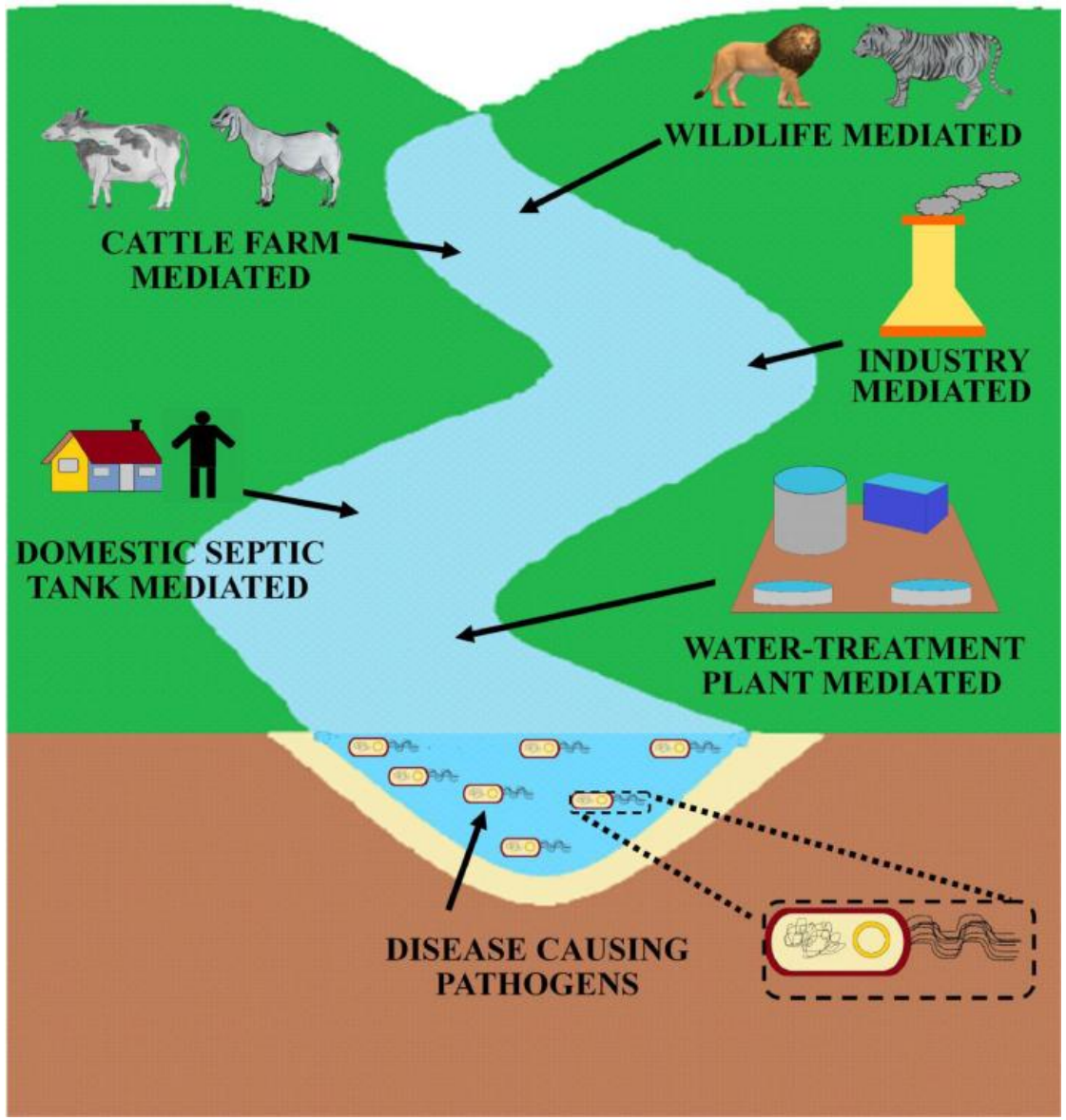
تست های تشخیصی میکروبی آب با رویکرد مولکولی

Microbial Water Contamination

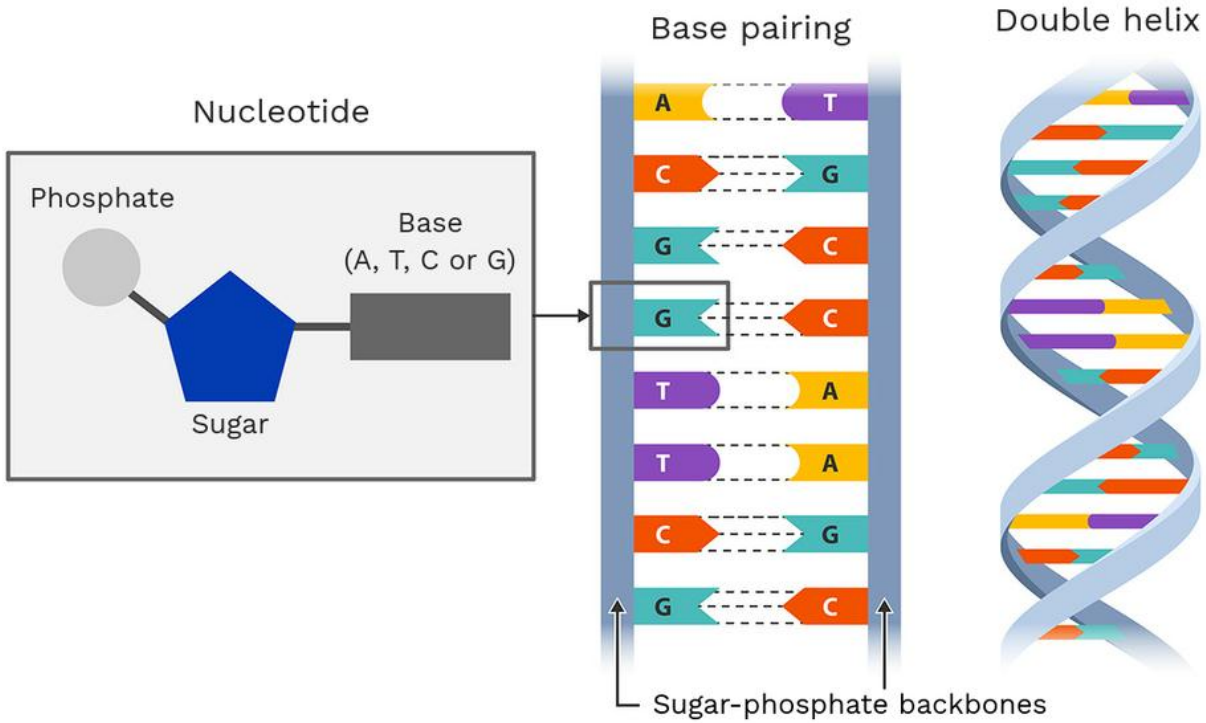


Molecular-based Method

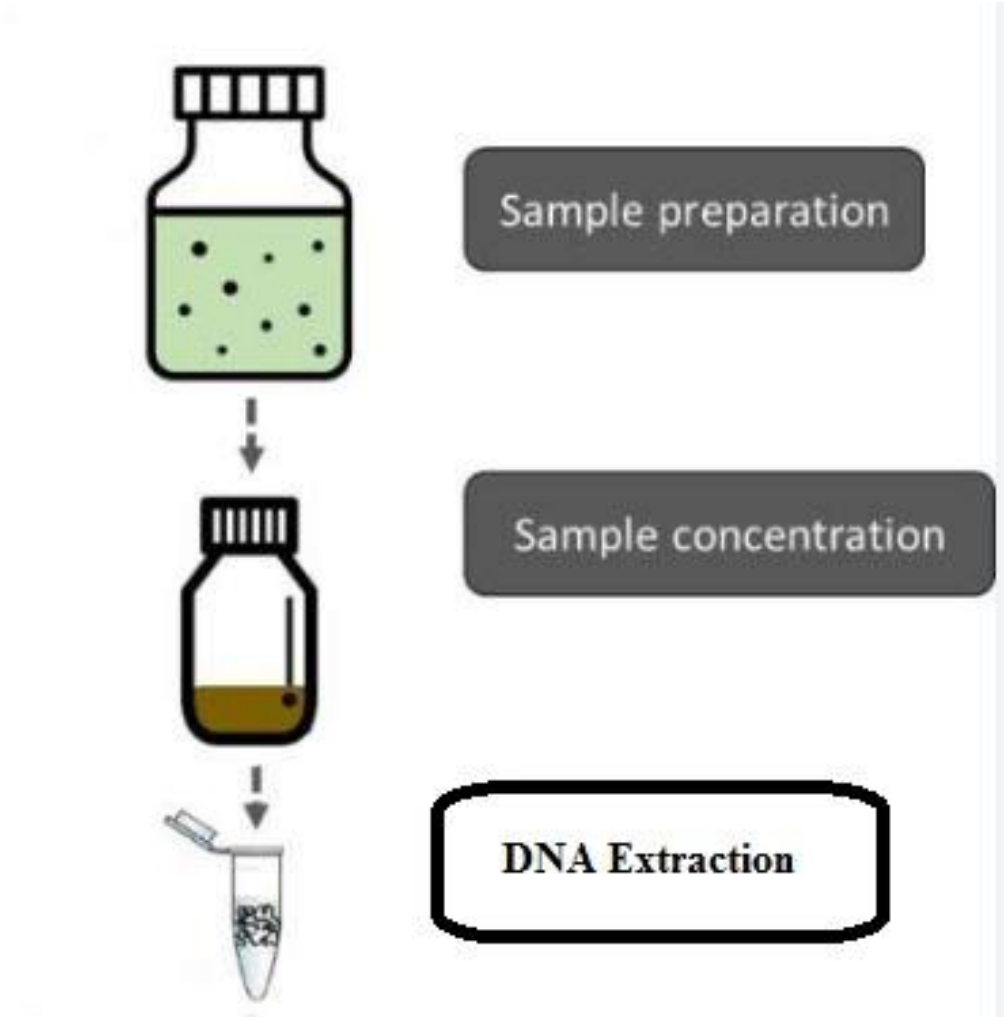
1

Molecular Methods

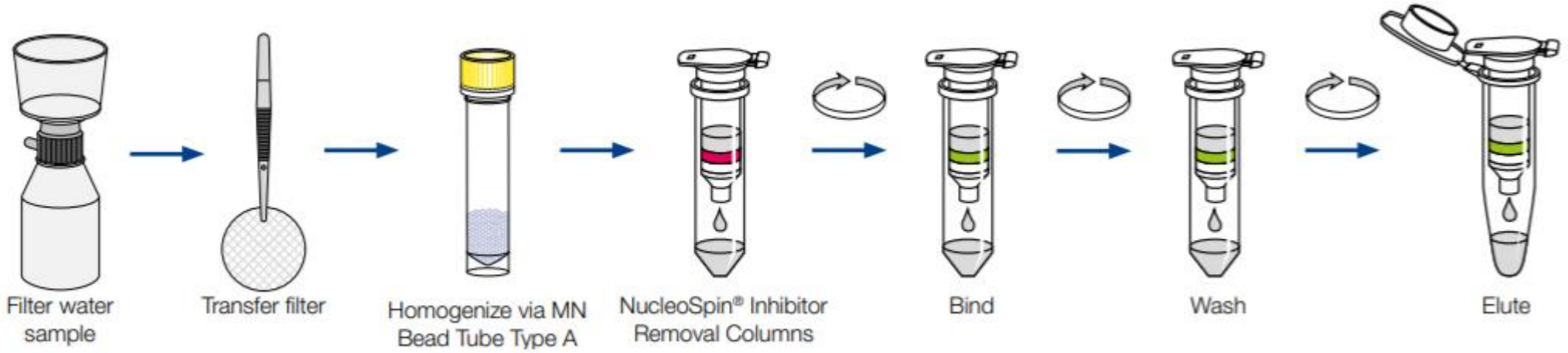
- **Samples Collection**
- **Concentration and DNA Extraction**
- **DNA amplification for:**
  - Bacterial Diagnosis (for example: gyrase PCR)
  - DNA Sequencing for Bacterial Diagnosis
    - 16S rDNA PCR- Sequencing (component of the 30S subunit of a prokaryotic ribosome)
  - Genotyping (for example: PCR- RFLP)
  - Plasmid and Phage identification (Antibiotic Resistance)
  - Virulence Factors, …

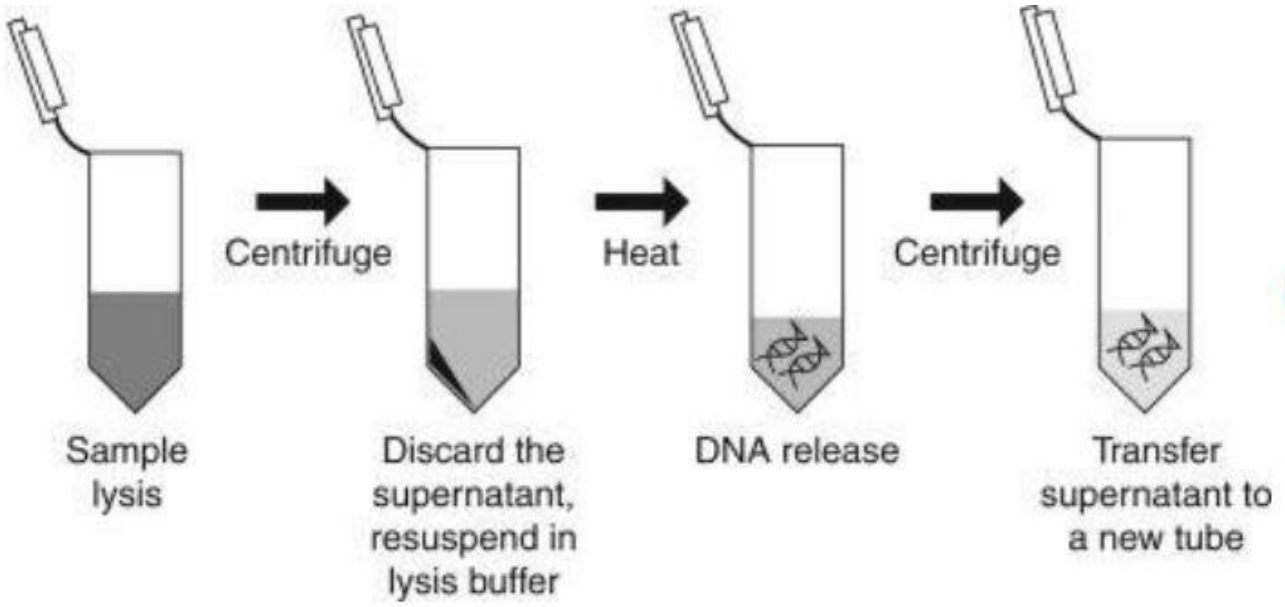


Sample Collection



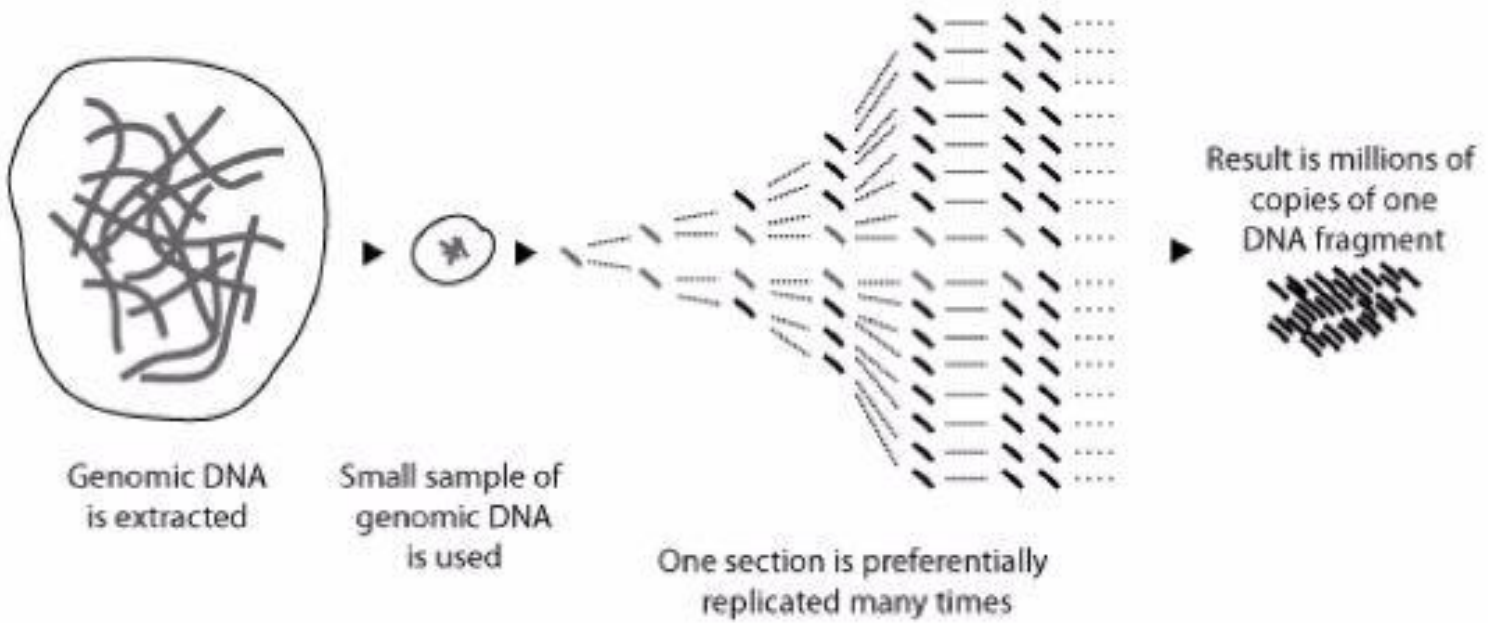
DNA Extraction



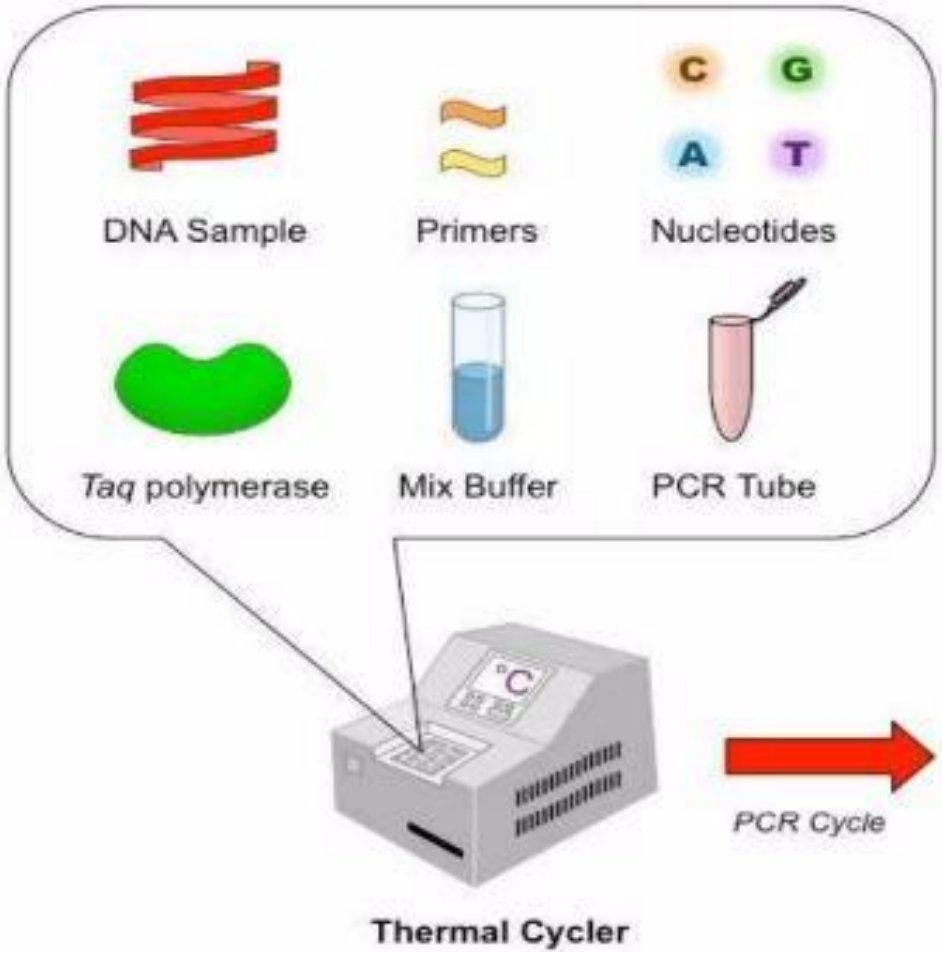


PCR (Polymerase Chain Reaction)

Exponential Amplification:
the DNA sequence between primers **doubles** after each cycle



PCR Components



PCR reaction contains

- Target DNA (example: environmental DNA)
- 2 primers (20-30 nts long)
- Thermostable DNA polymerase
- Nucleotides (dNTPs)
- Mg^{2+} (cofactor for DNA polymerase)

Mix is subjected to temperature cycling

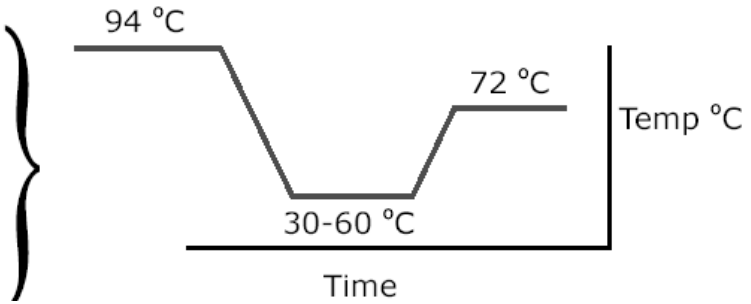
Each cycle

Denaturation
94 °C

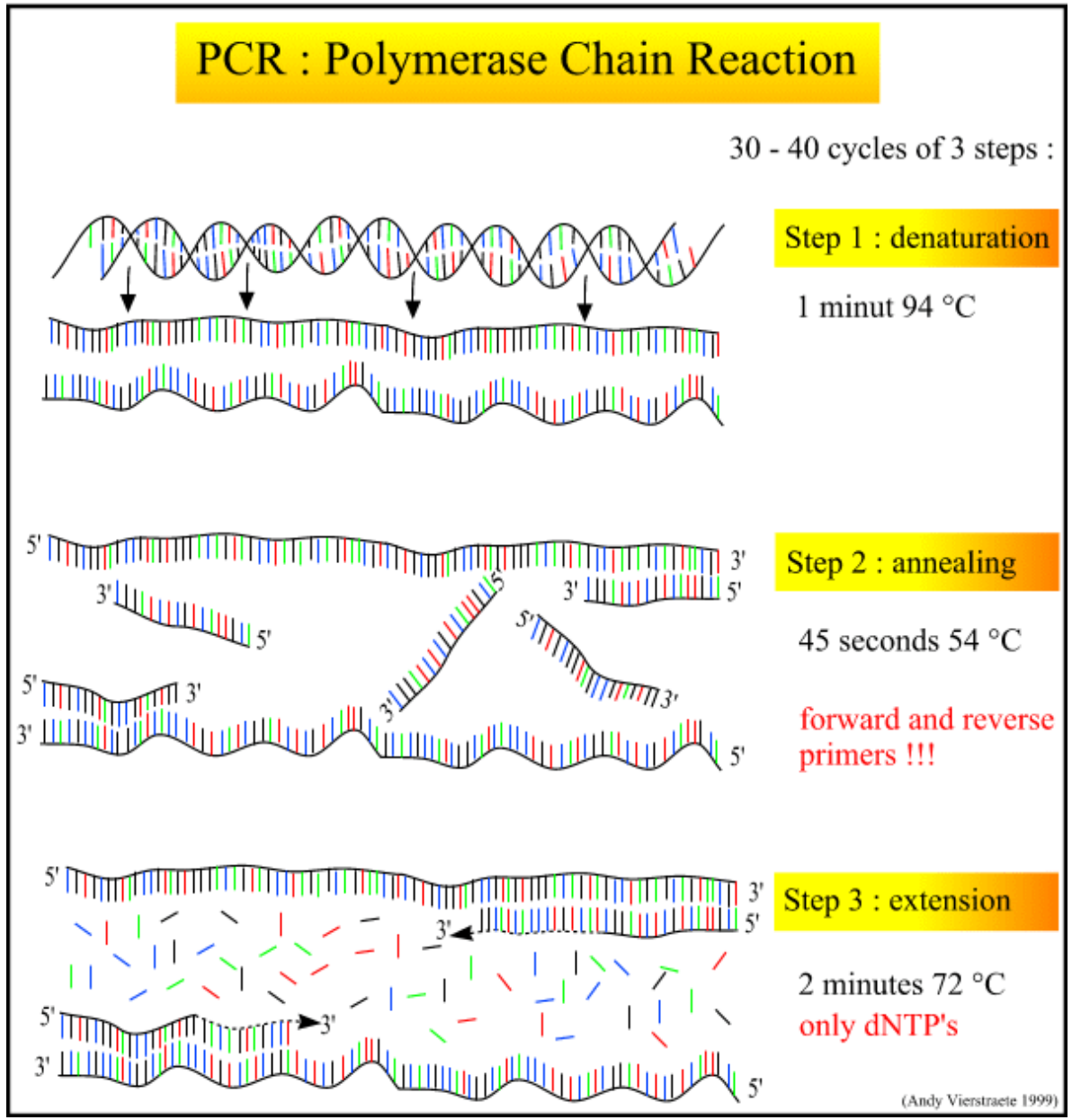
Annealing
30-60 °C

Extension
72 °C

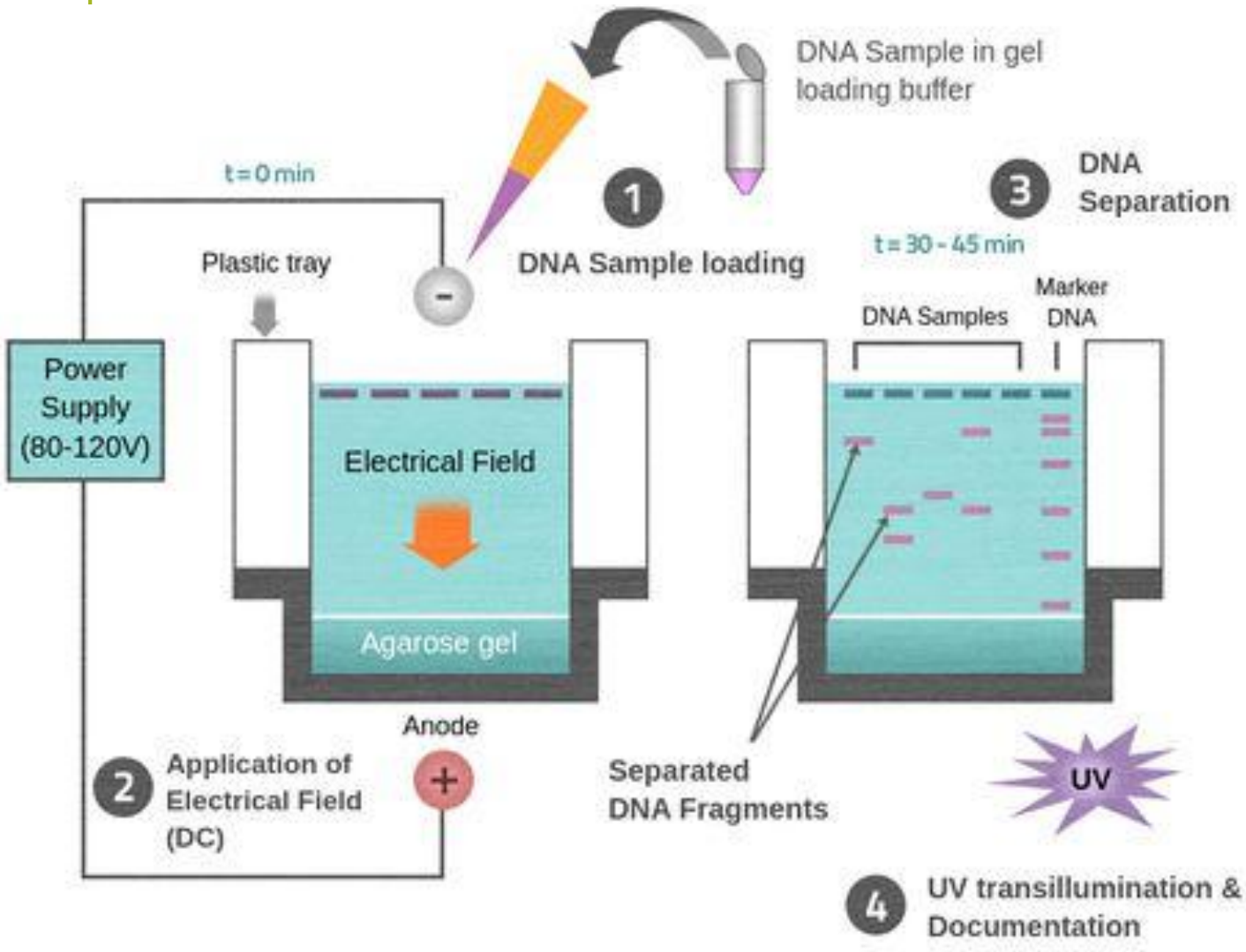
(adjust temperature to balance between specificity and amplification)



PCR Steps

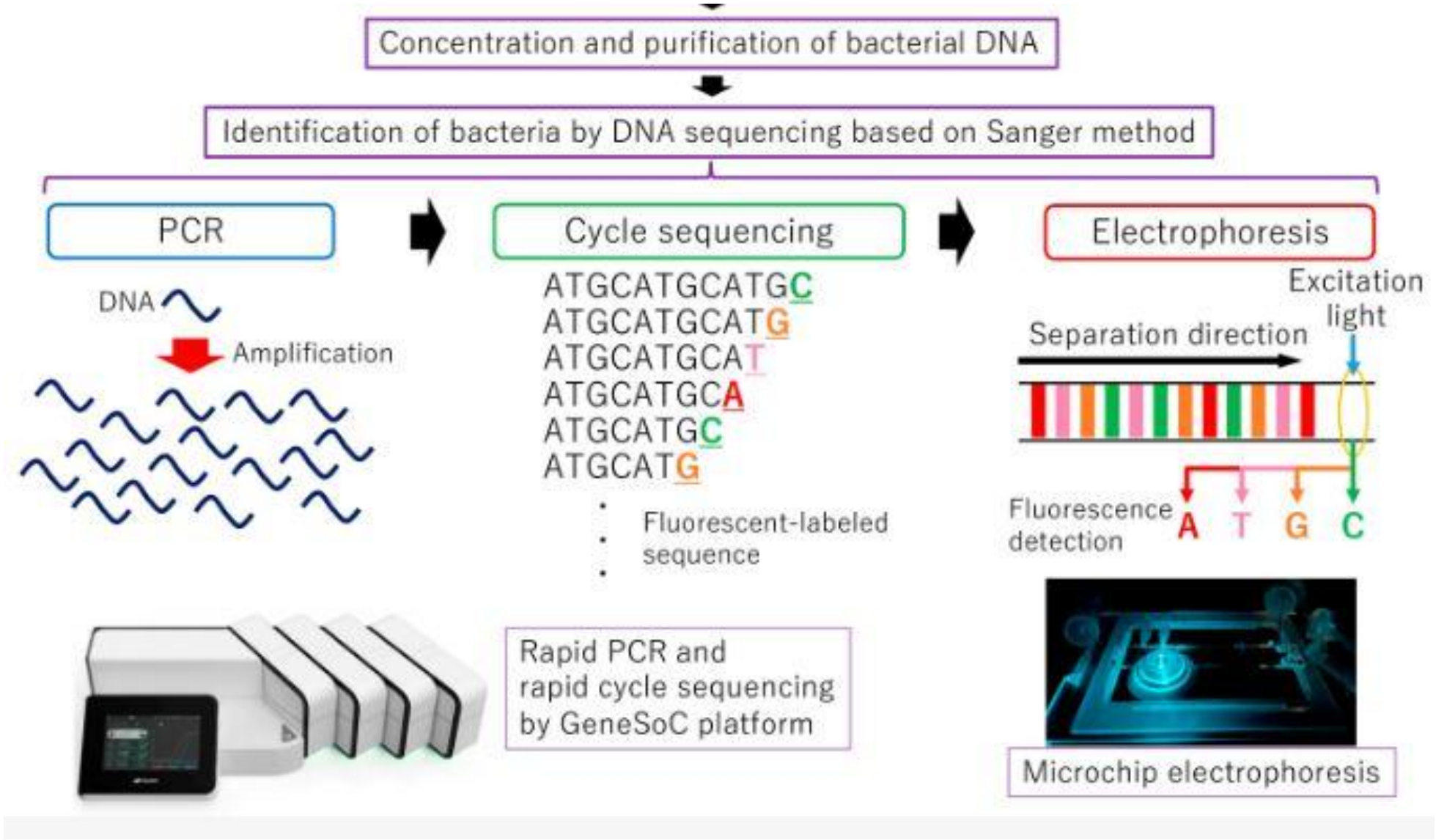

PCR : Polymerase Chain Reaction
30 - 40 cycles of 3 steps :
Step 1 : denaturation
1 minut 94 °C
Step 2 : annealing
45 seconds 54 °C
forward and reverse primers !!!
Step 3 : extension
2 minutes 72 °C
only dNTP's
(Andy Vierstraete 1999)

DNA Amplification

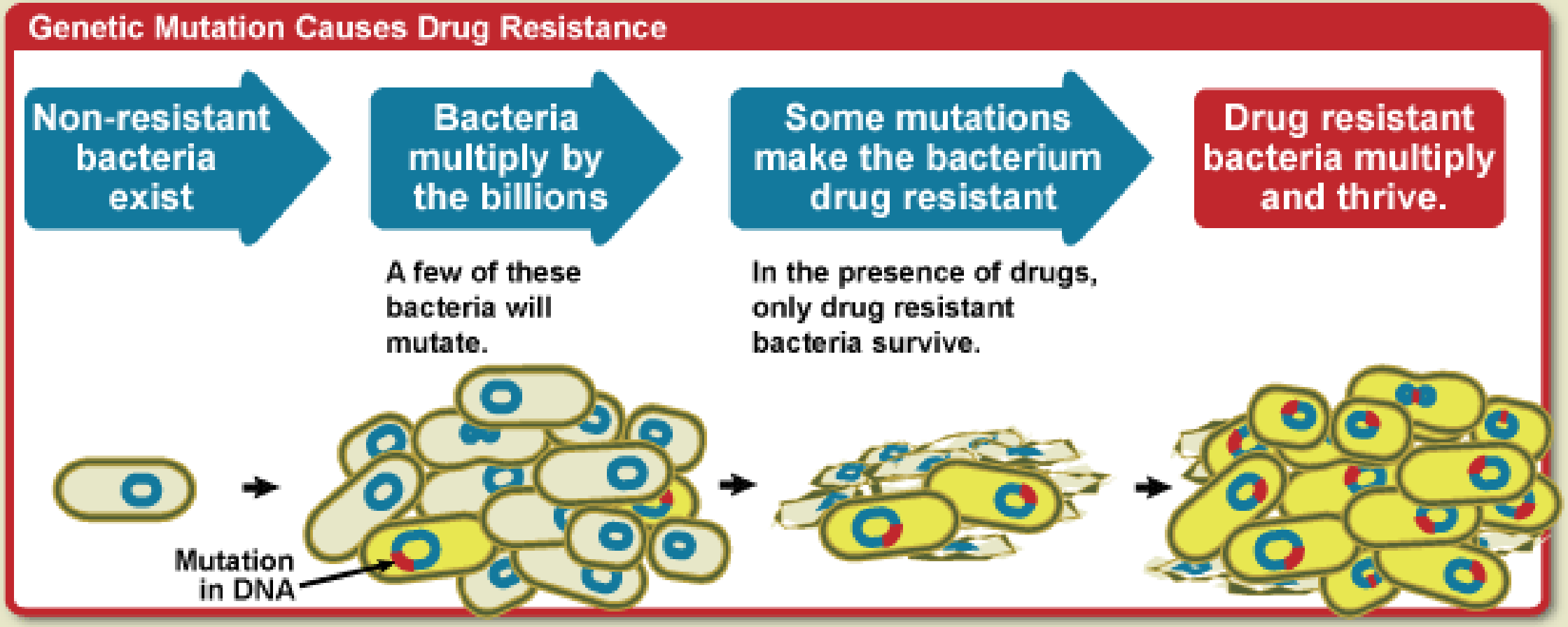


Gel Documentation System

DNA Sequencing For Bacterial Diagnosis



Antibiotic Resistance



Plasmid

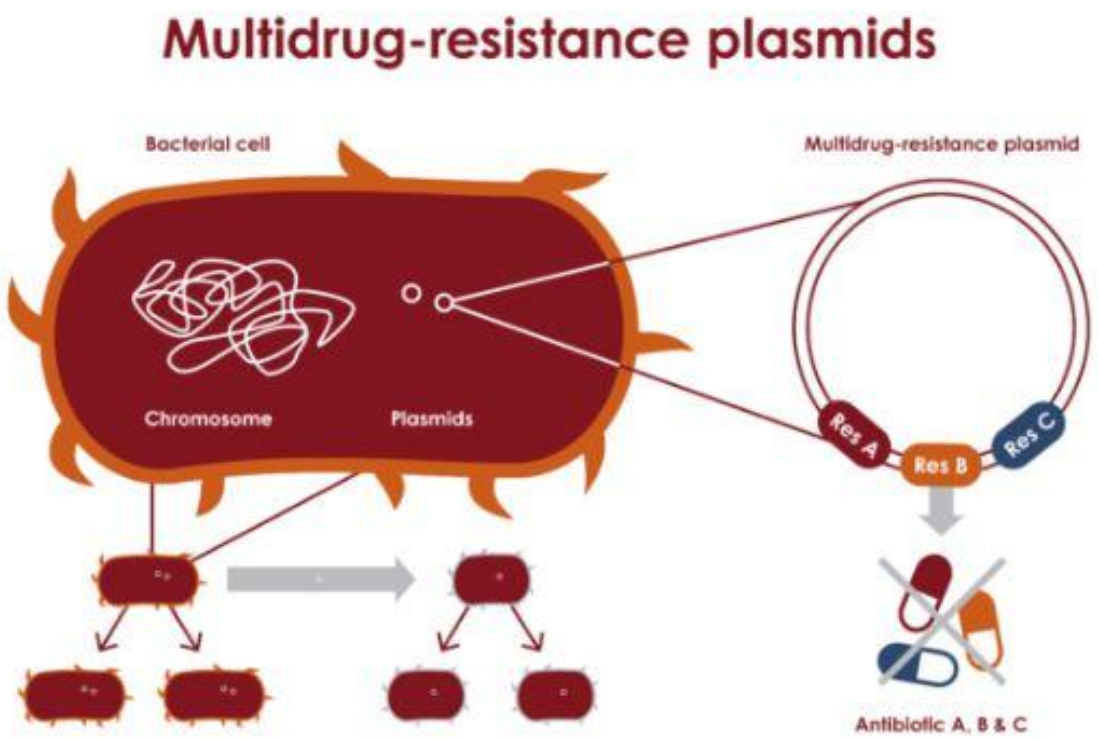
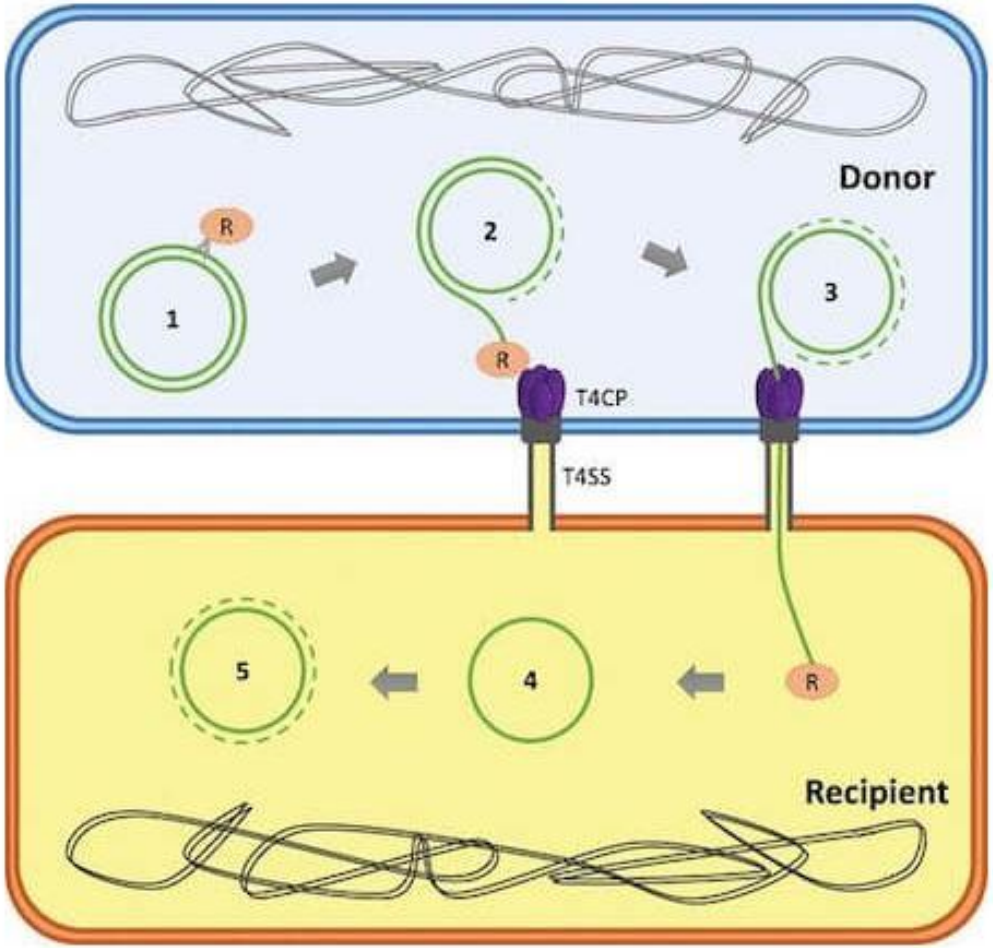


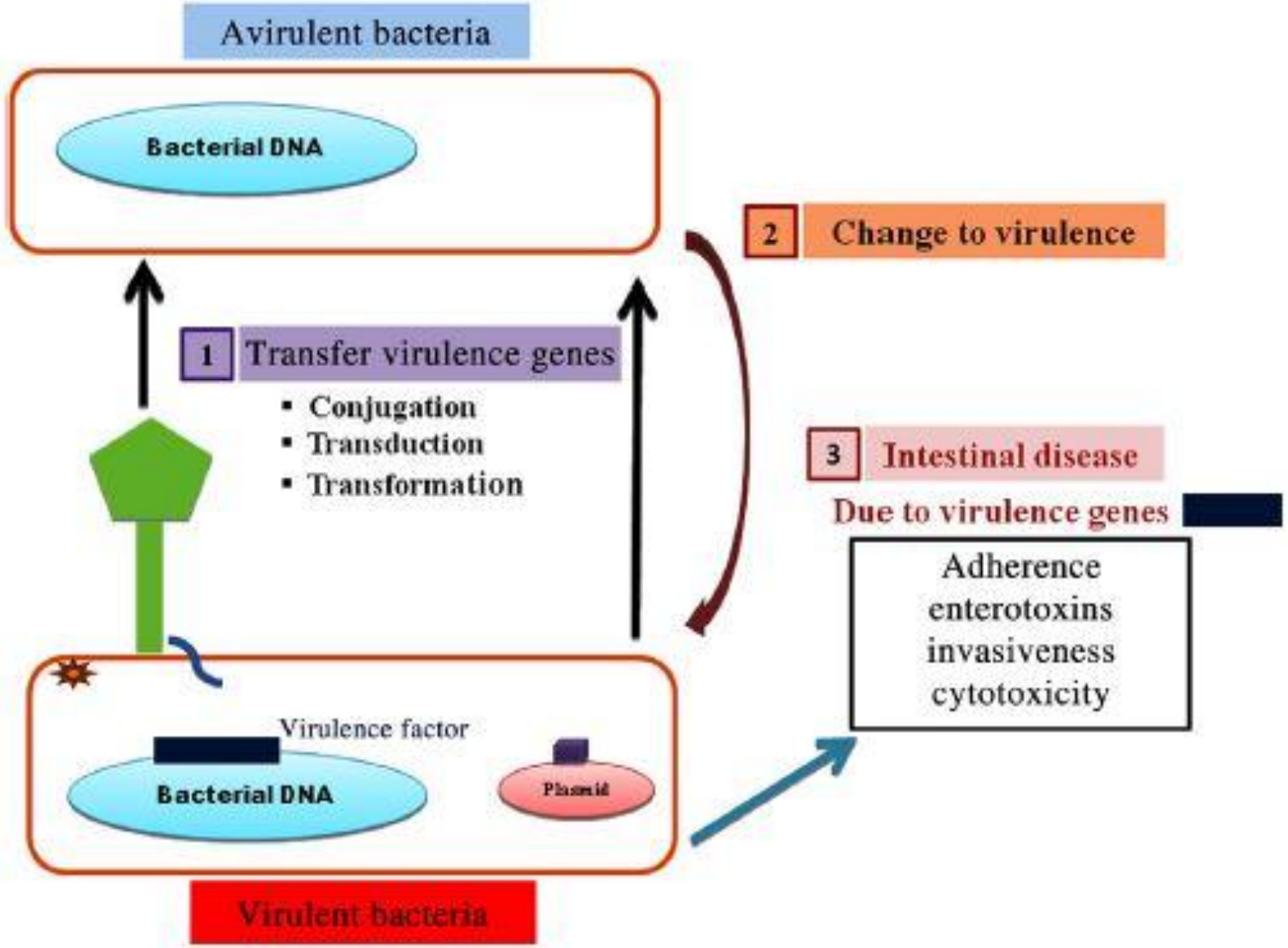
Figure 1. Multidrug-resistance plasmids. Plasmids are small DNA circles outside the bacterial chromosome. Several antibiotic resistance genes can be present on the same plasmid. In this example, they are called res A, res B and res C. Res A gives resistance to antibiotic A, res B to antibiotic B and so on. Adding antibiotic A (or antibiotic B or C) will select for all three resistance genes since they are on the same plasmid. Plasmids can in some cases be transferred to other types of bacteria, bringing the resistance along.



Transfer of a plasmid (green loop) between two bacterial cells through the process of conjugation.

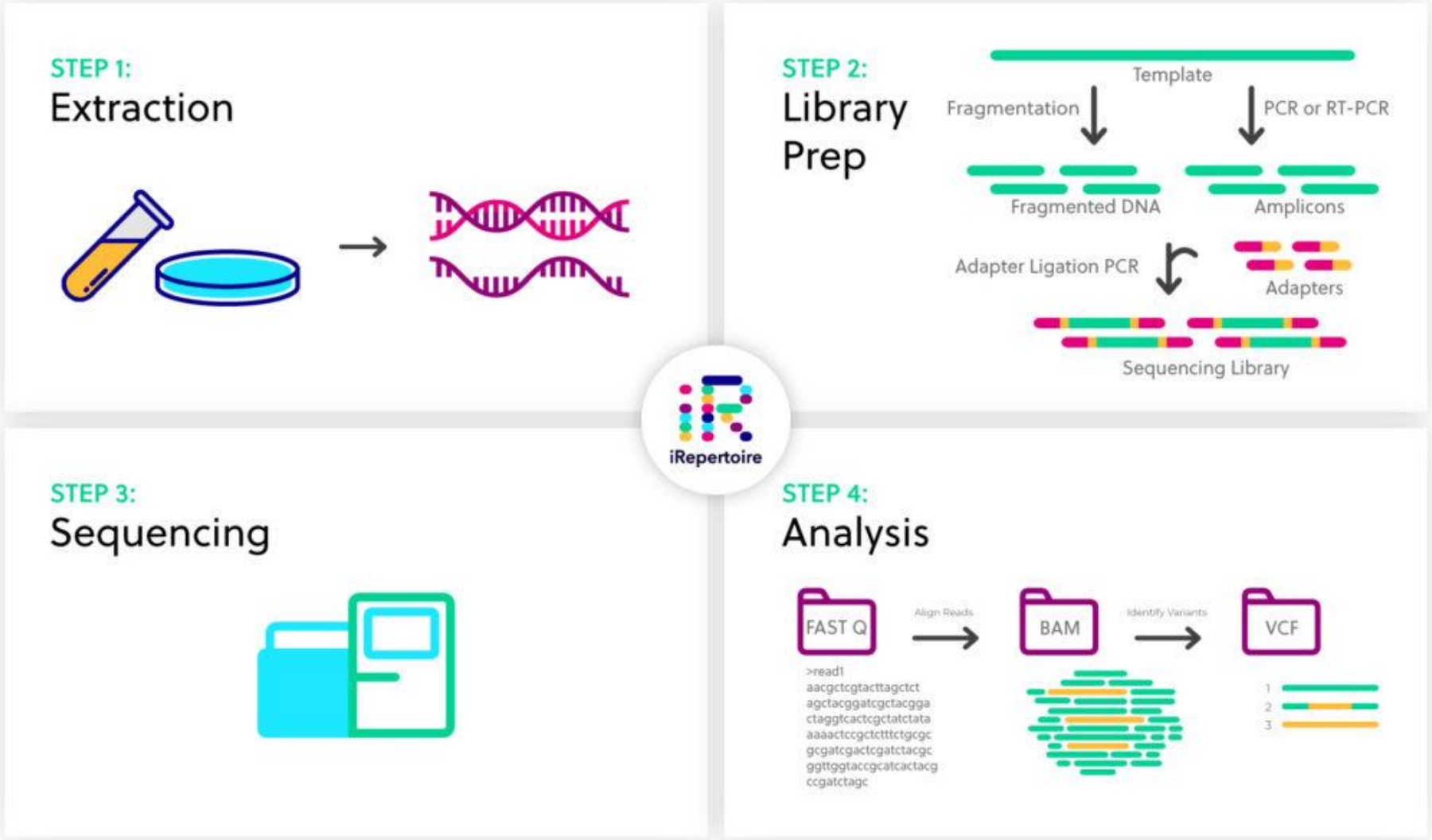
Source: Getting et al./Microbiology Spectrum, Jan. 2018

Virulence Gene

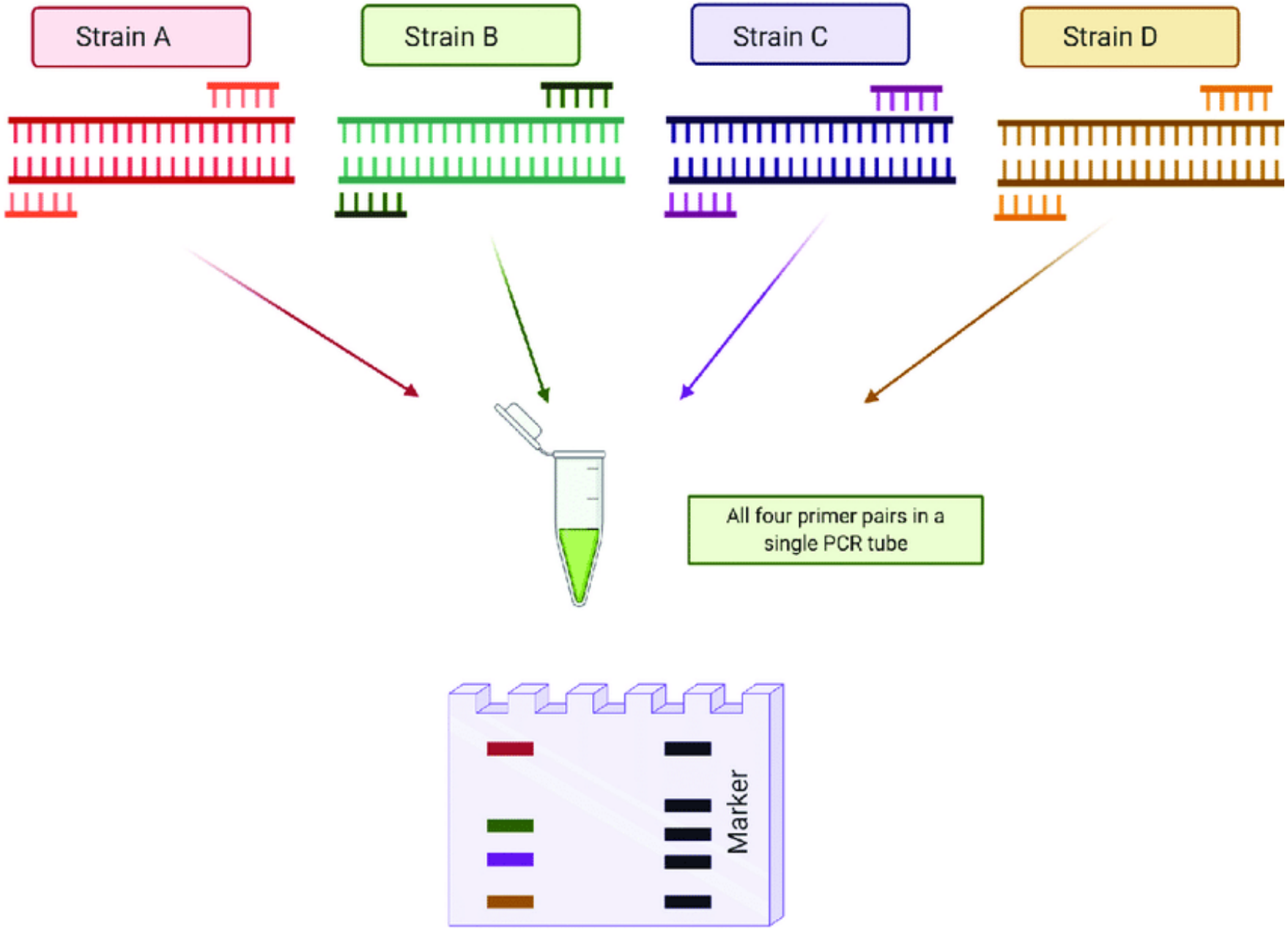


A general mechanism for acquiring virulence in a bacterial system.

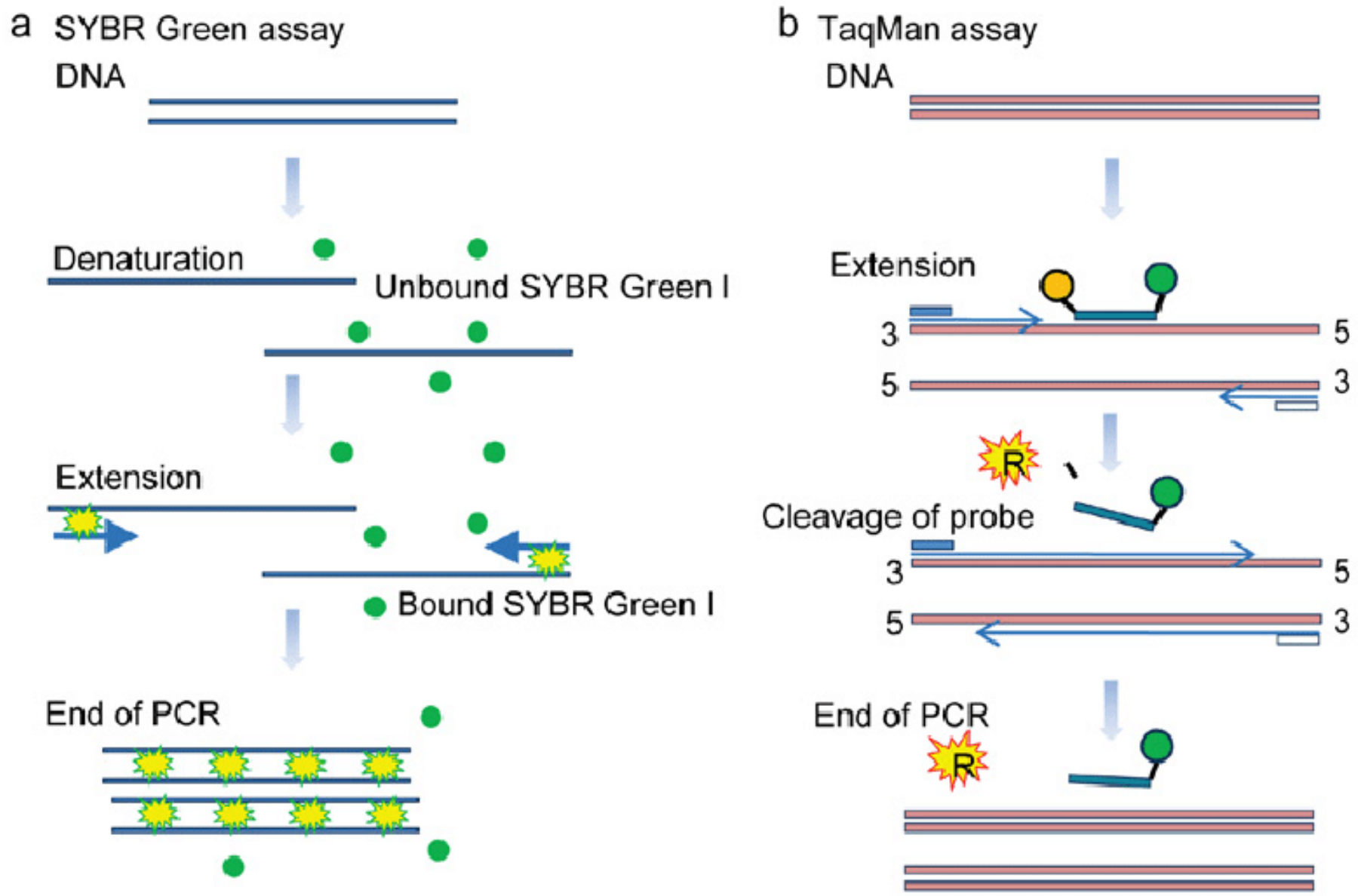
NGS (Next Generation Sequencing) For Bacterial Diagnosis



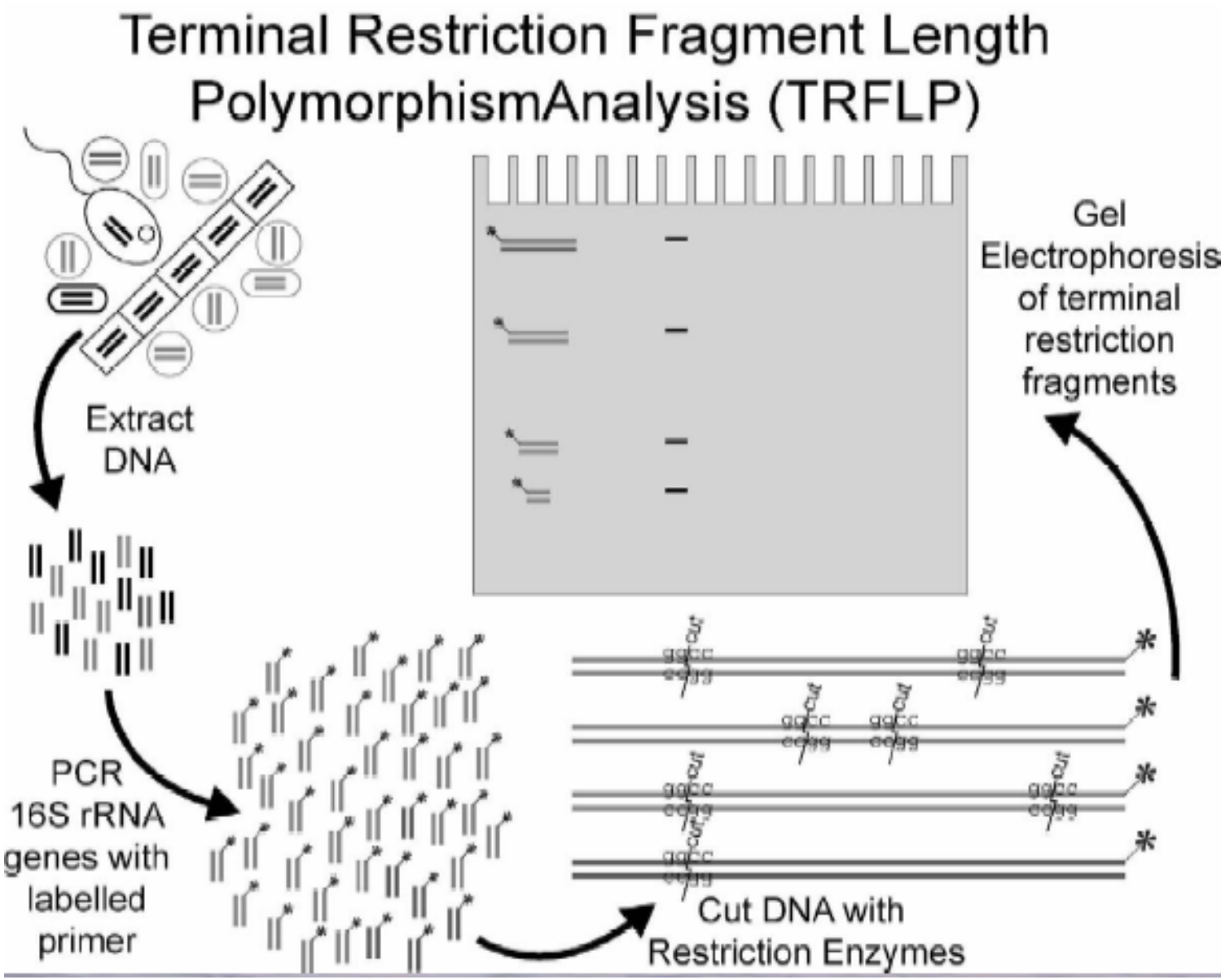
Multiplex PCR



Real-Time PCR



Genotyping


Terminal Restriction Fragment Length PolymorphismAnalysis (TRFLP)
Extract DNA
PCR 16S rRNA genes with labelled primer
Cut DNA with Restriction Enzymes
Gel Electrophoresis of terminal restriction fragments

Advantages And Disadvantages Of Molecular- Based Methods

Advantages	Disadvantages
Assesses DNA sequences (target-specific with PCR)	Risk of contamination
Potential to recover majority of microbes	Relies on established sequence identities
Modest skill level required	Relies on well-designed primer set
Relatively quick and straight-forward processing	DNA recovery influenced by cell lysis method
Can freeze samples for later processing	Unable to distinguish live from dead cells
High specificity	

Culture-based Method

2

Sampling for Bacteriological Examination:

- General Considerations:

✓ Clean sterilized glass bottle with glass stopper

✓ Capacity: 200-250 ml

✓ If sampled water is clorinated- add small quantity of **SODIUM THIOSULPHATE.**

✓ After collection immediately send to PHL.

Bacteriological Indicators

- Primary Bacterial Indicator:

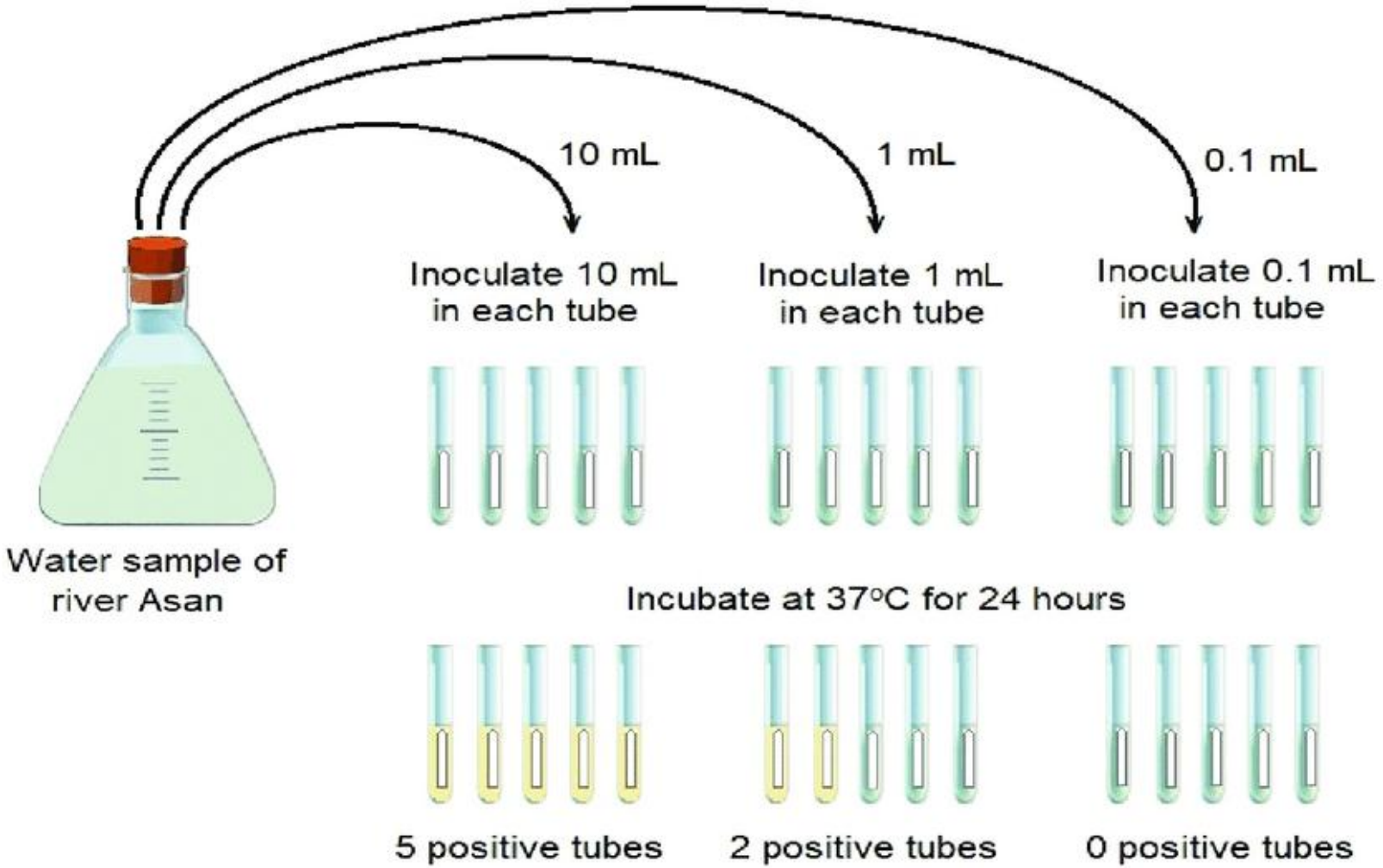
    1. **Coliform** group of organism

- Supplementary Bacterial Indicator:

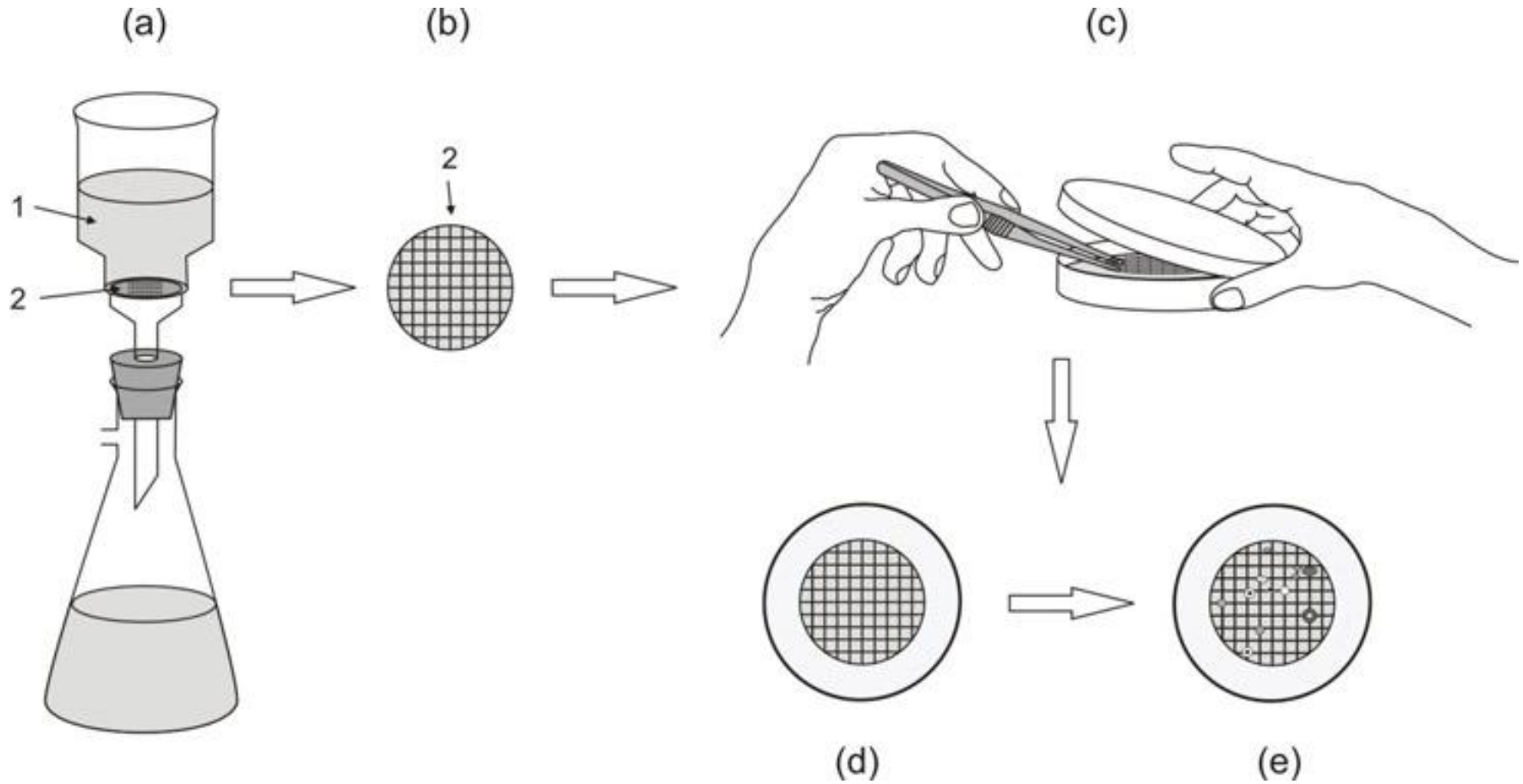
    2. **Faecal streptococci**

    3. **Cl. perfringens**

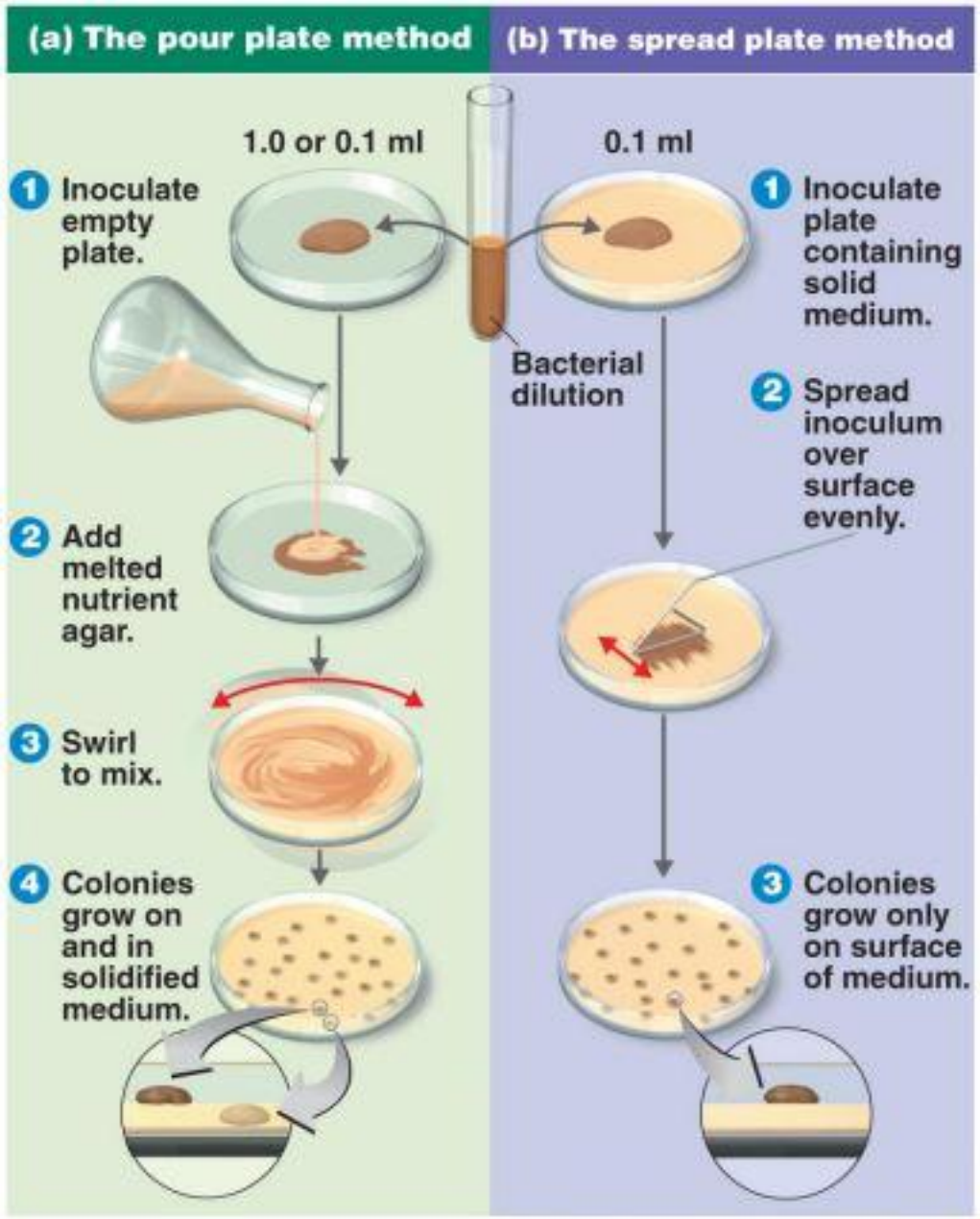
MOST PROBABLE NUMBER (MPN)



Membrane Filter



HPC


(a) The pour plate method
(b) The spread plate method
1.0 or 0.1 ml
0.1 ml
1 Inoculate empty plate.
Bacterial dilution
1 Inoculate plate containing solid medium.
2 Add melted nutrient agar.
2 Spread inoculum over surface evenly.
3 Swirl to mix.
4 Colonies grow on and in solidified medium.
3 Colonies grow only on surface of medium.

Presence/ Absence test

Bromcresol Purple: indicator dye

Advantages And Disadvantages Of Culture-based Methods

Advantages	Disadvantages
Assesses living (culturable) microbes	Risk of contamination
Able to recognize viable cells in a sample	High skill level is necessary for optimal results
Easy to quantitate cells in a sample	Time and resource intensive
High sensitivity with appropriate media	Relies on phenotypic biochemical characterization

Table 1. Techniques for the detection of pathogens and indicators in water environment.

Conventional Techniques		
Technique	**Merit**	**Demerit**
Microscopy	Simple, rapid and direct observation of microbial cells	Majority of bacterial population can not be identified
Culture dependent methods	Easy to identify the individual microbes	Majority of bacteria cannot be cultured on the general purpose-basic media
Microbial indicator based pathogen estimation	Easy to perform, current standard for coliform has been established	Labor intensive, time consuming and indirect estimation of pathogens rather than direct detection
Molecular Techniques		
Technique	**Merit**	**Demerit**
Ribotyping	Highly reproducible; classify isolates from multiple sources	Complex, expensive; labour intensive; database required; variation in methodology
Amplified ribosomal DNA restriction analysis (ARDRA)	Culture-independent technique for analysis of a variety of microbes	Not quantitative and require DNA extraction and PCR biases
Ribosomal RNA intergenic spacer analysis (RISA)	Culture-independent technique for analysis of a variety of microbes and give remarkable heterogeneity in length and sequence among bacteria	Not quantitative and require DNA extraction and PCR biases

Pulse-field gel electrophoresis (PFGE)	Extremely reproducible and highly sensitive to point genetic difference	Long assay time, too sensitive for broadly discriminate source, limited simultaneous processing and require database
Denaturing-gradient gel electrophoresis (DGGE)	Culture-independent technique for analysis of a variety of microbes and use rRNA gene sequence heterogeneity	DNA extraction and PCR biases
Terminal restriction fragment length polymorphism analysis (T-RFLP)	Fast, semi-quantitative, culture-independent technique for analysis of a variety of microbes	DNA extraction and PCR biases
Fluorescent In Situ Hybridization (FISH)	Quantitative and directly visualize the microbial cells including non-culturables	Inactive cells may not be detected
Quantitive PCR (qPCR)	Culture-independent technique for analysis of a variety of microbes	Expensive equipment; technically demanding
Repetitive DNA Sequences (Rep-PCR)	Simple and rapid	Reproducibility a concern; require cell culture and database required; variability increases as database increases
Length heterogeneity PCR (LH-PCR)	Culture-independent technique	Expensive equipment; technically demanding
Multiplex PCR (mPCR)	Fast and simultaneous detection of several target microorganisms	Combination of primer pairs must function in a single PCR reaction
	High throughput design with	Low sensitivity and

Nucleic acid microarrays	wider applications	processing complexities for environmental samples
Host-specific 16S rDNA	Does not require culturing or a database; indicator of recent pollution	Only tested on human and cattle markers; limited simultaneous processing; expensive equipment; technically demanding; little information about survival of *Bacteroides* spp. in environment
On-chip technology	Combination of PCR with nucleic acid hybridization on a single chip and less interference between parallel reactions	Integration and packaging