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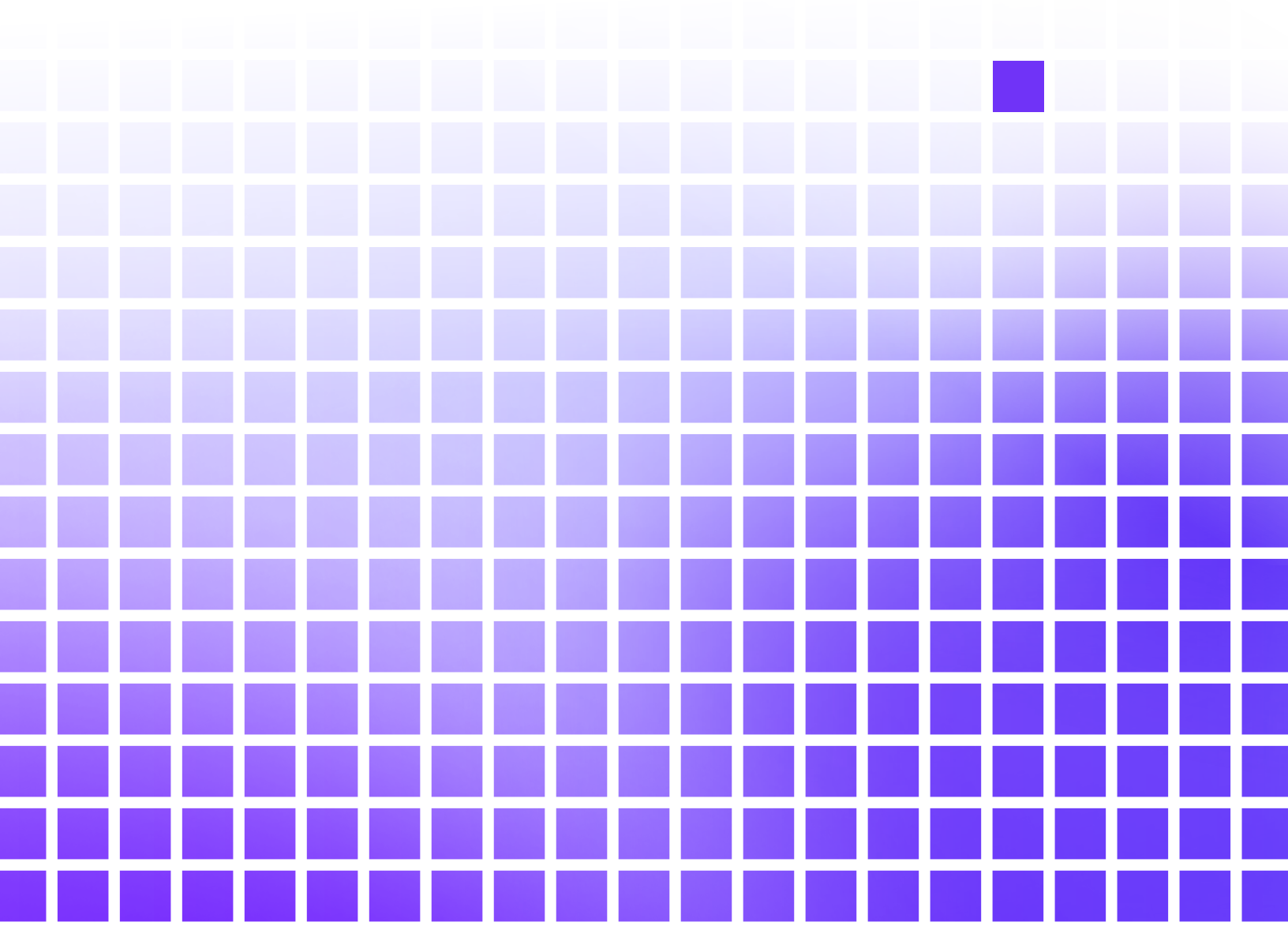
ADVANCES IN PCR QUANTIFICATION:

Choosing the right quantification technology for your experiment.



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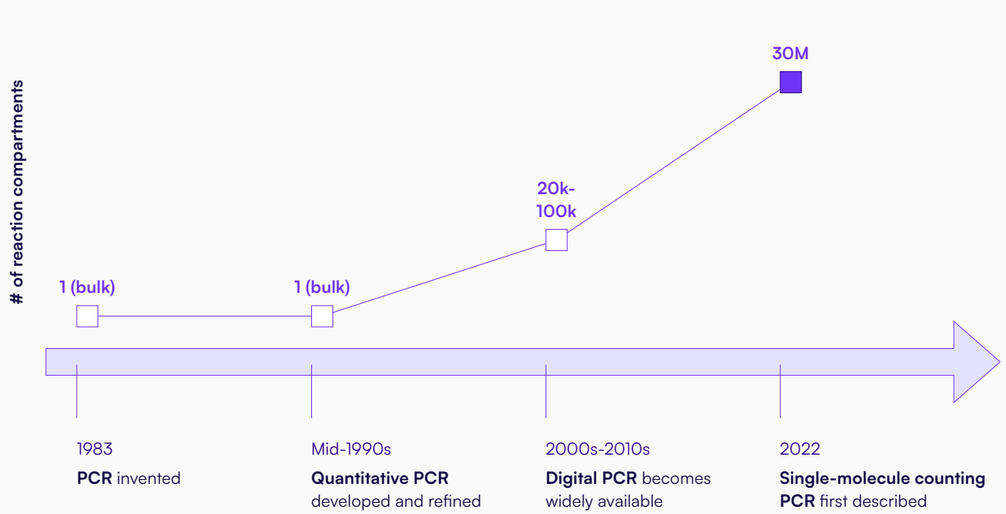
CHAPTER 1

Introduction

PCR is a foundational technique in molecular biology for amplifying specific DNA sequences, with applications spanning basic research, diagnostics, and clinical testing. Advances in PCR technology have expanded its utility beyond simple amplification to enable accurate quantification of target molecules — with uses ranging from measuring gene expression levels and detecting pathogens to determining DNA copy number. These quantification-based PCR platforms rely on fluorescent signal readout during or after amplification to achieve this. The key technological driver behind improved quantification has been the scale of partitioning: from a single bulk reaction in qPCR, to tens of thousands of compartments in dPCR, to over 30 million compartments in single-molecule counting PCR. With each generation addressing limitations of the last, selecting the right platform for a given application requires understanding where they differ in practice. Key parameters — including dynamic range, sensitivity, CVs, multiplexing capability, and analysis approach — are critical when considering quantification platforms. This article reviews each platform in turn and provides a framework for selecting the most appropriate method for a given experiment or study.



Evolution of PCR technology and increasing partitioning. Since 1983, PCR has advanced into a quantification method and subsequently increased sample partitioning for more accurate measurements — from the bulk reactions of quantitative PCR, to digital PCR (~20,000-100,000 compartmentalized reactions), to single-molecule counting PCR (~30 million compartmentalized reactions). Finer partitioning allows each target molecule to amplify independently, minimizing competition and preserving low-abundance signals.

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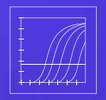
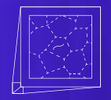
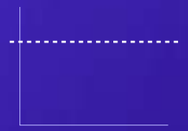
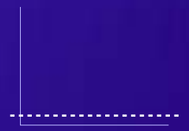
AT A GLANCE:

PCR quantification methods

	Quantitative PCR (qPCR)	Digital PCR (dPCR)	Single-molecule counting PCR
Quantification method	Real-time detection based on amplification kinetics  Relative to reference or standard curve	End-point detection based on binary signal "Absolute" signal with Poisson correction for multiple molecules per partition	End-point detection based on binary signal  Direct single-molecule counting
Dynamic range	10^6 — 10^7 range Theoretically broad, contingent on linear amplification	10^4 — 10^5 range Narrow range, better precision	10^6 range Broad range, highest precision
Precision	Low 50% — 100% CV	Moderate 10% CV	High 1% CV
Sensitivity	Limited  Typically >1% VAF	High, with effort About 0.03% VAF	Highest  0.004% VAF

CHAPTER 2

PCR methods for nucleic acid quantification

Quantitative PCR (qPCR)

Quantitative PCR is the most widely used method for nucleic acid quantification. First developed in the 1990s, qPCR detects the accumulation of fluorescent signal during PCR amplification in real time (Higuchi et al. 1992, Kubista et al. 2006). As DNA targets are copied each cycle, fluorescent intensity increases. The qPCR instrument measures this fluorescence each cycle and plots an amplification curve, producing a Cq (or Ct) value — the cycle number at which fluorescence crosses a set threshold. Samples with more starting target molecules reach that threshold earlier (lower Cq); samples with fewer take longer (higher Cq).

Two detection chemistries are available. Intercalating dyes (such as SYBR dyes) are sequence-nonspecific — they fluoresce whenever bound to double-stranded DNA, making them versatile and cost-effective, but limited to singleplex reactions due to background signal from primer dimers or non-specific amplification (Neta and Lapis 2009). Hydrolysis probes (such as TaqMan probes) hybridize to a specific target sequence and carry two fluorescent labels — a reporter dye and a quencher — held in close proximity so that the quencher suppresses fluorescent signal while the probe is intact (Heid et al. 1996). During PCR extension, Taq polymerase cleaves the probe, separating reporter from quencher and releasing a fluorescent signal proportional to target amplification. Because the signal is sequence-specific, multiple targets can be detected in the same reaction using probes with different reporter fluorophores, enabling multiplexing — though at significantly higher cost and assay optimization (Heid et al. 1996).

qPCR supports two analysis methods (Harshitha and Arunraj 2021). Relative quantification measures target abundance against a reference gene in the same sample, expressing the result as a fold-change. This is the standard approach used in gene expression studies. A more accurate analysis method converts Cq values into copy numbers using a standard curve — a dilution series of known concentrations run alongside test samples on every plate — from which the target abundance can be calculated for each sample.

qPCR limitations

At high target concentrations, the number of positive partitions and partitions with >1 molecule increases, and Poisson statistics become less reliable. When analyzing rare targets, dead volume inherent to microfluidic systems — which can account for up to 50% of sample input — limits sensitivity. Thresholding individual partitions as positive or negative can also be inconsistent across experiments, particularly in multiplexed assays, and this variability can significantly affect final quantification (Whale et al. 2016). Finally, multiplexing can be challenging and lead to partition-specific competition (PSC) when multiple DNA targets co-localize to the same partition and compete for PCR reagents (Whale et al. 2016).

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Digital PCR (dPCR)

Digital PCR was developed to address the central limitation of qPCR: inferential quantification. Rather than estimating target number from amplification kinetics, dPCR converts amplification into a direct digital readout by partitioning a sample into thousands of individual reactions — using droplets, microfluidic arrays, or nanoplates depending on the platform — and counting how many have positive fluorescent signal (end-point detection; Quan et al. 2018). This avoids the issue of PCR amplification bias seen in qPCR, which measures fluorescent signal in real-time. Both intercalating dyes and hydrolysis probes are compatible; hydrolysis probes are required for multiplexed applications.

dPCR divides a sample into approximately 20,000 individual reactions (platforms and configurations vary; commercialized platforms are typically limited to less than 100,000 partitions due to manufacturing and physical constraints). The instrument reads each partition as positive or negative, and the fraction of negative partitions is used with Poisson statistics to back-calculate the likely number of target molecules in the original sample. Compared to qPCR, dPCR is more robust to sample quality variation and PCR inhibitors.

dPCR limitations

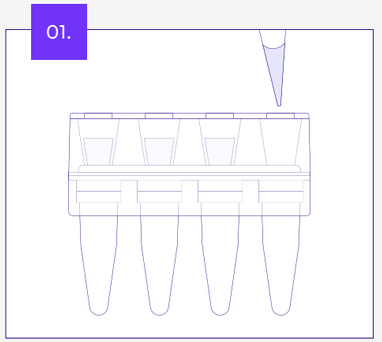
At high target concentrations, the number of positive partitions and partitions with >1 molecule increases, and Poisson statistics become less reliable. When analyzing rare targets, dead volume inherent to microfluidic systems — which can account for up to 50% of sample input — limits sensitivity. Thresholding individual partitions as positive or negative can also be inconsistent across experiments, particularly in multiplexed assays, and this variability can significantly affect final quantification (Whale et al. 2016). Finally, multiplexing can be challenging and lead to partition-specific competition (PSC) when multiple DNA targets co-localize to the same partition and compete for PCR reagents (Whale et al. 2016).

Single-molecule counting PCR

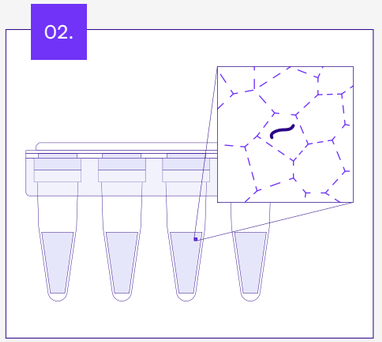
Single-molecule counting PCR (formerly known as UltraPCR) applies the same core principle as dPCR — dividing a sample into separate reactions and counting binary amplification events — but it scales the number of compartments by approximately 1,000-fold (~30 million compartments). At this scale, individual target molecules are much less likely to share a compartment with another target molecule. Platforms such as Countable PCR (https://countablelabs.com/resources/countable-pcr-6log-dynamic-range-validation) use a gel-like matrix generated via spin column to isolate single molecules into compartments; the transparent matrix allows molecules (detected via fluorescent probes) to be imaged directly in the PCR tube after amplification. Since molecules are effectively isolated one-per-compartment, Poisson statistics are not required — binary fluorescent signals are counted directly.

Single-molecule compartmentalization also eliminates PCR bias, increasing accuracy and also simplifying optimization for multiplex assays. The high number of compartments per reaction supports a broad dynamic range, detecting from ten to up to one million target molecules, and similarly to dPCR, is less susceptible to PCR inhibitors. Additional advantages include

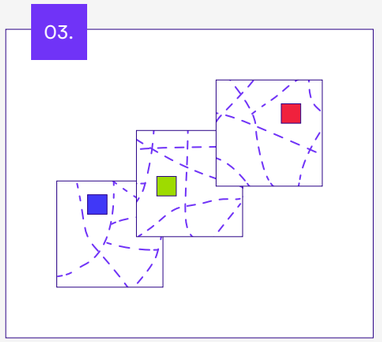
Single-molecule counting PCR workflow.



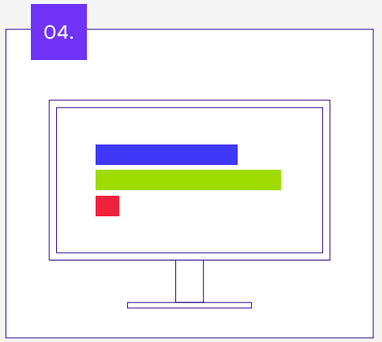
Prepare reaction
Add sample and reaction components to spin column



Generate matrix
Centrifuge to isolate single molecules



Amplify targets
Perform PCR to amplify target signal



Count targets
Quantify using 3D light sheet imaging

Single-molecule counting with a simple PCR workflow. Single molecules are isolated in the PCR reaction using a spin column to create a gel-like matrix, then amplified and imaged in the same tube for direct counting.

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the ability to design assays with Universal Multiplexing probes (see Highlight box) and the ability to perform single-molecule DNA linkage analysis.

Single-molecule linkage analysis

An additional feature of single-molecule counting PCR - not possible on other platforms without complex statistical frameworks - is direct DNA linkage analysis: the simultaneous detection of multiple regions on the same DNA molecule. This is uniquely enabled by the gel-like matrix, which physically stabilizes individual molecules in space. Because each molecule occupies a defined location without co-occupancy of a compartment by multiple target molecules or fragments, optical signatures from a given compartment can confirm the detected sequences originate from a single molecule - rather than representing independent molecules.

This capability has broad utility across applications where the physical relationship between genomic regions matters, not just their individual presence or abundance. In viral vector manufacturing, linkage analysis can confirm genome integrity by verifying that critical functional elements — such as inverted terminal repeats, transgene sequences, and regulatory elements — are present and intact on the same genome. In gene editing, it can distinguish between desired on-target edits and unintended insertions or deletions at adjacent sites. For CNV analysis, linkage provides resolution beyond copy number alone by confirming whether duplicated sequences are physically connected. The same principle applies to detecting gene fusions and characterizing insertion/deletion events, where confirming the structural relationship between sequences is essential to interpretation.

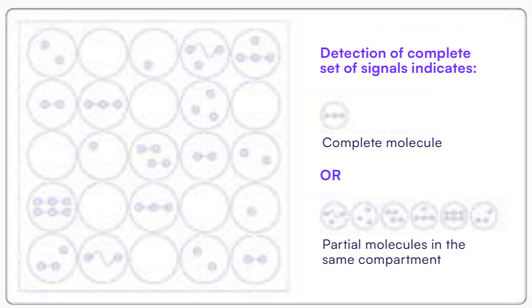
Single-molecule PCR limitations

The primary tradeoffs relative to qPCR and dPCR are platform novelty and availability. As a newer technology, the range of validated assays and third-party reagent support is more limited than for established platforms. Single-molecule counting PCR is also incompatible with intercalating dyes such as SYBR green — which rely on bulk signal from double-stranded DNA and are not suitable for single-compartment detection — meaning existing qPCR assays using dye-based chemistries would need to be converted to hydrolysis probes or Universal Multiplexing probes before use. Sample throughput is currently limited to 96 samples per run, compared to 384-well formats available on qPCR and some dPCR platforms. However, the ability to load higher sample volumes per reaction and multiplex multiple targets in a single tube can offset this in practice — for rare variant detection applications such as cfDNA, where dPCR often requires splitting samples across multiple reactions, or where several targets per sample are needed, the total number of reactions required may actually be lower on a single-molecule counting platform.

DNA linkage analysis across PCR technologies

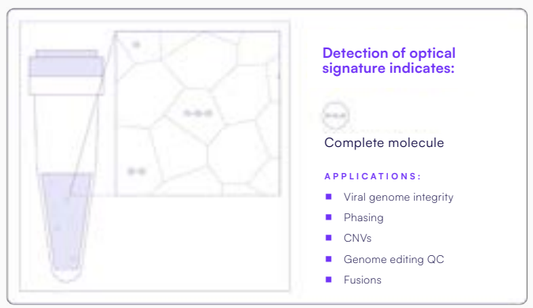
dPCR

Multiple molecules in a single partition



Single-molecule counting PCR

One molecule per compartment

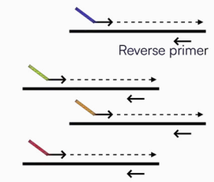


Measurement of DNA linkage across PCR technologies. Probes targeting multiple regions of a molecule are used to measure DNA linkage. In dPCR, where multiple molecules can occupy a single partition, detection of a complete set of signals could indicate either one complete molecule or several partial molecules in the same compartment (left). Single-molecule counting PCR isolates individual molecules, giving higher-confidence detection — a complete optical signature reliably reflects an intact molecule (right).

Highlight Box:

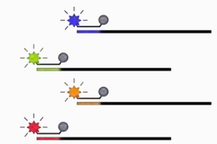
Universal Multiplexing

A **Universal adapter** sequence is appended to the primer.

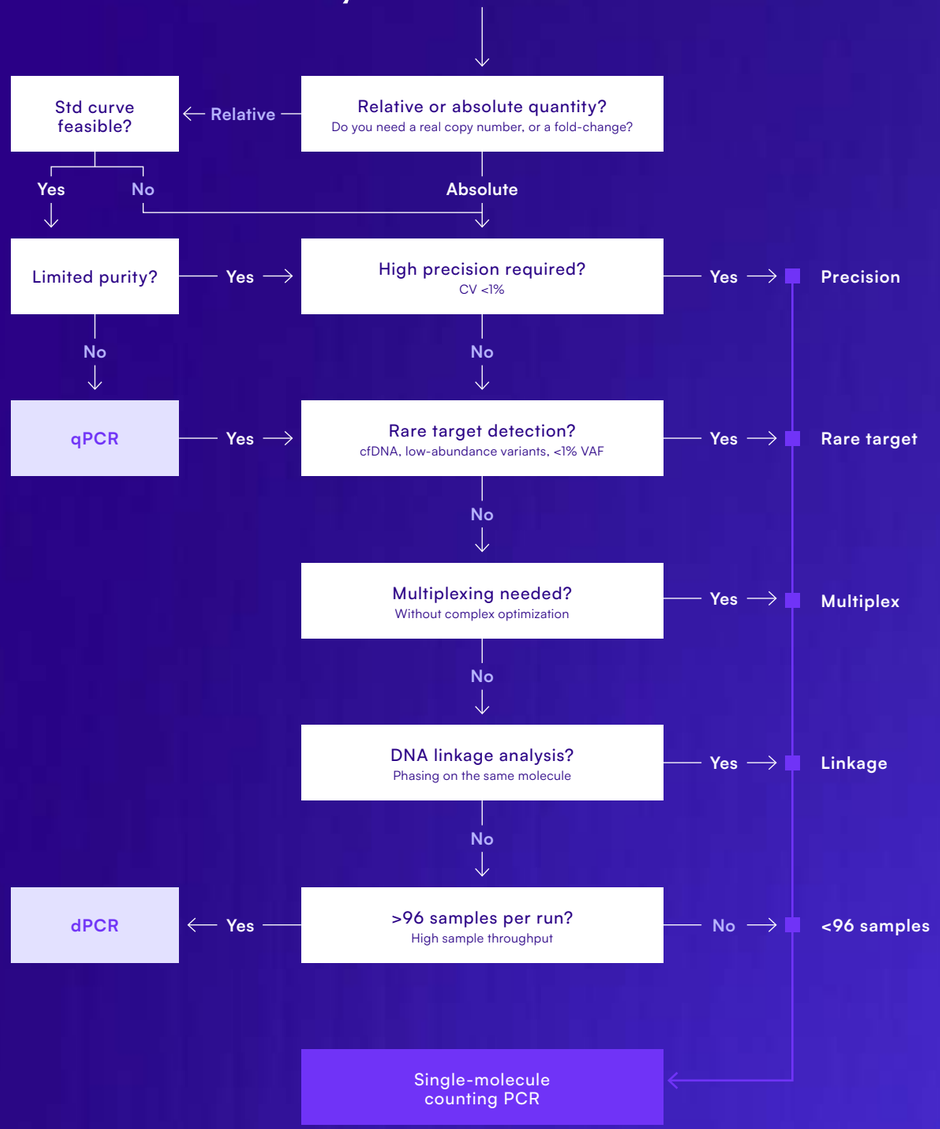


During PCR, an amplicon is generated that contains the adaptor complement for a **Universal probe** to hybridize to.

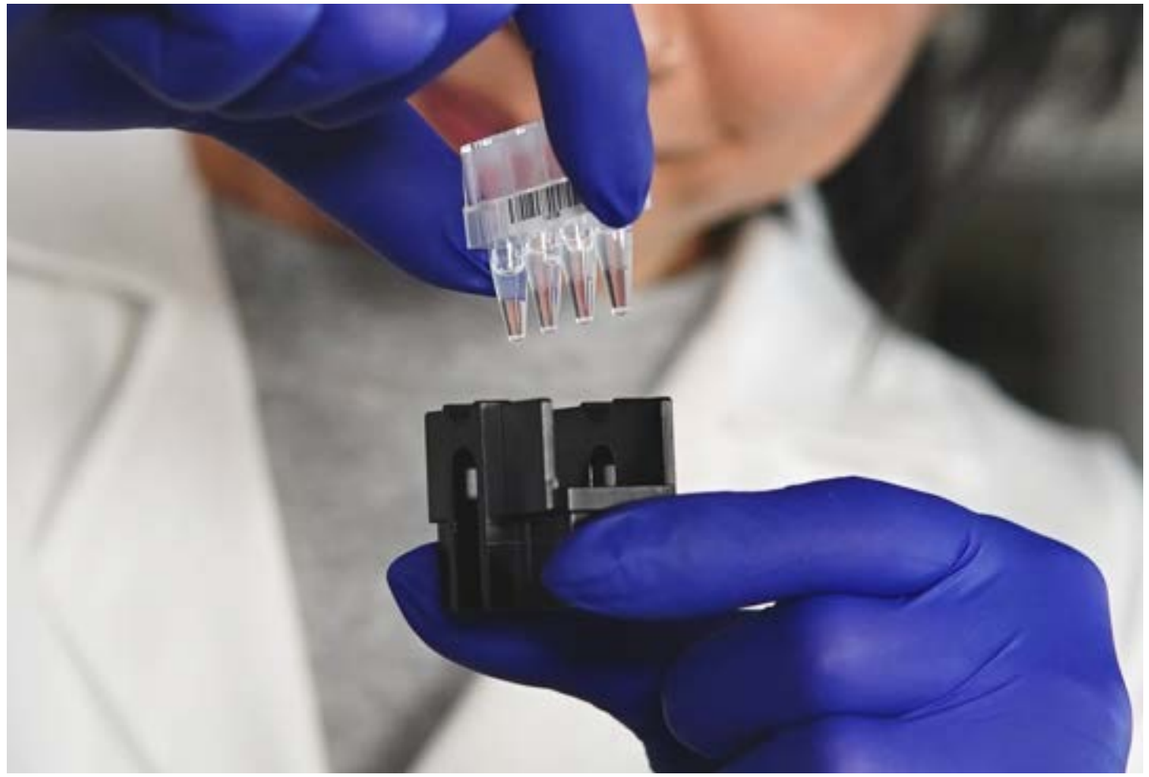
Fluorescent signal is imaged and counted.



What do you need to measure?



Choosing a PCR method by analytical requirement. Starting from whether the application needs absolute or relative quantification, this decision tree routes through precision, rare-target detection, multiplexing, linkage, and throughput requirements to the best-suited method—qPCR, dPCR, or single-molecule counting PCR.



CHAPTER 3

Choosing a PCR technology

Selecting the right PCR platform depends on what the experiment needs to measure and how precisely. A primary consideration is whether relative or absolute quantification is required. For gene expression studies where fold-change between conditions is the primary output, qPCR is often appropriate: it is well-established, cost-effective, and broadly supported across laboratory workflows. Where absolute quantification matters — viral titration, rare variant detection, copy number variation — qPCR's reliance on standard curve interpolation limits accuracy, and digital and single-molecule counting approaches are more reliable.

Sensitivity and reproducibility requirements further differentiate the platforms. dPCR and single-molecule counting PCR eliminate standard curve dependency and are less susceptible to PCR inhibitors, improving consistency across runs and operators. For rare target detection, the total partition count limits how much sample can be analyzed per reaction, often requiring sample splitting across multiple runs for dPCR analysis. Single-molecule counting PCR addresses this directly: the higher compartment count supports rare target detection without sample splitting, and the broad dynamic range accommodates target counts spanning several orders of magnitude within a single assay.

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Multiplexing is an important practical consideration. Detecting multiple targets in a single reaction reduces reagent consumption and streamlines workflow, but the most significant advantage may be internal standardization — co-detecting wild-type and variant sequences in the same tube, or normalizing a target against a reference sequence, which improves quantitative accuracy and experimental robustness. All PCR technologies discussed here support probe-based multiplexing, though differential amplification efficiency between targets can introduce bias in qPCR and dPCR, increasing the optimization burden for multi-target assays. Single-molecule counting PCR reduces this burden in two ways: compartmentalization of individual molecules means each target is amplified independently, minimizing inter-target competition, and Universal Multiplexing probe architecture eliminates the need to design and optimize target-specific probes for each assay, substantially reducing both cost and development time.

Finally, for applications where DNA linkage is important such as in viral vector manufacturing or looking at genomic rearrangements or genome editing fidelity, single-molecule counting PCR can analyze regions on the same molecule without complicated statistical deconvolution required on dPCR platforms.

CHAPTER 4

Lab management and best practices

When procuring a new PCR platform, several practical considerations beyond experimental performance also deserve attention.

Workflow integration is one: how the platform fits into existing laboratory data management systems, and what additional informatics or analysis pipelines are needed for adoption. Reproducibility across operators, labs, or sites is another — particularly for assays that need to generate comparable results over time or in multi-site studies. Calibration and maintenance requirements also vary considerably across platforms and should be evaluated alongside performance. These factors can be crucial to whether a PCR platform is able to meet the goals of the laboratory in addition to experimental performance.

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