MULTIPLEX PCR

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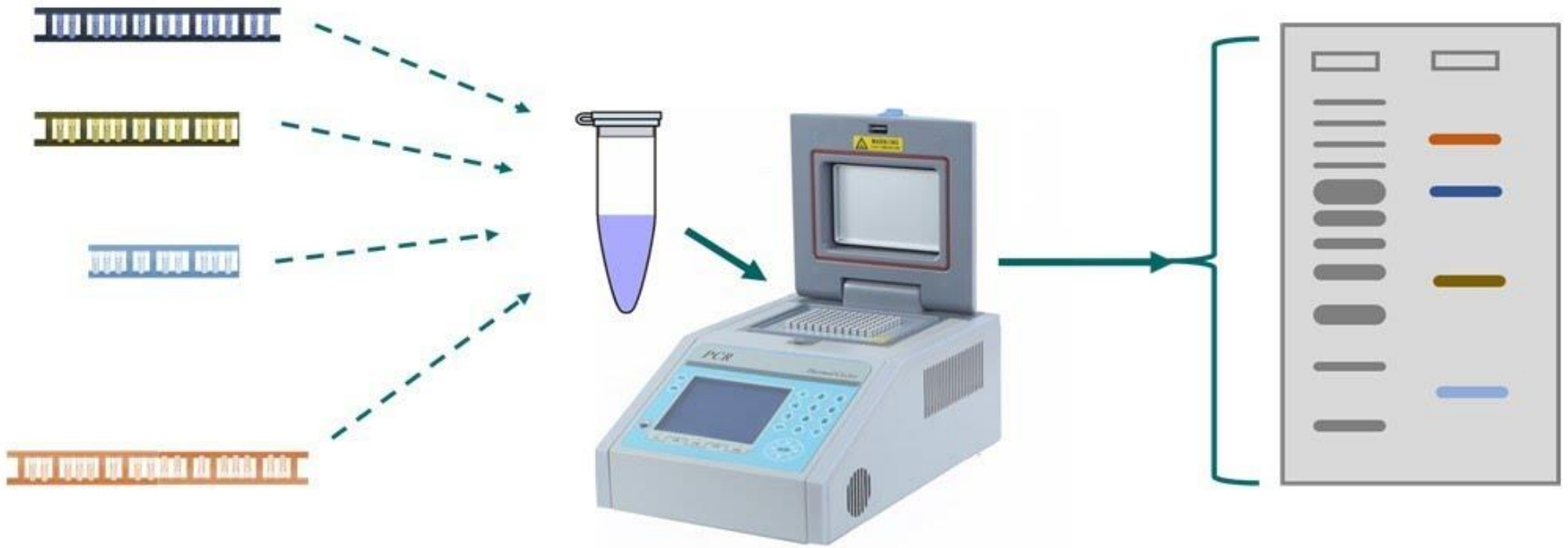
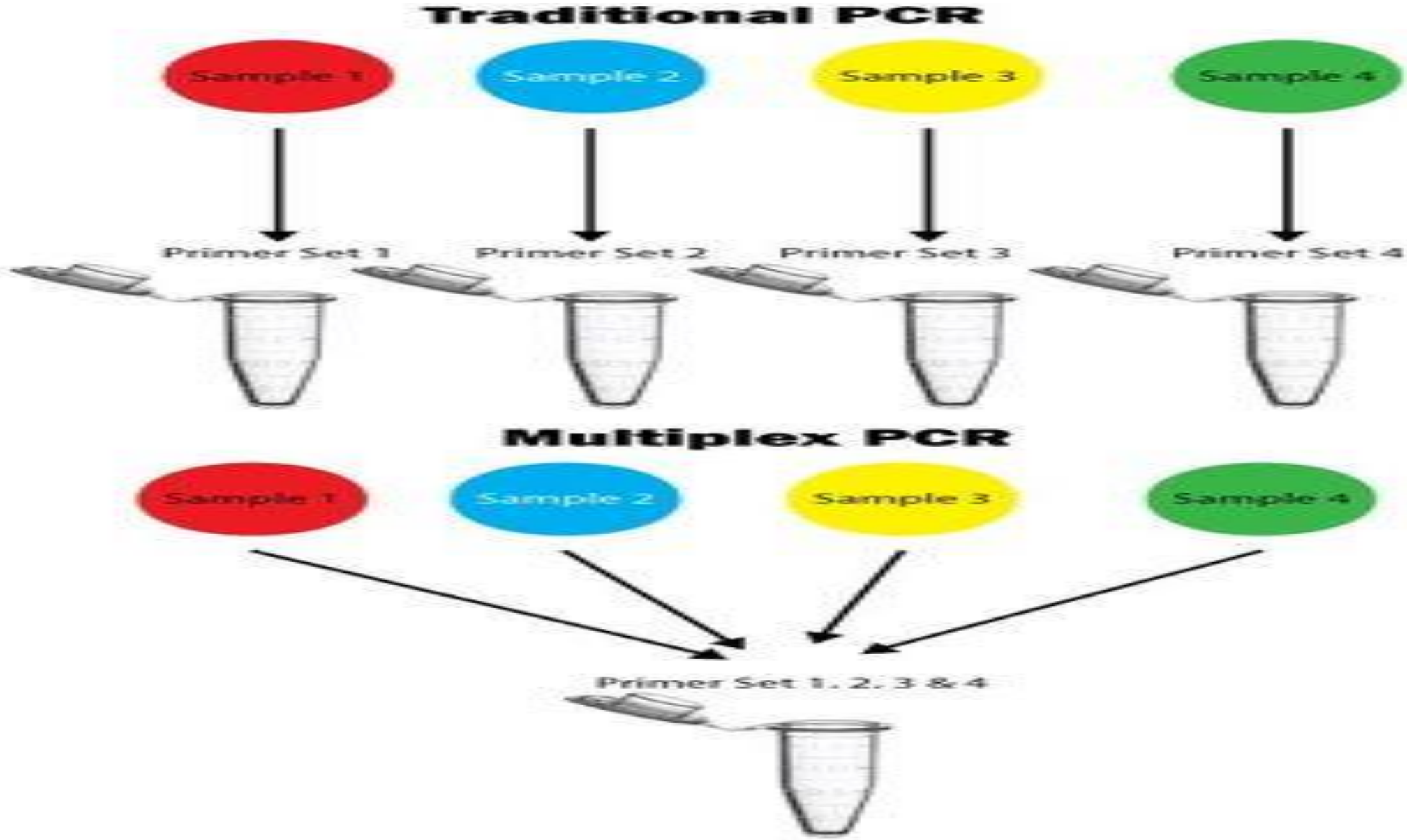
- Multiplex PCR is a variant of PCR methods in which more than one target sequence are amplified using multiple sets of primers within a single PCR mixture.
- This enables amplification of several gene segments at the same time, instead of specific test runs for each.
- This technology was first used by Chamberlain et al. for the diagnosis of Duchenne muscular dystrophy (1988).

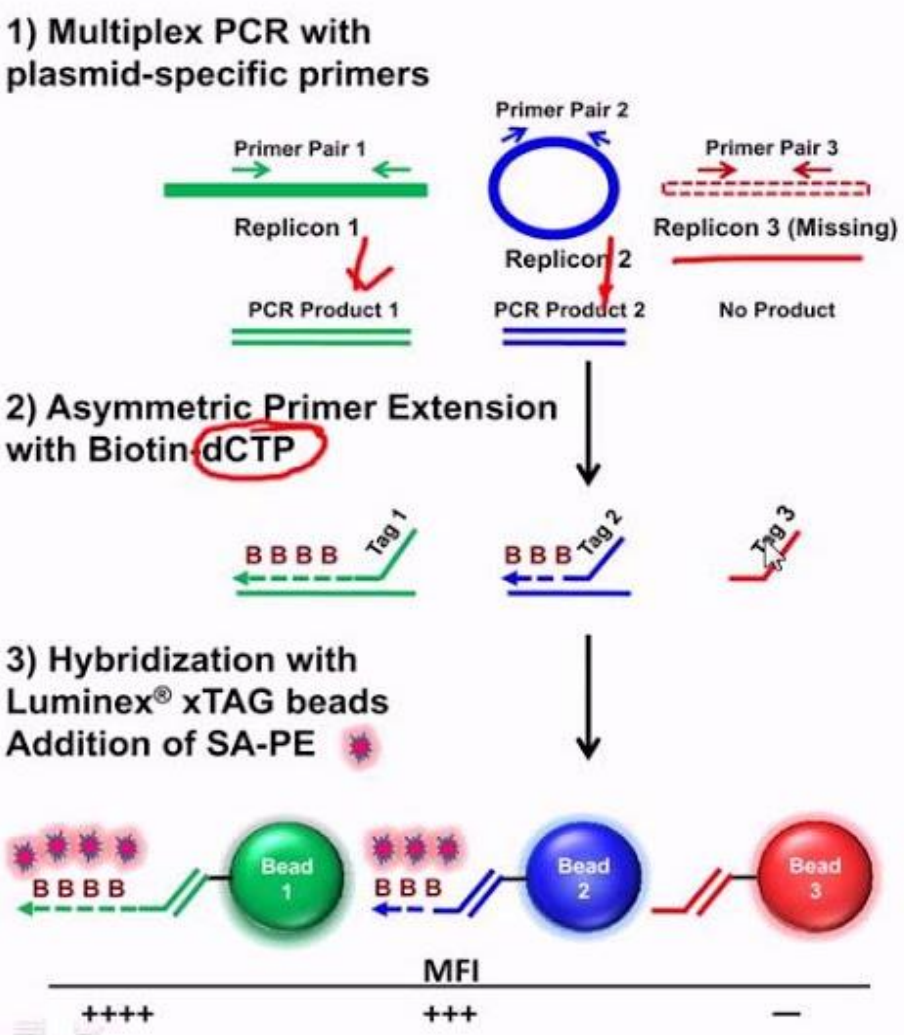
Multiplex PCR

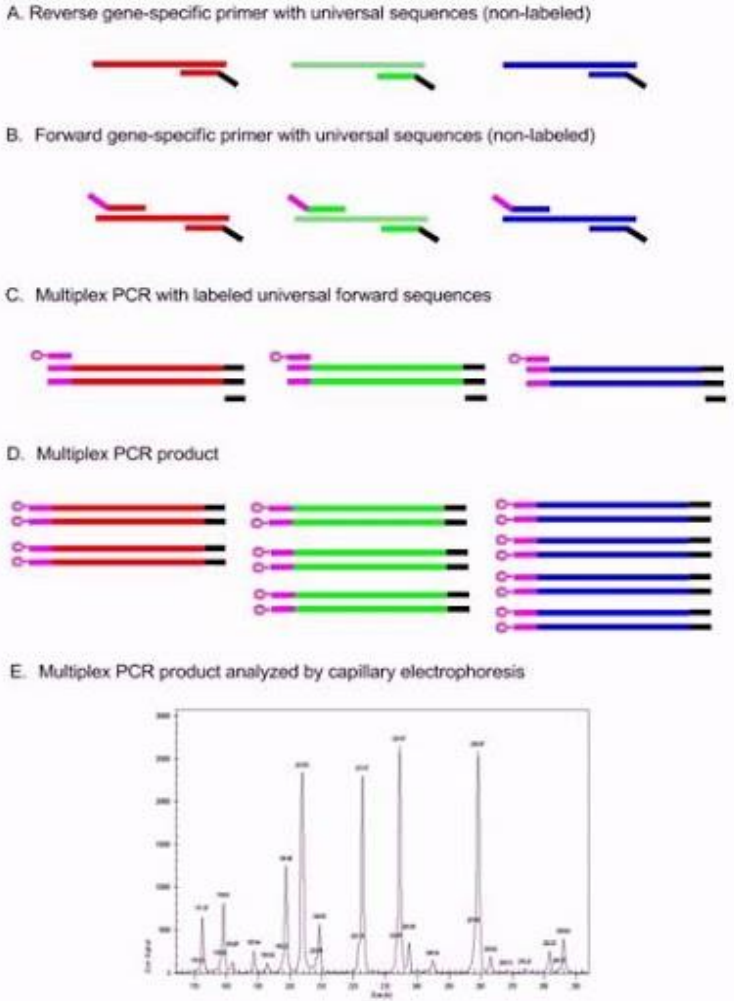


as if performing many separate PCR reactions all together in one reaction


Traditional PCR
Sample 1
Sample 2
Sample 3
Sample 4
Primer Set 1
Primer Set 2
Primer Set 3
Primer Set 4
Multiplex PCR
Sample 1
Sample 2
Sample 3
Sample 4
Primer Set 1, 2, 3 & 4

Multiplex PCR (process overview)





- Multiplex PCR is a space, time, and cost-effective method for genetic analyses that need to be repeated many times (e.g. sequencing).

- It requires a small amount of DNA (10–200 ng) as the starting **template** and can be performed on specimens with a suboptimal DNA quality.

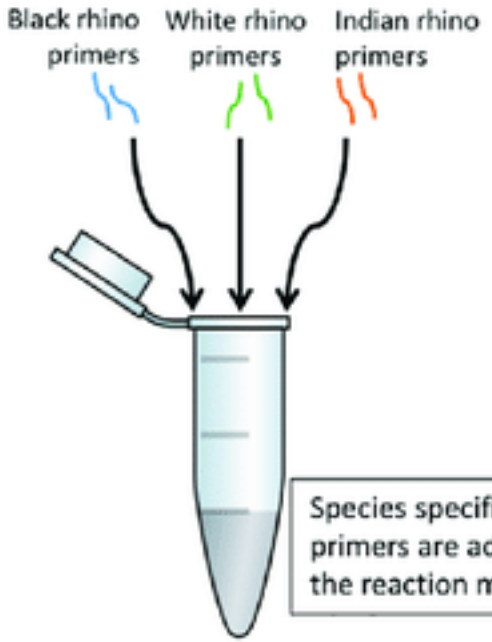
- Though multiplex PCR has many benefits, optimization of it is equally challenging. While using multiple primer pairs, primers from one pair can interact with primers from another one. As each primer pair could have different requirements, there is not a single optimum melting temperature (Tm) and ΔG.

Primer Designing

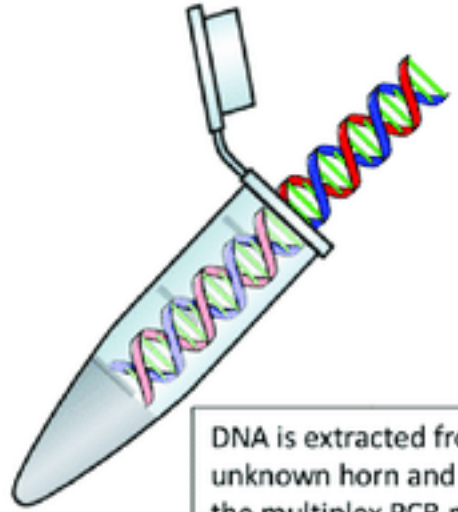
- When designing amplification primers for multiplex PCR, several factors must be considered including;

- **Primers length**: The primer lengths should be within 18–25 nucleotides,
- **Melting temperature (Tm):** Tm of the primers should be either identical or within 1–2°C,
- **GC content**: GC content of the primer should be appropriate (50–55%), and
- **Cross-complementarity**: To avoid interference, primers should lack cross-complementarity.

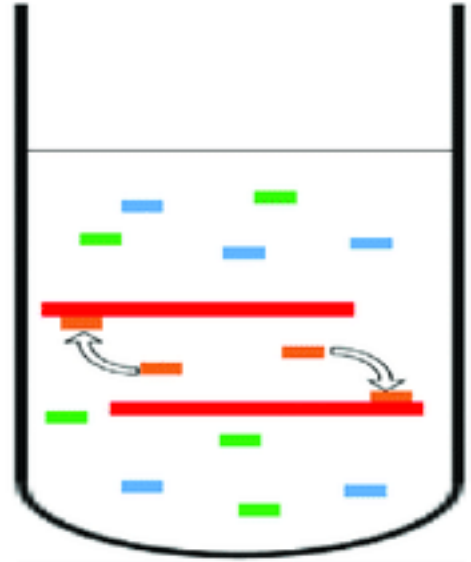
In addition, regions with repetitive sequences, known as germline single nucleotide polymorphisms (SNPs), and regions with high homology should be avoided because they may affect the efficiency of PCR amplification and create amplification bias.



Species specific primers are added to the reaction mixture.



DNA is extracted from the unknown horn and added to the multiplex PCR mixture.



One set of species specific primers will anneal to the DNA

Amplicon visualized on gel

A 2% agarose gel is used to visualize the length of the resultant amplicon.

A species ID can be conducted based on the length of the amplicon:
- Black rhino amplicon → 222 bp
- White rhino amplicon → 266 bp
- Indian rhino amplicon → 310 bp

Advantages of Multiplex PCR

- Multiplex PCR offers a couple of notable advantages such as:

1. Internal amplification controls ensure the accuracy of the negative PCR results

- **First,** strategies that include internal controls for PCR. For example, one primer pair can be directed at sequences present in all clinically relevant bacteria (i.e., the control or universal primers) and the second primer pair can be directed at a sequence-specific for the particular gene of interest (i.e., the test primers).

- **The control amplicon** should always be detectable after PCR. Absence of the control would indicate that PCR conditions were not met and the test would require repeating.

- When the control amplicon is detected, the absence of the test amplicon can be more confidently interpreted to indicate the absence of target nucleic acid in the specimen rather than a failure of the PCR system.

2. Numerous pathogens may be detected in a single reaction, even if these pathogens are from taxonomically different groups.

- Another advantage of multiplex PCR is the ability to search for different targets using one reaction. Primer pairs directed at sequences specific for different organisms or genes can be put together so that the use of multiple reaction vessels can be minimized. For example, detection of viral agents that cause meningitis or encephalitis (e.g., herpes simplex virus, enterovirus, West Nile virus) using multiplexed PCR assay.

Applications of Multiplex PCR

Multiplex PCR has many applications:

- It has been successfully applied in many areas such as genotyping, mutation and polymorphism analysis, detection of pathogens or genetically modified organisms, etc.

- In diagnostic laboratories, multiplex PCR is useful to detect different microorganisms that cause the same types of diseases. For example:

- Detection of *S. pneumoniae*, *H. influenzae*, and *N. meningitidis* (the most common causes of bacterial meningitis) in CSF sample.
- Detection of the viral agents of meningitis and meningoencephalitis.
- Detection and differentiation of polyomaviruses that infect humans.
- Detection of bacteria that cause middle ear infection, pneumonia, etc.

Commercial Applications

- ***BioFire FilmArray***

- The **BioFire FilmArray** technology uses a combination of nested, multiplex, and individual PCR reactions to detect a variety of pathogens.

- It uses a plastic pouch with automated capabilities, including sample preparation, reverse transcription for RNA viruses, and a two-stage nested multiplex PCR process thus simplifying molecular testing with a completely automated protocol.

- The System is used to identify dozens of viruses and bacteria, including emerging infectious diseases.

Commercial Applications

- ***Sensor technology***

- The **Sensor technology** utilizes multiplex PCR and/or RT-PCR to amplify a variety of nucleic acid targets.

- **Limitations of Multiplex PCR**

- Mixing different primers can cause some interference in the amplification process, especially as the number of different primer pairs used increases.

- Sequencing of large consecutive genomic regions by multiplex PCR can create a cross-reaction between primer pairs due to primer overlap.

- he Gibbs free energy change (ΔG) is a measure of the spontaneity of formation of the most stable duplex between the last 5 bases from the 3’ end and its exact complement in the template. **ΔG is the energy required to break the secondary structure,** and larger negative values indicate a higher propensity for false priming as the 3’ end can initiate polymerization even if the remainder of the primer does not bind well. Primers with a ΔG more negative than the specified value are not considered. Primers with a ΔG between -4 and the specified value are rated lower.