Chapter 3
Tools of the Laboratory:
The Methods for Studying Microorganisms
The 5 I’s of Culturing Microbes

Inoculation – introduction of a sample into a container of media to produce a culture of observable growth

Isolation – separating one species from another

Incubation – under conditions that allow growth

Inspection

Identification
Specimen Collection:
Nearly any object or material can serve as a source of microbes. Common ones are body fluids and tissues, foods, water, or soil. Specimens are removed by some form of sampling device: a swab, syringe, or a special transport system that holds, maintains, and preserves the microbes in the sample. Discussed on page 68.

A GUIDE TO THE FIVE “i”s: How the Sample Is Processed and Profiled

1 Incubation:
The sample is placed into a container of sterile medium containing appropriate nutrients to sustain growth. Incubation involves spreading a sample on the surface of a solid medium or introducing a sample into a flask or tube. Selection of media with specialized functions can improve later steps of isolation and identification. Some microbes may require a live organism (animal, egg) as the growth medium. Further discussion on pages 60-67.

2 Incubation:
An incubator creates the proper temperature and other conditions conducive to growth. This promotes multiplication of the microbes and usually takes a period of hours or days. Incubation gives rise to a culture—the visible growth of the microbe in or on the medium. Further discussion on page 68.

3 Isolation:
One result of inoculation and incubation is isolation of the microbe. Isolated microbes may take the form of separate colonies on solid media, or turbidity (cloudiness) in broth. Further isolation (subculturing) involves taking a bit of growth from an isolated colony and inoculating a separate medium. This makes a pure culture—one that contains only a single species of microbe. More detail on pages 60, 61, 65.

4 Inspection:
The colonies or broth cultures are observed macroscopically for growth characteristics (color, texture, size) that could be useful in analyzing the specimen contents. Slides are made to assess microscopic details such as cell shape, size, and motility. Staining techniques may be used to gather specific information on microscopic morphology. See pages 69, 66.

5 Identification:
A major purpose of the Five “i”s is to determine the type of microbe, usually to the level of species. Information used in identification can include relevant data already taken during initial inspection and additional tests that further describe and differentiate the microbes. These include biochemical tests to determine metabolic activities specific to the microbe, immunologic tests, and genetic analysis. See pages 69, 70.
Isolation

• If an individual bacterial cell is separated from other cells and has space on a nutrient surface, it will grow into a mound of cells--a colony.
• A colony consists of one species.
Figure 3.2 Isolation technique

- Mixture of cells in sample
  - Separation of cells by spreading or dilution on agar medium
  - Incubation
  - Growth increases the number of cells
  - Microbes become visible as isolated colonies containing millions of cells

- Microscopic view
  - Cellular level

- Macroscopic view
  - Colony level
• Isolation techniques include:
  – Streak plate technique
  – Pour plate technique
  – Spread plate technique
**Note:** This method only works if the spreading tool (usually an inoculating loop) is resterilized (flamed) after each of steps 1–4.

(a) **Steps in a Streak Plate:** this one is a four-part or quadrant streak.

(b) Image of a stained streak plate.

(c) **Steps in Loop Dilution:** also called a pour plate or serial dilution.

(d) Image of a loop dilution plate.

(e) **Steps in a Spread Plate**

(f) Image of a spread plate.

"Hockey stick"
Media can be classified according to three properties:

1. Physical state – liquid, semisolid, and solid
2. Chemical composition – synthetic (chemically defined) and nonsynthetic (complex)
3. Functional type – general purpose, enriched, selective, differential, anaerobic, transport, assay, enumeration
### Table 3.1 Three Categories of Media Classification

<table>
<thead>
<tr>
<th>Physical State (Medium’s Normal Consistency)</th>
<th>Chemical Composition (Type of Chemicals Medium Contains)</th>
<th>Functional Type (Purpose of Medium)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Liquid</td>
<td>1. Synthetic (chemically defined)</td>
<td>1. General purpose</td>
</tr>
<tr>
<td>2. Semisolid</td>
<td>2. Nonsynthetic (complex; not chemically defined)</td>
<td>2. Enriched</td>
</tr>
<tr>
<td>3. Solid (can be converted to liquid)</td>
<td></td>
<td>3. Selective</td>
</tr>
<tr>
<td>4. Solid (cannot be liquefied)</td>
<td></td>
<td>4. Differential</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Anaerobic growth</td>
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<td></td>
<td></td>
<td>6. Specimen transport</td>
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<tr>
<td></td>
<td></td>
<td>7. Assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8. Enumeration</td>
</tr>
</tbody>
</table>

*Some media can serve more than one function. For example, a medium such as brain-heart infusion is general purpose and enriched; mannitol salt agar is both selective and differential; and blood agar is both enriched and differential.
**Physical States of Media**

Liquid – broth; does not solidify

Semisolid – contains solidifying agent

Solid – firm surface for colony formation
  – Contains solidifying agent
  – Liquefiable and nonliquefiable
Most commonly used media:

- **Nutrient broth** – liquid medium containing beef extract and peptone
- **Nutrient agar** – solid media containing beef extract, peptone, and agar
Most commonly used solidifying agent is **agar**

- A complex polysaccharide isolated from red algae
  - Solid at room temperature, liquefies at boiling (100°C), does not re-solidify until it cools to 42°C
  - Provides framework to hold moisture and nutrients
  - Not digestible for most microbes
Chemical Content of Media

• **Synthetic** – contains pure organic and inorganic compounds in an exact chemical formula
• **Complex or nonsynthetic** – contains at least one ingredient that is not chemically definable
• **General purpose media** – grows a broad range of microbes, usually nonsynthetic
• **Enriched media** – contains complex organic substances such as blood, serum, hemoglobin, or special growth factors required by fastidious microbes
Figure 3.7 Examples of enriched media
• **Selective media** – contains one or more agents that inhibit growth of some microbes and encourage growth of the desired microbes

• **Differential media** – allows growth of several types of microbes and displays visible differences among those microbes
Mixed sample

General-purpose nonselective medium (All species grow.)

Selective medium (One species grows.)

(a)

Mixed sample

General-purpose nondifferential medium (All species have a similar appearance.)

Differential medium (All three species grow but may show different reactions.)

(b)
Miscellaneous Media

- **Reducing medium** – contains a substance that absorbs oxygen or slows penetration of oxygen into medium; used for growing anaerobic bacteria
- **Carbohydrate fermentation medium** – contains sugars that can be fermented, converted to acids, and a pH indicator to show this reaction
Figure 3.10 Media that differentiate
Figure 3.11 Carbohydrate fermentation in broths
Incubation, Inspection, and Identification

Incubation – temperature-controlled chamber
- Microbe multiplies and produces macroscopically observable growth

Inspection – observation; macroscopic and microscopic
- **Pure culture** – grows only single known species of microorganisms
- **Mixed cultures** – hold two or more identified species or microorganisms
- **Contaminated culture** – once pure or mixed culture that has unwanted microbes growing
Figure 3.12 Various conditions of cultures
Incubation, Inspection, and Identification

Identification – macroscopic and microscopic appearance, biochemical tests, genetic characteristics, immunological testing
Disposal of Cultures

Potentially hazardous cultures and specimens are usually disposed of in two ways:

– Steam sterilization
– Incineration
3.2 The Microscope

Key characteristics of a reliable microscope are:

- **Magnification** – ability to enlarge objects
- **Resolving power** – ability to show detail
Magnification in most microscopes results from interaction between visible light waves and curvature of the lens

– Angle of light passing through convex surface of glass changes – **refraction**

– Depending on the size and curvature of the lens, the image appears enlarged

– Extent of enlargement – **magnification**
Principles of Light Microscopy

• Magnification occurs in two phases –
  – The objective lens forms the magnified real image
  – The real image is projected to the ocular where it is magnified again to form the virtual image

• Total magnification of the final image is a product of the separate magnifying powers of the two lenses

\[
\text{power of objective} \times \text{power of ocular} = \text{total magnification}
\]
Resolution

Resolution defines the capacity to distinguish or separate two adjacent objects – resolving power

– Function of wavelength of light that forms the image along with characteristics of objectives

  • Visible light wavelength is 400 nm–750 nm
  • Numerical aperture of lens ranges from 0.1 to 1.25
  • Oil immersion lens requires the use of oil to prevent refractive loss of light
  • Shorter wavelength and larger numerical aperture will provide better resolution
  • Oil immersion objectives resolution is 0.2 μm
  • Magnification between 40X and 2000X
Figure 3.16 Effect of wavelength on resolution
Figure 3.17 Oil immersion lens
Variations on the Optical Microscope

• **Bright-field** – most widely used; specimen is darker than surrounding field; live and preserved stained specimens

• **Dark-field** – brightly illuminated specimens surrounded by dark field; live and unstained specimens

• **Phase-contrast** – transforms subtle changes in light waves passing through the specimen into differences in light intensity, best for observing intracellular structures
Figure 3.19 Three views of a basic cell
Phase Contrast and DIC Microscope

- The differential interference microscope is similar to the phase contrast but has more refinements.
Fluorescence Microscope

- Modified compound microscope with an ultraviolet radiation source and filter that protects the viewer’s eye
- Uses dyes that emit visible light when bombarded with shorter UV rays - fluorescence
- Useful in diagnosing infections
Figure 2.21 Fluorescent staining on cheek scrapings
Electron Microscopy

- Forms an image with a beam of electrons that can be made to travel in wavelike patterns when accelerated to high speeds
- Electron waves are 100,000 times shorter than the waves of visible light
- Electrons have tremendous power to resolve minute structures because resolving power is a function of wavelength
- Magnification between 5,000X and 1,000,000X
Figure 3.23 Comparison of microscopes

[Diagram showing the comparison between a Light Microscope and a Transmission Electron Microscope]
2 Types of Electron Microscopes

- Transmission electron microscopes (TEM) – transmit electrons through the specimen. Darker areas represent thicker, denser parts and lighter areas indicate more transparent, less dense parts.

- Scanning electron microscopes (SEM) – provide detailed three-dimensional view. SEM bombards surface of a whole, metal-coated specimen with electrons while scanning back and forth over it.
Figure 3.24b TEM

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Figure 3.25 SEM
Specimen Preparation for Optical Microscopes

Preparation of slide depends on
1. Condition of specimen
2. Aims of examiner
3. Type of microscopy available
Specimen Preparation for Optical Microscopes

- **Wet mounts and hanging drop mounts** – allow examination of characteristics of live cells: size, motility, shape, and arrangement.

- **Fixed mounts** are made by drying and heating a film of specimen. This *smear* is stained using dyes to permit visualization of cells or cell parts.
Staining

Dyes create contrast by imparting a color to cells or cell parts

- **Basic dyes** – cationic, positively charged chromophore
- **Acidic dyes** – anionic, negatively charged chromophore
- **Positive staining** – surfaces of microbes are negatively charged and attract basic dyes
- **Negative staining** – microbe repels dye, the dye stains the background
Figure 3.26 Staining reactions of dyes

(a) Basic Dye

(b) Acidic Dye

(c) Acidic Dye

Methylene blue

Eosin

Nigrosin

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Staining

• **Simple stains** – one dye is used; reveals shape, size, and arrangement

• **Differential stains** – use a primary stain and a counterstain to distinguish cell types or parts (examples: Gram stain, acid-fast stain, and endospore stain)

• **Structural stains** – reveal certain cell parts not revealed by conventional methods: capsule and flagellar stains
Figure 3.27 Microbiological stains

(a) Simple Stains
- Crystal violet stain of Escherichia coli (1,000×)

(b) Differential Stains
- Gram stain: Purple cells are gram-positive. Red cells are gram-negative (900×).

(c) Special Stains
- India ink capsule stain of Cryptococcus neoformans (500×)

- Methylene blue stain of Corynebacterium (1,000×)

- Acid-fast stain: Red cells are acid-fast. Blue cells are non-acid-fast (750×).

- Flagellar stain of Proteus vulgaris. A basic stain was used to build up the flagella (1,500×).

- Spore stain, showing spores (green) and vegetative cells (red) (1,000×).