RT-qPCR and Gene Expression Analysis

How fluorescence during amplification becomes quantitative information about RNA expression.

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PCR vs qPCR: what changes?

Endpoint detection versus real-time measurement

Conventional PCR

- Amplification occurs in cycles
- Product usually analyzed after the run
- Gel answers: band present? size correct?

qPCR / real-time PCR

- Fluorescence measured during cycling
- Amplification curve generated for each sample
- Cq/Ct provides quantitative information

Same amplification chemistry — different way of observing the reaction.

Do not confuse the terms

PCR, qPCR, RT-PCR and RT-qPCR

PCR	DNA amplification; usually endpoint detection
qPCR	real-time quantitative PCR; DNA/cDNA measured by fluorescence
RT-PCR	reverse transcription PCR: RNA → cDNA → PCR
RT-qPCR	reverse transcription + real-time qPCR; common for gene-expression analysis

Which molecule answers the biological question?

DNA abundance and RNA expression are different measurements

Question: Is the gene present?

Analyze DNA

Example: detection of acdS or a resistance gene.

Question: Is the gene being expressed?

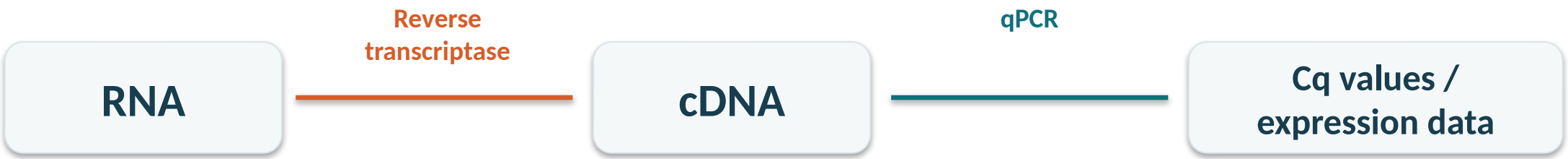
Analyze RNA → cDNA

Example: compare transcript level under control vs treatment.

Presence of a gene does not prove that the gene is actively transcribed.

Reverse transcription: RNA → cDNA

RNA itself is not amplified by standard DNA polymerase



- Use high-quality RNA.
- Include a no-RT control when genomic DNA contamination is a concern.
- Keep the reverse-transcription step consistent across samples.

One-step vs two-step RT-qPCR

Two valid workflows with different practical advantages

One-step

RNA → cDNA → qPCR in the same reaction tube

- Less handling
- Lower contamination risk
- Useful for targeted assays

Two-step

RNA → cDNA first then cDNA used in multiple qPCR assays

- Flexible cDNA archive
- Many genes from the same cDNA
- More pipetting steps

What is inside a qPCR reaction?

PCR chemistry plus a fluorescent detection system

- cDNA or DNA template
- Forward + reverse primers
- DNA polymerase
- dNTPs, Mg^{2+} and buffer
- Fluorescent chemistry: DNA-binding dye or target-specific probe
- Nuclease-free water





Accurate pipetting matters because qPCR detects small differences between samples.

What does a real-time PCR instrument add?

Thermal cycling + fluorescence excitation and detection



Conventional thermal cycler

Thermal block — **Optical system**

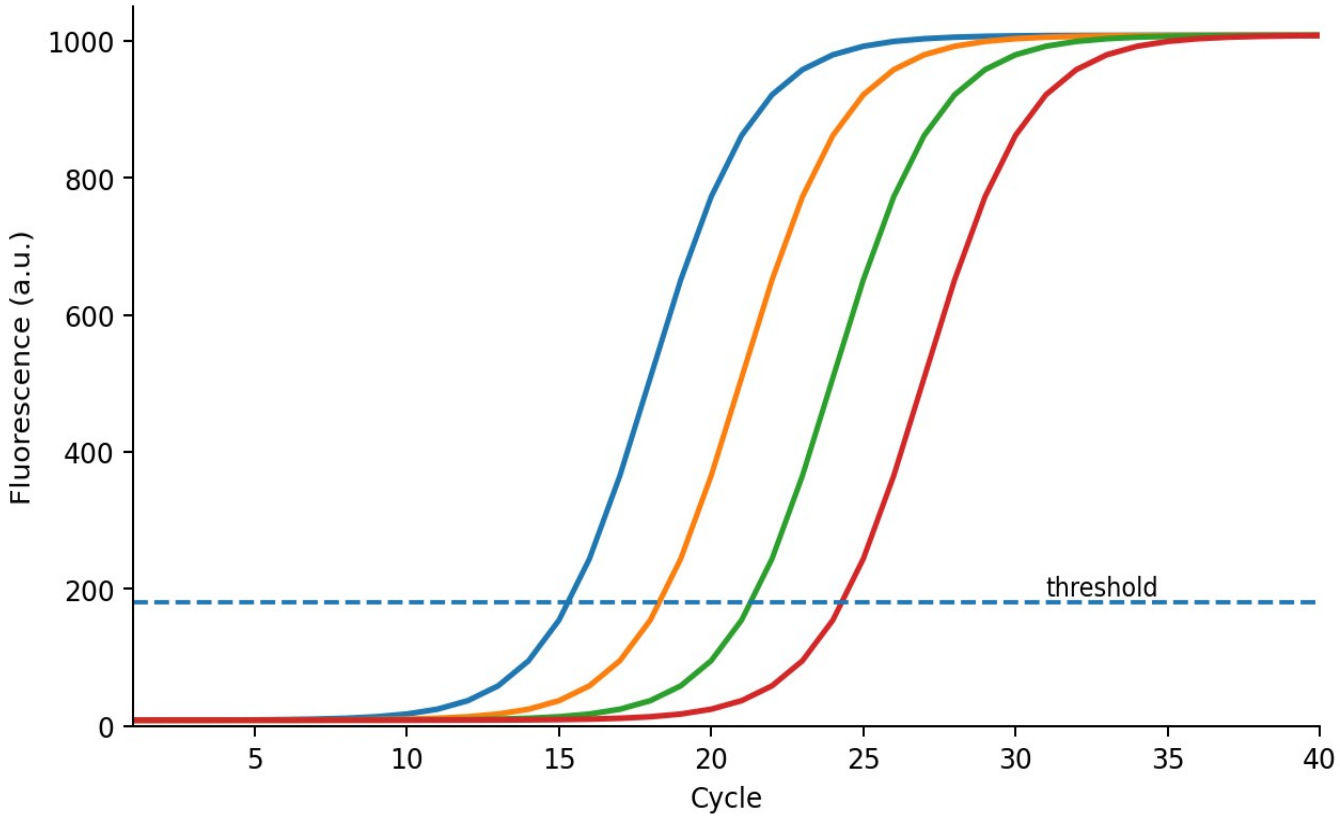
Excitation light — **Fluorescence detector**

qPCR instrument = cycling + optics + software

Real-time systems collect fluorescence data from each well during the run.

Fluorescence reports product accumulation

Signal should increase as the specific amplicon accumulates

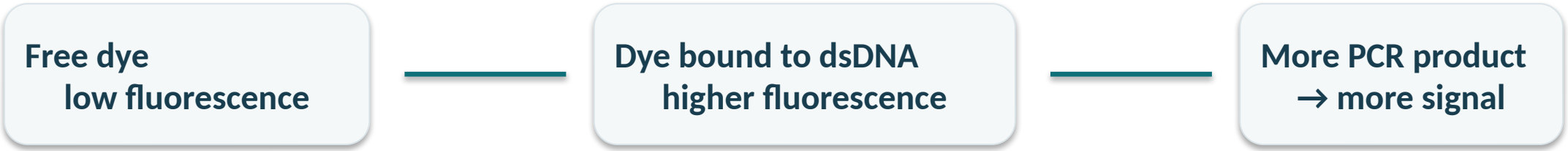


- Early cycles: signal close to background.
- Exponential phase: product and fluorescence rise rapidly.
- Late phase: reagents become limiting and the curve plateaus.

Quantification is based on the exponential region — not the plateau.

Detection chemistry 1 — DNA-binding dye

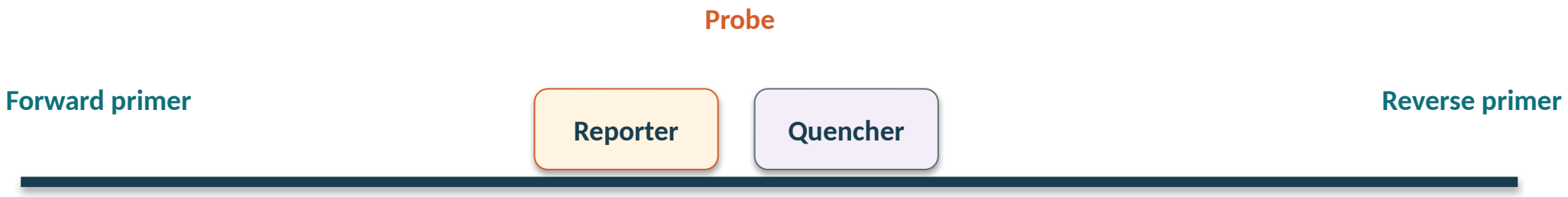
SYBR-type dyes report total double-stranded DNA



- Simple and economical.
- Signal is not sequence-specific: primer-dimers and non-specific products can also fluoresce.
- Melt-curve analysis is therefore especially useful.

Detection chemistry 2 — target-specific probe

Hydrolysis probes add a second level of sequence recognition



- Probe binds inside the target amplicon.
- Polymerase-mediated probe cleavage separates reporter from quencher.
- Fluorescence increases only when the target-specific probe is engaged.

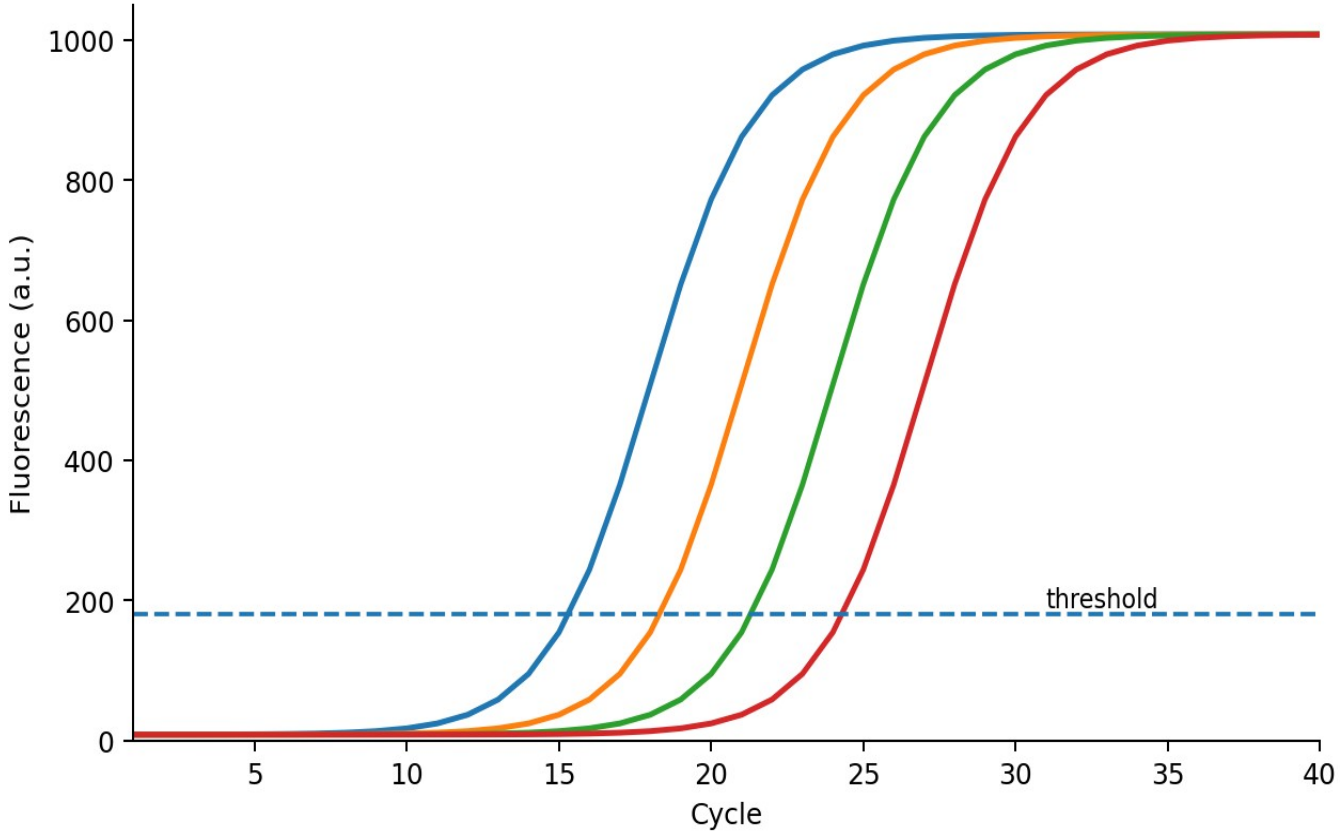
SYBR-type dye or probe?

Choose the detection chemistry for the experimental question

Feature	DNA-binding dye	Hydrolysis probe
Cost	Lower	Higher
Specificity	Primers only	Primers + probe
Melt curve	Very useful	Usually not required for probe identity
Multiplexing	Limited	Strong advantage
Assay design	Simpler	More complex

Anatomy of an amplification curve

Baseline → exponential rise → plateau



Baseline

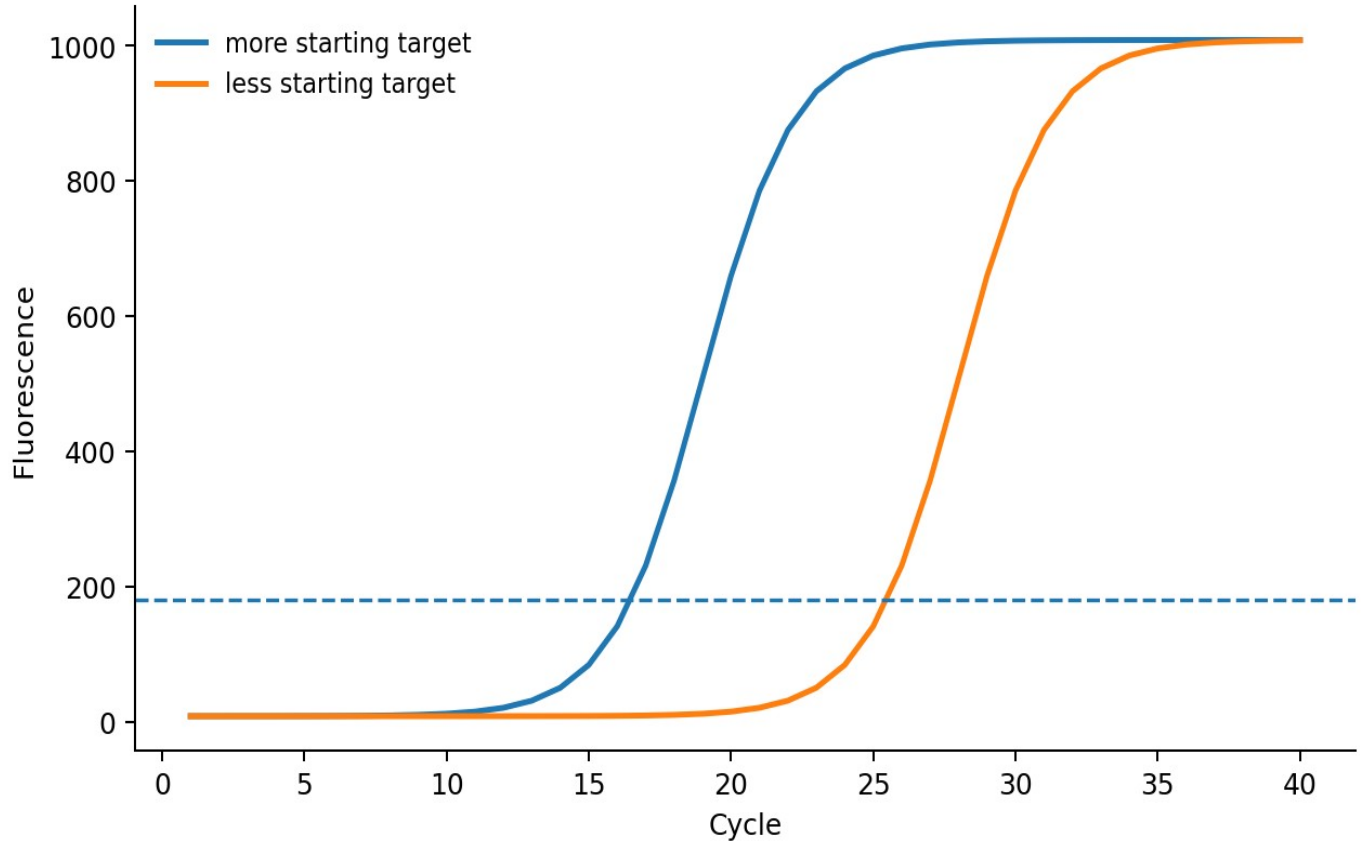
Exponential phase

Threshold crossing

Plateau

Baseline and threshold are analytical concepts

The Cq depends on where the amplification curve crosses the threshold

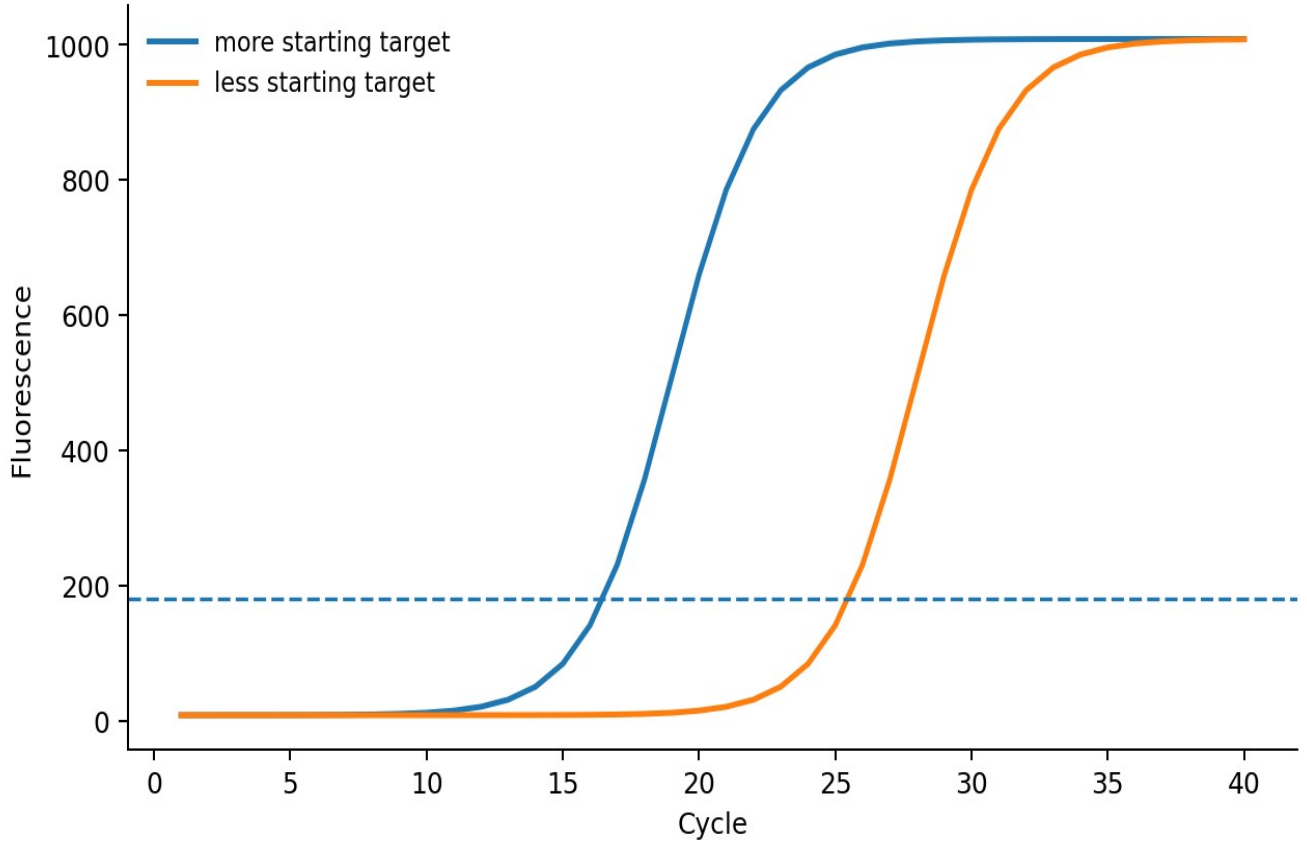


- Baseline = background fluorescence before clear amplification.
- Threshold = fluorescence level used to define the crossing cycle.
- Cq/Ct = cycle at which the amplification curve crosses that threshold.

Compare samples only when the assay and analysis settings are appropriate and consistent.

How to read Cq/Ct values

Lower Cq usually means more starting target



Cq 19

More starting target

Cq 28

Less starting target

Do not interpret an isolated Cq without controls, replicates and normalization.

Technical replicates measure technical precision

Replicates should agree before biological interpretation

Example technical triplicate

Rep 1	Rep 2	Rep 3
Cq 22.1	Cq 22.2	Cq 22.0

Tight agreement → good technical precision

Technical replicates

same biological sample
repeated wells / reactions

Biological replicates

independent biological samples
used to estimate biological variation

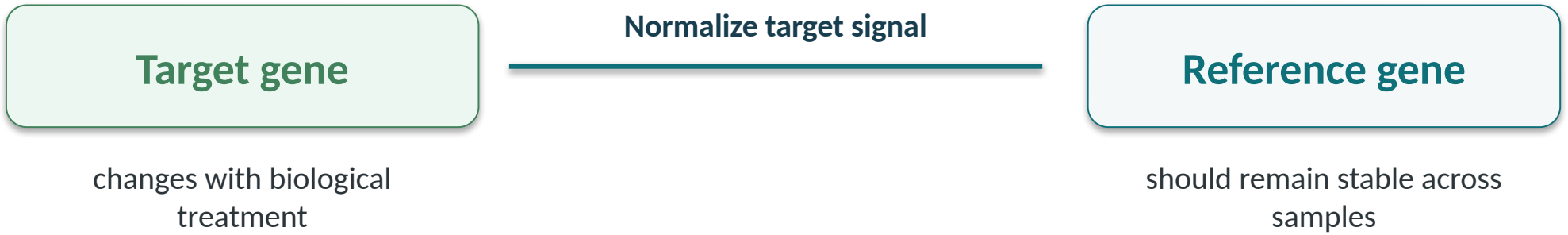
Controls in RT-qPCR

A quantitative result is only as strong as its controls

NTC	**No template added**	Detect reagent/carry-over contamination or primer-dimer
No-RT	**RNA sample without reverse transcriptase**	Detect genomic DNA contribution
Positive control	**Known amplifiable target**	Confirm assay and reaction can work

Reference genes: the normalization anchor

A reference gene should be stable under the tested conditions



- Do not assume that a “housekeeping gene” is always stable.
- Validate reference-gene behavior for the organism, tissue and treatment.
- Using more than one validated reference gene can improve robustness.

Step 1 — calculate ΔCq (ΔCt)

Normalize target gene to the reference gene within each sample

$$\Delta Cq = Cq(target) - Cq(reference)$$

Example sample

Target Cq = 25.0

Reference Cq = 20.0

ΔCq = 5.0

ΔCq is a normalized value for that sample.

Step 2 — calculate ΔΔCq

Compare normalized treatment with normalized control

$$\Delta\Delta Cq = \Delta Cq(\text{treatment}) - \Delta Cq(\text{control})$$

Control
$\Delta Cq = 5$

Treatment
$\Delta Cq = 2$

$\Delta\Delta Cq = 2 - 5 = -3$

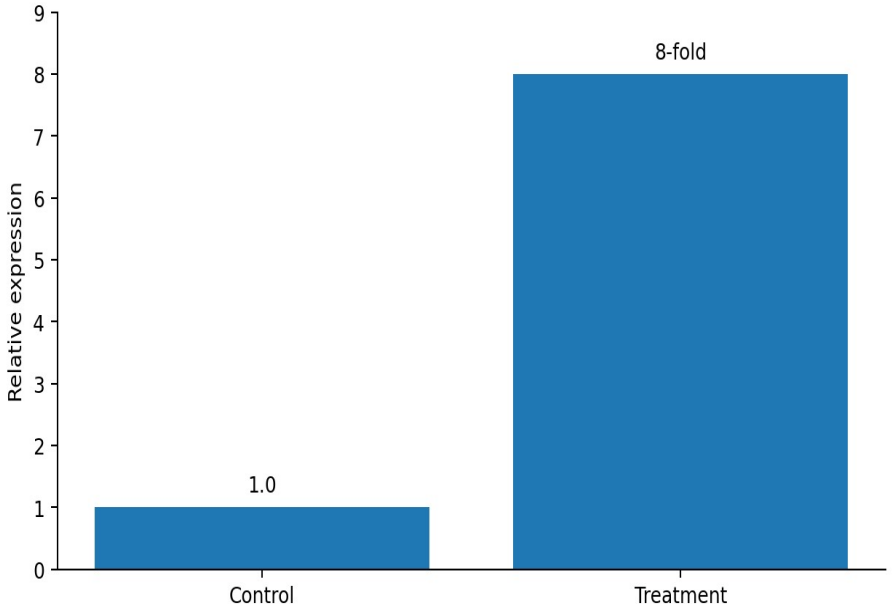
Negative ΔΔCq here means the treatment reached threshold earlier after normalization.

Step 3 — convert ΔΔCq to relative expression

For an assay with appropriate comparable amplification efficiency

Relative expression = 2^(−ΔΔCq)

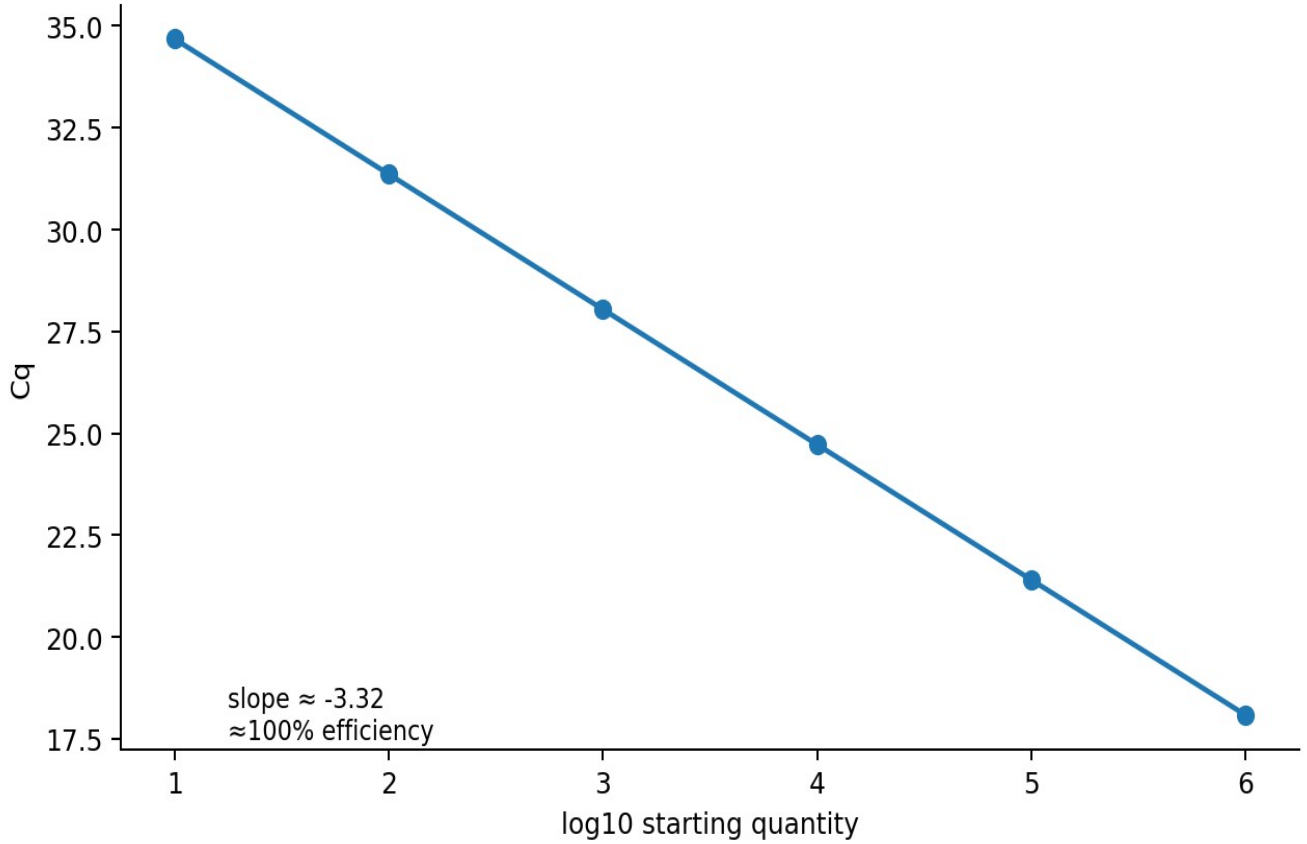
$\Delta\Delta Cq = -3 \rightarrow 2^{3} = 8$



Interpretation: treatment shows ~8-fold higher relative expression than control.

Amplification efficiency matters

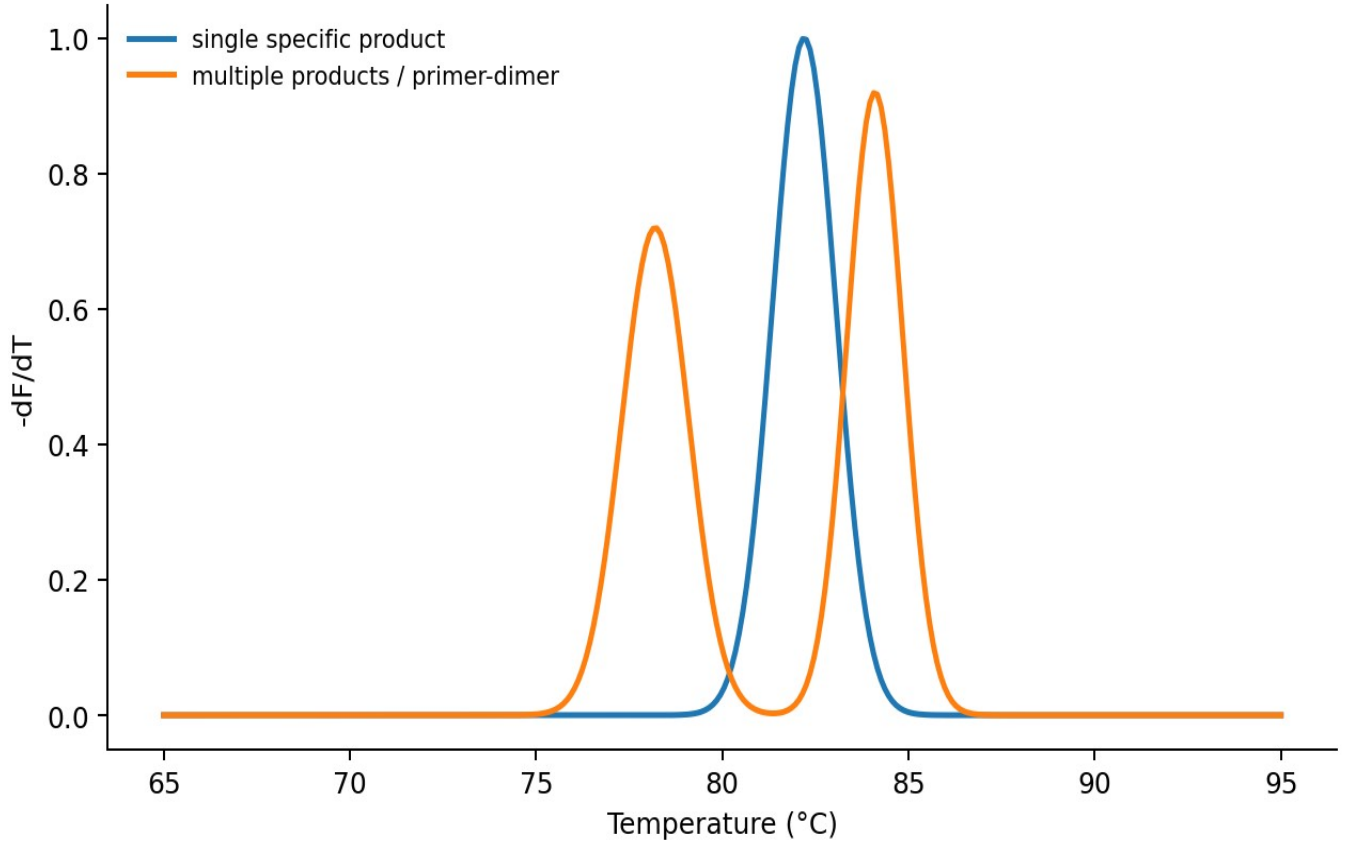
The 2^−ΔΔCq approach assumes comparable efficient amplification



- Serial template dilutions generate a standard curve.
- Slope near −3.32 corresponds to ~100% efficiency.
- A practical validated range often used is approximately 90–110%.
- Target and reference assays should have suitable, comparable efficiencies for simple ΔΔCq analysis.

Melt-curve analysis checks dye-based specificity

A single sharp peak supports one dominant amplicon



- Single dominant peak: consistent with one main product.
- Extra peaks or shoulders: possible non-specific products.
- Low-temperature peak: may indicate primer-dimer.
- Confirm questionable results with gel or sequencing when needed.

Common qPCR warning signs

Interpret the pattern before interpreting the biology

No amplification	poor template, inhibition, wrong assay, missing reagent
Late NTC Cq	contamination, primer-dimer or artifact — investigate
Wide technical replicates	pipetting / mixing / well problem
Reference gene shifts	normalization may be unreliable
Multiple melt peaks	non-specific amplification

Quality checklist before reporting expression

A small dataset can only be interpreted when the experiment is valid

- RNA quality and reverse-transcription workflow documented
- Primer/assay specificity and efficiency evaluated
- Technical and biological replicates defined
- Reference-gene stability justified
- NTC and no-RT controls checked
- Cq data interpreted before fold-change claims

Selected sources and video material

Use official technical resources for further study

RT-qPCR / qPCR

- Bustin SA et al. (2009). MIQE guidelines for quantitative real-time PCR experiments.
- Bio-Rad. Real-Time PCR Applications Guide and amplification-efficiency resources.
- Thermo Fisher Scientific. Real-time PCR and RT-qPCR learning resources.
- New England Biolabs. qPCR technical and educational resources.
- Use assay-specific validated protocols for primers, controls, efficiency and analysis.

Suggested video: qPCR amplification and Cq interpretation from an established manufacturer.
https://www.neb.com/en/tools-and-resources/video-library/overview-of-qpcr?utm_source=chatgpt.com