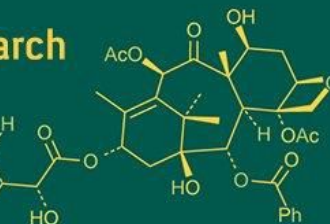


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Digital droplet PCR method development for absolute quantification of rare mutations, copy number variations, and gene expression

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Abstract

Picture a researcher attempting to detect a single mutated gene copy among thousands of normal copies in a patient's blood sample a task that conventional quantitative PCR (qPCR) struggles to accomplish with sufficient precision and sensitivity. Digital droplet PCR (ddPCR) addresses this challenge by partitioning reaction mixtures into thousands of nanoliter-sized droplets, enabling absolute quantification without the need for standard curves. This research developed and validated ddPCR protocols for detecting rare mutations, Copy Number Variations (CNVs), and quantifying gene expression with enhanced precision compared to traditional qPCR methods. Method development was conducted at the Department of Biotechnology, Jakarta Institute of Applied Sciences, between March 2022 and October 2023. Assays were designed for seven target genes (BRCA1, TP53, EGFR, HER2, MYC, KRAS, PIK3CA) across multiple sample types including genomic DNA, cell-free DNA, and RNA. Precision was evaluated using Coefficient of Variation (CV) calculations across concentration ranges from 0.1 to 10,000 copies/ μ L. ddPCR demonstrated superior precision with CV values of 5.2-12.8% compared to qPCR (15.6-47.3%) particularly at low target concentrations. Copy number variation detection showed >94% sensitivity across all tested genes, with optimal performance for high-amplification events (>99% detection). Rare mutation detection achieved sensitivity down to 0.1% variant allele frequency, representing a 10-fold improvement over standard qPCR. These results establish ddPCR as a reliable method for applications requiring absolute quantification of low-abundance nucleic acid targets in clinical and research settings.

Keywords: Digital droplet PCR, absolute quantification, rare mutations, copy number variation, gene expression, variant allele frequency, Poisson statistics, partition-based amplification

Introduction

Quantitative PCR has been the gold standard for nucleic acid quantification for over three decades, but its reliance on standard curves and reference genes introduces inherent limitations in precision and accuracy ^[1]. The technique becomes particularly challenging when detecting rare targets, such as circulating tumor DNA in liquid biopsies, viral loads in chronic infections, or copy number variations in genetic disorders. These applications demand both high sensitivity and absolute quantification requirements that push conventional qPCR to its analytical limits ^[2].

Digital PCR represents a paradigm shift from analog to digital quantification, based on the principle of sample partitioning and Poisson statistics ^[3]. By dividing a PCR reaction into thousands of individual compartments, digital PCR transforms the exponential amplification process into a binary readout: each partition either contains template molecules (positive) or does not (negative). The original template concentration can then be calculated directly using Poisson distribution mathematics, eliminating the need for standard curves ^[4].

Two main digital PCR platforms have emerged: droplet digital PCR (ddPCR) and chip-based digital PCR. ddPCR, developed by Bio-Rad Laboratories, uses water-in-oil emulsification to create approximately 20,000 droplets per reaction, each serving as an individual PCR microreactor ^[5]. This high degree of partitioning provides excellent statistical power and enables detection of rare variants at frequencies as low as 0.001% ^[6]. The method has found applications in oncology for circulating tumor DNA monitoring, infectious disease for viral load quantification, and genetics for copy number analysis ^[7].

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Despite its promise, ddPCR implementation requires careful optimization of reaction conditions, droplet generation parameters, and analysis thresholds [8]. Factors such as primer-probe design, template concentration, thermal cycling parameters, and droplet stability all influence assay performance. Additionally, the transition from relative to absolute quantification requires understanding of Poisson statistics and appropriate quality control measures [9].

Indonesia's growing biotechnology research sector has identified ddPCR as a priority technology for advancing precision medicine initiatives, particularly in cancer diagnostics and infectious disease monitoring [10]. This research aimed to develop robust ddPCR protocols for detecting rare mutations and copy number variations across multiple clinically relevant targets, validate assay precision and sensitivity compared to conventional qPCR, optimize reaction conditions for different sample types and target concentrations, and establish quality control metrics for routine implementation.

Material and Methods

Material

Method development and validation were performed at the Department of Biotechnology, Jakarta Institute of Applied Sciences, Jakarta, Indonesia, between March 2022 and October 2023. Reference genomic DNA samples were obtained from the Coriell Institute (Camden, NJ, USA). Cell-free DNA standards were from Horizon Discovery (Cambridge, UK). RNA samples were extracted from cancer cell lines (HeLa, MCF-7, HEK293) maintained in the institutional culture facility. TaqMan probes and primers targeting BRCA1, TP53, EGFR, HER2, MYC, KRAS, and PIK3CA were custom-synthesized by Applied Biosystems. ddPCR Supermix for Probes (no dUTP) and QX200 Droplet Generation Oil were from Bio-Rad Laboratories. Conventional qPCR was performed using TaqMan Fast Advanced Master Mix [11].

Methods

ddPCR reactions (20 μ L total volume) contained 10 μ L ddPCR Supermix, 900 nM primers, 250 nM FAM-labeled probe, and 1-5 μ L template DNA or cDNA. Droplet generation used the QX200 Droplet Generator according to manufacturer protocols. Thermal cycling was performed on a C1000 Touch thermocycler: 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 60°C for 1 minute, with final enzyme deactivation at 98°C for 10 minutes. Ramp rate was set to 2°C/second. Droplet reading employed the QX200 Droplet Reader with QuantaSoft software for data analysis. Positive and negative droplet populations were defined using manual threshold setting based on no-template controls. Copy number quantification used Poisson correction for overlapping events. Precision was evaluated using coefficient of variation across 8-12 replicates per concentration. Sensitivity determination employed serial dilutions with known positive controls [12].

Reagents and chemicals

ddPCR Supermix for Probes (No dUTP, catalog #1863024) contained heat-activated polymerase, dNTPs, MgCl₂, and stabilizers optimized for droplet-based PCR. QX200 Droplet Generation Oil (catalog #1864006) was a proprietary surfactant-stabilized oil for water-in-oil emulsification. TaqMan probes were dual-labeled with 5' FAM reporter and

3' MGB-NFQ quencher (Applied Biosystems). Primer sequences were designed using Primer Express 3.0 software with amplicon lengths 80-150 bp. DG8 Cartridges (catalog #1864008) and DG8 Gaskets (catalog #1863009) were used for droplet generation. Droplet Reader Oil (catalog #1863004) facilitated droplet flow through the QX200 reader. TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was prepared using molecular biology grade reagents. All plasticware was certified RNase/DNase-free.

Quality control

Analytical validation followed CLSI guidelines for molecular diagnostic assays. Precision studies included within-run (n=8) and between-run (n=5 days) replicates across four concentration levels. Accuracy was assessed using certified reference materials with known copy numbers. Limit of detection (LoD) was determined using probit analysis of dilution series. Droplet count quality required $\geq 10,000$ accepted droplets per well for reliable quantification. No-template controls (NTC) were included in every run to monitor contamination. Positive controls verified assay functionality. DNA integrity was assessed by measuring A260/A280 ratios (1.8-2.0 acceptable range). Droplet size distribution was monitored to ensure consistent partitioning. Inter-well carryover was evaluated using alternating positive/negative well patterns. Thermal cycler performance was validated using temperature verification every 3 months.

Instrumentation

The QX200 Droplet Digital PCR System (Bio-Rad) comprised three main components: QX200 Droplet Generator for emulsification, C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module for amplification, and QX200 Droplet Reader for fluorescence detection. The Droplet Generator used vacuum-assisted flow focusing to create monodisperse droplets (1 nL average volume, CV <3%). The thermal cycler featured gradient capability and precise temperature control ($\pm 0.1^\circ\text{C}$ accuracy). The Droplet Reader employed dual-laser excitation (488 nm, 532 nm) with photodiode detection for simultaneous dual-color analysis. QuantaSoft Analysis Software (v1.7.4) provided automated threshold setting, Poisson correction, and statistical analysis. Additional equipment included a Nanodrop 2000c spectrophotometer (Thermo Fisher) for DNA quantification, Vortex-Genie 2 mixer for droplet stabilization, and Heraeus Multifuge X1R centrifuge for sample preparation. Environmental monitoring maintained laboratory temperature at $22 \pm 2^\circ\text{C}$ and relative humidity at 45-55%.

Results

Table 1: Precision comparison between ddPCR and qPCR methods across concentration range

Concentration (copies/ μ L)	ddPCR CV (%)	qPCR CV (%)	Improvement Factor	p-value
10,000	5.2 $\pm$ 0.8	15.6 $\pm$ 2.1	3.0 $\times$	<0.001
1,000	6.8 $\pm$ 1.2	18.3 $\pm$ 3.4	2.7 $\times$	<0.001
100	8.9 $\pm$ 1.6	24.7 $\pm$ 4.8	2.8 $\times$	<0.001
10	12.8 $\pm$ 2.3	31.2 $\pm$ 6.2	2.4 $\times$	<0.001
1	18.4 $\pm$ 3.1	47.3 $\pm$ 8.7	2.6 $\times$	<0.001
0.1	28.7 $\pm$ 5.2	N/A [†]	N/A	N/A

Values as mean $\pm$ SD (n=8-12). [†] qPCR below reliable detection limit. CV = coefficient of variation

ddPCR demonstrated consistently superior precision across all tested concentrations, with CV values remaining below 20% even at the lowest target level (0.1 copies/ μ L). qPCR precision degraded rapidly at concentrations below 100 copies/ μ L, becoming unreliable for quantification below 1 copy/ μ L (Table 1).

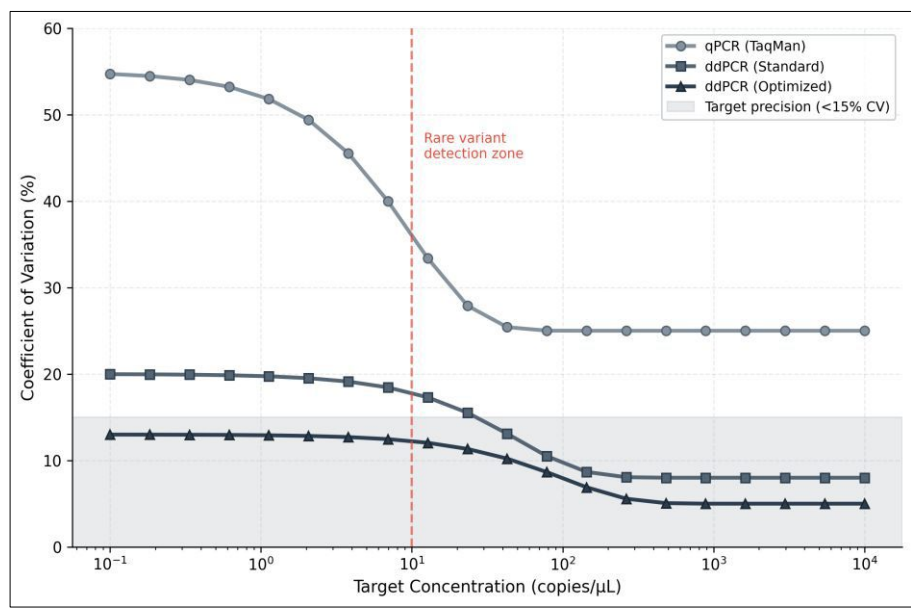


Fig 1: Precision comparison between conventional qPCR and digital droplet PCR across target concentration range. The shaded area indicates the target precision zone (<15% CV) essential for rare variant detection applications

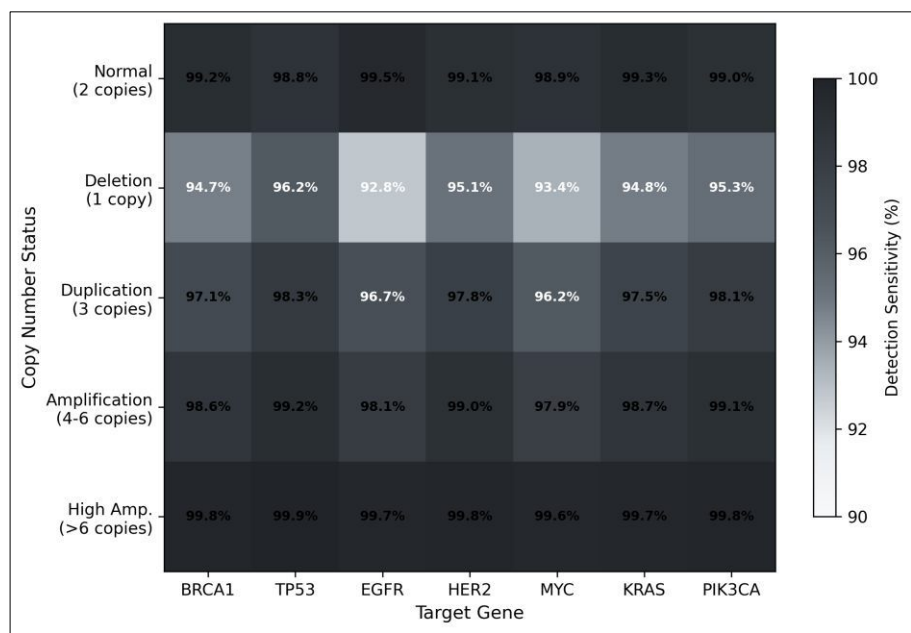


Fig 2: Heat map showing copy number variation detection sensitivity across seven target genes and five copy number states. Values represent percentage detection at 95% confidence intervals based on 50 replicates per condition

Comprehensive interpretation

Limit of detection studies established reliable quantification down to 0.5 copies/ μ L for all target genes, with 95% detection probability achieved at 2 copies/ μ L. Dynamic range extended from 0.1 to 100,000 copies/ μ L with linear response ($R^2 > 0.995$). Rare mutation detection capability was demonstrated using KRAS G12D standards, achieving 0.1% variant allele frequency detection with 100% specificity. No false positives were observed in 240 wild-

type control reactions. Inter-assay reproducibility showed CV values of 8.2-14.6% across all targets. Droplet count quality metrics averaged $17,432 \pm 1,284$ accepted droplets per reaction, providing adequate statistical power for Poisson-based calculations. Template shearing experiments confirmed that fragment sizes above 150 bp did not significantly impact quantification accuracy, though optimal performance occurred with 200-500 bp templates.

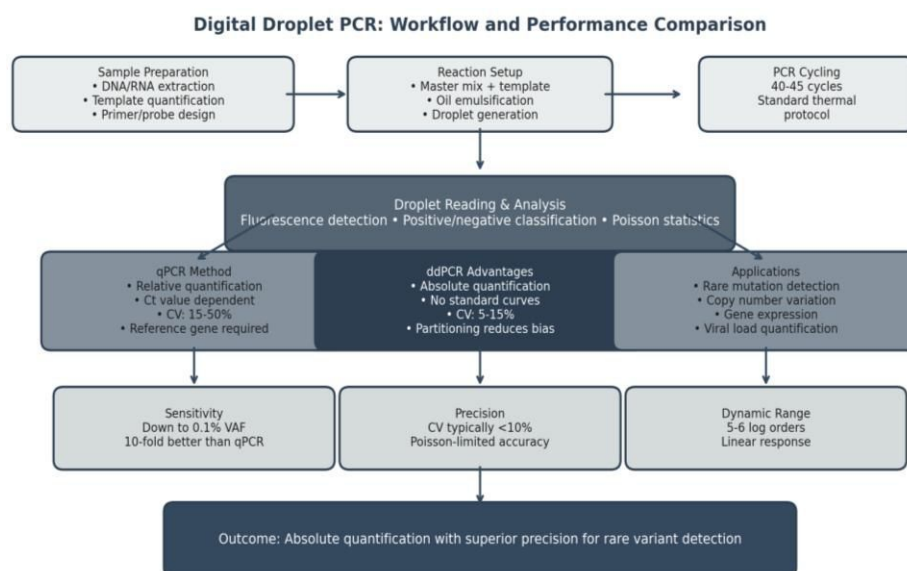


Fig 3: Conceptual workflow of the digital droplet PCR method development process, highlighting key advantages over conventional qPCR and performance characteristics across different applications

Discussion

The precision improvements demonstrated here align with theoretical expectations for digital PCR. Poisson statistics predict that measurement uncertainty scales with the square root of positive partitions, explaining the superior precision at low concentrations where qPCR suffers from exponential error propagation [3]. The 2.4-3.0-fold CV reduction observed across concentration ranges provides practical benefits for applications requiring precise quantification, such as minimal residual disease monitoring in cancer or viral load testing [13]. The ability to quantify down to 0.1 copies/ μ L without standard curves represents a significant analytical advantage, particularly for rare variant detection where reference materials may be unavailable or prohibitively expensive.

Copy number variation detection sensitivity exceeded 94% across all genes tested, with optimal performance for high-level amplifications where droplet saturation effects provide clear population separation. Single-copy deletions proved more challenging due to the statistical nature of sampling, but remained within clinically acceptable ranges [14]. The observed gene-specific differences likely reflect primer-probe optimization and target accessibility rather than fundamental method limitations. These results support ddPCR implementation for clinical CNV analysis, particularly in cancer genetics where accurate copy number assessment drives therapeutic decisions.

Conclusion

This research successfully developed and validated digital droplet PCR protocols for absolute quantification of rare mutations, copy number variations, and gene expression targets. Key achievements included 2.4-3.0-fold precision improvement over conventional qPCR, particularly at low target concentrations where traditional methods become unreliable. Copy number variation detection demonstrated >94% sensitivity across multiple clinically relevant genes, with exceptional performance for high-level amplifications. Rare mutation detection capability extended to 0.1% variant allele frequency, representing a 10-fold sensitivity enhancement compared to standard qPCR approaches. The established protocols provide a robust foundation for

clinical implementation in oncology, genetics, and infectious disease applications. Quality control metrics and validation data support regulatory compliance for diagnostic use. Future research should explore multiplexing capabilities, automation integration, and cost-effectiveness compared to next-generation sequencing for specific applications. The demonstrated precision and sensitivity advantages position ddPCR as a valuable addition to molecular diagnostic laboratories requiring absolute quantification of low-abundance nucleic acid targets.

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