Enhanced Data Quality and Simplified NGS Workflows of FFPE Samples Using iconPCR™ with AutoNorm™

Austin W, Jouvenot Y, Patel P
n6, Pleasanton, California, 94566

Abstract:

Traditional PCR workflows require users to predefine the number of amplification cycles based on input quantity and assay type. This rigid framework is particularly problematic for formalin-fixed, paraffin-embedded (FFPE) samples, where nucleic acid degradation leads to highly variable input quality and unpredictable amplification efficiency. As a result, accurate quantification becomes difficult, often necessitating multiple thermocycler runs, grouped by input amount and integrity, along with labor-intensive pre- and post-PCR quality control steps. iconPCR, developed by n6, introduces a paradigm shift in PCR amplification through its AutoNorm technology. By monitoring fluorescence in real-time, iconPCR automatically terminates each individual reaction once a predefined amplification threshold is reached. This per-sample, real-time control eliminates the need for fixed-cycle settings and standardizes output across samples, regardless of input variability or degradation level. For FFPE and other challenging inputs, iconPCR dramatically simplifies workflows, reduces batch effects, and enhances the consistency and quality of sequencing libraries—enabling more efficient and accurate downstream analysis in both research and clinical settings.

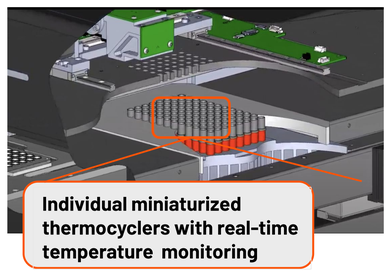
Here we present two studies showcasing workflow and data quality improvements for FFPE NGS preparations:

Study 1: Optimized whole genome sequencing of FFPE samples

Study 2: Effect of overamplification during RNAseq of FFPE samples

iconPCR with AutoNorm overview

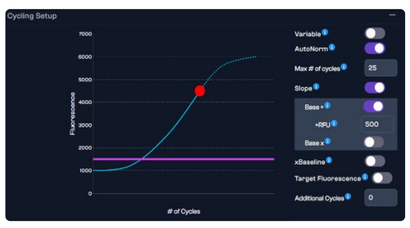
Engineering revolution



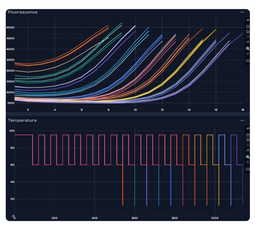
iconPCR
The world's first thermocycler with individually controlled wells

With a fundamental change in hardware design, iconPCR can independently control the temperature and cycling of each well.

Use of AutoNorm for controlling amplification



Within the iconPCR software, user-defined parameters can be used to ensure target amplification is achieved without over-amplification or drop-outs due to under amplification.



Once the threshold is reached the well stops cycling and goes to a cold hold. The remaining wells continue to cycle until their threshold is reached.

Workflows reimagined

Old workflow

Sample quant ▸ Add adaptors ▸ PCR ▸ SPRI cleanup ▸ Library quant ▸ Library normalization ▸ Sequence

New workflow

Add adaptors ▸ PCR ▸ 1 cleanup ▸ Sequence

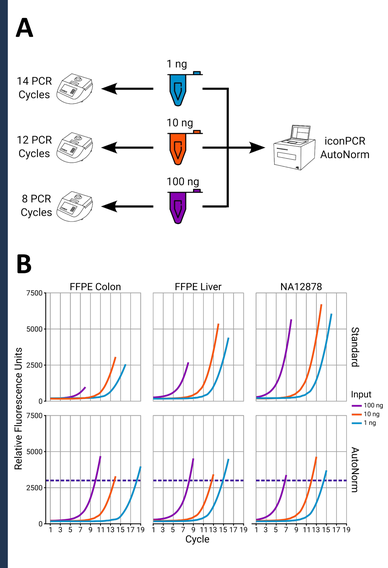
iconPCR workflow

- Saves time
- Eliminates need for quantification, dilution, or normalization reagents
- Boosts sequencing throughput
- Reduces PCR duplicates and library artifacts

Study 1: Optimized whole genome sequencing of FFPE samples

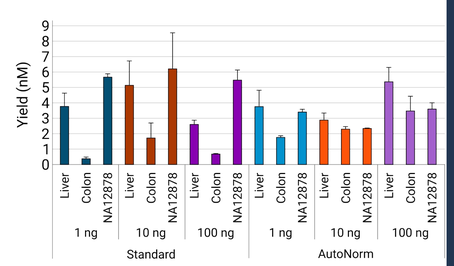
Experimental Details: Two FFPE-derived DNA samples (colon and liver) and a high-quality genomic DNA control (NA12878) were used to generate whole-genome sequencing (WGS) libraries at three input amounts: 1 ng, 10 ng, and 100 ng. Library construction was performed using either the manufacturer's standard PCR protocol or iconPCR with AutoNorm. Post-PCR, libraries underwent SPRI bead purification and quantification via Qubit fluorometry. Final libraries were sequenced on an Illumina NovaSeq X platform. Quality metrics—including library yield, coverage uniformity, and duplication rates—were calculated to assess the impact of AutoNorm compared to standard fixed-cycle PCR.

Figure 1. AutoNorm-enabled iconPCR accounts for sample diversity and streamlines complex workflows



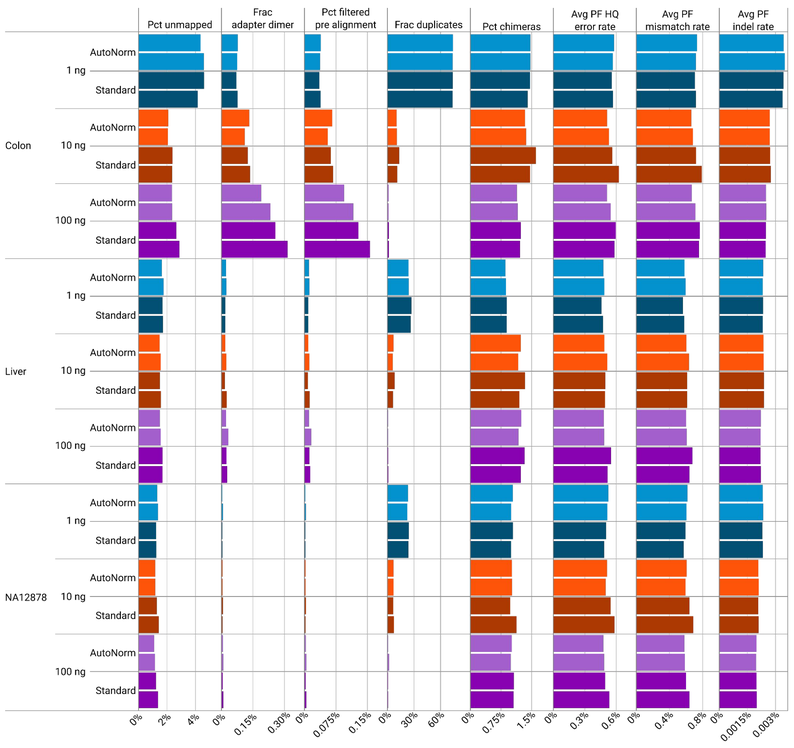
C

	Standard	AutoNorm		
Input	All	Colon	Liver	NA12878
1 ng	16	19	16	15
10 ng	14	14	13	13
100 ng	8	11	9	7



A) Whole-genome sequencing (WGS) libraries were generated using 1 ng, 10 ng, or 100 ng of input DNA. Traditional PCR workflows would require three separate instruments to optimize cycle numbers for each input, whereas iconPCR enables all libraries to be processed on a single instrument through AutoNorm. **B)** Representative amplification curves for each sample/input combination. Even with cycle adjustments for input amount, endpoint fluorescence values differ across inputs when using standard PCR. Sample-specific variation is more pronounced than input variation. The colon FFPE sample exhibits the lowest amplification signal, followed by liver, with NA12878 showing the highest signal. **C)** Summary of PCR cycle requirements. NA12878 consistently required fewer cycles than recommended, while the colon sample needed additional cycles across inputs. The liver sample displayed mixed results, requiring extra cycles for 100 ng but fewer for 10 ng input. **D)** Final library yields. Standard PCR conditions produced high variability across samples, with the colon sample generating low yields. In contrast, AutoNorm generated more uniform library concentrations. By dynamically applying additional cycles to the colon sample, AutoNorm increased yields and improved normalization prior to sequencing.

Figure 2. AutoNorm maintains sequencing quality metrics

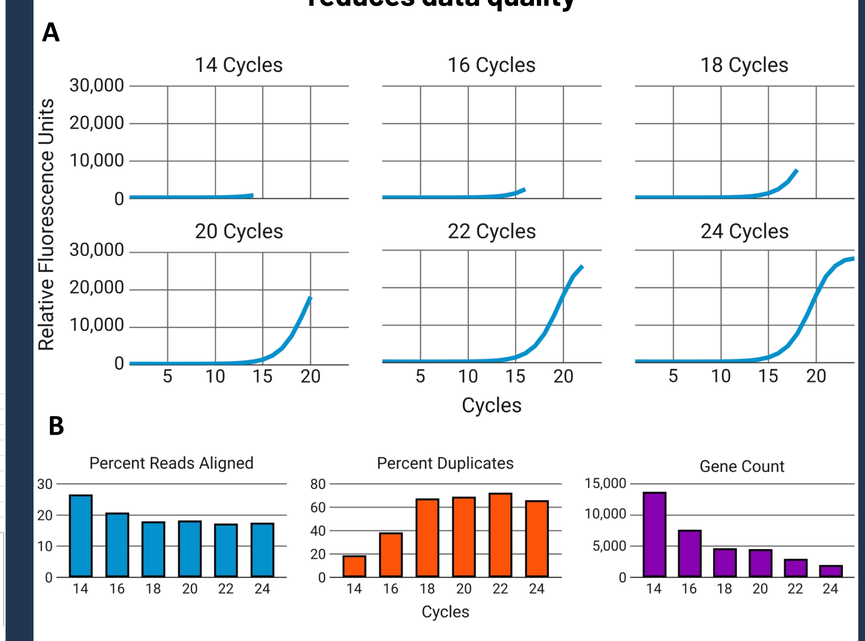


Sequencing metrics for all libraries. Quality metrics varied by both input amount and sample type. Libraries generated from 1 ng input exhibited the highest levels of PCR duplicates and unmapped reads. Among sample types, the colon FFPE samples showed a higher percentage of unmapped reads and adapter dimers compared to liver or NA12878. Notably, AutoNorm did not negatively affect any sequencing quality metrics, maintaining performance comparable to or better than standard PCR conditions.

Study 2: Effect of overamplification on FFPE RNAseq data

Experimental Details: For Figure 1, RNA-seq libraries were prepared from 50 ng of RNA derived from a single FFPE sample using a range of PCR cycle numbers. Libraries were sequenced and aligned, and quality metrics (e.g., read mapping rates, duplication levels) as well as gene counts were determined. For Figure 2, RNA-seq libraries were prepared using 1 ng, 10 ng, and 100 ng of RNA from four different FFPE samples. Libraries were amplified using either the manufacturer's recommended fixed PCR cycles (standard) or iconPCR with AutoNorm. Following sequencing and alignment, the same quality metrics and gene count analyses as described for Figure 1 were performed.

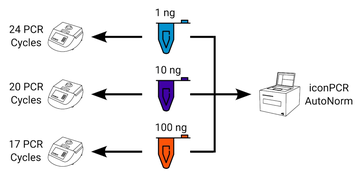
Figure 3. Overamplification of RNAseq libraries reduces data quality



A) RNA-seq libraries were generated from 50 ng of RNA from a single FFPE sample, with amplification ranging from 14 to 24 PCR cycles in 2-cycle increments. Each panel represents the point along the amplification curve where cycling was terminated. **B)** Libraries were sequenced, subsampled to 1 million reads, and analyzed. As the number of PCR cycles increased, there was a decrease in the percentage of aligned reads (left), an increase in PCR duplicates (center), and a reduction in detected gene counts (right).

Figure 4. AutoNorm preserves data integrity by preventing overamplification

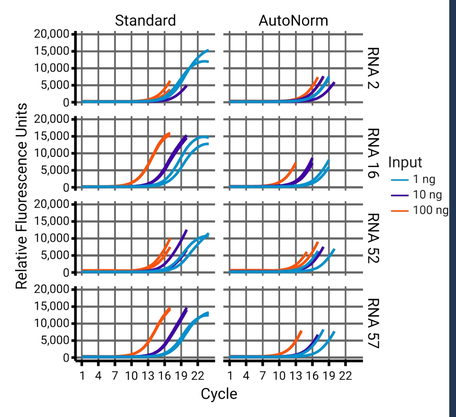
A



B

Sample	Conc (ng/uL)	DV200
RNA 2	98.6	45.80%
RNA 16	60.6	67.40%
RNA 52	33.2	23.30%
RNA 57	46.5	63.40%

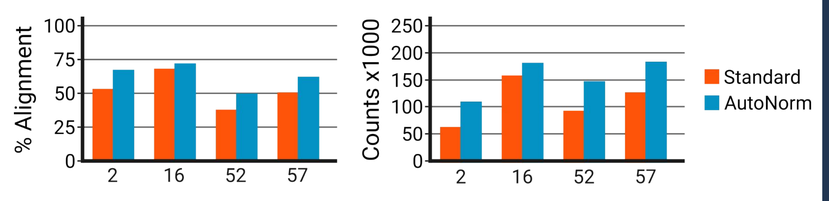
C



D

	1ng		10ng		100ng	
	Standard	AutoNorm	Standard	AutoNorm	Standard	AutoNorm
Sample 2	24	19	20	18/20	17	17/18
Sample 16		19		16		13
Sample 52		17/20		17/18		15/17
Sample 57		18/20		17		14

E



A) Standard PCR workflows require dividing samples of different input amounts across multiple thermocyclers, each programmed with a different number of PCR cycles. In contrast, iconPCR with AutoNorm)enables all libraries—regardless of input amount—to be amplified on a single instrument. **B)** Table summarizing the FFPE samples used in this study, including initial RNA concentrations and DV200 values as a measure of RNA integrity. **C)** Real-time PCR amplification curves using standard PCR cycling (left) versus iconPCR with AutoNorm (right). Standard cycling results in variable endpoint fluorescence due to differences in input and sample quality. With AutoNorm, each sample terminates at the same amplification threshold, independent of input or degradation level. **D)** Number of PCR cycles required for each sample. **E)** AutoNorm improves data quality. Libraries from 1 ng input were sequenced and subsampled to 1M reads. Graphs show the percentage of reads aligning to the reference genome and the number of deduplicated gene counts, averaged across two technical replicates.