool is useful for forming bends in glass.

Before each use, sterilize the spreader by immersing the triangular end in a beaker of 95% ethanol, passing the spreader through a flame, then allowing the flame to burn out. Be careful not to let the burning ethanol drip into the beaker or onto flammable objects. Cool the spreader by touching to an unused portion of the agar surface. Sterilize again after use by immersing in ethanol.

Presterilized, disposable plastic culture spreaders are also available from a variety of sources. As with plastic inoculating loops (see above), disposable spreaders are expensive over the long run and generate considerable waste, but may be preferable in certain situations.

CULTURE TECHNIQUES

Oxygen Environment

Bacteria differ in their ability to grow in the presence of oxygen. Not all species can utilize oxygen, and many are unable to survive exposure to it. Be sure to follow guidelines described in this book for each species.

Growth of *E. coli* is generally enhanced by the presence of oxygen, with the Krebs cycle and oxidative phosphorylation facilitating complete catabolism of complex carbon sources to carbon dioxide. For this reason, *E. coli* cultures on solid media should be incubated in standard (aerobic, not CO_2) incubators. Liquid cultures should be oxygenated by shaking during incubation. Special Erlenmeyer flasks with baffles on the bottom are ideal, as the baffles perturb liquid flow and help dissolve air into the medium. Caps should be loosely attached, or vented caps or other enclosures used. To maximize exposure of liquid to air, do not fill culture containers more than one-third full.

Monitoring Growth in Liquid Medium

A simple method for quantifying growth is to measure the turbidity of the culture using a spectrophotometer. To perform this technique, measure the absorbance at an appropriate wavelength, e.g., 600 nm is often used for *E. coli*. Zero the spectrophotometer with a blank containing unused culture medium. Klett meters are inexpensive, simple spectrophotometers designed for this purpose. Correlation between absorbance ("optical density" or "OD") and bacterial density varies between species and sometimes between strains due to differences in bacterial sizes and shapes, and so must be determined empirically for each organism.

A hemacytometer, Petroff-Hausser counting chamber, or similar cell counter can be used to determine culture density under a microscope. Such counters consist of a glass slide with a fine grid etched in the glass and a second slide that is suspended above the first; the two hold a specific volume of liquid between them by capillary action. An example of a hemacytometer is given in Figure A.4A.2. Volumes and mathematical ratios vary between units, so be sure to thoroughly read the manufacturer's instructions for the chamber prior to use.

Measurement of colony-forming units (cfu) is probably the most accurate method to determine the number of live bacteria in a culture. To perform this procedure, 10-fold serial dilutions of the culture are individually spread on agar plates (see below) and incubated, and numbers of colonies arising per plate are counted. For accuracy, two to three plates per dilution should be inoculated. This technique is similar to the enumeration of plaque-forming units (pfu) used for quantification of bacteriophage or other virus suspensions.

Positioning Plates within the Incubator

For most bacteria, petri dishes are incubated inverted, with the agar side at the top. The reason for this is that condensation often forms in dishes during incubation. If the agar side is at the bottom, water may drip from the lid onto the agar surface, spreading bacteria around the plate, thus preventing isolation of individual colonies. However, for some bacteria it is actually necessary that plates be incubated with the agar side down, as the extra moisture is beneficial. Be sure to consult the appropriate units of this manual for specific guidelines.

Spreading a Culture on a Plate

Many species of bacteria can be efficiently spread across the surface of an agar plate using a culture spreader (see Commonly Used Tools, above). To do this, place a small

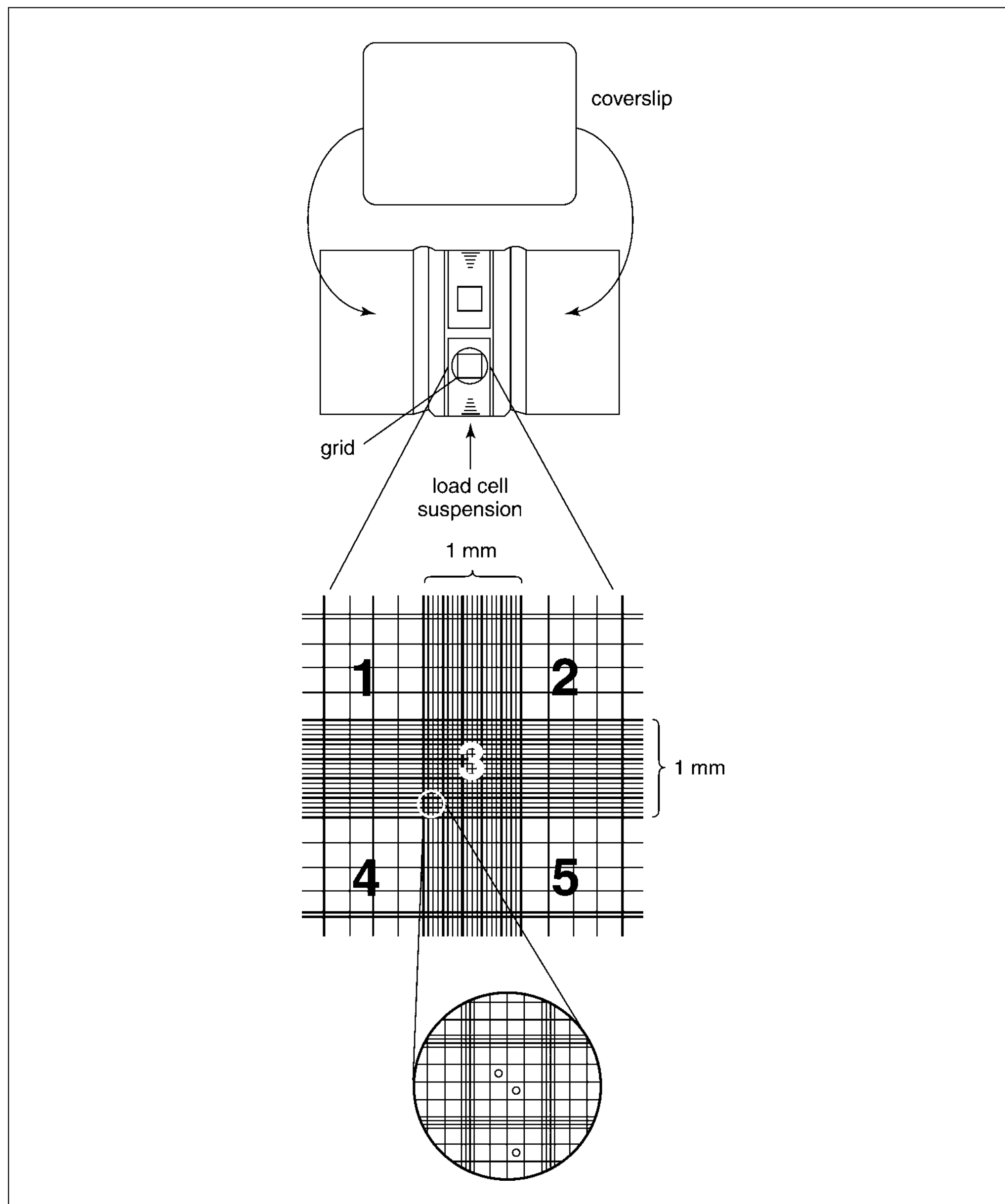

Figure A.4A.2 Example illustrating a gridded counting chamber, a hemacytometer slide (Improved Neubauer), and a coverslip. The coverslip is applied to the slide and the cell suspension is added to the counting chamber using a mechanical pipettor or a Pasteur pipet. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

volume of culture (50 to 500 µl) in the middle of the agar surface. Sterilize the spreader by immersing the hooked end into 95% ethanol in a small beaker, then burn off the ethanol by passing the spreader through a Bunsen burner flame. Cool the spreader by touching it lightly to a sterile agar surface, then spread the bacterial culture uniformly around the plate. Return the spreader to the beaker with the ethanol and incubate the plate at the appropriate temperature.

Rotating "lazy Susan" devices, which spin the plate, are readily available from many sources, and can aid uniform spreading of cultures. To use such a device, place the petri dish, agar side down, in the center of the rotating device. Flame and cool the spreader, lift the lid off the dish, spin the device with a free hand, and touch the spreader to the agar surface. After a dozen or so rotations, stop the spinning by hand and replace the lid.

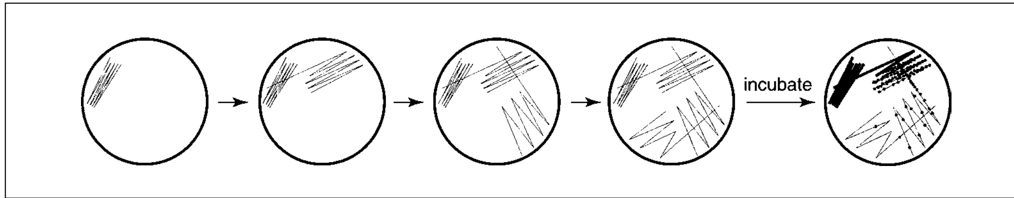

Figure A.4A.3 Proper technique for streaking bacteria on solid medium.

Streaking a Culture on a Plate

For many species of bacteria, clonal populations can be derived by streak plating. Each successive streak dilutes the previous streak, such that isolated colonies (clones) can be obtained (Fig. A.4A.3). To perform this technique, first dip the loop into liquid medium containing the organism of interest or touch the edge to a single colony growing on solid medium. Streak the culture across a small (∼2- to 3-cm-long) area near one side of an agar plate. Flame the loop to sterilize. Touch the loop to an unoccupied area of the plate to cool. Lightly drag the loop *once* through the first streak, then continue to drag in a zigzag manner over a section of the agar surface. Be careful not to cross previous lines. Flame the loop again and cool by touching the agar surface. Again, drag the loop *once* through the previous streak, then continue to make a second streak. Repeat for a total of four to five streaks. Incubate plate at appropriate temperature.

Note that some bacteria, such as *Proteus* species, are very motile and will not form isolated colonies on solid medium, but will instead swarm across the entire surface. Other bacterial species will not grow on the surface of solid medium and thus cannot be cultured by this procedure. Consult the appropriate unit of this manual for information specific to the bacterium under investigation.

COMMONLY USED BACTERIAL MEDIA

Several common media used to culture *E. coli*, which are also suitable for cultivation of certain other species, are described below and in Table A.4A.1. Media are generally sterilized by autoclaving; however, certain media or supplements cannot be autoclaved and must instead be sterilized by passage through 0.22-μm filters. Refer to the appropriate units in this manual for details regarding culture medium preparation specific to each organism. *APPENDIX 2C* provides a list of media used in this manual and the corresponding units in which their formulation can be found.

For certain media, components must be sterilized independently, then combined later. Certain mixtures of salts and other compounds can yield toxic byproducts when subjected to autoclave conditions. Other components, such as sugars, are generally filter sterilized to prevent burning (caramelization) that could occur during autoclaving.

For most bacteria, deionized or distilled water is used to make culture media. For less fastidious bacterial species such as *E. coli*, tap water is satisfactory or even preferable for making culture media, as the trace minerals in such water can be beneficial.

CAUTION: Loosen caps of bottles before autoclaving, to prevent explosions.

Liquid Media

LB (Luria-Bertani) medium

10 g tryptone
5 g yeast extract
5 g NaCl
Adjust volume to 1 liter with H_2O

Table A.4A.1 Media Commonly Used for Culture of *E. coli*

Medium	Comments
LB medium	Sometimes also referred to as Luria broth or L broth; a good general-purpose medium for culturing *E. coli*
M9 minimal salts	A simple defined medium useful for selective culture of auxotrophs
SOC medium	A rich medium with high osmolarity; ideal for the recovery step following transformation of *E. coli* and similar bacteria
Terrific broth (TB)	*E. coli* grows rapidly in this rich medium: a very small inoculum in 100 ml will grow to stationary phase overnight, making it a good choice for growing bacteria for plasmid preparations

Sterilize by autoclaving
Store indefinitely at room temperature

Some researchers adjust the pH to ∼7 by titrating with 1N NaOH, but this is not necessary.

M9 minimal salts, 5×

30 g Na_2HPO_4
15 g KH_2PO_4
5 g NH_4Cl
2.5 g NaCl
15 mg $CaCl_2$ (optional)
Adjust volume to 1 liter with H_2O
Add ∼50 ml chloroform to 5× stock solution as a preservative
Store 5× concentrate up to many months at 4°C

Just before use, dilute 1:5 with water, and sterilize by autoclaving. Cool to <50°C and add the following:

1 ml 1 M $MgSO_4$: filter sterilize; store indefinitely at room temperature
10 ml 20% carbon source (e.g., glucose, lactose, glycerol): filter sterilize; store indefinitely at room temperature
Store indefinitely at room temperature

The chloroform added to the concentrated medium separates into an organic layer at the bottom of the bottle. Be careful not to transfer any of the chloroform when diluting 5× concentrated stock.

SOC medium

20 g Bacto tryptone
5 g Bacto yeast extract
10 ml 1 M NaCl
2.5 ml 1 M KCl
Adjust volume to 980 ml with H_2O
Sterilize by autoclaving
Cool to <50°C and add the following:
10 ml 2 M $MgCl_2$
20 ml 20% (w/v) glucose
Store indefinitely at room temperature

TB (Terrific broth)

12 g Bacto tryptone
24 g Bacto yeast extract
4 ml glycerol

Adjust volume to 900 ml with H_2O
Dispense 90-ml aliquots into screw-cap bottles and autoclave
Store indefinitely at room temperature
Just prior use, add 10 ml TB-potassium salts (see recipe) to each bottle containing a 90-ml aliquot

TB potassium salts

125.5 g K_2HPO_4
23 g KH_2PO_4
Dissolve in 800 ml H_2O, then adjust to a final volume to 1 liter with H_2O
Dispense 100-ml aliquots into screw-cap bottles and autoclave
Store indefinitely at room temperature

Preparing Plates

Media can generally be solidified by addition of 1.5% (w/v) agar or agarose prior to autoclaving. It is easiest to dissolve all other ingredients as described in the appropriate recipes (see, e.g., Liquid Media, above) and then add the agar or agarose immediately before autoclaving, as the autoclaving process will melt the solidifying agent. After autoclaving, swirl gently to evenly disperse ingredients. If solid medium is to be used in plates, cool the medium to ~50°C in a water bath before pouring as this will prevent melting of plastic petri dishes, permit easier handling of the container flask, and reduce condensation in the solidified plates. Dry off any residual water on the outside of the flask before pouring to avoid contamination; briefly passing the lip of the flask through a Bunsen burner flame between plate pours can also help prevent introduction of contaminants. Pour 20 to 25 ml medium per 100-mm diameter petri dish. Swirl plates, if necessary, to cover the entire dish. Bubbles on the medium surface can be removed by quickly passing a burner flame over the surface prior to solidification. Solidified plates should be left at room temperature for 1 to 2 days to allow evaporation of excess moisture; this will reduce condensation during subsequent culture incubations. Alternatively, plates can be dried by incubating at 37°C with the agar side on the bottom for ~30 min with the lids ajar. A perfect plate for growth of *E. coli* has a slightly rippled surface (also see Positioning Plates within the Incubator). Plates can be stored almost indefinitely at 4°C if wrapped in plastic to prevent desiccation.

Table A.4A.2 Antibiotic Stock Solutions for Use with *E. coli* Cultures

Antibiotic	Stock concentration	Working concentration for *E. coli*
Ampicillin[a] (Na salt)	10 mg/ml in H_2O	50 μg/ml
Carbenicillin[a]	10 mg/ml in H_2O	50 μg/ml
Chloramphenicol	6 mg/ml in 100% ethanol	30 μg/ml
Gentamicin	3 mg/ml in H_2O	15 μg/ml
Kanamycin	10 mg/ml in H_2O	50 μg/ml
Rifampicin	20 mg/ml in 100% methanol	100 μg/ml
Spectinomycin	20 mg/ml in H_2O	100 μg/ml
Streptomycin	10 mg/ml in H_2O	50 μg/ml
Tetracycline·HCl	2.5 mg/ml in 50% ethanol	12.5 μg/ml

[a]Carbenicillin is more stable than ampicillin.

If not to be used immediately for pouring plates, solid media can be stored indefinitely at room temperature in tightly capped bottles. To use, loosen cap, melt by autoclaving or by heating in a microwave oven at a high setting (microwave oven times vary according to oven and volume of medium, and must be determined empirically), then proceed as described above.

Antibiotic Supplements for Media

To prevent destruction by excess heat, antibiotics should be added only after the medium has cooled to $<50°C$. If solid medium is being prepared, cool in a 50°C water bath, add antibiotic(s), then pour plates.

Aqueous antibiotics should be sterilized by passage through 0.22-μm filters. Antibiotics dissolved in ethanol solutions do not require additional sterilization.

For simplicity of use, concentrated stock solutions of each antibiotic should be prepared (Table A.4A.2). Concentrated solutions should be kept no more than 1 month at 4°C or 6 months at −20°C. Media containing antibiotics should be kept for no more than 1 month. Protect media containing light-sensitive antibiotics (e.g., tetracycline) from light by wrapping in aluminum foil or storing in a dark place.

Note that other bacteria/species are often more or less sensitive than *E. coli* to some antibiotics. Refer to appropriate units in this manual for antibiotic requirements when working with other bacteria.

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