Nested PCR

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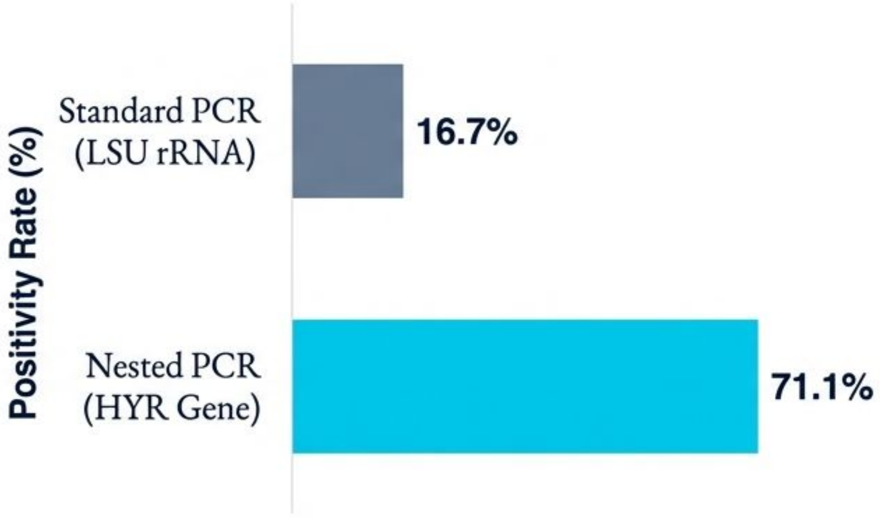
The Detection Threshold Challenge

Standard PCR is a powerful tool, but it faces significant limitations when dealing with diagnostic 'edge cases':

1. **Low-Copy Templates:** Target DNA is too scarce to reach the detection threshold.
2. **High Background Noise:** In complex samples (feces, soil, tissue), non-specific binding drowns out the signal.
3. **False Negatives:** Asymptomatic or sub-microscopic infections are missed.

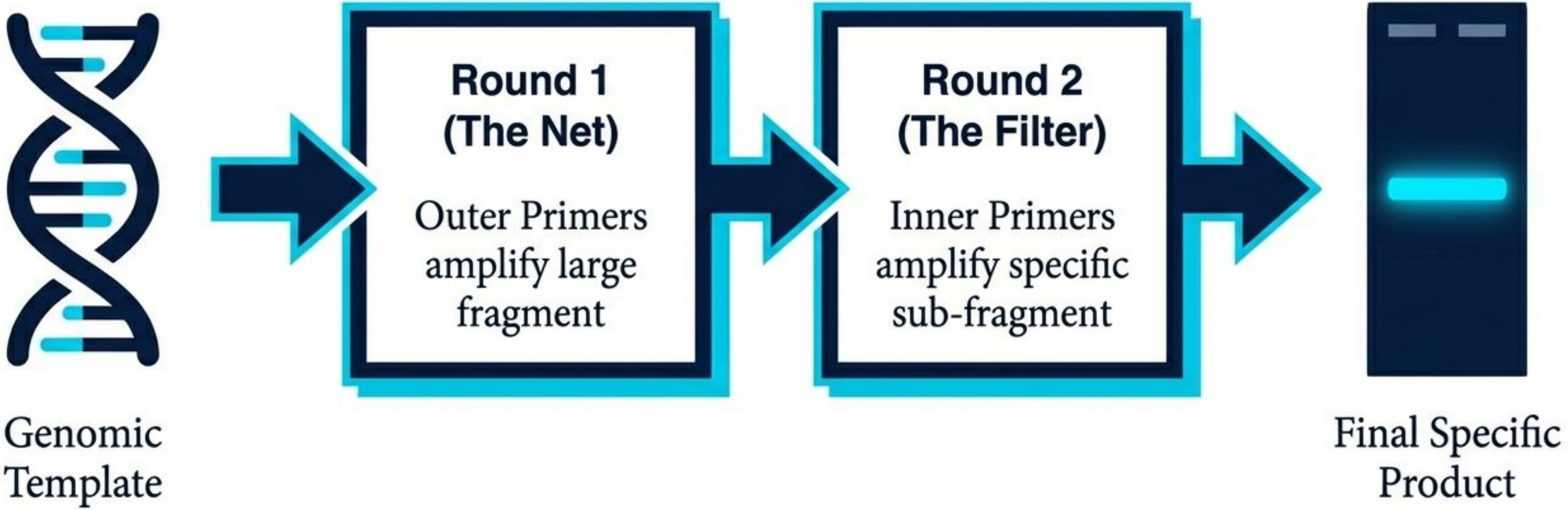
Detection of *Metschnikowia bicuspidata* in Crabs



Standard methods missed >80% of infections.

Defining Nested PCR

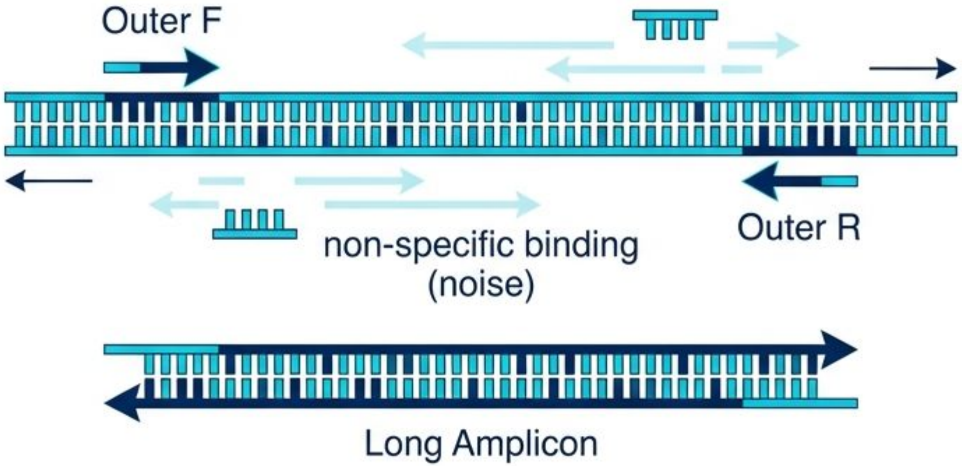
A modified Polymerase Chain Reaction technique utilizing two sets of primers (Outer and Inner) in two successive reaction rounds to amplify a single specific target.



Mechanism Round 1: The Outer Run

Process:
– 'Outer' Forward and Reverse primers bind to flanking regions.
– Runs for 25–30 cycles.

Result:
– Generates a large quantity of the primary target fragment.
– *Risk*: Product may include non-specific amplicons (noise) due to binding at unexpected locations.



Mechanism Round 2: The Inner Run

Process:

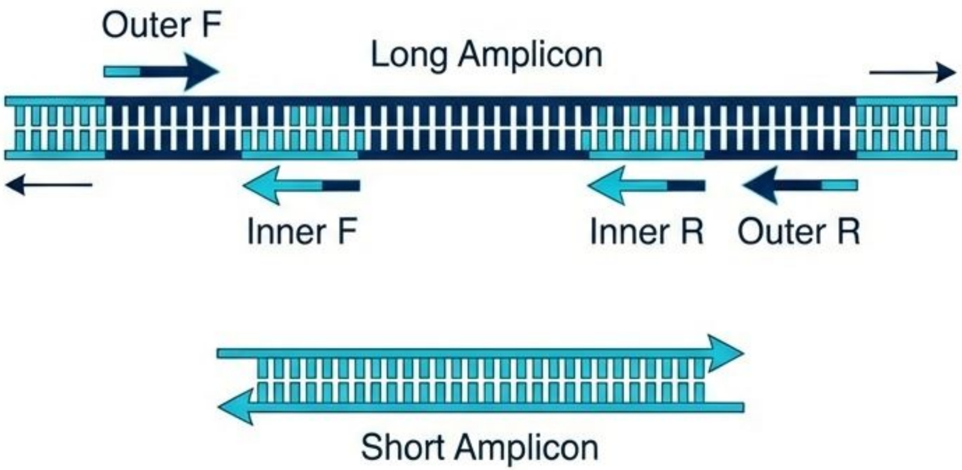
- Product of Round 1 serves as the template.
- 'Inner' primers bind to sequences *internal* to the first fragment.

Selectivity:

- Non-specific products from Round 1 lack the Inner binding sites and are ignored.

Outcome:

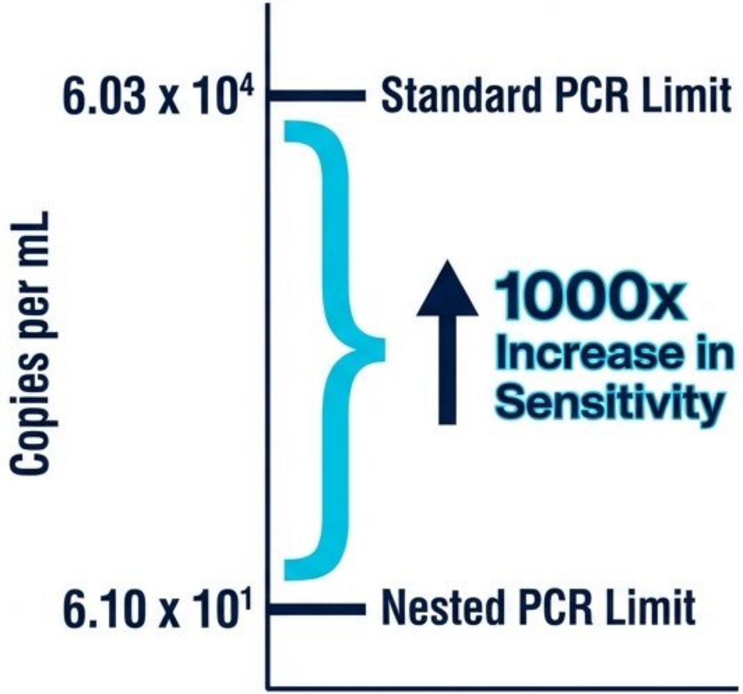
- Exponential amplification of *only* the specific target sequence.



The Sensitivity Advantage: A Mathematical Reality

Why go through the trouble?

The probability of non-specific binding occurring in *both* the outer and inner regions simultaneously is astronomically low.



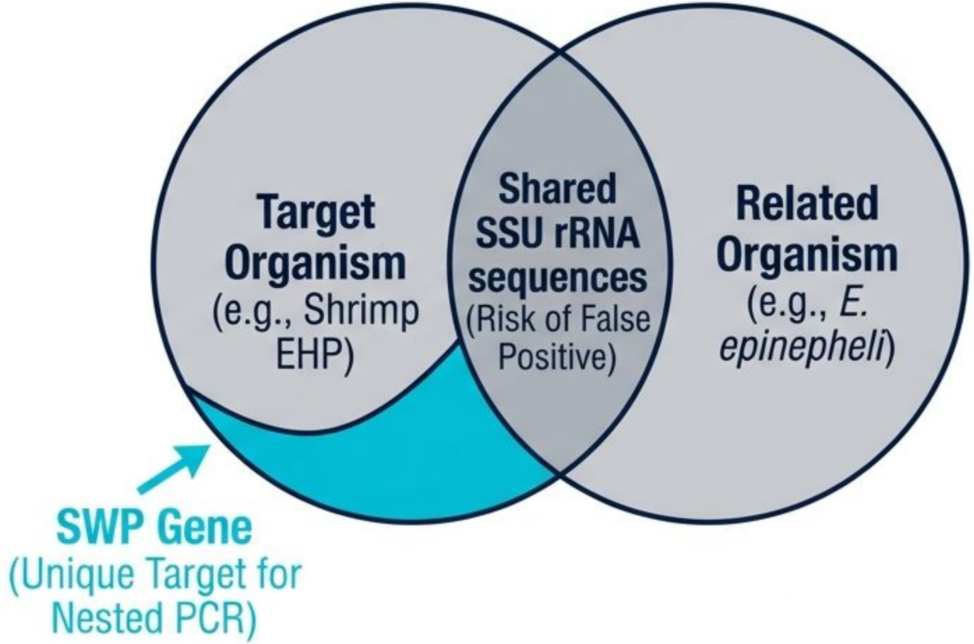
Data: Bao et al., Frontiers in Cellular and Infection Microbiology, 2022

Module 2: Pros, Cons & Optimization

Specificity and The Avoidance of Cross-Reaction

Challenge: Related organisms often share ribosomal DNA (rDNA), leading to false positives in standard PCR.

Solution: Nested PCR targets unique genes (e.g., SWP) or uses the **dual-primer lock** to eliminate cross-reaction.



The Disadvantages: Cost and Complexity

Time Intensive

Requires two consecutive thermal cycling runs. Total time: 4–5 hours (vs. 2 hours for standard PCR).

Resource Heavy

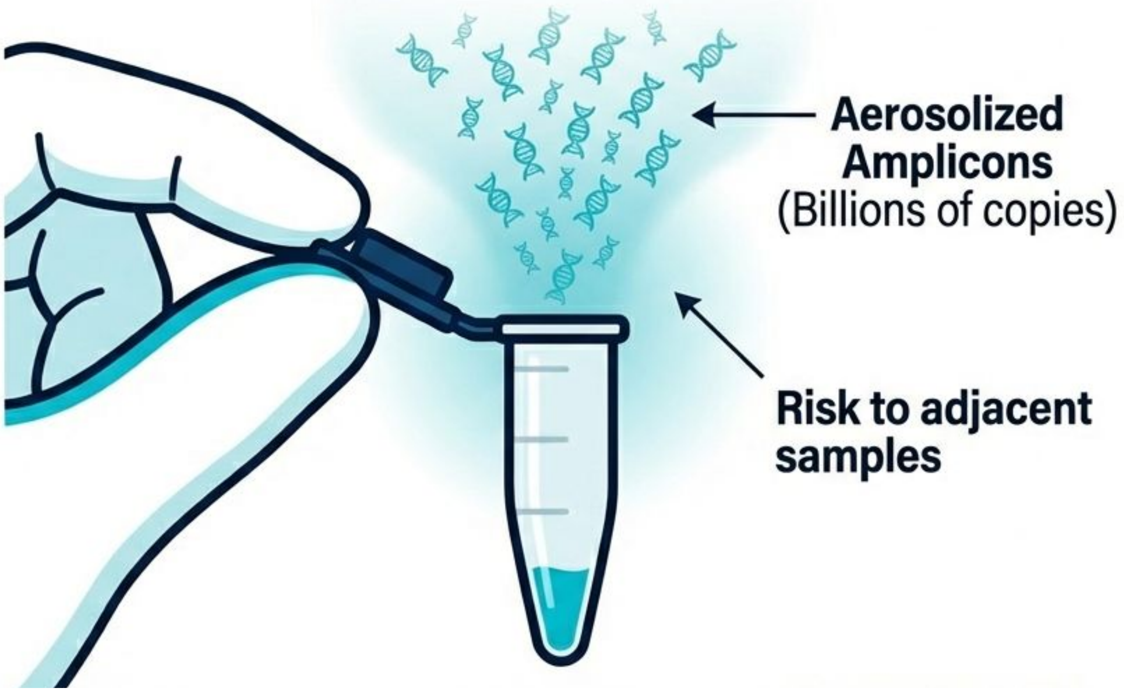
Doubles the consumption of reagents (Taq polymerase, dNTPs) and requires synthesis of two distinct primer sets.

Operational Risk

Not a 'quick fix' for high-throughput screening. Requires experienced handling to avoid error.

The Major Risk: Contamination

The 'Open Tube' Problem is the Achilles' heel of Nested PCR.

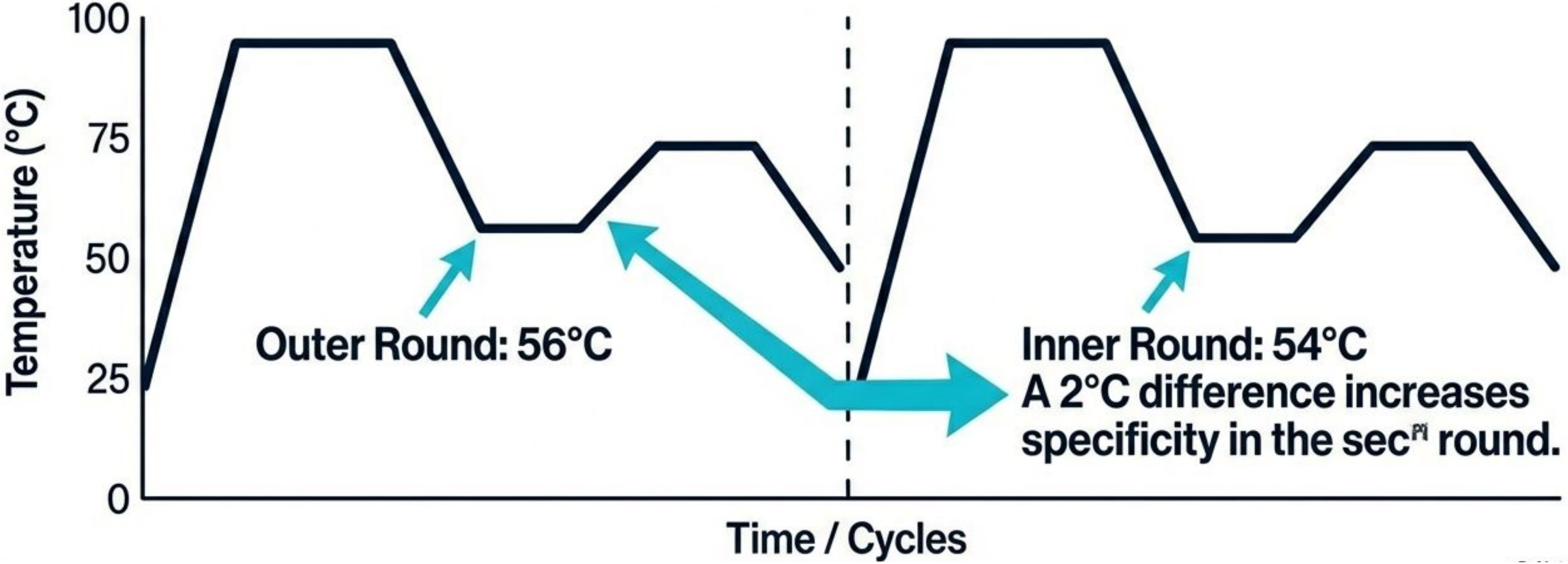


Between Round 1 and Round 2, tubes must be opened to transfer template.

Even microscopic droplets can contaminate reagents, leading to false positives in future runs.

Optimization Strategy 1: Thermodynamics

Optimizing annealing temperatures is critical to prevent dimer formation between the two rounds.



Optimization Strategy 2: Chemistry

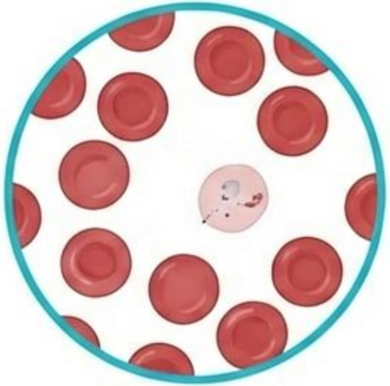
Balancing reagents is as important as thermal cycling.

Variable	Impact	Optimization Goal
MgCl2 Concentration	Cofactor for Taq Polymerase	Empirical testing (e.g., 25 mM stock). Too high = non-specific binding.
Primer Concentration	Reaction Efficiency	Adjusting (e.g., 10 µM) to balance sensitivity against primer-dimer formation.

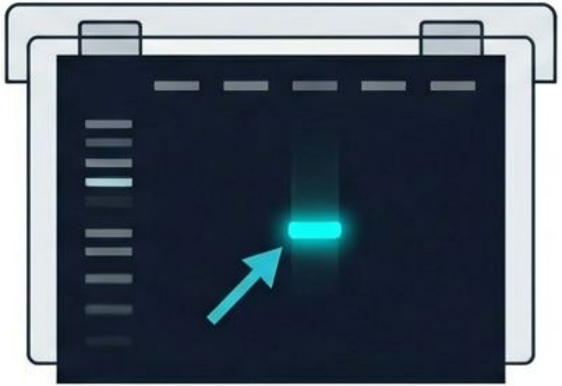
"Distinct protocols (variations in Buffer, dNTPs, and $MgCl_2$) can determine success or failure in detecting specific genotypes."

Module 3: Real-World Applications

Case Study 1: Malaria Diagnosis & Epidemiology



Microscopy: Misses low-level parasitemia (asymptomatic carriers).



Nested PCR: Detects sub-microscopic infection.

The Clinical Need: Differentiating between recrudescence (treatment failure) and reinfection.

Strategy: - **Outer Primers:** Genus-specific (*Plasmodium* 18S rRNA).
- **Inner Primers:** Species-specific (*P. falciparum* vs. *P. vivax*).

Aquaculture: The Shrimp Crisis (*EHP*)

False positives in aquaculture lead to massive economic losses.

Gene 1: SSU rRNA
(Cross-reacts with harmless *Enterospora epinepheli*)

False Positive Target

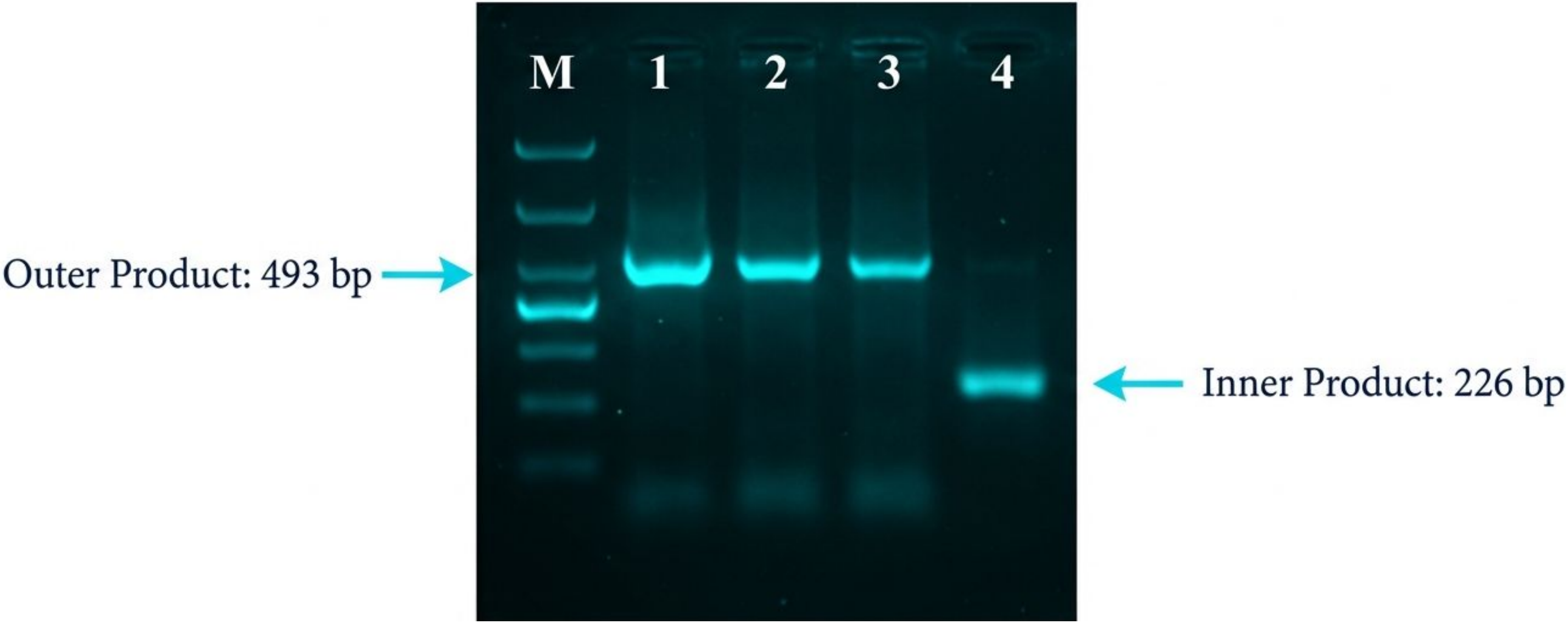
Gene 2: Spore Wall Protein (SWP) Gene

Specific EHP Target

- Pathogen: *Enterocytozoon hepatopenaei* (EHP).
- Outcome: Nested PCR targeting SWP achieves high sensitivity (10^3 copies) with ZERO cross-reactivity.

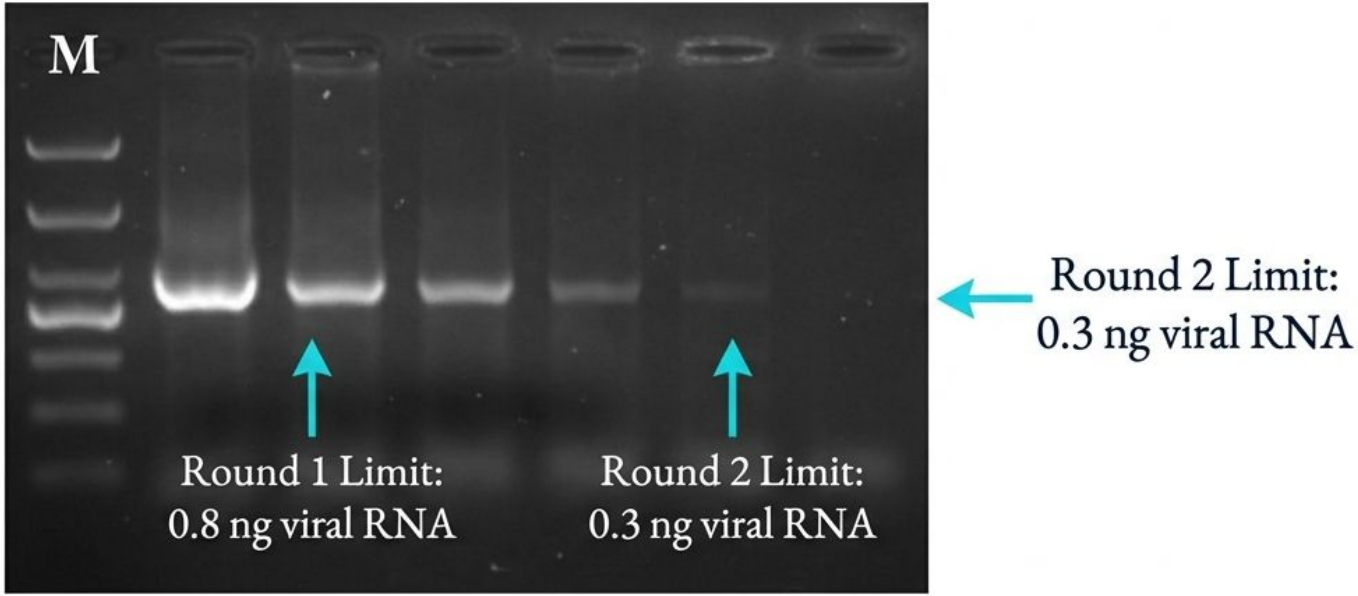
Aquaculture: Tracking *Metschnikowia bicuspidata* in Crabs

Used to discover and block transmission routes in reservoir hosts.



Veterinary Virology: SARS-CoV-2 in Cats

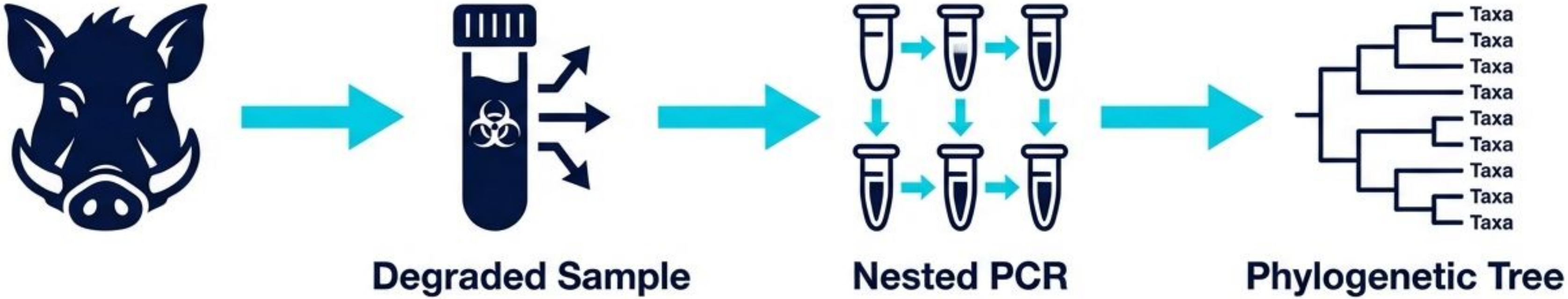
Companion animals often have low viral loads, making standard IVD kits unreliable.



nPCR serves as a reliable confirmation tool for doubtful veterinary cases.

Epidemiology: Hepatitis E in Wild Boars

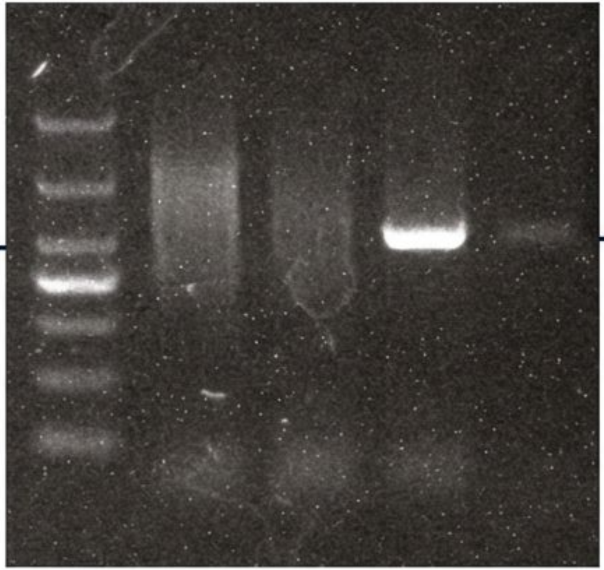
Surveillance of zoonotic diseases often relies on degraded samples (liver, bile, feces) from the wild.



- **Target:** ORF1 and ORF2 regions.
- **Protocol:** Optimized Program B (56°C/54°C annealing).
- **Result:** Enabled tracking of transmission routes to humans via game meat.

Handling Difficult Samples

Inhibitors in feces and tissue can ruin the reaction. The solution is dilution.



Diluting the Round 1 product reduces inhibitors significantly more than it reduces the amplicon count.

Summary of Key Advantages

Extreme Sensitivity

1000x

More sensitive than standard PCR.

Verification

Double-Check Mechanism

Inner primers confirm the authenticity of the outer product.

Sequenceability

Clean Amplicons

Produces specific bands suitable for Sanger sequencing.

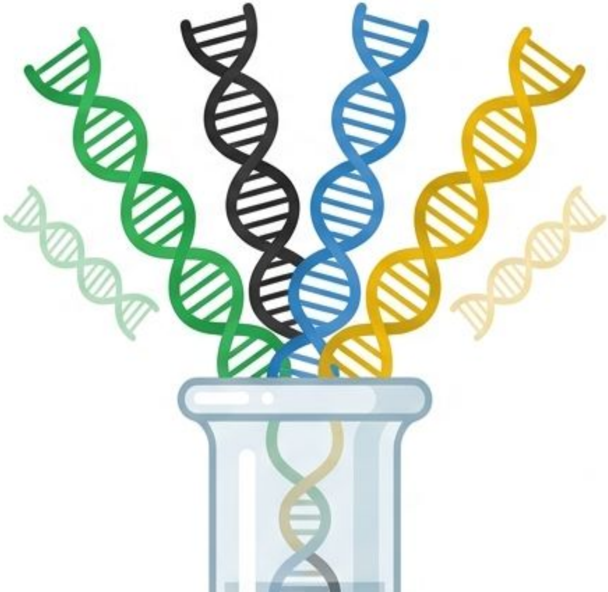
Tolerance

Robustness

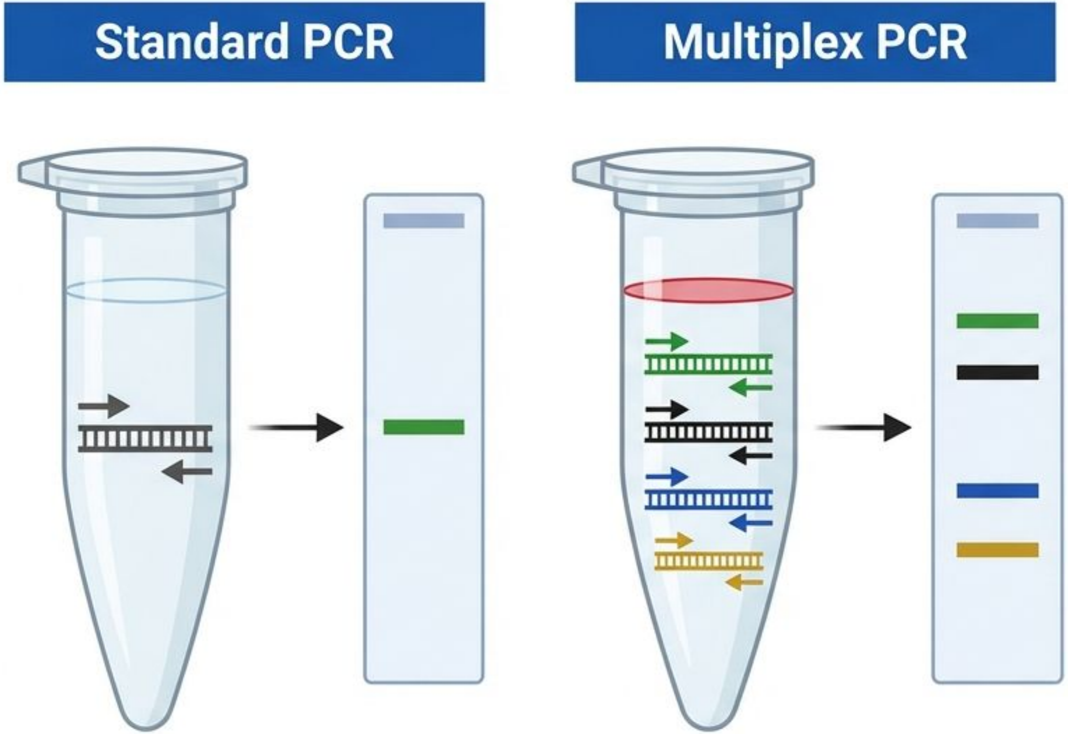
Effective even with degraded DNA or presence of inhibitors.

Multiplex PCR: High-Throughput Detection Principles

Optimization, Design Strategies, and Clinical Applications



Defining Multiplex PCR



- **Core Concept:** Simultaneous amplification of two or more DNA targets (loci) in a single reaction vessel.
- **Mechanism:** Utilizes multiple unique primer pairs to target distinct genomic sequences.
- **Clinical Value:**
  - High-throughput screening (Pathogen panels)
  - Cost and time efficiency
  - Sample conservation (Critical for low-volume samples)

The Challenge: Managing Competitive Amplification

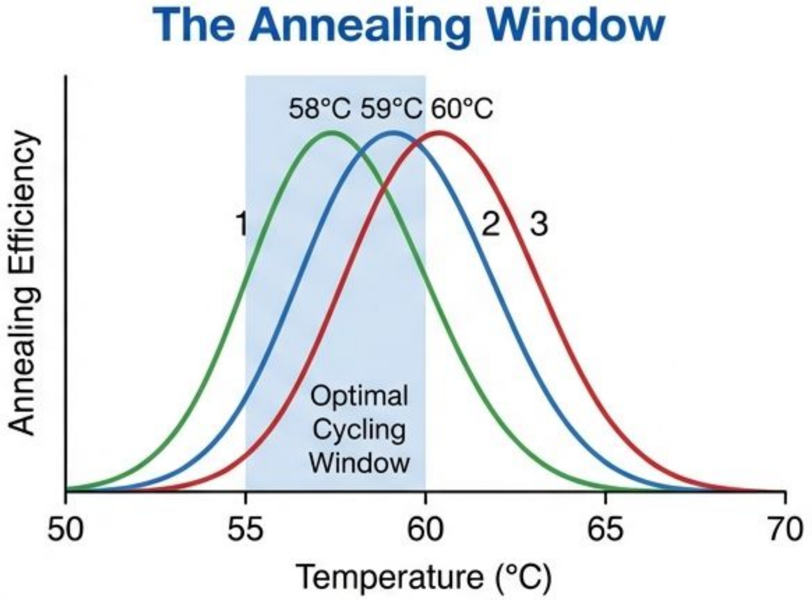
Dynamic Equilibrium.

The Problem: All targets compete for a limited pool of Taq polymerase and dNTPs.

The Risk: "Strong" targets deplete resources, causing "weak" targets to drop out (False Negatives).

Critical Design Rule 1: Tm Compatibility

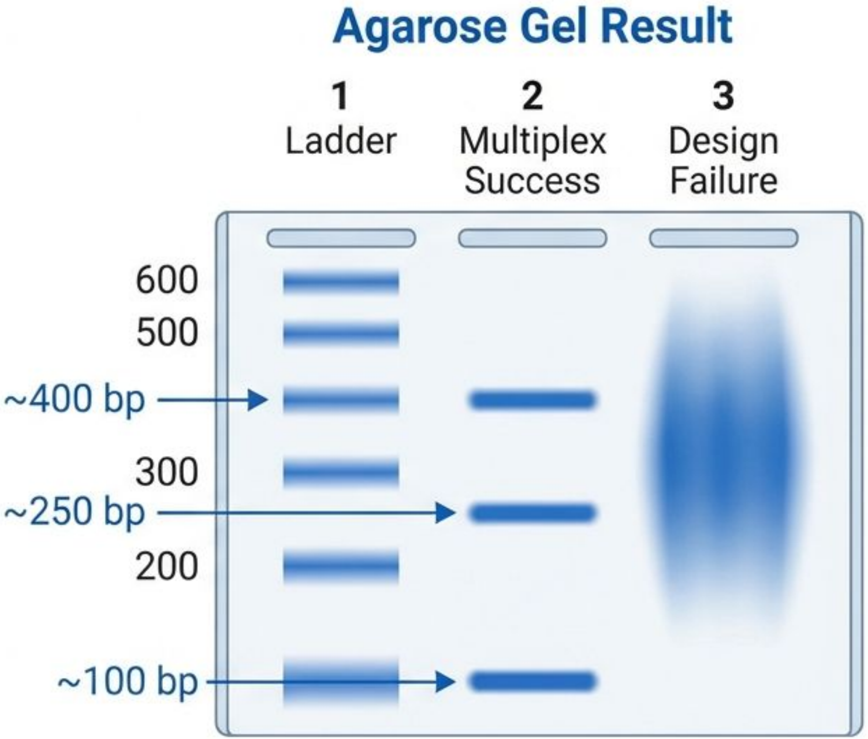
- **The Rule:** All primers must share compatible annealing temperatures.
- **Specification:** Tm variance must be < 3–5°C.
- **Calculation:** Tm ≈ 4(G+C) + 2(A+T).



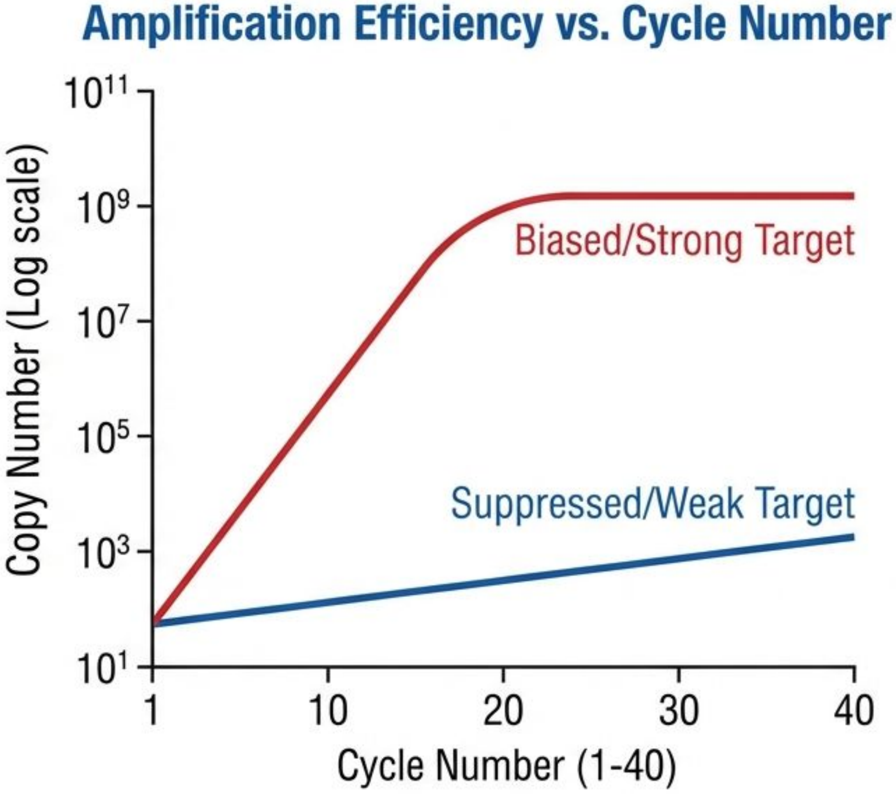
Multiplex reactions often require lowering annealing temp by 4–6°C compared to singleplex.

Critical Design Rule 2: Distinct Amplicon Sizes

- **Requirement:** Each target must produce a PCR product of a distinct size for gel separation.
- **Separation Strategy:**
  - Target A: ~100 bp
  - Target B: ~250 bp
  - Target C: ~400 bp
- **Resolution:** Sizes must differ by >30–50 bp.



The Phenomenon of “PCR Bias” (Helvetica Now Display)

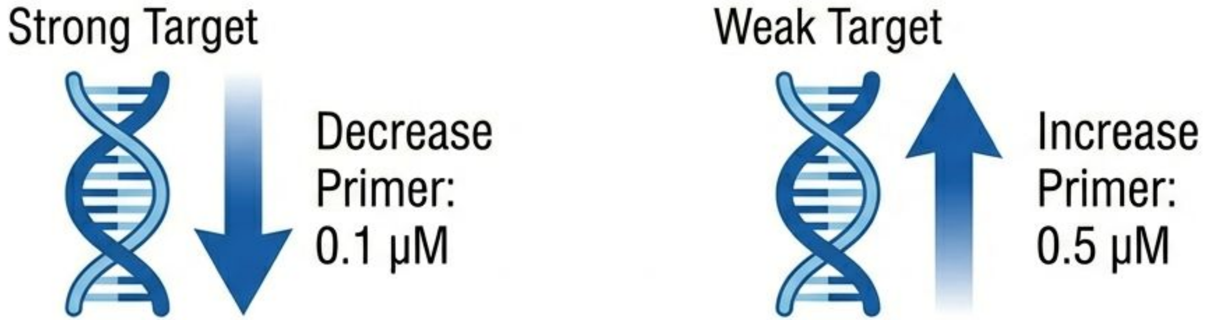


1. PCR Selection (Deterministic):
   - Short amplicons amplify faster.
   - High copy number templates dominate.
2. PCR Drift (Stochastic):
   - Random fluctuations in early cycles.
   - Critical impact on low-concentration samples.

Optimization Strategy 1: Adjusting Primer Ratios

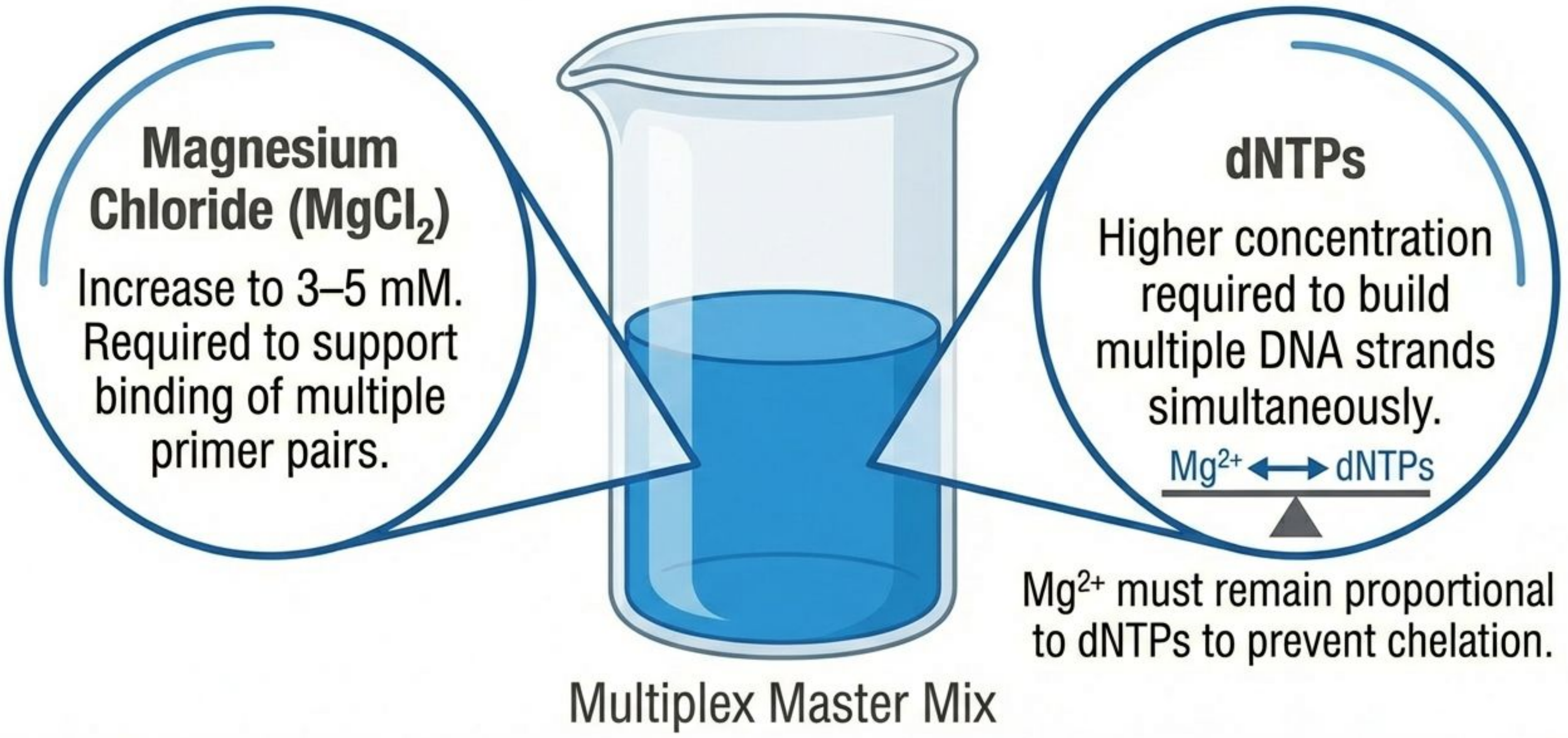
Problem (The Lever)	Solution (The Fix)
Equimolar concentrations (e.g., 0.2 µM) favor strong targets.	Imbalance primer concentrations to throttle strong reactions.

Primer Balancing

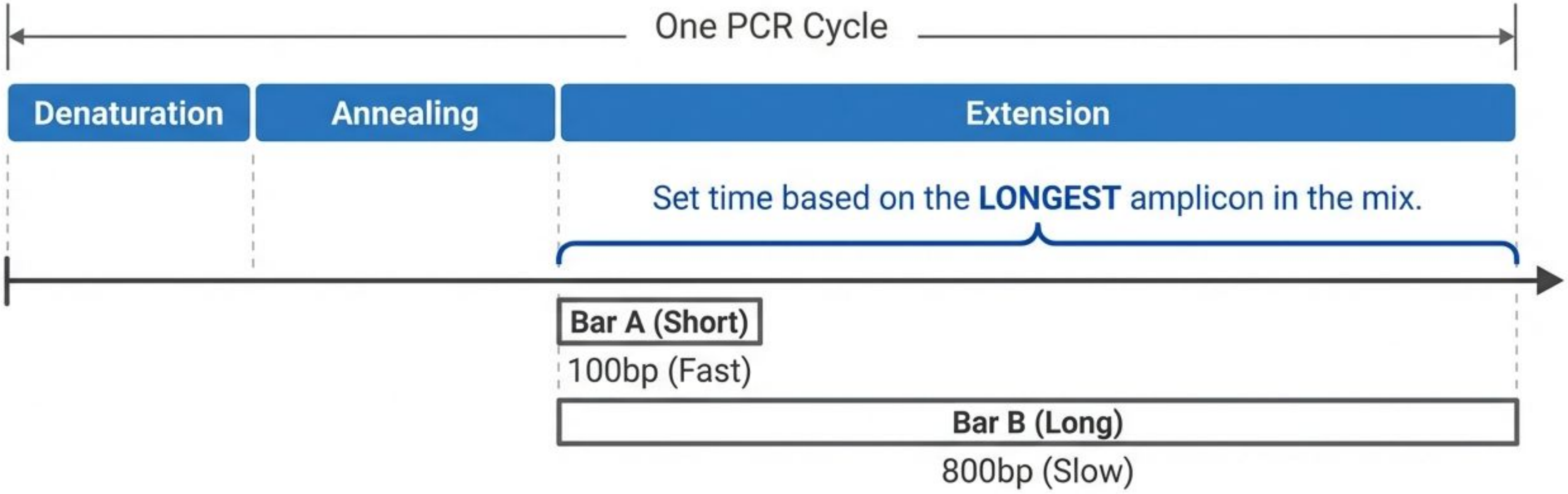


Outcome: Limits exponential growth of strong targets to preserve reagents for weak targets.

Optimization Strategy 2: Buffer Components



Optimization Strategy 3: Extension Time & Cycling



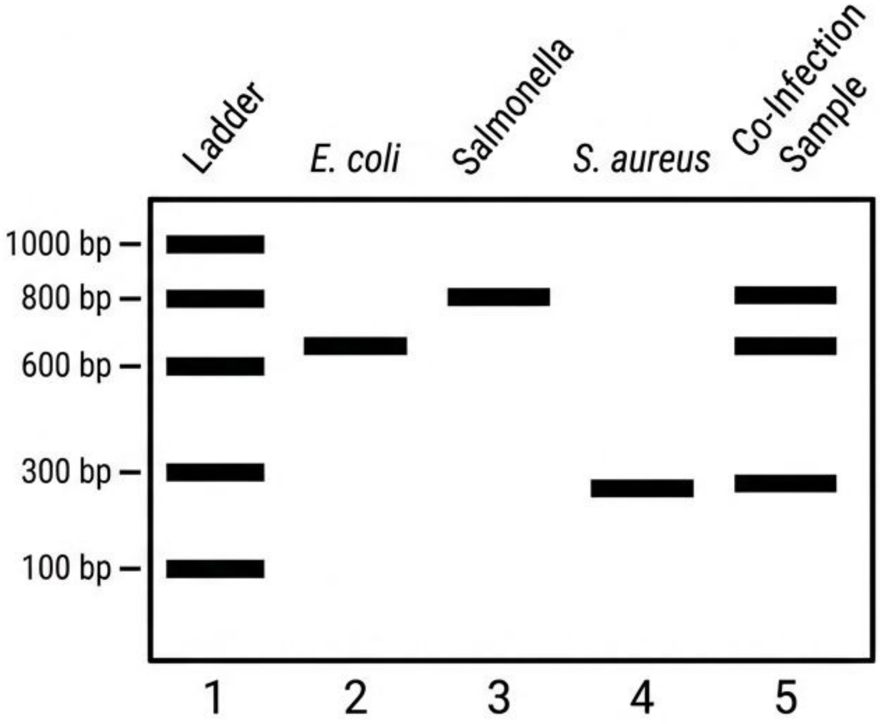
Key Technique

Hot Start PCR: Essential to prevent primer-dimer formation before cycling begins.

Case Study: Pathogen Detection in Mink

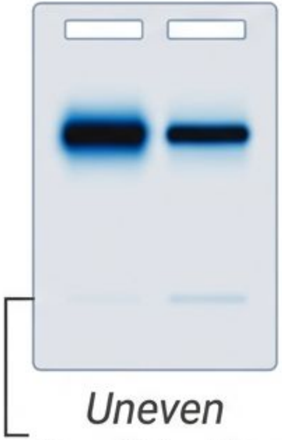
Differentiation of bacterial co-infections

- Target Profile
  1. *Escherichia coli*: 622 bp
  2. *Salmonella* spp.: 801 bp
  3. *Staphylococcus aureus*: 280 bp



Troubleshooting: Dropout & Uneven Amplification

Symptom



Uneven Amplification

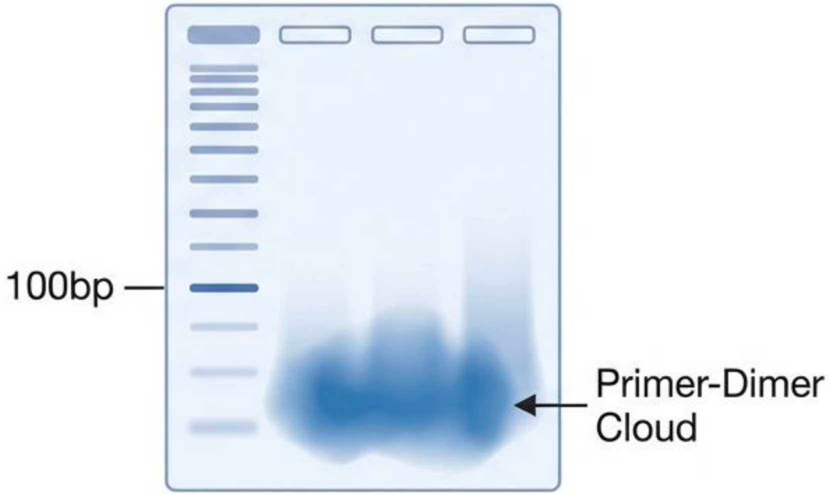
Root Cause: PCR Drift/Selection favoring abundant targets or reagent depletion.

Solution

Rebalance Primers	Increase [ ] for faint targets; Decrease [ ] for bright targets.
Buffer Strength	Switch from 1X to 2X Buffer (Increase $MgCl_2$).
Adjuvants	Add DMSO or Glycerol to relax secondary structures.

Troubleshooting: Primer-Dimers

Symptom



Root Cause: Primers annealing to each other (heterodimers) or themselves.

Solution

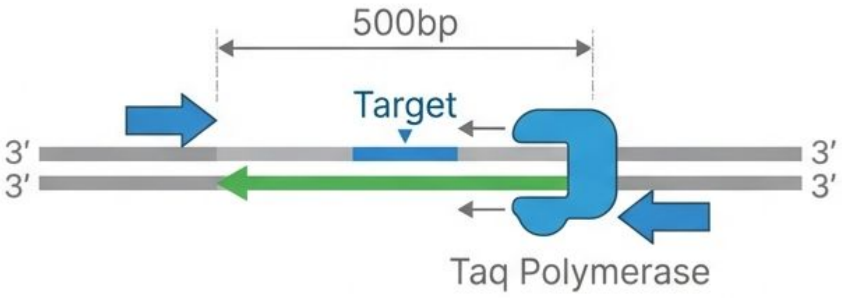
Design Check	Ensure no 3' complementarity in silico.
Hot Start	Prevent low-temp annealing during setup.
Concentration	Use minimum effective primer dose.

Gap-PCR: Detecting the Invisible Deletions

Principles, Methodology, and Clinical Applications for Large Deletions

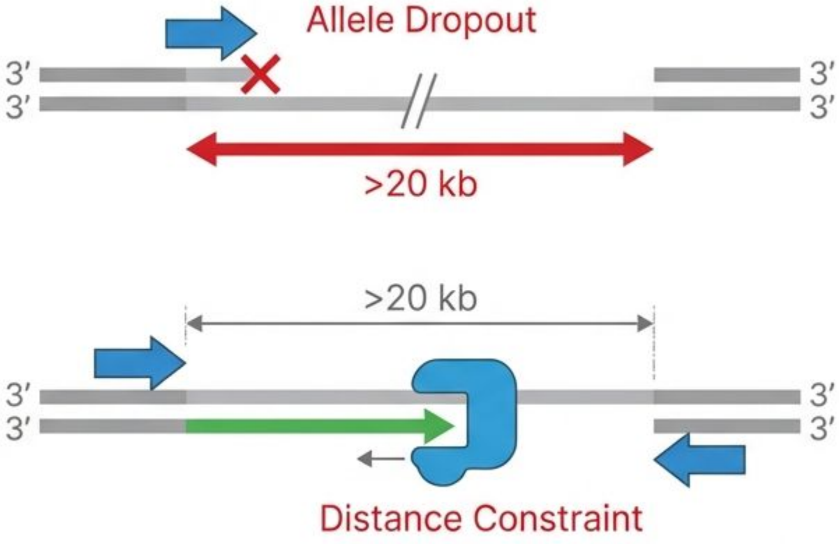
The Limitation of Standard PCR

Point Mutation (Detectable)



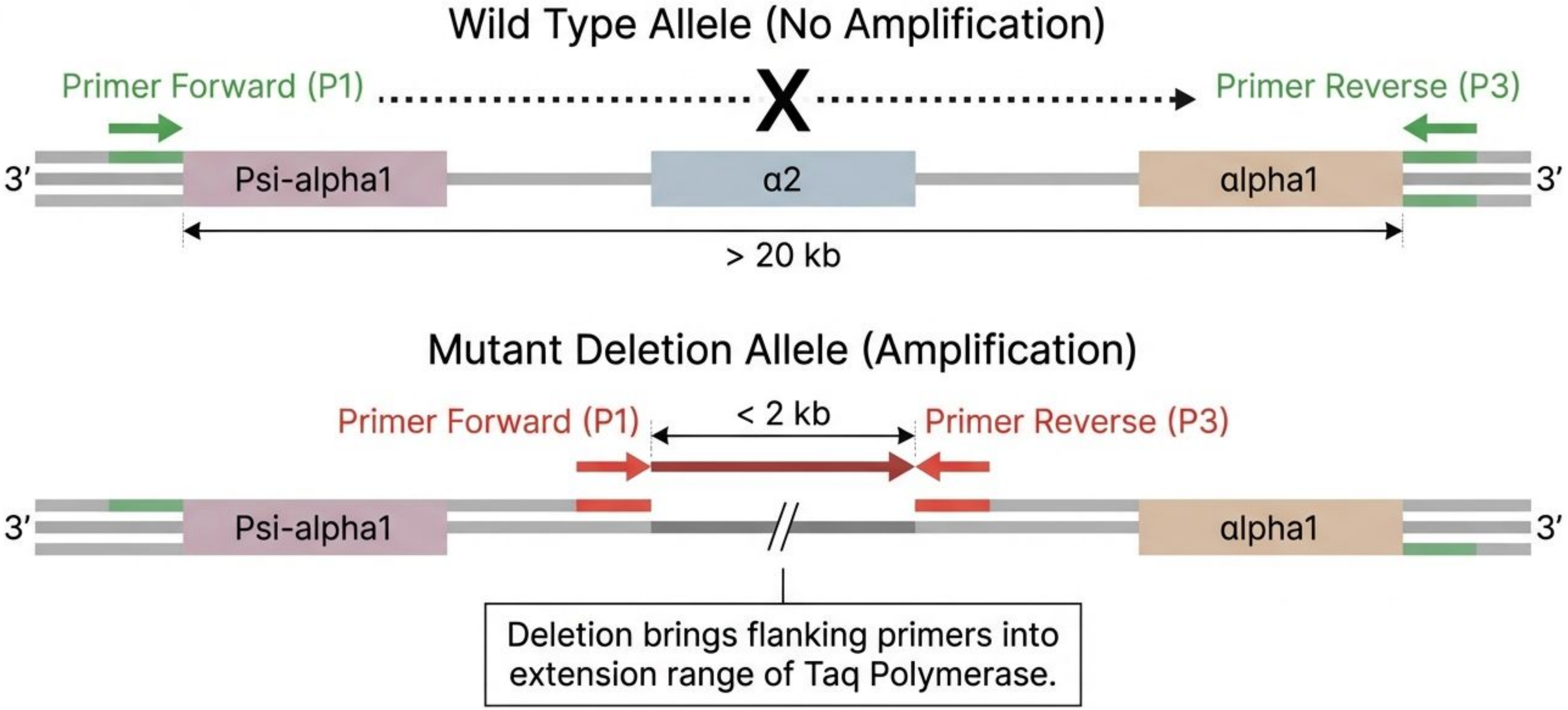
Successful Amplification

Large Deletion (Undetectable)



No Amplification / False Normal

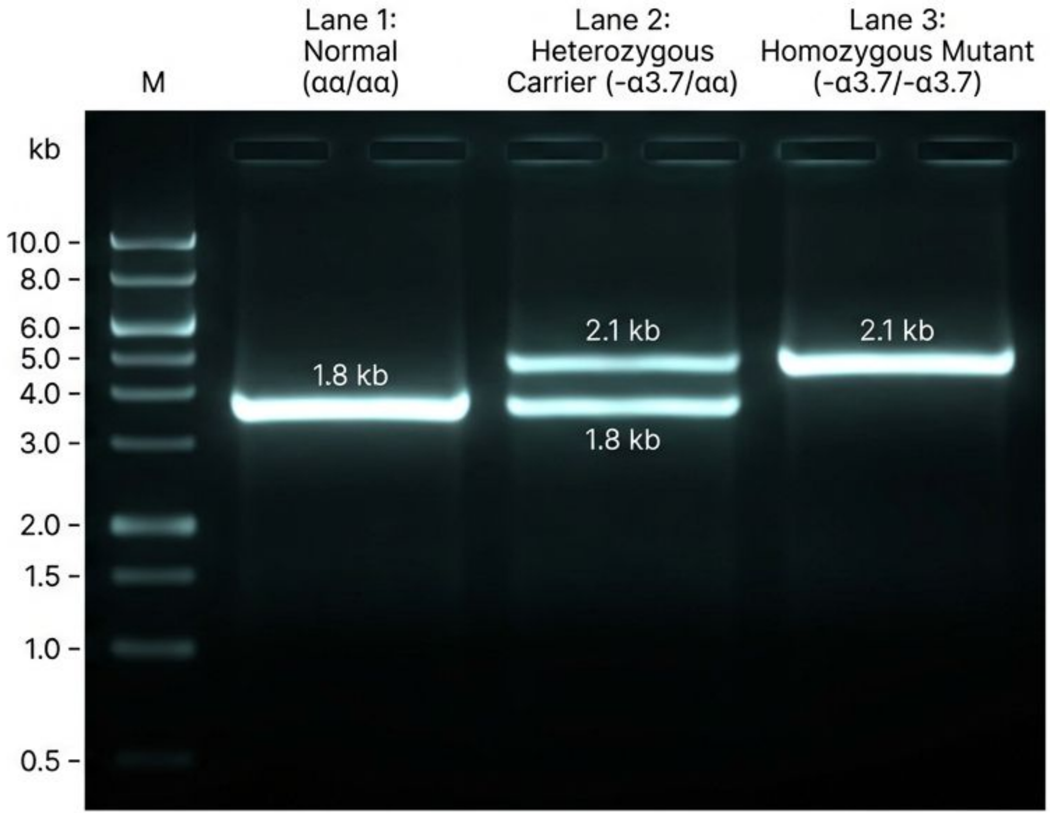
The ‘Bridging the Gap’ Principle



The 3-Primer Genotyping System

Genotype	Primer Interaction	Distance	Result
Wild Type	P1 + P2 bind	Short	Normal Band (Green)
	P1 + P3 bind	Too far	No Band
Homozygous Mutant	P1 + P2 (Site deleted)	N/A	No Band
	P1 + P3 bind	Short (due to deletion)	Mutant Band (Red)
Heterozygous	P1 + P2 amplify	Short	Normal Band (Green) Two Bands
	P1 + P3 amplify	Short (due to deletion)	Mutant Band (Red)

Interpreting Gel Electrophoresis Results (Case Study 1)



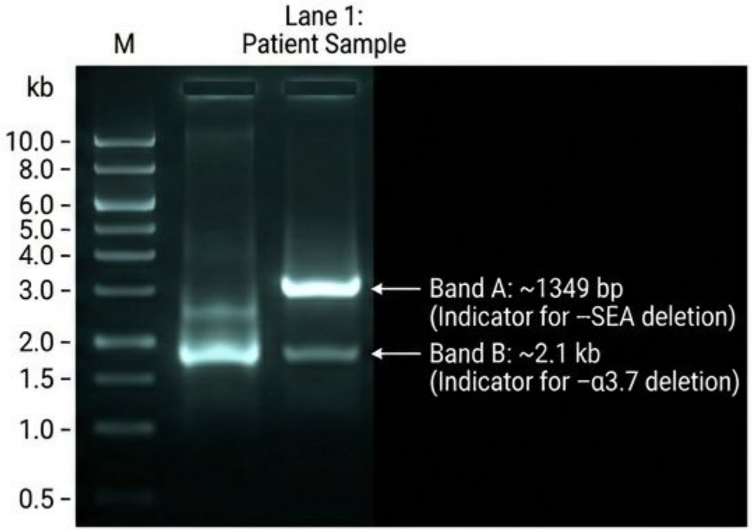
Detecting Hemoglobin H (HbH) Disease

Clinical Presentation

Patient: Microcytic hypochromic anemia.

Diagnosis: Compound Heterozygosity.

Genotype Result

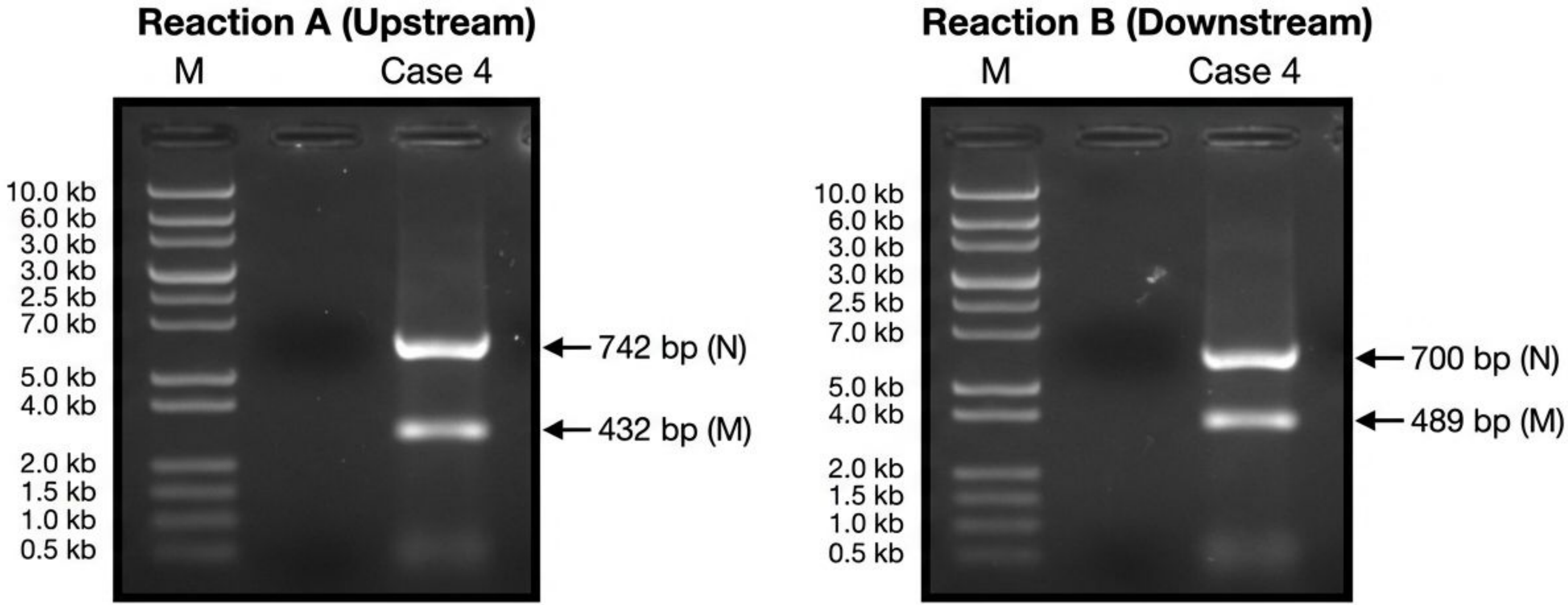


Genotype: --SEA / -α3.7

Conclusion: Gap-PCR successfully resolves compound states essential for prenatal counseling.

Beta-Globin Cluster: Turkish Inversion/Deletion (δβ)0

Demonstrating Gap-PCR Application Beyond Alpha-Thal.



Two-reaction confirmation defines the breakpoints of the inversion/deletion, resolving ‘unexplained’ thalassemia intermedia cases.

THANKS FOR LISTENING