Chapter 5: Development of microbial resistance

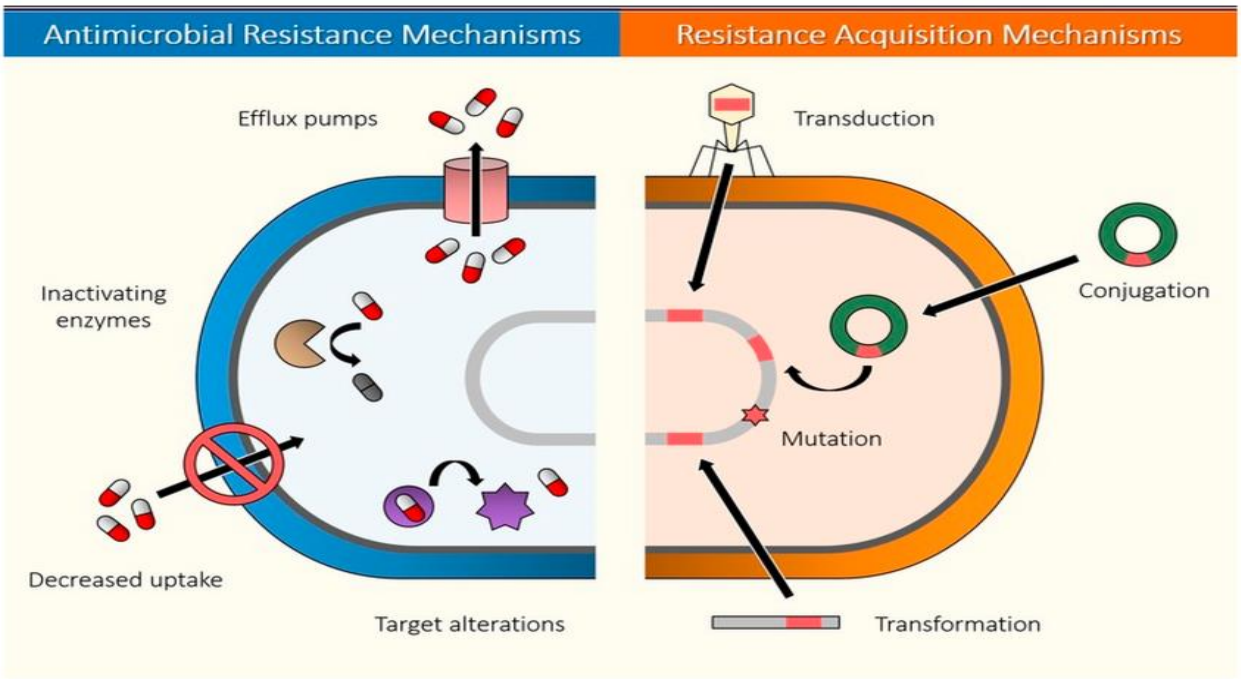
Microbial resistance is a dynamic phenomenon, ranging from physiological adaptation (transient) to genetic evolution (permanent). Methodological study relies on stress induction techniques, survival measurement, molecular analysis, and population genetics. Mastering these approaches is essential to predict, prevent, or bypass microbial inactivation.

1. Importance of resistance responses

- Microbial resistance compromises the efficacy of inactivation treatments (heat, acids, disinfectants, radiation).
- It is a major issue in **food safety, health** (antibiotic resistance), and **biotechnology**.
- Two main categories of resistance:
  - ✓ **Transient responses** (inducible, reversible)
  - ✓ **Permanent responses** (genetic, heritable)

2. Main categories of resistance

Feature	Transient response	Permanent response
Origin	Phenotypic, non-heritable	Genetic (mutation, gene transfer)
Duration	As long as stress is present	Stable even without stress
Example	Induction of heat shock proteins	Mutation conferring antibiotic resistance
Reversibility	Yes	No (except rare back-mutation)



3. Genetic regulation of resistance development

3.1 Sigma factors (σ) and stress response

Sigma factors are subunits of bacterial RNA polymerase that direct transcription of specific genes. For example:

- **σ^{70}**: Housekeeping genes.
- **σ^{32} (RpoH)**: Heat shock response.
- **σ^{5} (RpoS)**: General stress response (acid, oxidative, cold, starvation).

General mechanism:
Activation of a sigma factor → transcription of stress operons → synthesis of protective proteins (chaperones, antioxidant enzymes, efflux pumps).

❖ **Key mechanisms of action**

✓ **ECF Sigma factors (Extracytoplasmic function):** These act as sensors for external threats like antimicrobials. When antibiotics or stress damage the bacterial cell envelope, ECF sigma factors are released (often via regulated intramembrane proteolysis) to launch transcription of resistance genes.

✓ **Alternative Sigma factor activation:**

- **σ^{S} (RpoS):** Known as the general stress sigma factor in gram-negative bacteria, it acts during the stationary phase and stress to increase survival against antibiotic treatment.
- **σ^{B}(SigB):** Acts in gram-positive bacteria to activate stress-response genes and, in some cases, regulate membrane rigidity to enhance survival.

✓ **Anti-Sigma factor sequestration:** Sigma factors are usually kept inactive by binding to an anti-sigma factor. External stress disrupts this pairing, releasing the sigma factor to bind to the RNA polymerase core enzyme.

✓ **Competition for RNA Polymerase:** Under stress, alternative sigma factors compete with the housekeeping sigma factor (**σ^{70}**) for limited RNA polymerase, altering the overall gene expression profile to favor resistance.

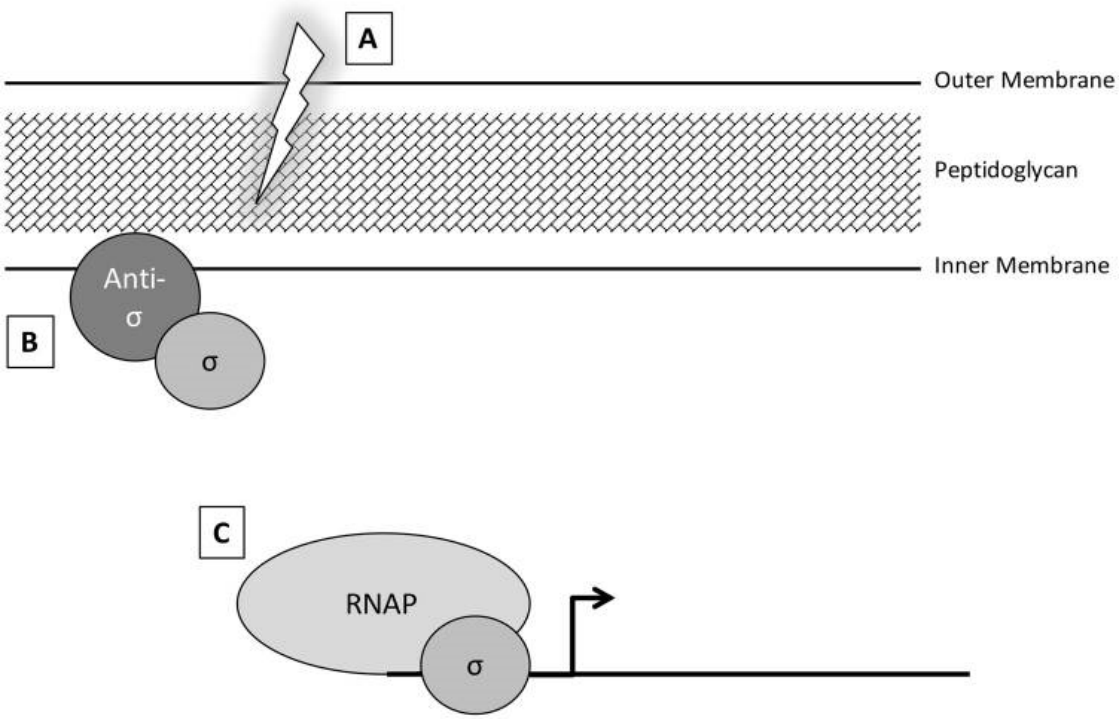


Figure 1. Model of ECF sigma factor activation.

(A) ECF sigma factors (labeled σ) are typically kept inactive via sequestration at the cell membrane by an anti-sigma factor (labeled anti-σ). **(B)** An extracellular signal or perturbation initiates a proteolytic cascade that cleaves the anti-sigma factor to release the sigma factor. **(C)** Upon release, the sigma factor is free to bind to DNA and direct the RNA polymerase (RNAP) to transcribe specific genes.

3.2 Selection of resistant mutants

- Under selective pressure (antimicrobial agent), pre-existing mutants survive and proliferate. Example: mutation in RNA polymerase or topoisomerase gene leads to antibiotic resistance.
- Permanent resistance often arises from point mutations, deletions, or acquisition of mobile genetic elements (plasmids, transposons).

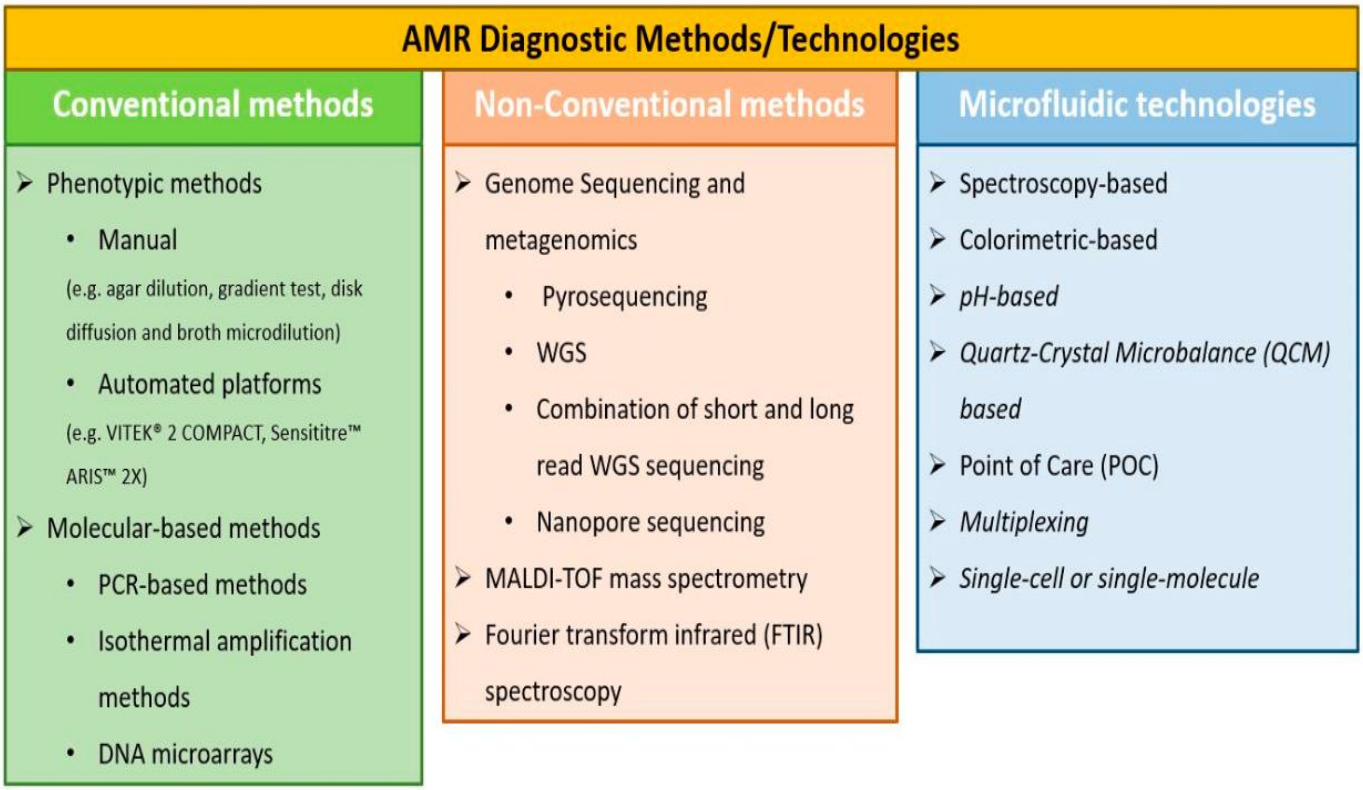
4. Responses to different stresses

Bacteria are constantly exposed to fluctuations in their environment, which can impose various forms of stress threatening their survival and integrity. In order to cope with these challenges, they have evolved sophisticated and highly regulated adaptive responses. Depending on the nature of the stress—whether thermal, pH-related, or oxidative—bacteria activate specific resistance mechanisms that are often transient and energy-intensive. These mechanisms involve the coordinated expression of key genes and regulatory pathways, enabling cells to maintain homeostasis, protect macromolecules, and restore normal functions once the stress subsides. The table below summarizes the major stress types encountered by bacteria, along with the corresponding resistance strategies and the genetic determinants involved.

Table: Responses to different stresses

Stress type	Resistance mechanisms (transient)	Key genes/pathways
Heat shock	Production of chaperones (GroEL, DnaK) and proteases	σ^{32}, Hsp70, Hsp60
Cold stress	Synthesis of cold shock proteins (CspA); membrane modification	σ^{24}, Csp family
Acid stress	Proton pumps (ATPase), decarboxylases (arginine, glutamate)	σ^{5}, ADI system, GAD
Alkaline stress	Intracellular pH regulation via Na^{+}/H^{+} antiporters, membrane adaptation	σ^{5}, NhaA
Oxidative stress	Activation of catalase, superoxide dismutase (SOD), peroxidases, thioredoxin/glutathione systems	OxyR, SoxRS, σ^{5}

5. Techniques for studying the development of microbial resistance



5.1 Methods for induction and monitoring of transient responses

- Adaptive pretreatment

Cells are first exposed to a sub-lethal stress to induce protective mechanisms, then challenged with a lethal stress. Survival rates are compared with non-pretreated controls to quantify acquired tolerance.

- Inactivation kinetics (survival curves) with/without pretreatment

Cell viability is monitored over time during lethal stress exposure, with or without prior adaptation. Survival curves reveal how transient responses delay death (shoulder effect) or reduce inactivation rates (D-values).

- Gene expression measurement

- **RT-qPCR** : Quantifies mRNA levels of stress genes (e.g., *groEL*, *rpoS*) at different time points, providing a dynamic and sensitive measure of transcriptional induction.
- **Reporter gene assays (e.g., *rpoS-lacZ* fusion)** : A stress promoter is fused to a reporter gene (e.g., *lacZ*). Promoter activity is measured via colorimetric or fluorescent signals, allowing easy monitoring without RNA extraction.

5.2 Techniques for studying permanent resistance

• Spontaneous mutation assay

- **Fluctuation test (Luria–Delbrück)** : Estimates the mutation rate toward resistance by comparing the variance in resistant mutant numbers across parallel cultures. Demonstrates that mutations arise randomly, not in response to selection.

• Whole-genome sequencing (WGS)

Identifies the specific genetic mutations (SNPs, indels, etc.) responsible for acquired permanent resistance by comparing resistant mutants to the ancestral sensitive strain.

• Experimental evolution

- **Continuous culture (chemostat) under selective pressure** : Bacteria are grown for many generations under constant stress (e.g., antibiotic, acid), allowing resistant mutants to emerge and take over the population.
- **Population monitoring** : Tracks changes in fitness (growth rate, competitive ability) and cross-resistance (resistance to other unrelated stresses) throughout evolution.

5.3 Techniques specific to Sigma factors

• Promoter mapping (RNA-seq, ChIP-seq)

- **RNA-seq** : Compares transcriptomes of wild-type and sigma factor mutant strains to identify genes whose expression depends on the sigma factor.

- **ChIP-seq** : Maps genome-wide binding sites of a sigma factor to directly identify which promoters it recognizes.

• Directed mutagenesis of the Sigma factor gene

The sigma factor gene is knocked out or mutated, causing loss of function. The mutant strain is then tested for stress sensitivity, revealing the sigma factor's role in protecting against specific stresses.

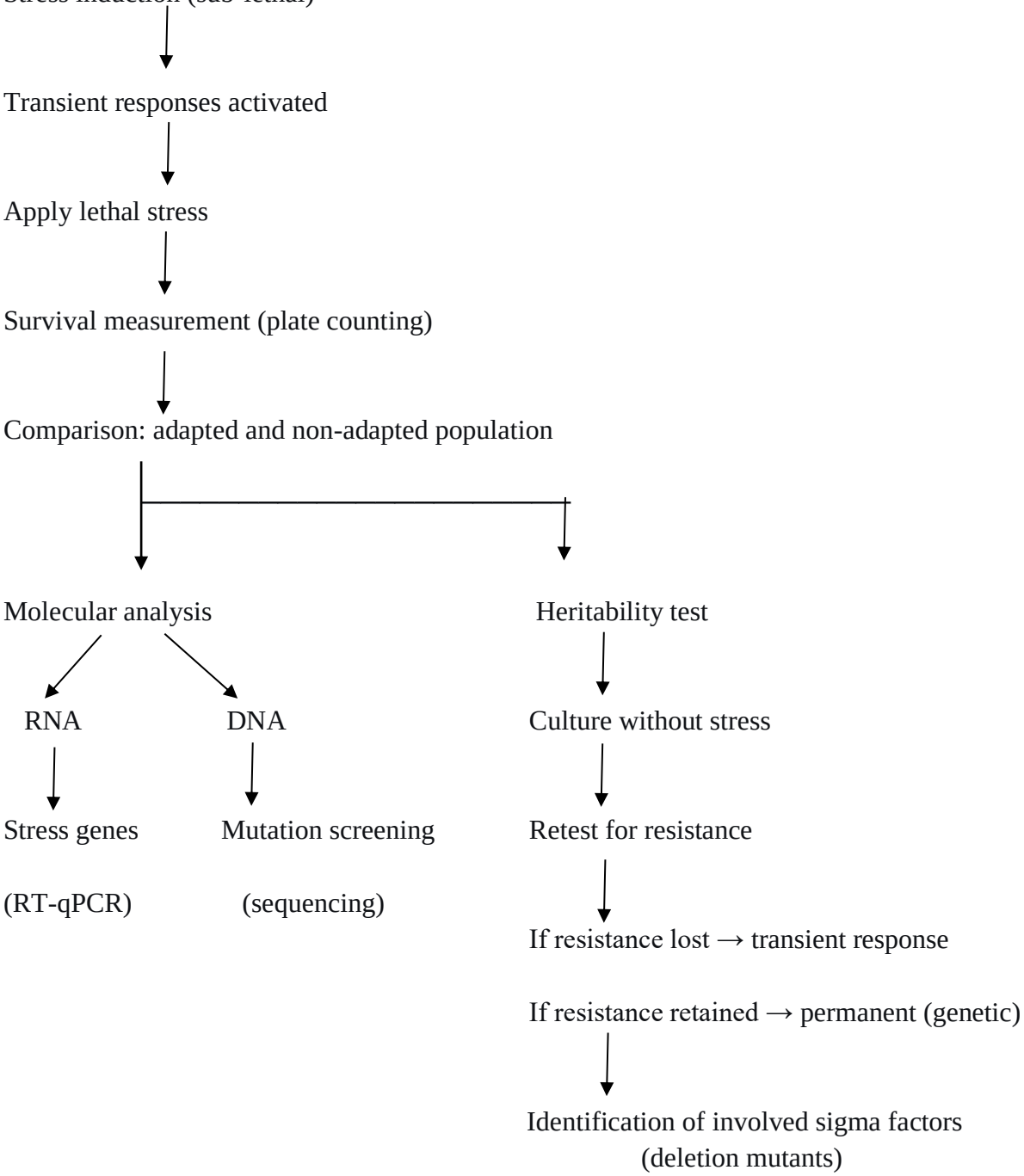
5.4 Examples of techniques for each stress type

Stress	Specific technique
Heat	Sub-lethal heat shock (42–45°C) then lethal temperature (55–60°C)
Cold	Incubation at 4–10°C then lethal temperature or freezing
Acid	Maintenance at pH 4–5 then shock at pH 2–3 (gastric simulation)
Alkaline	Adaptation at pH 9–10 then shock at pH 11–12
Oxidative	Sub-lethal H_2O_2 (0.5 mM) then lethal H_2O_2 (10–50 mM)

5.5 Complementary Techniques

- **Flow cytometry** with viability probes (propidium iodide, CFDA) to assess resistance heterogeneity.
- **Fluorescence microscopy** (e.g., GFP expression under stress promoter).
- **Efflux measurement** (ethidium bromide accumulation).
- **Antimicrobial susceptibility testing** (MIC, MBC) before/after adaptation.

6. Methodological workflow for studying resistance



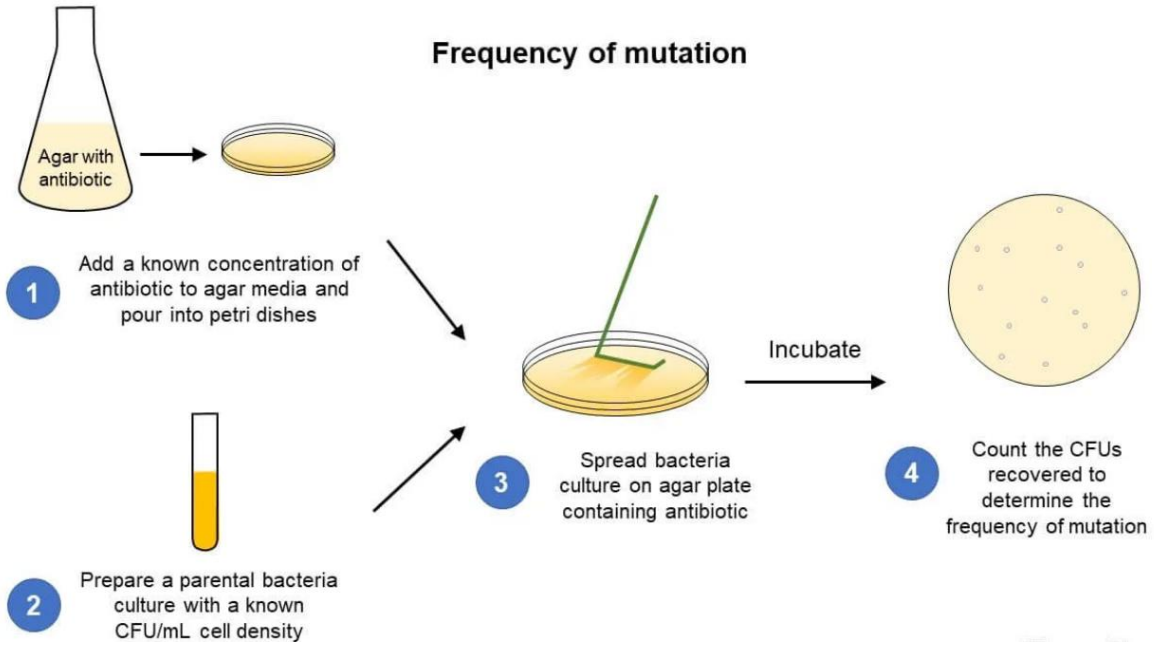
7. Example: Antibiotic resistance development

Antibiotic resistance development is studied using two main approaches:

1. Single-step resistance studies

Resistance emerges after a single exposure to an antibiotic. This includes:

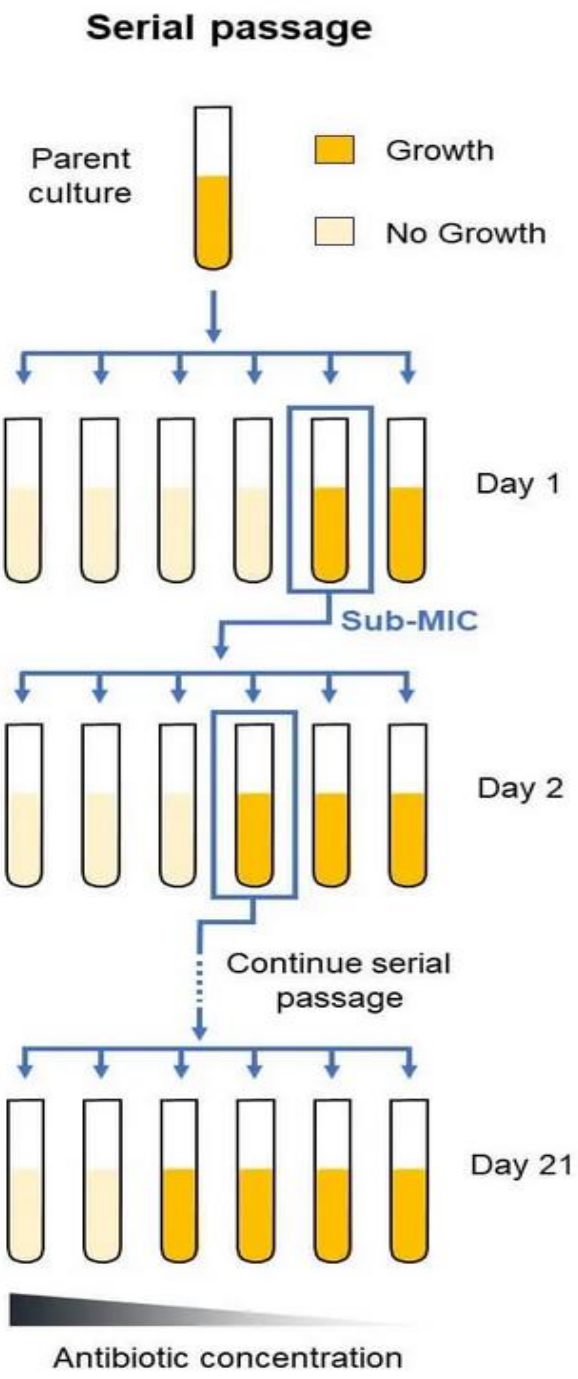
- ✓ **Spontaneous mutation frequency** : Measures how often resistant mutants appear.
- ✓ **Mutant characterization** : Confirms resistance level and genetic mechanisms.
- ✓ **Mutant Prevention Concentration (MPC)** : The lowest antibiotic concentration that prevents resistant colony formation.



Spontaneous Mutation Frequency. This figure illustrates the general workflow for conducting a single-step, spontaneous mutation frequency assay. (1) Liquid agar containing a known concentration of antibiotics (typically 4-fold or 8-fold the MIC value of the bacteria being assayed) is prepared, poured into petri dishes, and allowed to solidify. (2) A parental bacteria culture is prepared and the bacterial density in CFU/mL is determined. (3) The parental bacteria culture is spread onto the agar plate containing antibiotic and incubated for up to 3 days. (4) The number of CFUs recovered is enumerated and this value is compared to the parental bacterial density to generate a frequency of mutation.

2. Multi-step resistance studies

Bacteria are exposed to gradually increasing antibiotic concentrations over multiple passages (subcultures). The main method is **serial passage**, where bacteria are grown in sub-inhibitory antibiotic concentrations for up to 21 days to track how resistance develops over time.



These studies help predict how frequently resistant mutants arise and what types of resistance might emerge in clinical settings.