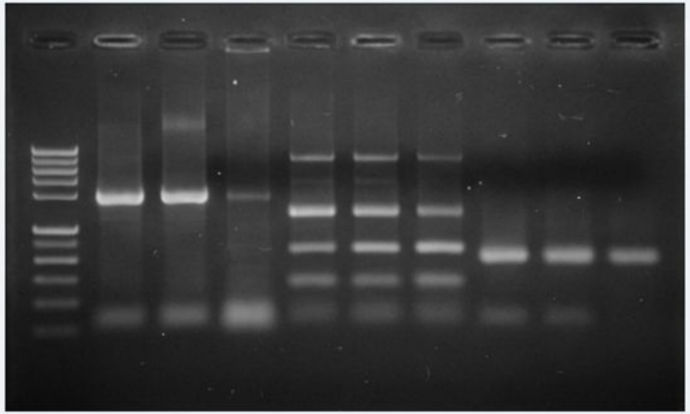
Real-time PCR (qPCR)

Dr. Luu Phuc Loi, PhD.
Presenter: Hoang Kim

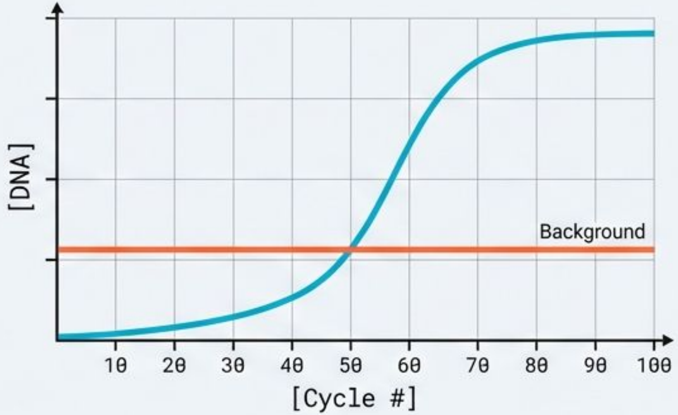
The Evolution: From End-Point to Real-Time Detection

The Past: Conventional PCR



- **Method:** End-point detection (Agarose Gel)
- **Nature:** Qualitative / Semi-quantitative
- **Drawbacks:** Labor-intensive, Open-tube system (Contamination risk)
- **Insight:** Analysis of the 'remains' after the reaction.

The Present: Real-Time PCR (qPCR)



- **Method:** Kinetic detection (Fluorescence)
- **Nature:** Quantitative, High-throughput
- **Advantages:** Closed-tube system, Wide dynamic range
- **Insight:** Observation of the reaction 'live' as it happens.

The Problem with End-Point Analysis: The Plateau Effect

The Phenomenon

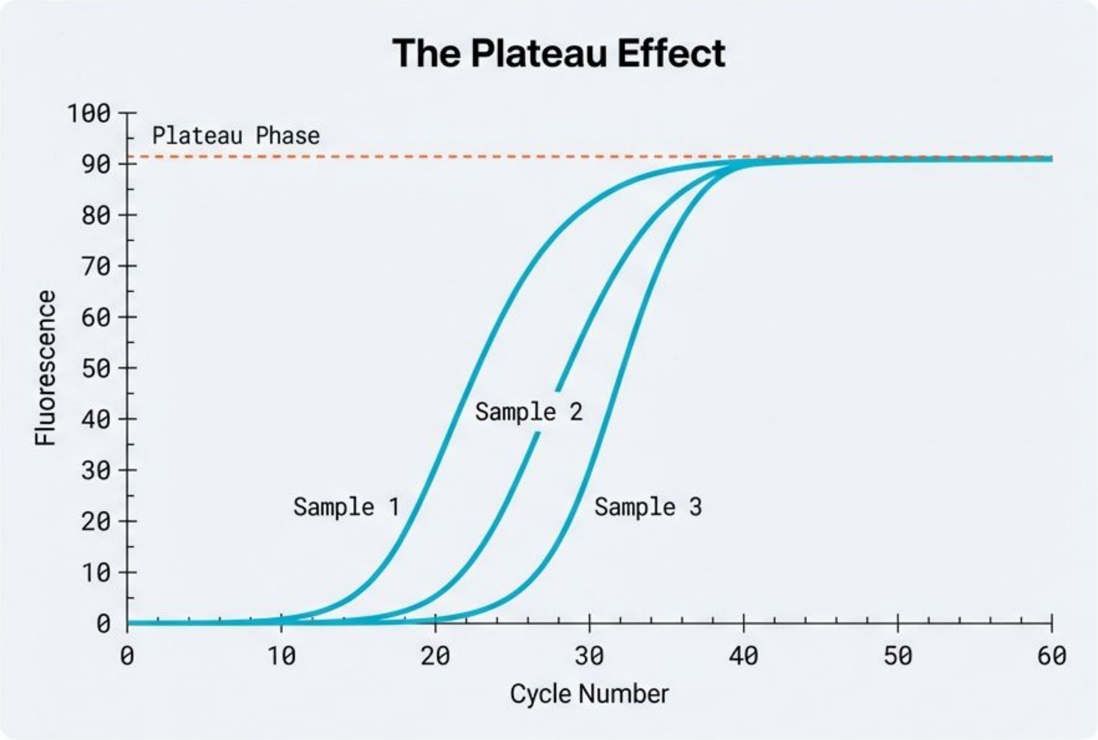
As reagents (dNTPs, primers) deplete and enzyme activity slows, the reaction stops generating template exponentially.

The Flaw

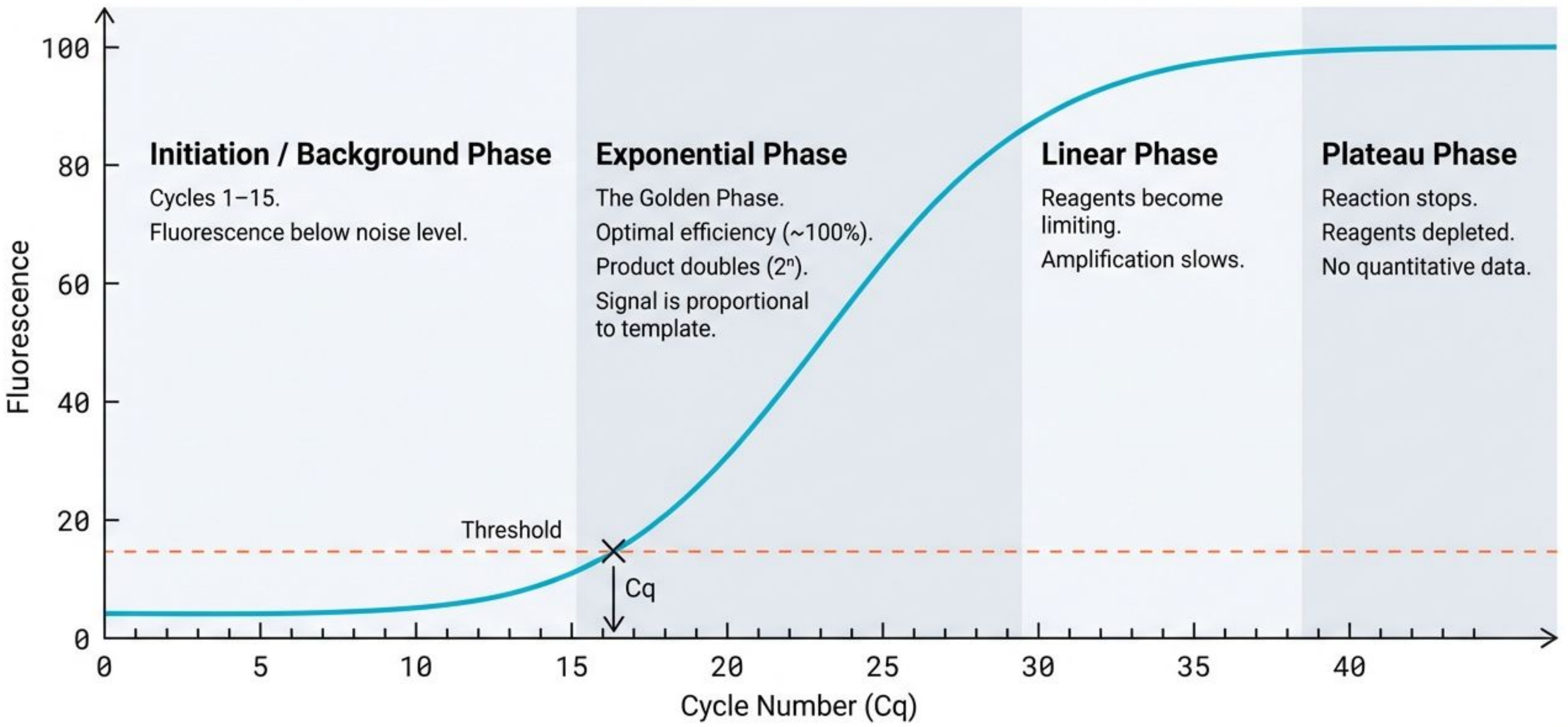
End-point band intensity correlates with *yield*, not the *initial input*. Looking at Cycle 40 (the plateau), Sample 1 and Sample 3 appear identical despite different starting quantities.

The Analogy

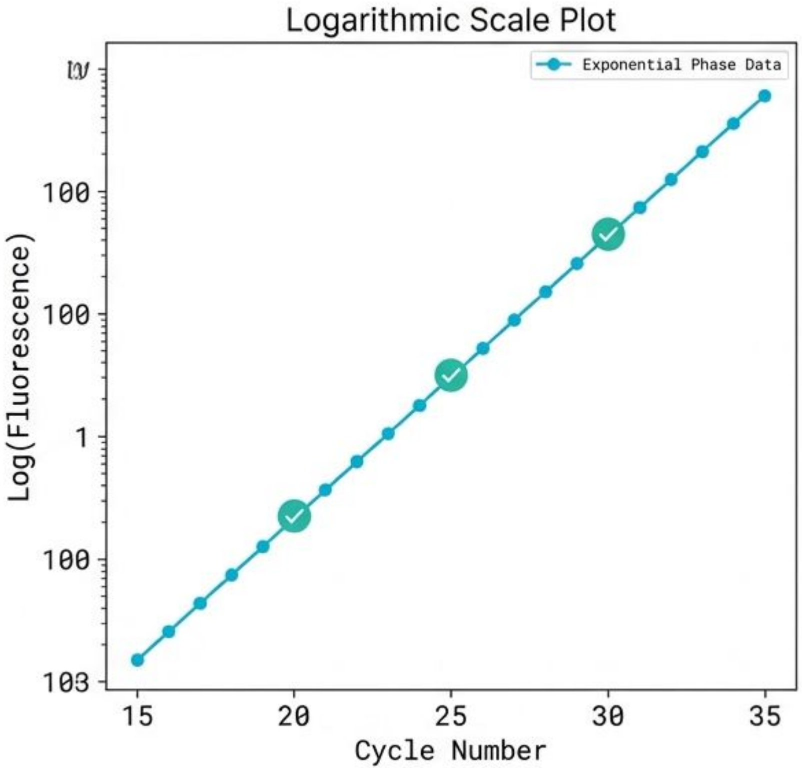
You cannot judge the speed of runners by taking a photo at the finish line after everyone has stopped running.



Decoding the Kinetics: The Four Phases of PCR



The Exponential Phase: The Window of Quantification



Why do we measure here?

1. Non-Limiting Reagents

dNTPs and primers are in excess. The reaction is not starving.

2. Peak Efficiency

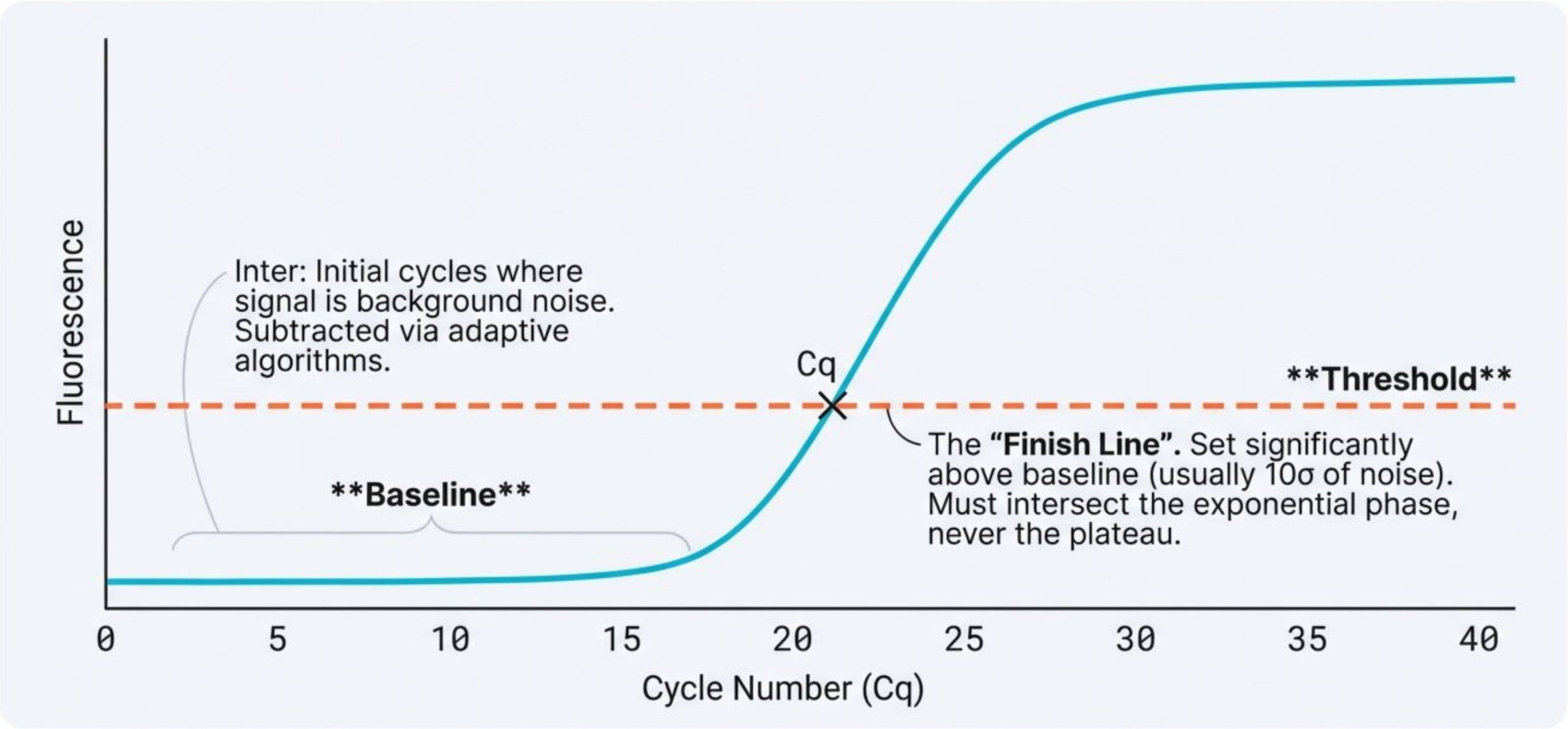
The polymerase is working at maximum capacity.

Equation: $Amount = Start \times 2^{cycles}$

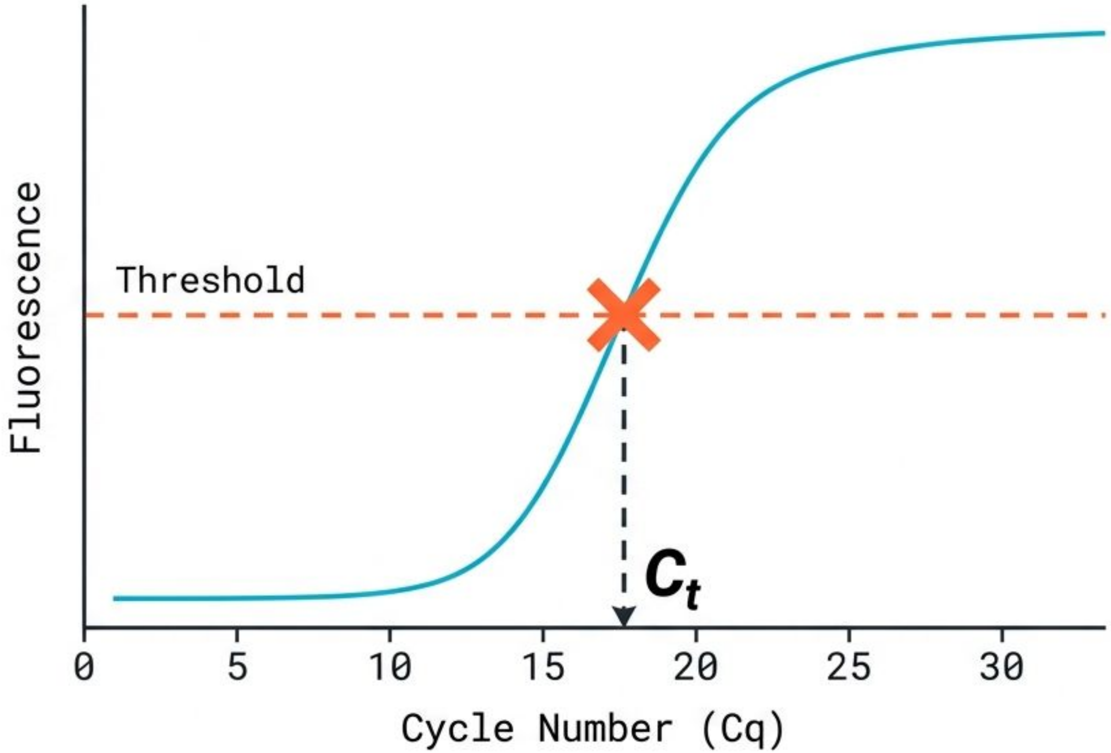
3. Precision

The relationship between Fluorescence (R_n) and Template Amount is linear on a log scale ($R^2 > 0.99$). We ignore the plateau and the baseline.

Setting the Parameters: Baseline and Threshold



The Fundamental Metric: The Ct Value



Definition

The fractional cycle number at which the fluorescence signal crosses the threshold line.

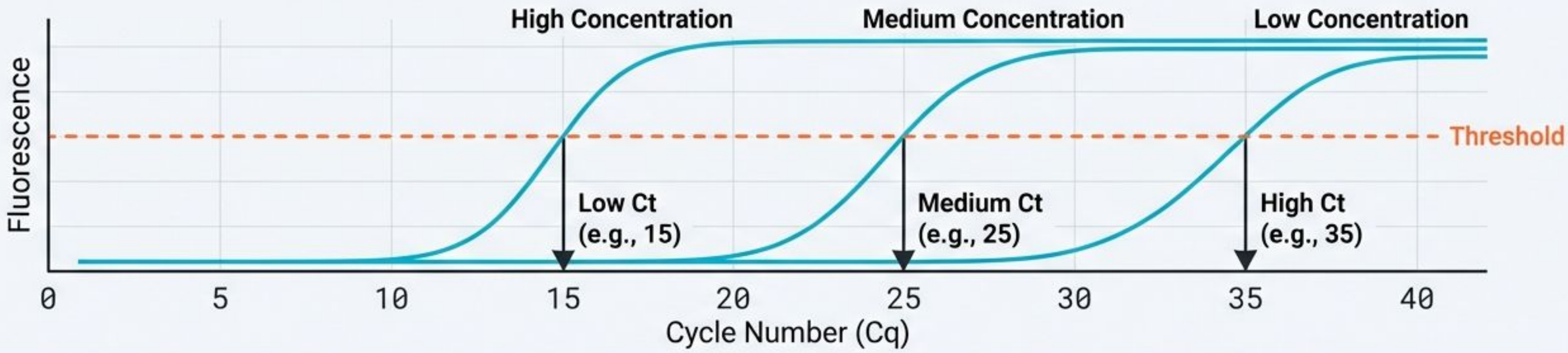
Synonyms

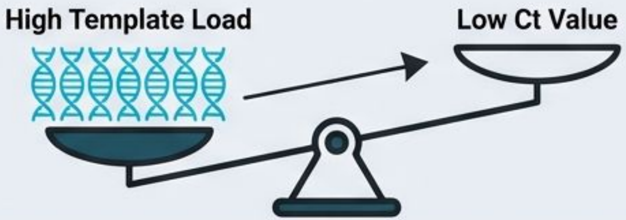
- C_q (Quantification Cycle) – MIQE preferred
- C_p (Crossing Point) – Roche LightCycler

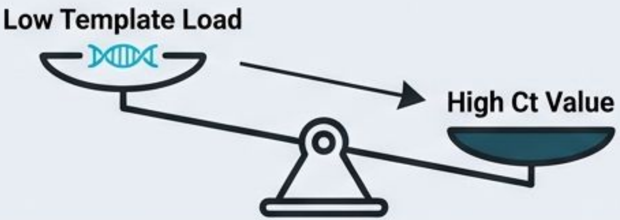
Key Concept

C_t is a time-based measurement (when did it happen?), not an intensity-based measurement.

The Inverse Relationship



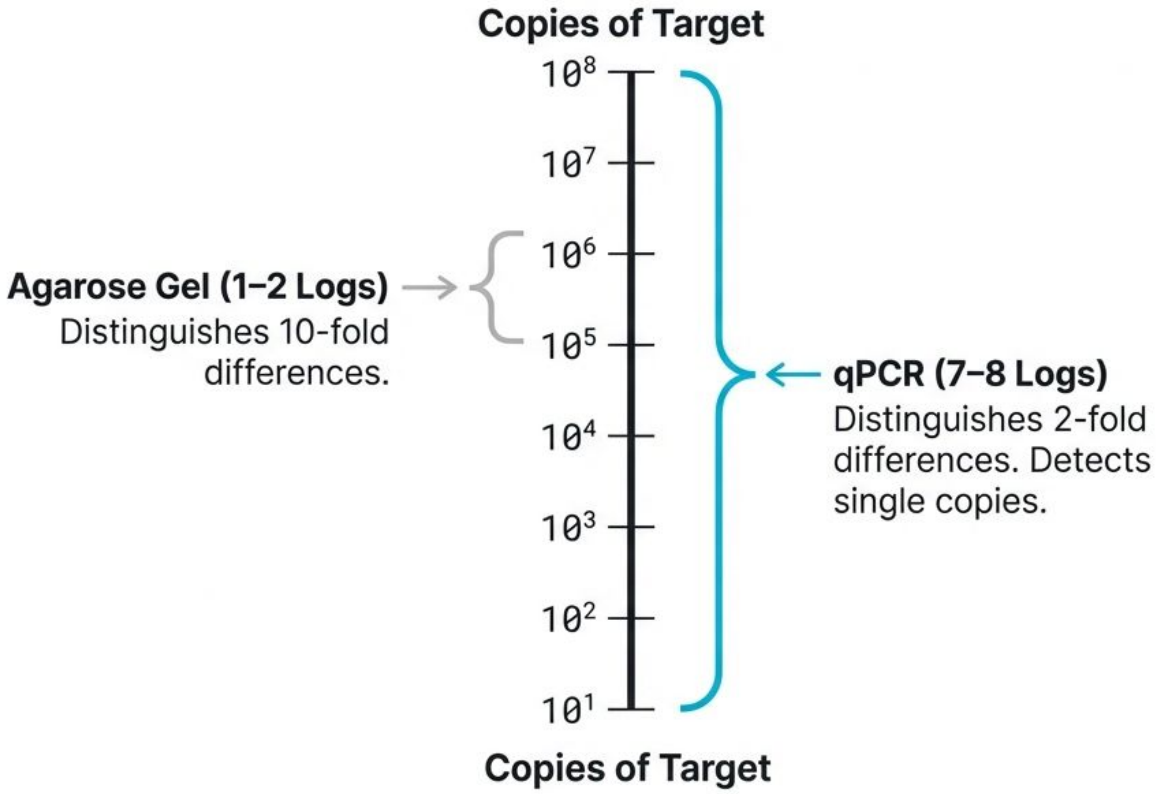




Rule: Ct is inversely proportional to the log of the initial template copy number.

$\Delta 3.3$ cycles ≈ 10-fold difference.

Dynamic Range and Sensitivity

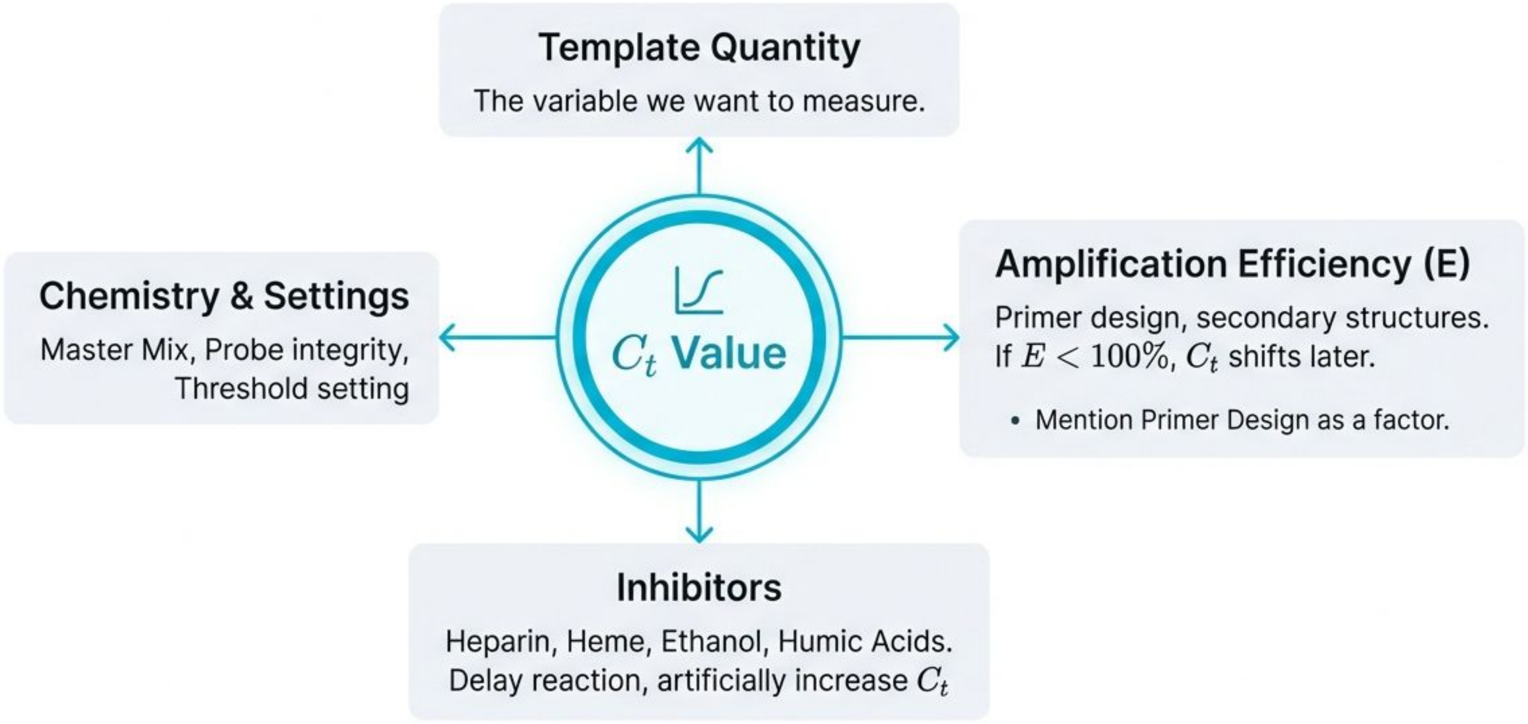


Sensitivity:
Detection down to 1 copy.

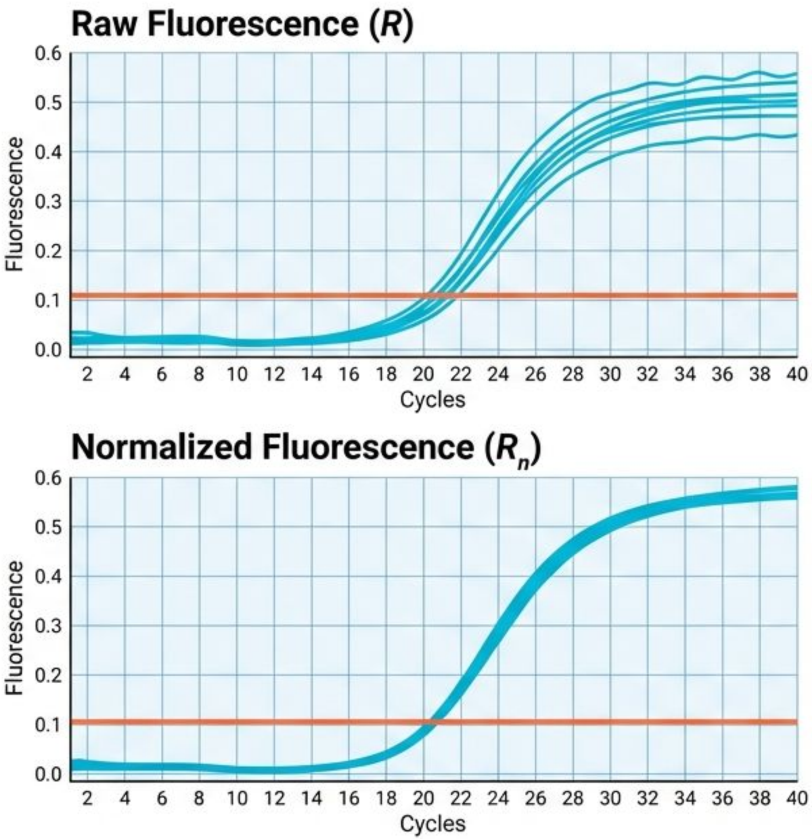
Precision:
Reliable differentiation between 10,000 and 20,000 copies.

Linearity:
Linear results across 8 orders of magnitude.

Factors Influencing the C_t Value



Normalization: The Role of Passive Reference Dyes (ROX)



The Problem:
Pipetting errors, air bubbles, and optical variations cause signal fluctuations.

The Solution:
Passive Reference Dye (ROX). A dye that does not participate in PCR provides a constant internal control.

The Formula:

$$R_n = \frac{\text{Emission of Reporter Dye (FAM)}}{\text{Emission of Passive Reference (ROX)}}$$

STRATAGENE

Module 1 Summary: The Theoretical Pillars

Quantification Window

Analysis must occur in the **Exponential Phase**, where reagents are non-limiting and efficiency is constant.

The Metric

The C_t (or C_q) **value** is the gold standard for quantification, representing the cycle where signal exceeds noise.

The Relationship

C_t is **inversely proportional** to the starting template quantity. (`$High Load = Low` C_t).

Sensitivity

qPCR offers a dynamic range of 7–8 logs, vastly superior to end-point gel analysis.

Normalization

Reference dyes (ROX) and baseline correction are essential for removing non-biological noise.

Two Strategies to Visualize the Invisible

Classification of Detection Chemistries

1. Non-Specific Detection
(The “Floodlight” Strategy)

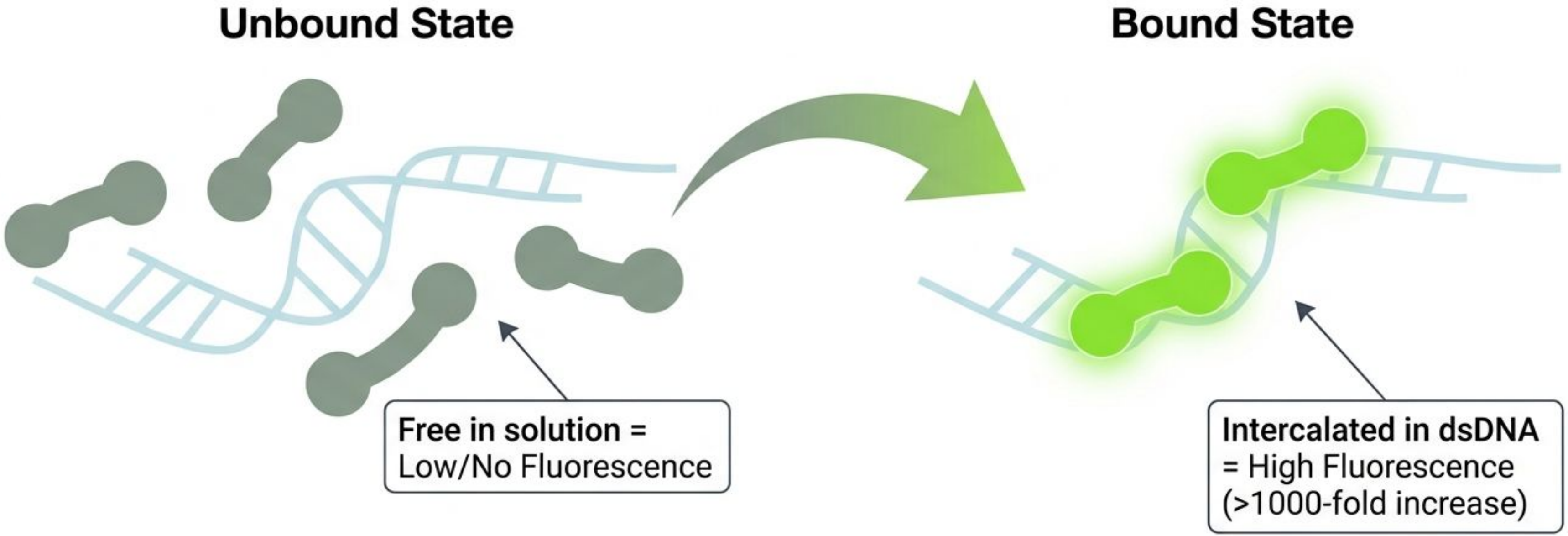
- **Mechanism**: DNA-binding dyes intercalate into any double-stranded DNA (dsDNA).
- **Principle**: ‘If it is double-stranded, it glows.’
- **Key Example**: SYBR® Green I

2. Specific Detection
(The “Sniper” Strategy)

- **Mechanism**: Sequence-specific oligonucleotide probes labeled with fluorophores.
- **Principle**: ‘Glows only when the specific target code is found.’
- **Key Examples**: Hydrolysis Probes (TaqMan®), Molecular Beacons

The Mechanism of SYBR® Green I

From Darkness to Light: Intercalation



The Correlation: Fluorescence intensity is directly proportional to the amount of dsDNA amplicon accumulated.

Why Choose SYBR Green?

The Advantages of DNA-Binding Dyes

Cost-Effective

Significantly less expensive than labeled probes. Ideal for large-scale screening or limited budgets.

Simplicity

Requires only standard PCR primers. No need to design complex internal probes or labeled oligos.

Universality

“One dye fits all.” The same master mix works for any gene target, from GAPDH to viral pathogens.

Ideal Use Case: Initial expression validation, primer optimization, and high-throughput screening.

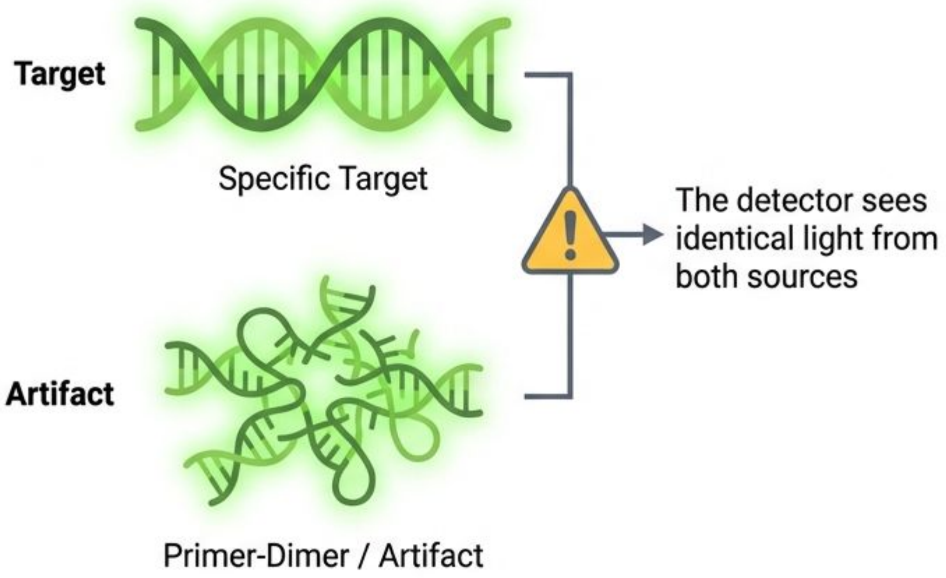
The ‘Blindness’ of SYBR Green
Limitations: Non-Specificity and Artifacts

The Problem:
SYBR Green binds indiscriminately to *any* double-stranded DNA. It cannot distinguish between the target gene and amplification artifacts.

Common Artifacts:
- **Primer-Dimers**: Primers binding to each other.
- **Non-Specific Products**: Mis-priming elsewhere in the genome.

The Consequence:
False positives and overestimation of target quantity (Ct values appear too early).

What the Machine Sees vs. Reality



Crucial Requirement: Users must verify reaction specificity post-amplification using Melt Curve Analysis.

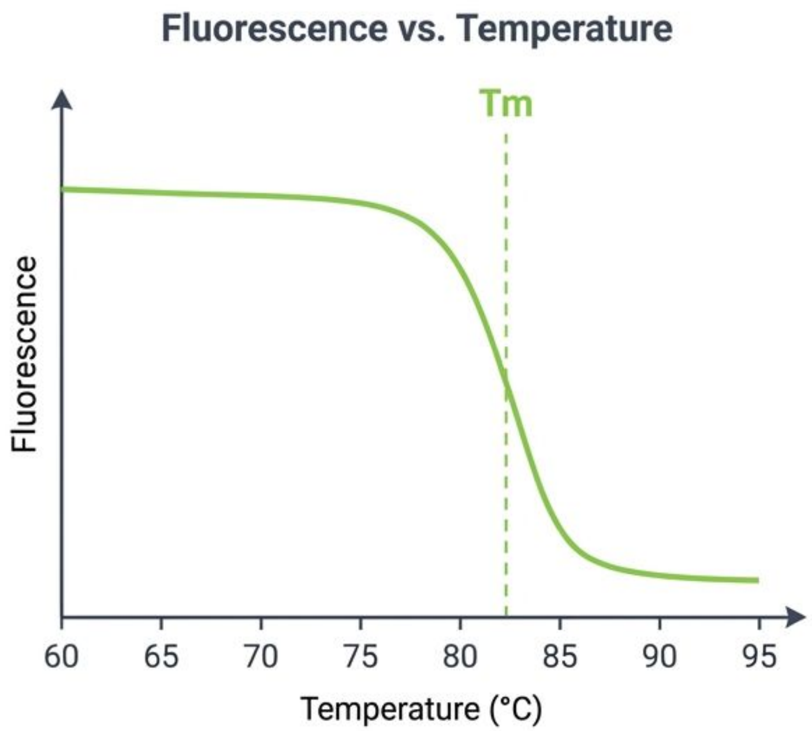
The Solution: Melt Curve Analysis

Using Temperature to Determine Identity

The Protocol: Performed immediately after PCR cycles.

1. **Ramp**: Temperature is slowly increased (60°C → 95°C).
2. **Denaturation**: dsDNA destabilizes and separates into single strands.
3. **Release**: Dye is released back into solution.
4. **Signal Drop**: Fluorescence decreases rapidly.

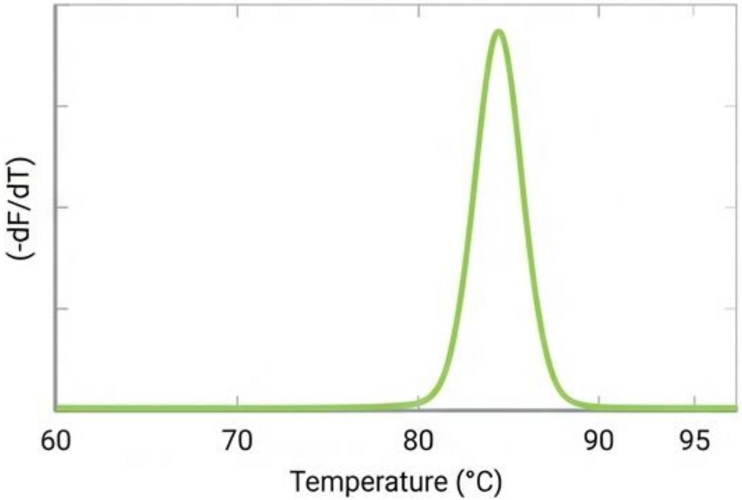
Definition: Tm (Melting Temperature) is the point where 50% of the amplicon is denatured.



Interpreting the Dissociation Curve

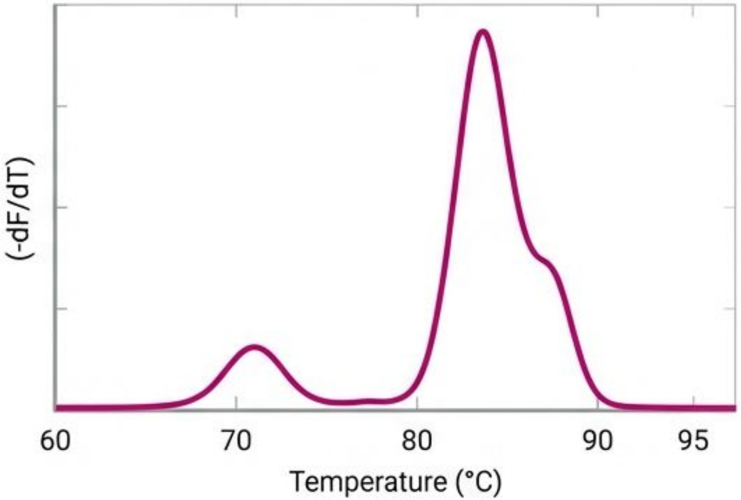
Identifying Success vs. Contamination

Scenario A: Clean Reaction (Pass)



Specific Amplification. Single peak indicates one pure product.

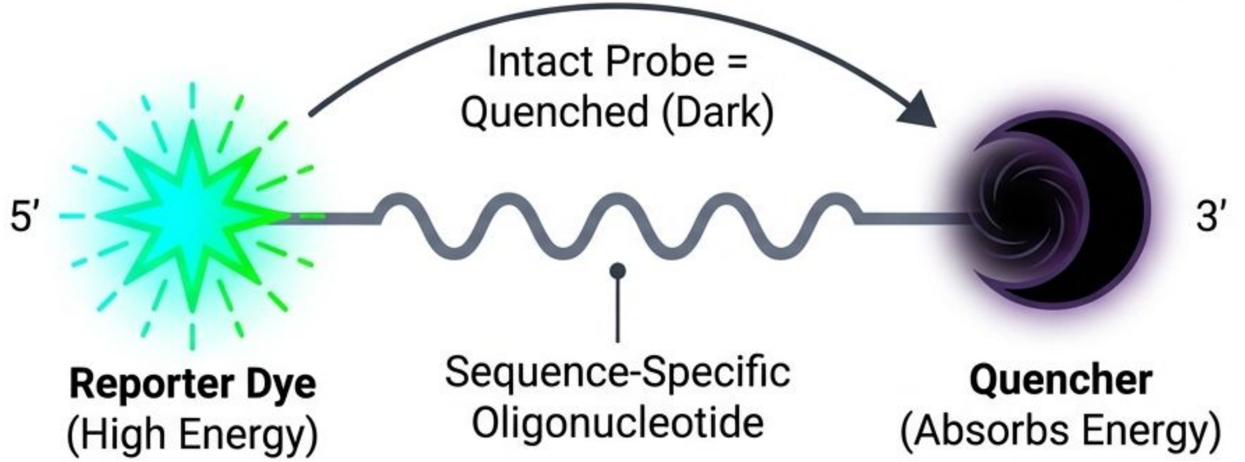
Scenario B: Contamination (Fail)



Non-Specific Binding / Primer-Dimers. Multiple peaks or shoulders indicate artifacts.

Hydrolysis Probes (TaqMan®) Structure

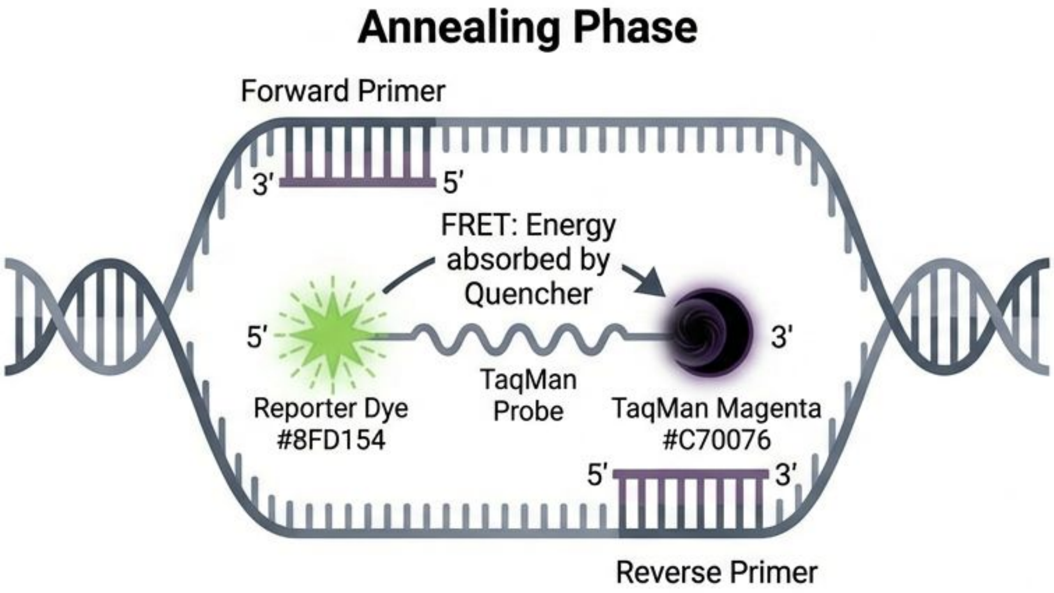
The Components of Specificity



Design: The probe binds specifically to the target DNA between the forward and reverse primers.

The Mechanism: FRET

Fluorescence Resonance Energy Transfer

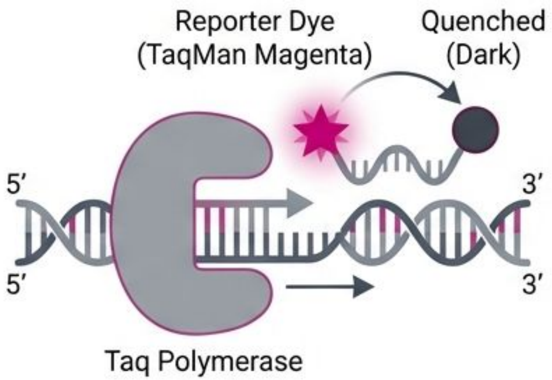


The Setup: During annealing ling (55-65°C), the probe binds specifically to the target. Because the probe is intact, the Quencher suppresses the Reporter. The signal is silent.

Signal Generation: The Role of Taq Polymerase

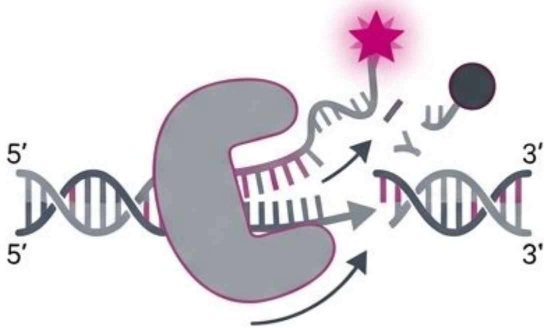
5′ → 3′ Exonuclease Activity

Step 1: Encounter



Polymerase Extends

Step 2: Hydrolysis



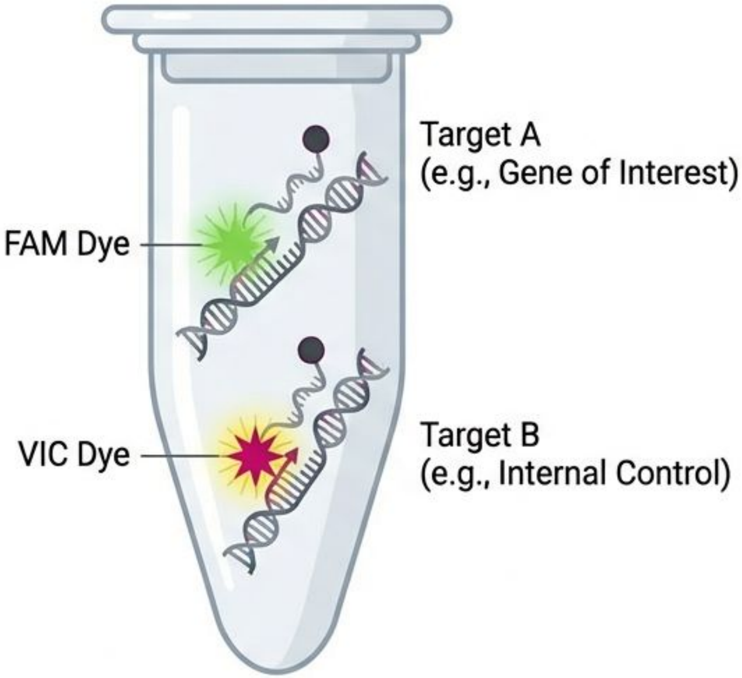
5′ → 3′ Exonuclease Activity

Step 3: Signal Release

FRET Broken = **Fluorescence**

Multiplexing Capability

Multiple Targets, One Tube



The Power of Color: Distinct probes with different reporter dyes allow simultaneous detection.

Benefits:

- **Internal Controls**: Normalize data within the same well.
- **Efficiency**: Conserves sample and reagents.
- **Throughput**: Detect multiple pathogens at once.

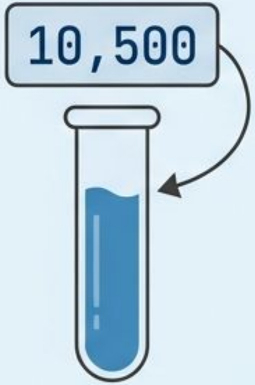
Comparison: SYBR® Green vs. TaqMan®

Choosing the Right Chemistry

Feature	SYBR® Green (Dye)	TaqMan® (Probe)
Specificity	Low (Primers only)	**High** (Primers + Probe)
Cost	**Low** (Entry-level friendly)	High (Requires custom probe)
Assay Design	**Simple** (Primers only)	Complex (Probe design needed)
Multiplexing	No	**Yes** (Multicolor)
Post-PCR Analysis	Required (Melt Curve)	**Not Required**

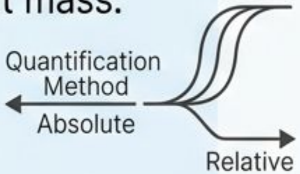
Defining the Goal: Absolute vs. Relative Quantification

Absolute Quantification

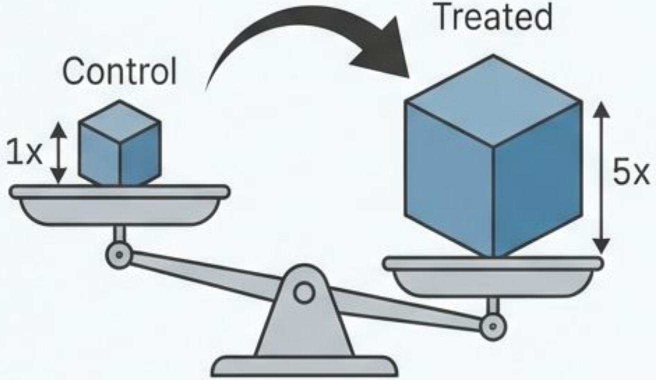


Question: "How many copies are there?"

- Determines exact copy number or unit mass.
- Requires interpolation against a Standard Curve.
- **Example:** Viral Load (Copies/mL).



Relative Quantification

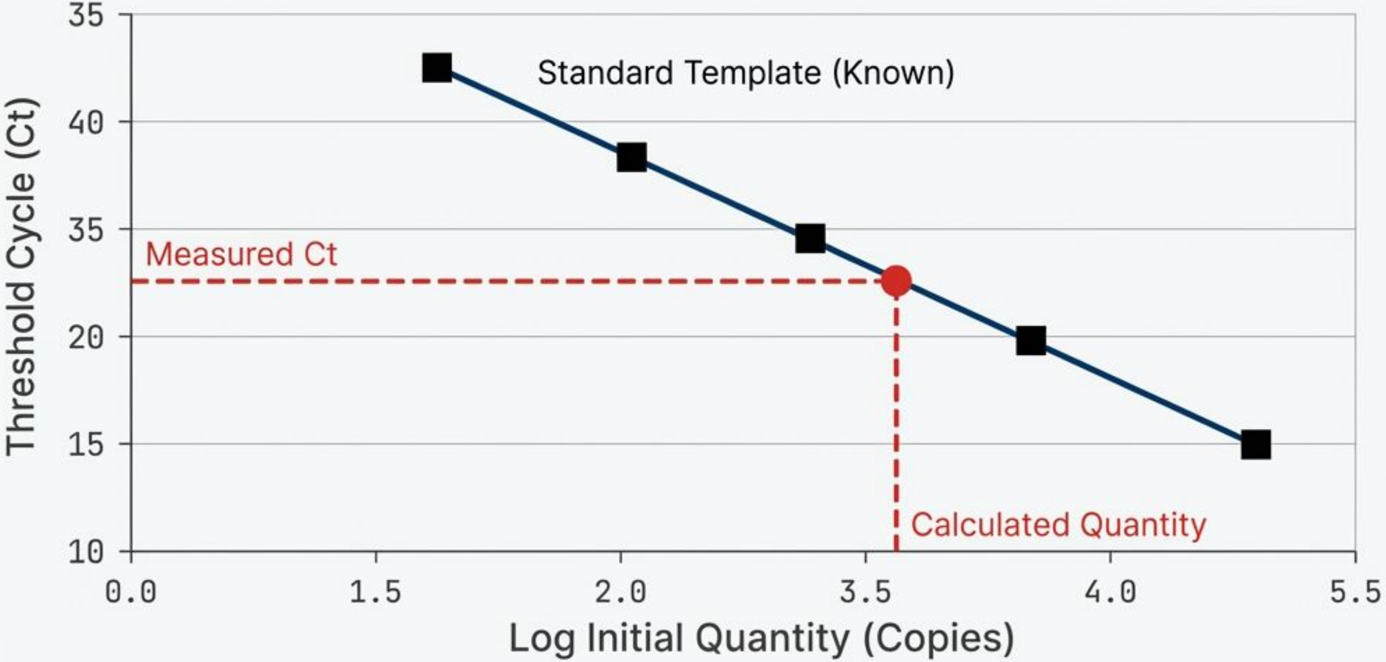


Question: "How much did it change?"

- Calculates fold-change ratios.
- Requires normalization to Reference Gene & Calibrator.
- **Example:** Gene Expression (5-fold increase).

Absolute Quantification: Calibrating the Ruler

Measuring the Unknown against the Known

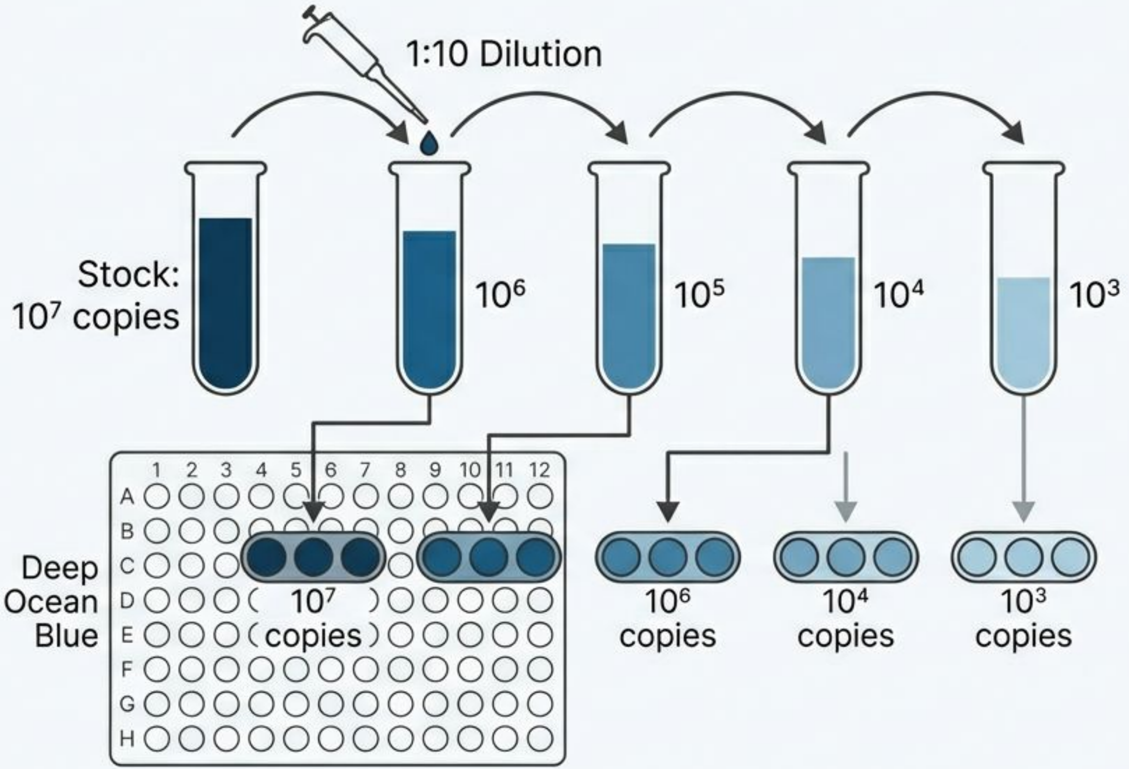


Standard Templates:
Plasmid with cloned GOI
Synthetic Oligonucleotides
Genomic DNA
In Vitro Transcripts

Critical Rule: Quantification must be by Interpolation (within the curve), never Extrapolation.

Constructing the Standard Curve

The Serial Dilution Workflow

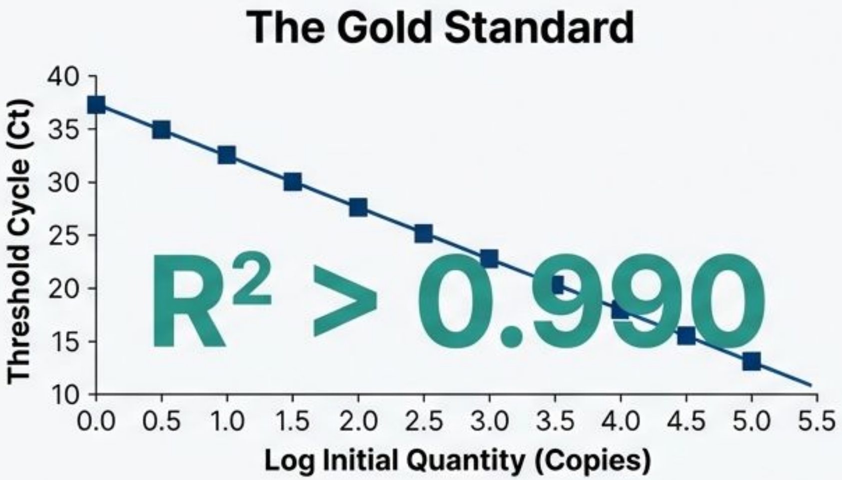


Design Requirements:

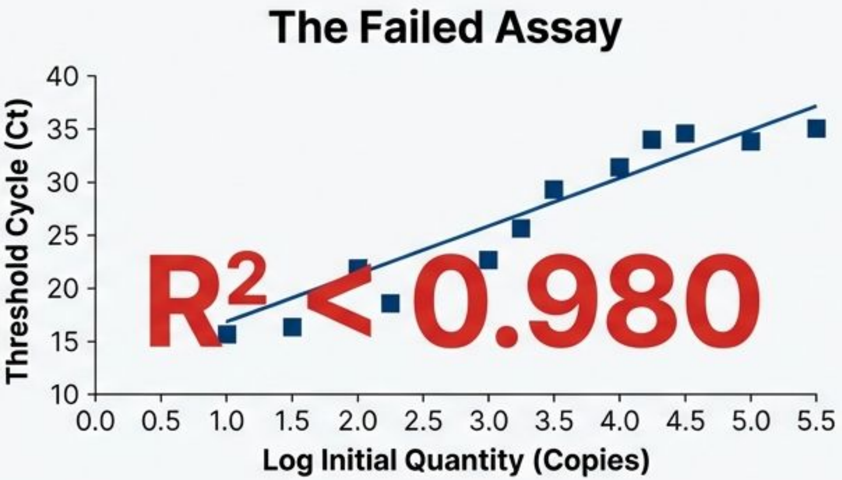
- **Dynamic Range:** Must cover the expected range of unknowns.
- **Points:** Minimum 4 concentration points.
- **Replicates:** Duplicates or Triplicates to reduce pipetting error.

Quality Control: Evaluating Linearity (R^2)

Is the ruler straight?



Excellent Linearity



Poor Fit / Non-Linear

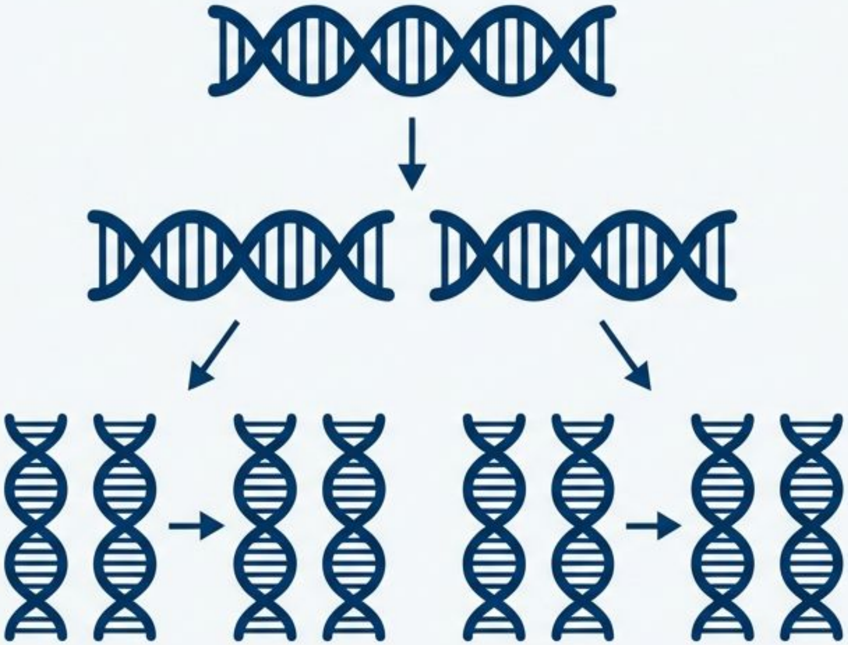
R^2 (Pearson Correlation Coefficient): Measures fit quality.

Acceptance Criteria: > 0.980

⚠ **Warning:** Bending at high Ct indicates Limit of Detection (LOD).

PCR Efficiency (E): The Doubling Principle

Theoretical Perfection vs. Biochemical Reality



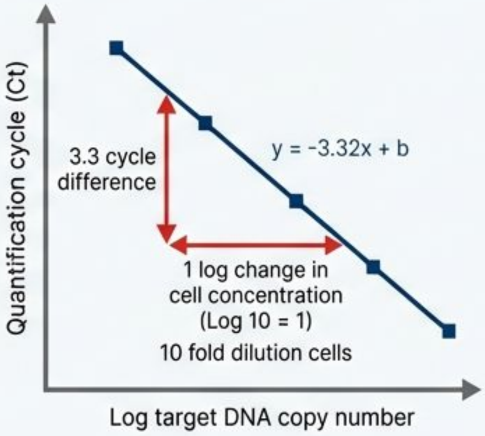
1 Cycle = 2x Copies (100% Efficiency)

The Ideal Slope: -3.32

Calculated from:
10-fold increase = 3.32 cycles ($2^{3.32} \approx 10$)

Acceptable Range:
90% – 105%

Slope Range:
−3.1 to −3.6



10 fold change = 2^3.3 i.e. a 3.3 cycle difference.
PCR double each cycle = 2^X where x is number cycles

Calculating Efficiency from Slope

Deriving the percentage from the regression line

$$\text{Efficiency}\,(E) = 10^{-1/\text{slope}} - 1$$

Derived from Standard Curve

Worked Example

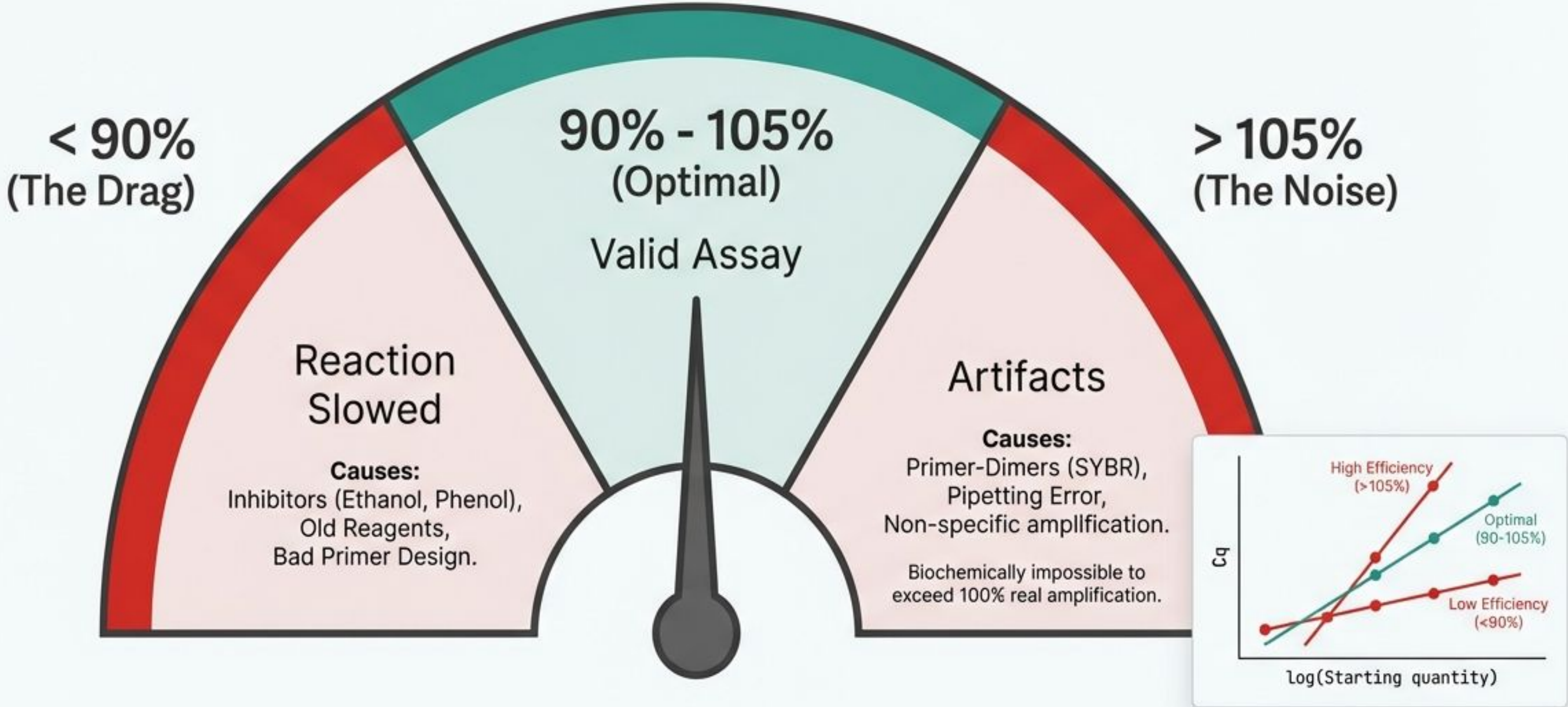
Given Slope $= -3.32$

1. Step 1: Divide -1 by $-3.32 = 0.301$
2. Step 2: Calculate $10^{0.301} = 2.0$
3. Step 3: Subtract $1 = 1.0$

Result: 100% Efficiency

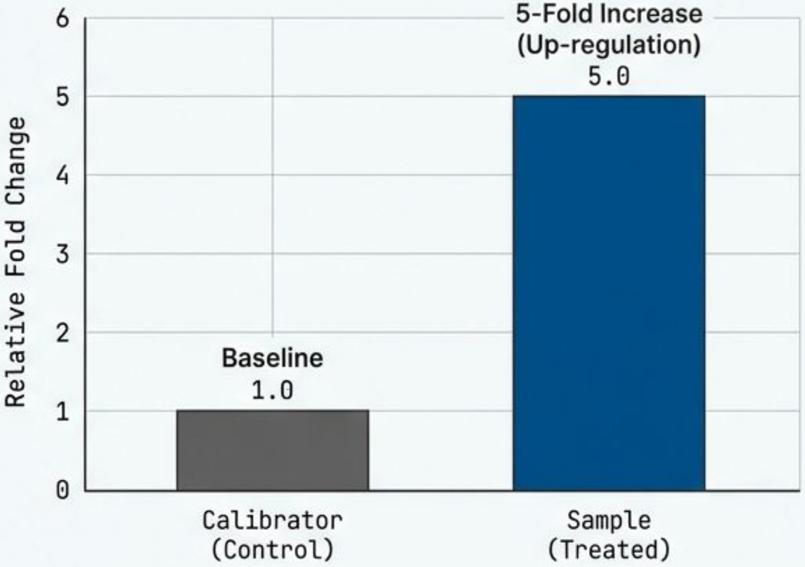
Diagnosing Efficiency Issues

What the numbers reveal about reaction health



Relative Quantification: Measuring Gene Expression

Focus: Fold-Change Ratios

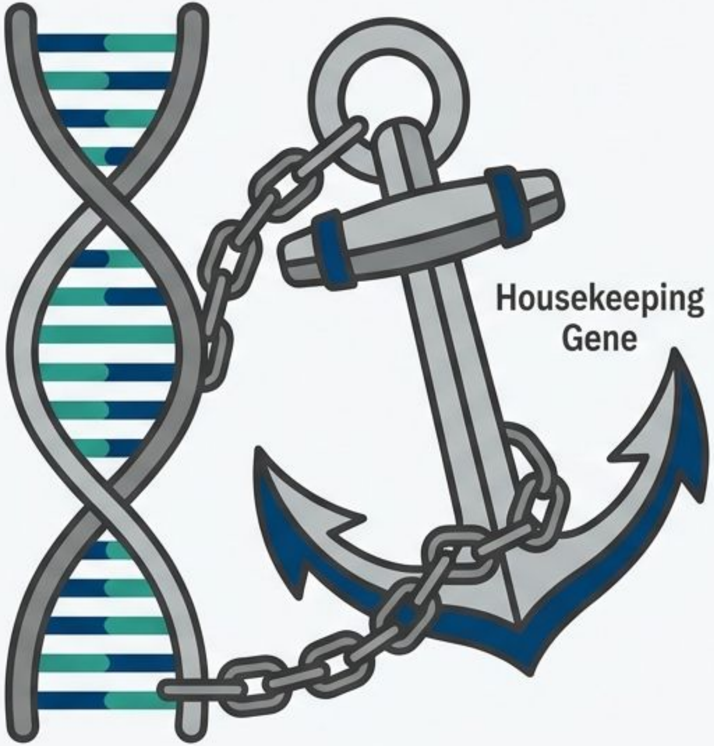


Objective:

- Determine up-regulation or down-regulation.
- Requires a Calibrator (Untreated/Time Zero).
- CRITICAL: Must be normalized to a Reference Gene to account for loading errors.

The Anchor: Selecting Reference Genes

Normalizing for Error



The Role:

- Corrects for variations in RNA input.
- Corrects for Reverse Transcription efficiency.

The Criteria:

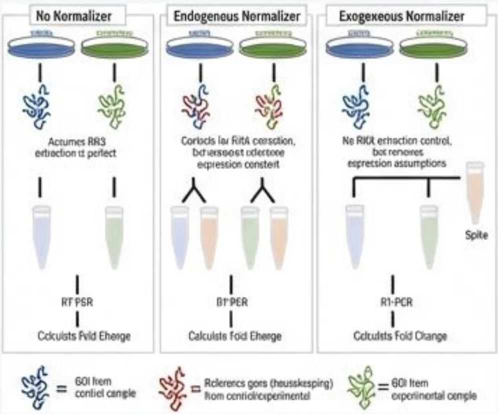
- Constitutive expression.
- **STABLE** across all treatments (Drug, Temp, Time).

Common Anchors:

- GAPDH
- Beta-Actin
- 18S rRNA

Validation is key.

If the anchor moves (expression changes), the data is invalid.



Step 1: The Delta Ct Calculation

Normalizing Within the Sample

$$\Delta Ct = Ct(\text{Target Gene}) - Ct(\text{Reference Gene})$$

- This calculation is performed for **every** sample individually.
- Goal: Eliminate loading bias. If you loaded 2x RNA, both Ct values shift, but the difference (ΔCt) remains constant.

Sample	Target Ct	Ref Ct	ΔCt
Treated	24.5	18.0	**6.5**
Control	26.5	18.0	**8.5**

Step 2: The Delta-Delta Ct Calculation

Comparing Sample vs. Calibrator

$$\Delta\Delta Ct = \Delta Ct(\text{Sample}) - \Delta Ct(\text{Calibrator})$$

Usually the Untreated Control

- This subtracts the baseline noise of the control from the sample signal.
- If Result is 0: No difference.
- If Result is Negative: Target is Up-regulated (Lower Ct = More Template).

Example Continuation

`ΔΔCt = 6.5 (Treated) - 8.5 (Control) =` **`-2.0`**

Step 3: The Fold Change Formula (Livak Method)

The $2^{\wedge-\Delta\Delta Ct}$ Method

$$\text{Fold Change} = 2^{-\Delta\Delta Ct}$$

Assumption of 100% Efficiency (Doubling)

The difference in cycles calculated in Step 2.

Example Calculation

$$\begin{aligned}\text{Fold Change} &= 2^{-(-2.0)} \\ &= 2^{2} \\ &= \textbf{4-Fold Increase}\end{aligned}$$

Founders Grotesk

- Efficiencies of Target and Reference must be approx. EQUAL and near 100%.

Crucial Assumption

- Efficiencies of Target and Reference must be approx. EQUAL and near 100%.

⚠ If efficiencies differ, this method fails.

The Pfaffl Method

When Efficiencies Differ

What if Target E = 90% and Reference E = 100%?
The Livak method will be inaccurate.

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct(control-sample)}}{(E_{ref})^{\Delta Ct(control-sample)}}$$

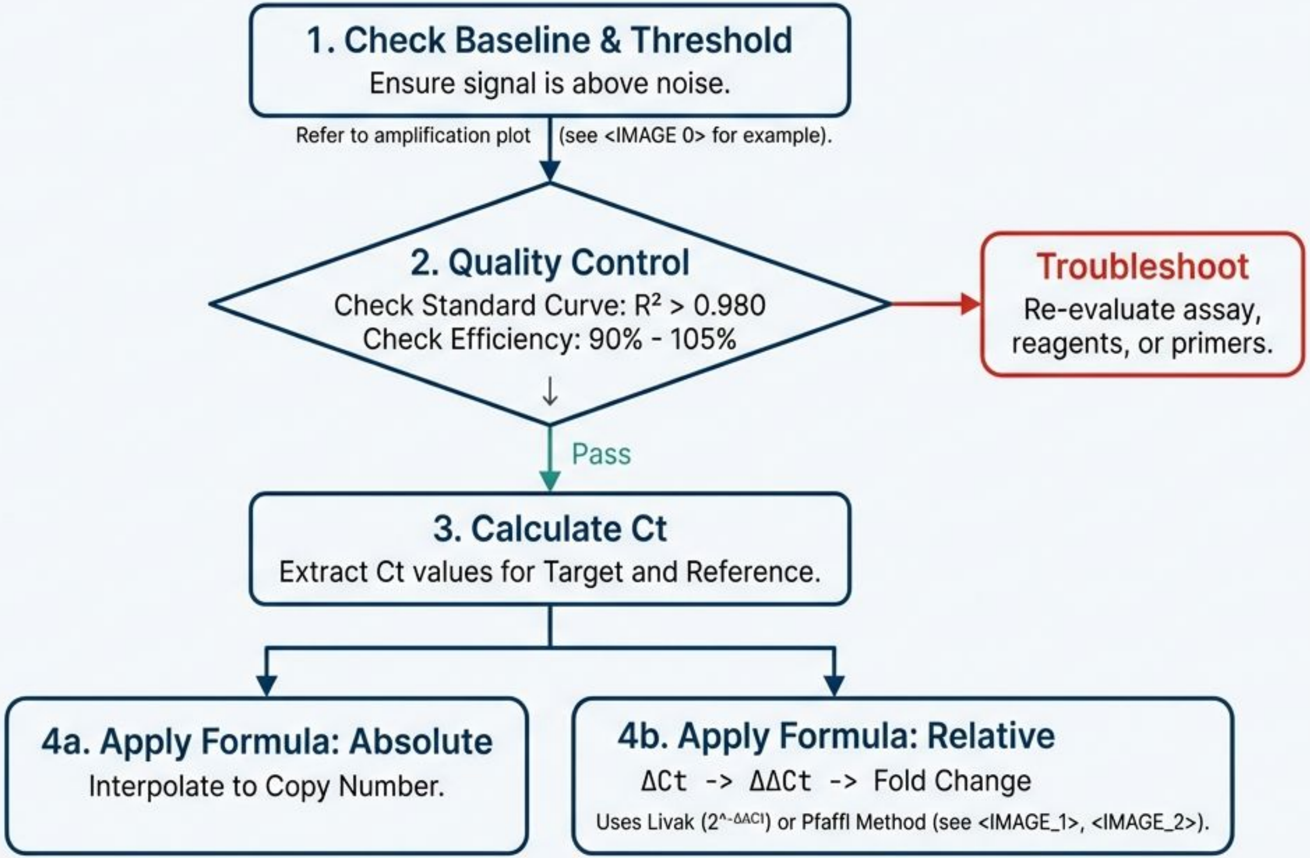
Uses specific Efficiency values calculated from standard curves instead of assuming “2”.

Takeaway

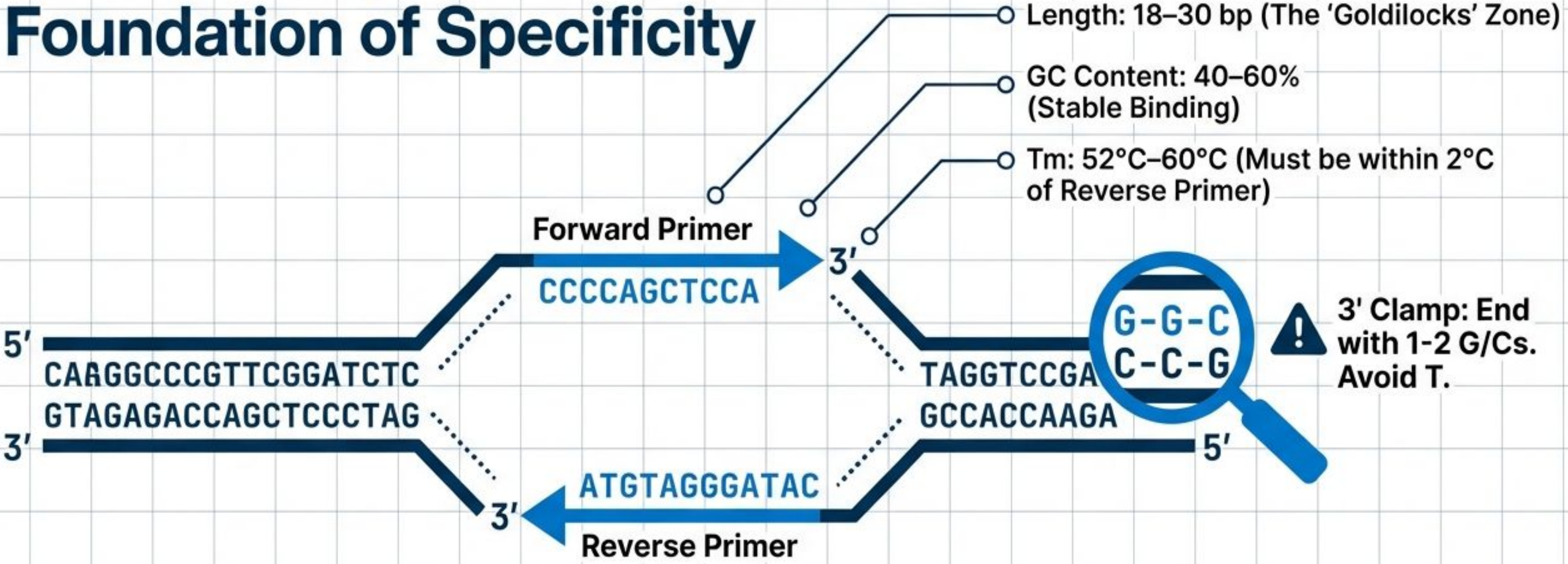
Use Pfaffl for complex biological samples or when perfect PCR conditions are impossible.

Data Analysis Workflow Summary

From Validation to Calculation



Primer Design 101: The Foundation of Specificity



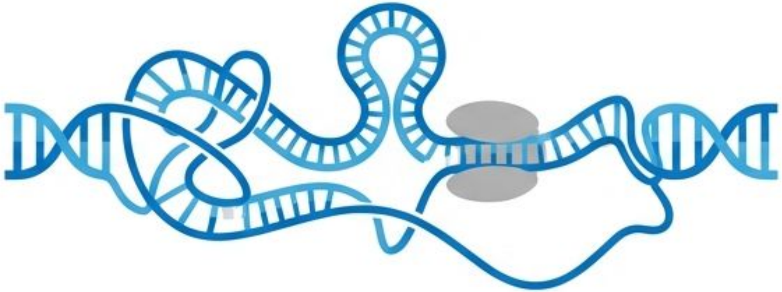
Ideally: ΔG of Dimer Formation > -4 kcal/mol (Avoid Self-Binding).

Amplicon Design: Optimization for Efficiency

OPTIMAL: The Sprinter

- **Target Length:** 70–150 bp (Probe) / 100–300 bp (SYBR)
- **Benefit:** Polymerase completes extension before cycle ends.
- **Condition:** Spans Exon-Exon Junction (cDNA only).

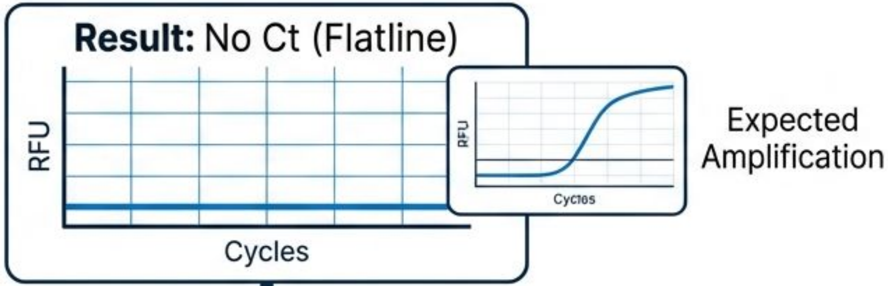
SUB-OPTIMAL: The Marathon Runner



- **Target Length:** > 300 bp
- **Risk:** Secondary structures (hairpins) block binding.
- **Result:** Reduced Efficiency.

PRO TIP: Use ‘mfold’ software to predict and avoid secondary structures.

Troubleshooting: The ‘Flatline’ (No Amplification)



REAGENTS (Chemistry)

Missing Taq, dNTPs, or Primers?

Action: Check Master Mix. Run Positive Control.

TEMPLATE (Sample)

Degraded or Low Concentration?

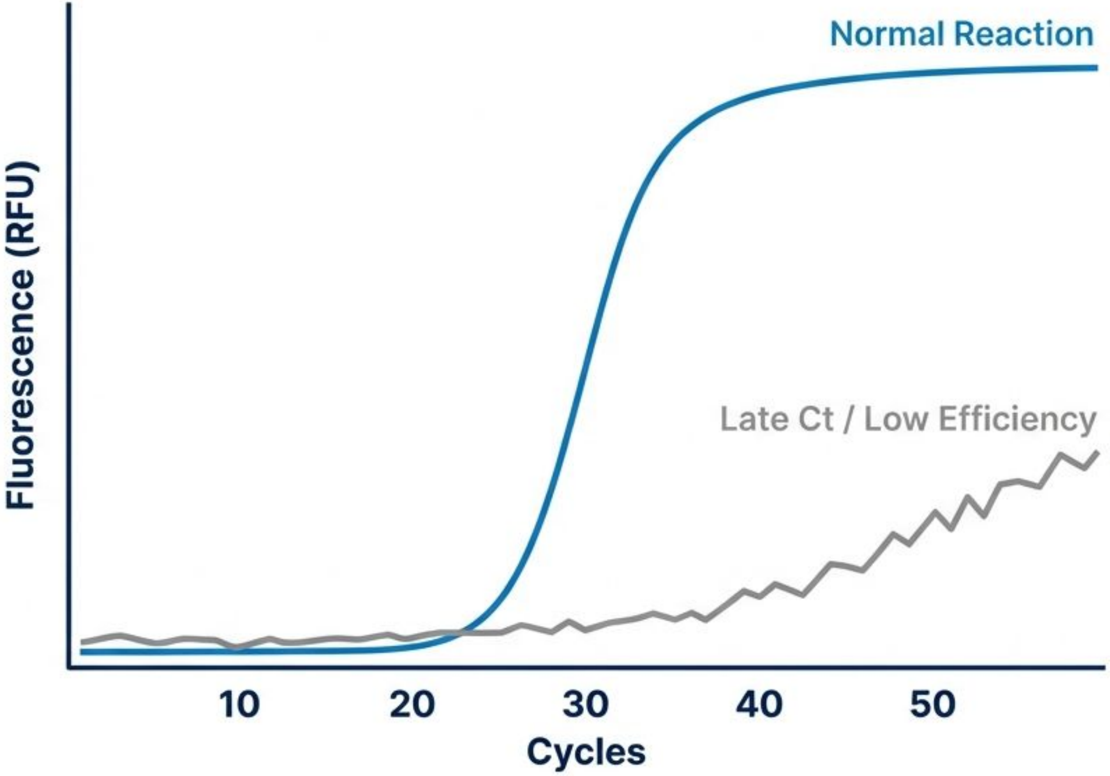
Action: Check integrity on Bioanalyzer.

INHIBITION (Environment)

Ethanol, Salt, or Hemoglobin carryover?

Action: Dilute Template (1:10). If Ct improves, inhibition is present.

Troubleshooting: Delayed Amplification (Late Ct)



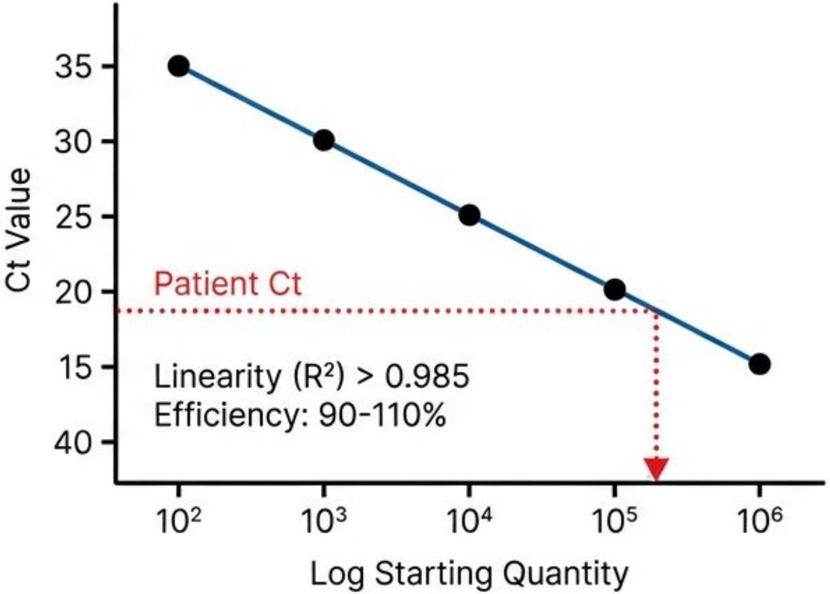
Low Target Abundance:
Not enough template to detect early.
(Solution: Increase input).
JetBrains Mono

Poor Efficiency:
Primers annealing poorly.
(Solution: Optimize Tm Gradient).

Partial Inhibition:
Reaction struggling against contaminants.
(Solution: Re-purify).

Clinical Application: Absolute Quantification (Viral Load)

The Standard Curve



The Patient Report

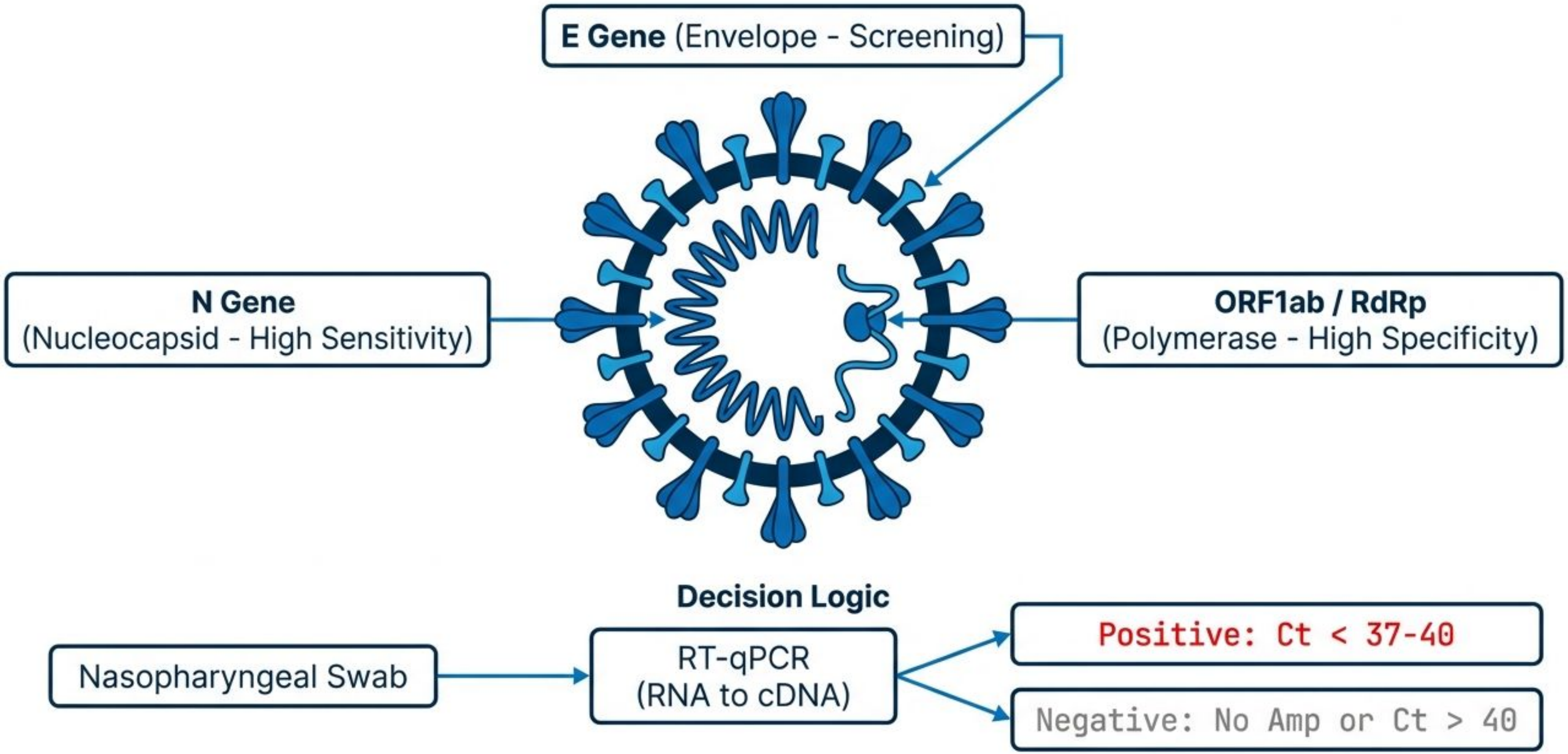
Viral Load Assay: Hepatitis B (HBV)

Sample ID: #89204-X

1.5×10^5 copies/mL

Status: Detected - Monitor Therapy Response

Diagnostic Gold Standard: COVID-19 Detection

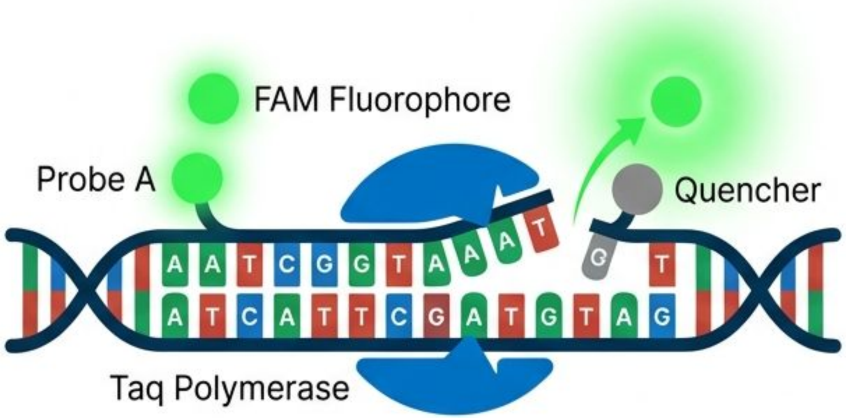


Clinical Precision meets High-End Scientific Publishing

Genotyping: The Mechanism of SNP Detection

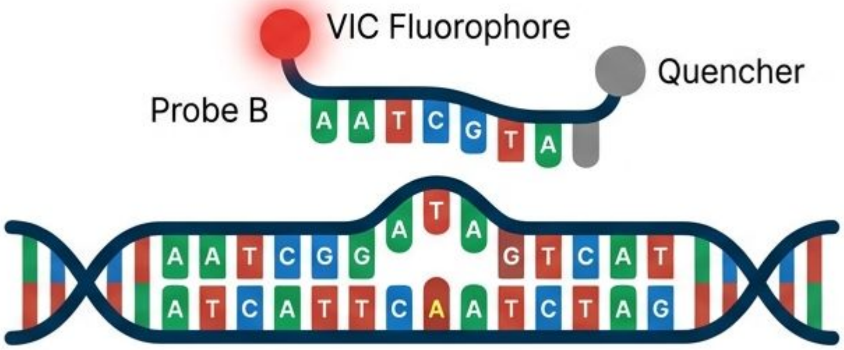
TaqMan Probe Competition (Allelic Discrimination)

The Perfect Match (Wild Type)



Match = Cleavage = Signal

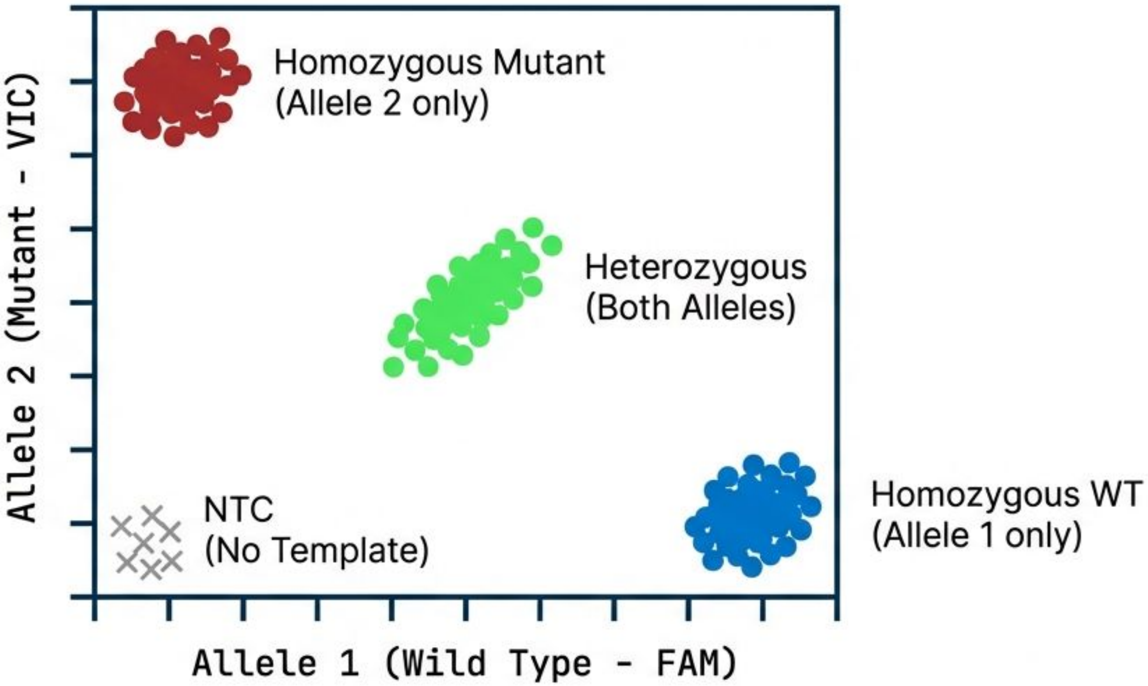
The Mismatch (Mutant)



Mismatch = Displacement = No Signal

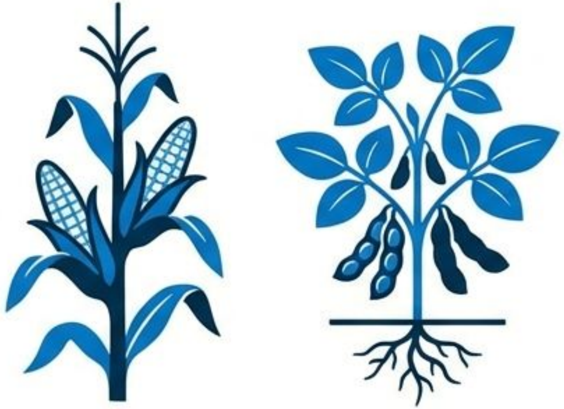
MGB Probes allow for shorter sequences (~13bp), increasing mismatch sensitivity.

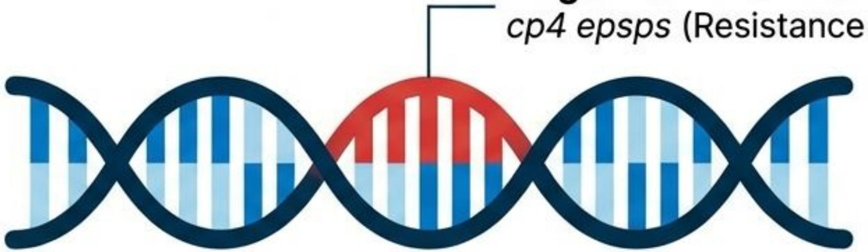
Data Analysis: Reading the Allelic Discrimination Plot



Endpoint Analysis: Position on the plot determines genotype identity.

Agricultural Application: GMO Quantification





$$\% \text{ GMO} = \frac{\text{Copy Number of Target Gene}}{\text{Copy Number of Reference Gene}} \times 100$$

(e.g., Lectin - Total Organism DNA)

Environmental Monitoring: Water & Soil Safety

Water Quality

Target: Fecal Coliforms (*Enterococcus*)

↳ **Benefit:** Speed: Result in hours (vs. 24+ days for culture).

Context: Beach & Recreational Safety.

Bioremediation

Target: *alkB* gene (Alkane Hydroxylase)

↳ **Benefit:** Tracking oil-eating bacteria populations.

Context: Confirming cleanup efficacy.

THANKS FOR LISTENING