Improving Next-Generation Sequencing Workflows with Droplet Digital PCR

Next-generation sequencing (NGS) has become a central technology in translational research due to its ability to interrogate a broad range of genetic mutations in a single assay. For cancer research, NGS can support tumor profiling, biomarker discovery, and resistance mechanism identification. While NGS throughput and accessibility have been expanded substantially, it remains challenging to achieve consistent read depth and uniform coverage, particularly when using low-input or heterogeneous samples such as formalin-fixed, paraffin-embedded (FFPE) tissue and liquid biopsies.

Key Stages and Common Bottlenecks in the NGS Workflow

Although commercially available NGS platforms use several different chemistries, a typical NGS workflow can be separated into four stages: (i) sample preparation and nucleic acid extraction, (ii) library preparation, (iii) sequencing, and (iv) data analysis (Figure 1).[1]

Sample Preparation and Nucleic Acid Extraction

The first stage of NGS involves isolating DNA or RNA from a biological sample (e.g., tissue, FFPE or liquid biopsy sample) in a way that preserves nucleic acid integrity and removes the contaminants that could inhibit downstream enzymatic reactions. This process may involve cell lysis to extract nucleic acids, removal of cellular components, and purification to ensure sample purity. For RNA-based workflows (e.g., RNA-Seq), the RNA extracted is often assessed for integrity and degradation because fragmentation and chemical modification can impact library construction efficiency and introduce coverage bias.[2]

Library Preparation

During library preparation, nucleic acids are fragmented into sequencing-ready molecules using enzymes or physical methods and ligated to platform-specific adapter sequences. These adapters provide sequences required for downstream amplification and sequencing and may incorporate index sequences to enable multiplexing of multiple samples in a single sequencing run. The ligated fragments are then PCR-amplified to generate millions of template molecules for the sequencing reaction. This step is strongly influenced by sample quality and quantity and is susceptible to experimental bias, including overrepresentation of some fragments and duplication artifacts.[3]

The libraries are then assessed for concentration and fragment size distribution, ensuring that they meet the platform specifications prior to pooling and sequencing. This is a critical step because over- or underloading affects cluster density, read yield, and the probability of achieving sufficient depth for low-frequency variant detection.

Sequencing

During the sequencing reaction, each nucleotide position on the template molecule generates an optical (e.g., fluorescence) or chemical (e.g., pH) signal in response to a process, such as nucleotide addition on a growing complementary strand.

Data Analysis

Data analysis is often divided into three distinct steps, each producing different outputs that allow for data interpretation.

- Primary analysis: Raw instrument signal outputs are converted into sequence reads and associated quality metrics, often producing standard read files for downstream analysis
- Secondary analysis: Sequence reads are aligned to genomic coordinates using a reference genome (or by performing de novo assembly if a reference genome is unavailable); this provides the full sequence for a sample, from which genetic variants can be determined
- Tertiary analysis: Secondary outputs are translated into biological data that provide insights into cellular processes, revealing novel biomarkers and identifying causes of disease

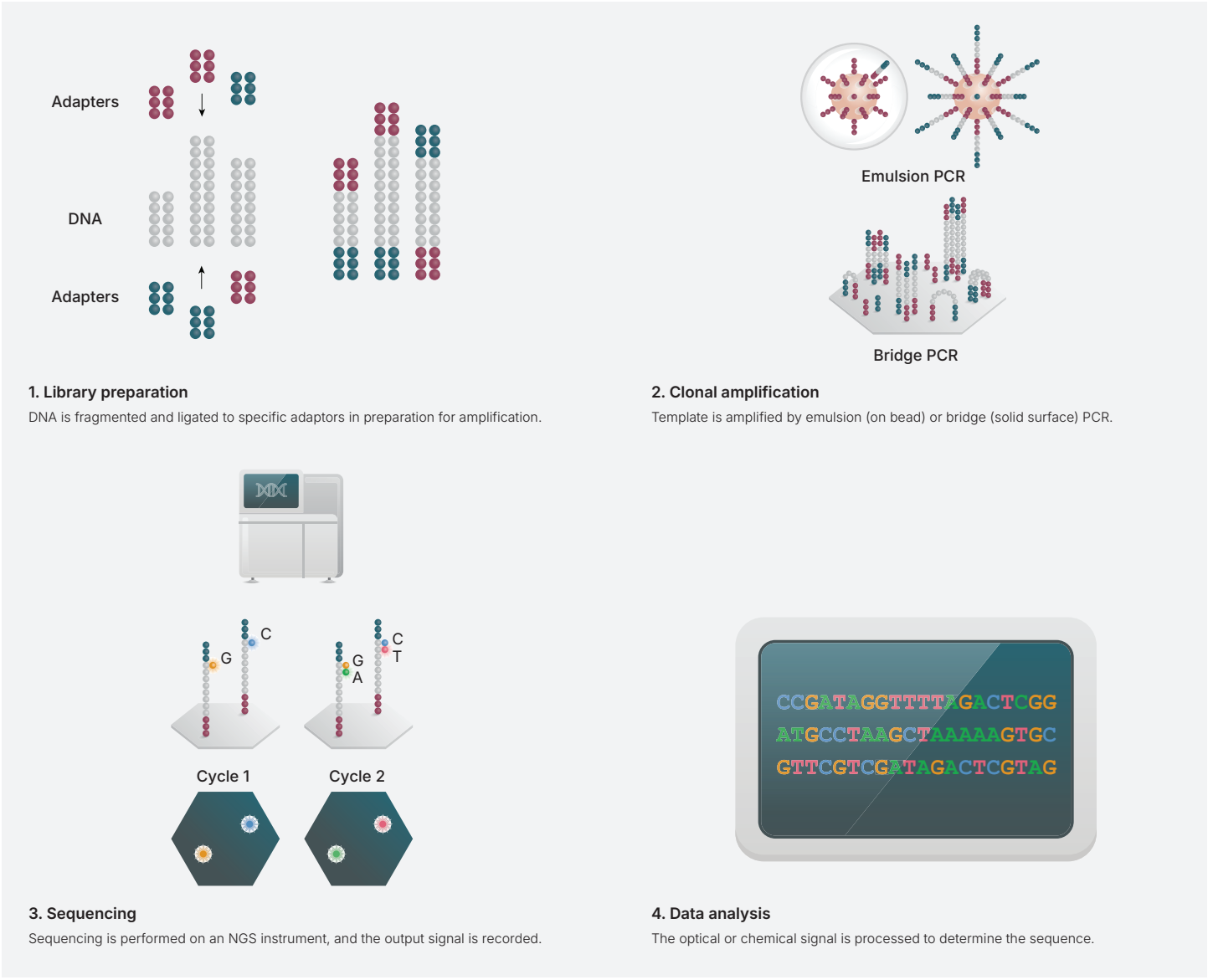

Fig. 1. The basic NGS workflow.

How Digital PCR Supports the NGS Workflow

Despite ongoing improvements in sequencing chemistry and informatics, consistent performance, particularly for challenging samples, can be negatively affected by factors that occur upstream of sequencing. These factors include library preparation quality, amplification bias, and variability in library quantification and pooling.

Accordingly, Droplet Digital™ PCR (ddPCR™) has emerged as a complementary technology that can be integrated into NGS workflows to improve sequencing efficiency and confidence in its results. By partitioning a sample into tens of thousands of nanoliter-scale water-in-oil droplets, ddPCR technology enables absolute quantification of nucleic acids using Poisson statistics. This supports highly sensitive detection using low sample volumes and concentrations, making the technique well suited for applications such as rare variant detection, copy number variation analysis, and DNA methylation studies.

Rather than replacing sequencing, ddPCR technology is most commonly used to enhance NGS workflows by supporting library preparation, enabling accurate library quantification, and providing orthogonal verification of sequencing calls.

Sample QC and Library Preparation

During library preparation, fragmented DNA is ligated to platform-specific adapters and subsequently amplified to enrich fragments that contain adapters on both ends. Excessive or poorly controlled amplification can distort library composition, leading to loss of sequence heterogeneity, preferential enrichment of shorter fragments, GC-content bias, and reduced representation of rare variants. By isolating template molecules into droplets, ddPCR technology reduces competition between high-abundance and low-abundance templates during amplification, thereby maintaining the original representation of molecules in a sample.[4] In general, preservation of sequence diversity during early amplification steps results in libraries that are more likely to yield uniform read coverage, improved representation of GC-rich or structurally complex regions, and fewer ambiguous or undercovered loci.

Accurate Library Quantification

A critical step for achieving optimal sequencing workflows is accurately quantifying the true concentration of functional library molecules. This has practical implications because library overloading can compromise data quality; conversely, underloading reduces read yield and may create coverage gaps.[3] Quantitative PCR (qPCR) can be used to selectively and rapidly amplify adapter-ligated fragments. However, its reliance on external standards can introduce additional variability and complicates comparisons across sequencing runs.

ddPCR technology addresses these limitations by providing absolute quantification without reliance on standard curves. By measuring target copies directly, ddPCR technology offers a more reliable estimate of sequencing-ready library concentration, supporting consistent performance, particularly in studies in which repeating runs is cost-prohibitive or limited by sample availability.[4,5]

Orthogonal Validation of NGS Findings

In addition to offering upstream workflow support, ddPCR technology can be used to validate and confirm NGS findings. This is particularly important when variants will inform downstream decisions, such as cohort stratification, assay development, or longitudinal monitoring, and when calls fall near reporting thresholds. In these cases, ddPCR technology can provide sensitive and precise quantification of known low-abundant variants.[6]

For example, in a recent study on the *MYD88* L265P mutation associated with lymphoplasmacytic lymphoma, ddPCR technology and NGS showed comparable analytical performance, with both methods detecting the variant at allele fractions as low as 0.5% variant allele frequency (VAF). The authors also found that using ddPCR results to set an NGS reporting threshold eliminated false-positive NGS calls in their dataset, demonstrating how ddPCR technology can improve confidence in low-frequency sequencing results and help define analytical cutoffs.[7]

Translational Applications of NGS and ddPCR Technology

Both NGS and ddPCR technology address practical needs in oncology research: NGS offers comprehensive genome profiling and new target discovery; and ddPCR delivers sensitive, absolute quantification of predefined targets using a workflow that is robust, reproducible, and well-suited for routine analytical use (Table 1). Additionally, compared to NGS, ddPCR technology provides more rapid results, and it is both cost-effective and approachable in terms of workflow and data analysis.[8]

Table 1. NGS vs. ddPCR technology.

Feature	NGS	ddPCR Technology
Turnaround time	5–40 days	1–3 days
Sensitivity	~0.1% VAF (with UMIs)	≤0.01% VAF
Cost per sample	$$$	$
Workflow complexity	High	Simple, same day
Multiplexing	Hundreds of genes	Up to 20 targets per run*

UMI, unique molecular identifier; VAF, variant allele frequency.
*Up to 20 targets per run for discrimination and higher multiplexing for screening.

Liquid Biopsy and ctDNA Analysis

In translational research, and specifically for liquid biopsy, NGS can be used early to map a tumor's genomic profile via circulating tumor DNA (ctDNA) analysis to identify resistance mechanisms, disease-driving mutations, and therapeutic targets. However, once relevant targets are defined, research often shifts from discovery to longitudinal monitoring in an attempt to track how a known marker is changed over time. In this case, ddPCR technology is commonly employed because it supports absolute quantification and sensitive detection of predefined variants at very low allele fractions, with case studies reporting performance in the ~0.01% VAF range (assay and input dependent).[9]

Monitoring MRD

Oncology research employing molecular residual disease (MRD) monitoring to identify cancer recurrence and evaluate treatment response in real time requires assays capable of detecting low-abundance biomarkers in liquid biopsy samples with high sensitivity and accuracy. ddPCR technology is ideal for such analyses. Custom multiplexed ddPCR assays, designed based on tumor cell biomarkers identified through NGS, enable MRD monitoring with low-complexity workflows with faster turnaround times and reduced costs, all while delivering exceptional sensitivity, precision, and absolute quantification.[6]

As an example, a recent study addressed a gap in MRD monitoring approaches for rare leukemia markers by developing custom ddPCR MRD assays that targeted atypical fusion transcripts and uncommon mutation patterns.[10] The authors demonstrated sensitivity and quantitative performance at very low target levels and showed that those assays could be used for reliable serial MRD monitoring over time.

Companion Diagnostics

In addition to being used as an alternative to NGS for target detection, ddPCR technology can be employed alongside NGS to validate and refine sequencing findings. This is particularly important when variants are present at low levels or when additional quantitative confidence is required.

For instance, a recent multicenter study illustrated this complementary approach in non-squamous non-small-cell lung cancer (NSCLC).[11] The authors assessed ctDNA mutations using a targeted ddPCR panel (including *EGFR*, *KRAS*, and *BRAF*) and compared the results with tissue NGS when available. Among 142 NSCLC patients for whom tissue NGS data were available, ddPCR technology detected 32 of the 45 (71%) driver mutations identified by NGS. Notably, ddPCR technology identified mutations in two cases not detected by the initial tissue NGS. In one case, repeat biopsy followed by NGS confirmed the *EGFR* Ex19Del mutation first detected only by ddPCR technology, underscoring how plasma-based testing can capture the heterogeneity missed by using a single tissue sample. Overall, the authors concluded that taking a “plasma-first” approach (i.e., screening ctDNA by ddPCR) and reserving tissue NGS for ctDNA-negative cases increased the total number of mutations detected by 17%, reducing the number of tissue NGS tests by 40% and, consequently, the need for multiple biopsies.

Conclusions

The translational research examples described above reflect a consistent workflow pattern in which ddPCR technology complements NGS in two main ways: (1) by supporting library quality control (QC) through absolute quantification and by helping to limit amplification-driven artifacts via droplet partitioning; and (2) by offering an orthogonal approach to confirm and precisely quantify low-frequency variants near reporting thresholds. Together, these methods support a hybrid workflow in which NGS can be used for genomic profiling and ddPCR technology can be used for targeted validation and longitudinal monitoring once targets have been identified.

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