AutoNorm™ of FFPE NGS Libraries Using iconPCR™ Technology Reduces Dropouts and Workflow Complexity

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Abstract

Next-generation sequencing (NGS) library preparation faces three persistent challenges: 1) sample qualification uncertainties, 2) inefficient library purification/normalization processes, and 3) sequencing artifacts from PCR duplicates/adapter dimers. Current normalization methods relying on individual quantification (Qubit, Tapestation) and liquid handling robotics incur substantial costs, while failing to address sample-specific amplification needs. This proves particularly problematic for low-input FFPE samples where degradation and crosslinking create variable template quality undetectable by conventional quantification methods.

We present a solution powered by n6 iconPCR technology that enables:
- Real-time amplification monitoring through fluorescent tracer incorporation
- AutoNorm during PCR via cycle threshold adjustment
- Direct pooling from amplification plates

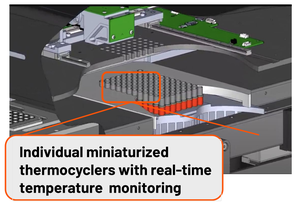
In FFPE samples, this approach:
- Reduced dropout rates
- Decreased PCR duplicates
- Eliminated pre-pooling quantification steps

This workflow enables reliable processing of challenging samples while reducing per-library costs through reagent and time savings. The technology's dynamic adaptation to sample quality parameters demonstrates particular value for degraded clinical specimens requiring maximal data recovery.

iconPCR with AutoNorm overview

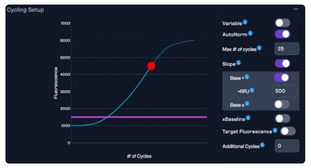
Engineering revolution

iconPCR
The world's first thermocycler with **i**ndividually **con**trolled wells

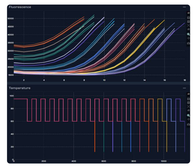


With a fundamental change in hardware design, iconPCR can independently control the temperature and cycling of each well.

Use of AutoNorm for optimizing workflows



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.

Old workflow

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

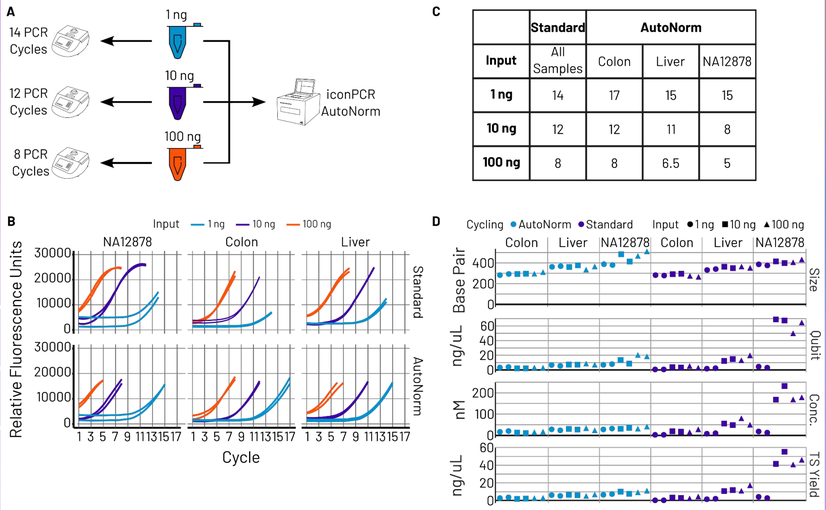
New workflow

Add adaptors ► PCR ► 1 cleanup ► Sequence

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 prepare whole-genome sequencing (WGS) libraries at three input levels (1 ng, 10 ng, and 100 ng). Libraries were constructed using the manufacturer's standard fixed-cycle PCR protocol or iconPCR with AutoNorm. Following amplification, libraries were purified with SPRI beads and quantified. Final libraries were sequenced on an Illumina NextSeq 550. Quality metrics, including library yield, mutation rates, and duplication rates, were evaluated to compare AutoNorm with standard PCR.

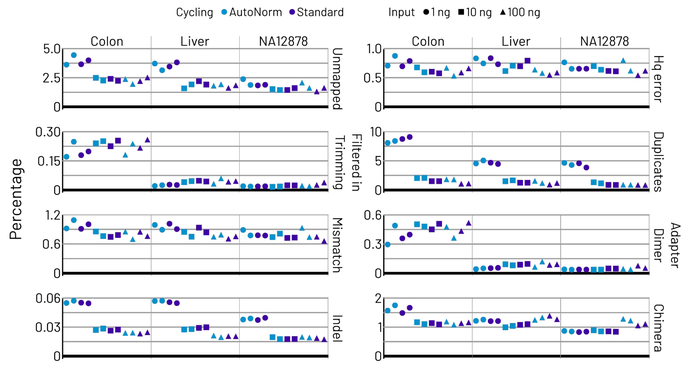
Figure 1. AutoNorm-enabled iconPCR accounts for sample diversity and streamlines complex workflows



C	Standard	AutoNorm		
Input	All Samples	Colon	Liver	NA12878
1 ng	14	17	15	15
10 ng	12	12	11	8
100 ng	8	8	6.5	5

(A) Whole-genome sequencing (WGS) libraries were prepared from 1 ng, 10 ng, and 100 ng of input DNA. Traditional PCR workflows require separate instruments or optimization for each input, whereas iconPCR with AutoNorm enables all libraries to be processed on a single instrument.
(B) Amplification curves for each sample/input combination. With standard PCR, endpoint fluorescence varies by input amount, even when cycle numbers are adjusted. AutoNorm achieves consistent endpoint fluorescence across inputs and samples.
(C) PCR cycle requirements. NA12878 required fewer cycles than the standard protocol, while each FFPE sample reached the AutoNorm threshold at different cycle numbers, illustrating sample-specific adaptation.
(D) Standard PCR produced highly variable yields across inputs and samples. In contrast, AutoNorm generated consistent concentrations and fragment profiles, with Qubit and Tapestation (Conc. and TS Yield) measurements showing uniformity across all libraries.

Figure 2. AutoNorm maintains sequencing quality metrics

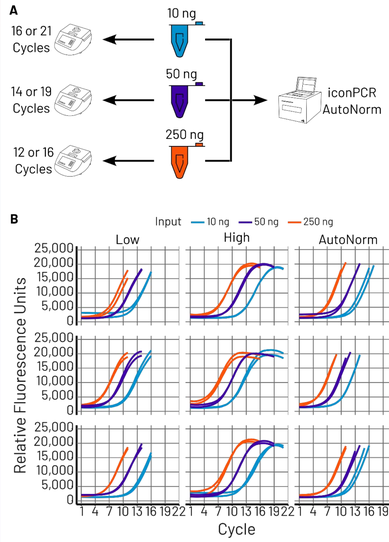


Sequencing quality metrics. Quality varied with both input amount and sample type. Libraries from 1 ng inputs showed more PCR artifacts than those from 10 ng or 100 ng. Colon samples had an increased percent of reads filtered during trimming and more adapter dimers compared to liver or NA12878. Notably, AutoNorm preserved sequencing performance, yielding quality metrics comparable to standard PCR across all inputs and samples.

Study 2: Effect of overamplification on FFPE RNA-seq data

Experimental Details: RNA-seq libraries were prepared from 10 ng, 50 ng, and 250 ng of RNA derived from two FFPE samples and Universal Human Reference (UHR) RNA. Libraries were amplified using either the manufacturer's recommended cycle number (Low), the recommended number plus five cycles to induce overamplification (High), or iconPCR with AutoNorm. After amplification, libraries were purified with SPRI beads and sequenced on an Illumina NextSeq 550. Following alignment, sequencing quality metrics were calculated, and differential expression analysis was performed.

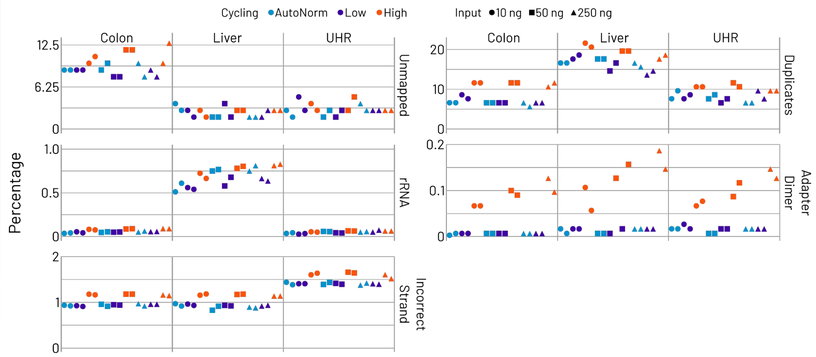
Figure 3. AutoNorm enables optimal amplification across diverse inputs and sample types



C	Standard		AutoNorm		
	Low	High			
Input	All Samples	All Samples	Colon	Liver	UHR
10 ng	16	21	14	15.5	16.5
50 ng	14	19	11.5	13.5	14
250 ng	11	16	9	11	10.5

(A) Standard PCR requires distributing libraries of different input amounts across multiple thermocyclers programmed with different cycle numbers. AutoNorm allows all libraries to be amplified on a single instrument.
(B) Real-time amplification curves. Standard fixed-cycle PCR produces variable endpoint fluorescence depending on input and sample quality. AutoNorm terminates amplification at a consistent threshold across all inputs and degradation levels.
(C) PCR cycle requirements. AutoNorm dynamically adjusts cycle number to achieve optimal amplification, closely aligning with the low-cycle condition.
(D) Final library yields. Library fragment sizes were comparable across both cycling methods. However, yield measurements by Qubit and Tapestation (Conc. and TS Yield) revealed that standard PCR gave inconsistent concentrations, whereas AutoNorm produced reproducible yields across samples and inputs.

Figure 4. AutoNorm preserves data integrity by preventing overamplification



Sequencing quality metrics. Overcycled libraries exhibited elevated levels of adapter dimers, PCR duplicates, and mis-stranded alignments. In colon samples, overcycling also increased the proportion of unmapped reads. By contrast, AutoNorm libraries maintained sequencing quality, closely matching the metrics observed with low-cycle fixed PCR.