Application Note

Optimize RNA-Seq with **iconPCR™**

Overview

Standard PCR systems require users to set a predetermined number of cycles, which is dependent on the assay and the amount of input. This requires quantifying input material and splitting samples of varying inputs across multiple PCR runs. Optimal cycling of RNA-Seq libraries is critical, as over or under cycling of libraries can result in changes to gene expression. Further challenges arise when using samples of varying quality, such as FFPE, where similar input amounts may no longer follow similar amplification profiles.

Figure 1. iconPCR, the world's first real-time thermocycler with 96 individually controlled wells.

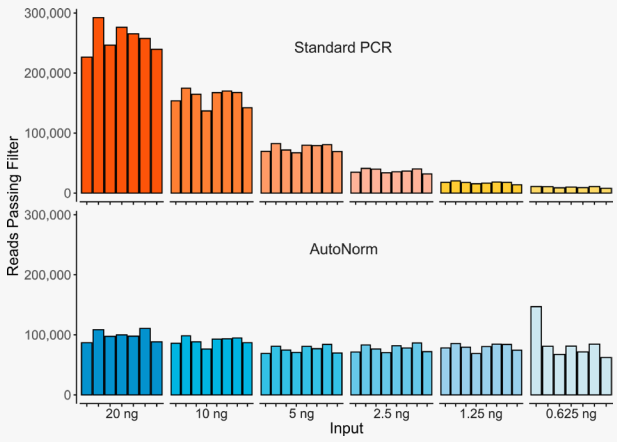

Figure 2. Here we show the significant yield variance when using a single PCR instrument with a fixed number of PCR cycles compared where each sample is amplified to similar levels.

iconPCR, developed by n6, represents a transformative shift in PCR technology. Its core innovation lies in the concept of AutoNorm™, wherein each individual PCR reaction is monitored and terminated in real-time based on fluorescence thresholds. This eliminates the guesswork of standard PCR and ensures every library is amplified optimally.

To evaluate performance, we prepared RNA-Seq libraries from FFPE samples at various numbers of cycles and, in doing so, illustrated that overamplification of RNA-Seq libraries results in decreased data quality. Furthermore, use of AutoNorm prevents overamplification, restoring data quality, while simplifying the processing of a diverse set of samples.

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Effect of PCR Cycles on RNA-Seq Data Quality

50 ng from a single FFPE sample was used as input for RNA-Seq library preparation across a wide range of PCR cycles. Following sequencing and downsampling to normalize read counts, sequencing metrics were compared across cycle number.

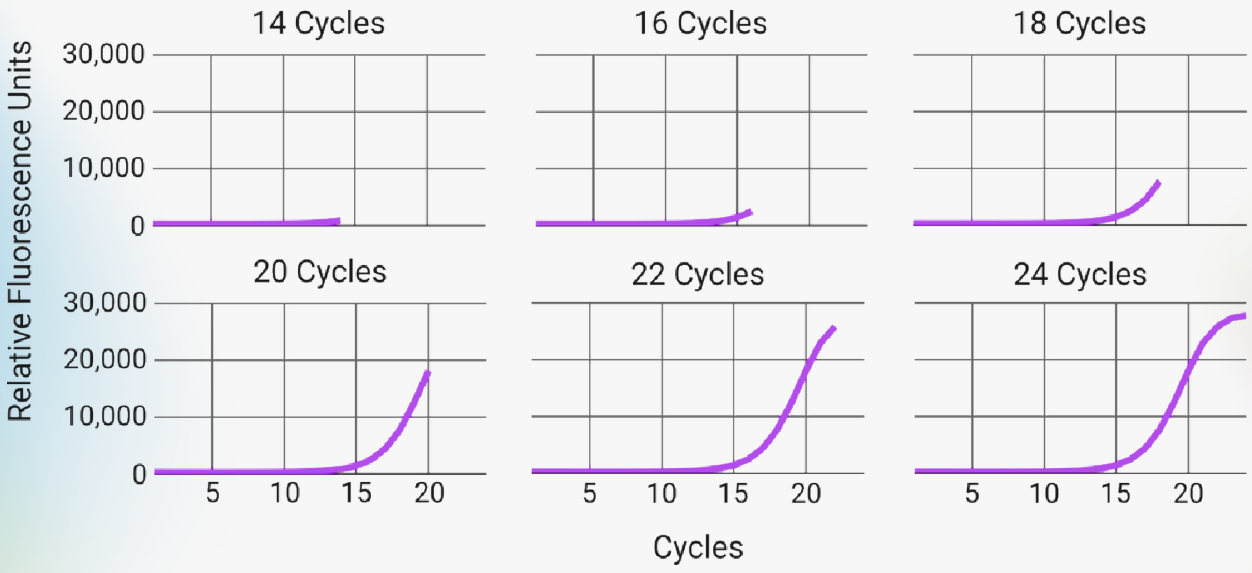

Figure 3. PCR amplification curves across differing cycle numbers. Plots depict where along the PCR amplification curve each condition was stopped. The conditions tested represent those from the early exponential phase (14 cycles) up the the establishment of the plateau (24 cycles) in 2 cycle increments.

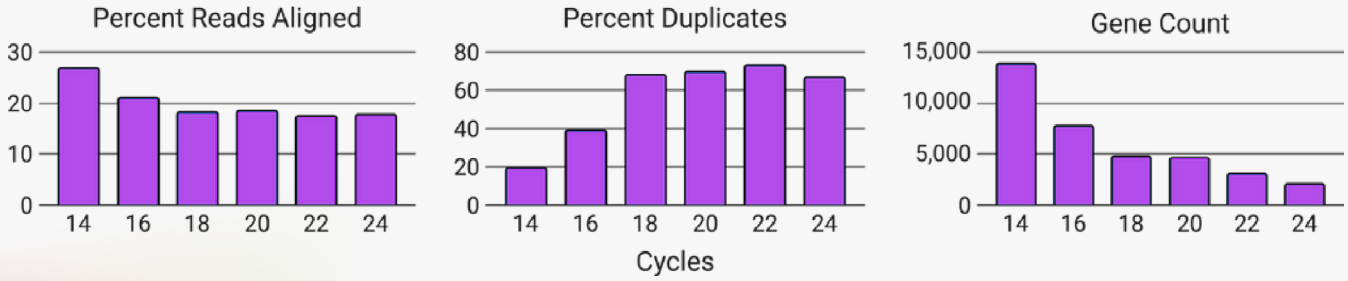

Figure 4. Increasing PCR cycles decreases data quality. Each condition was downsampled to 1M reads passing filter and subsequently aligned to the human reference genome. As the number of PCR cycles increased there was a decreasing in the percent of aligned reads and an increase in the percentage of PCR duplicates identified. Combined, this led to a decrease in the total number of genes detected.

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Optimized RNA-Seq Workflows

To demonstrate how iconPCR can improve RNA-Seq workflows, RNA-Seq libraries were generated from 4 different FFPE samples from 1ng, 10 ng, or 100 ng of input using either standard PCR conditions or iconPCR with AutoNorm.

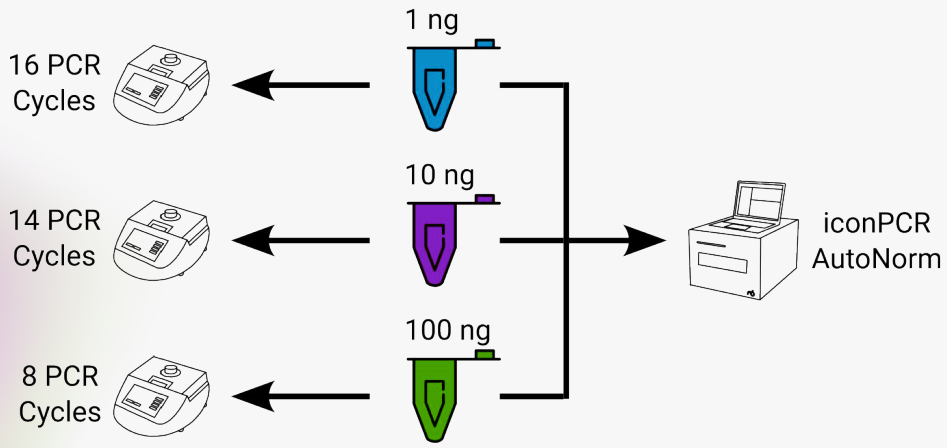

Figure 5. Experimental design showing sample distribution across workflows. For each sample tested, 1 ng, 10 ng, or 100 ng of RNA was used as input. For standard PCR, each input amount was used with a fixed number of PCR cycles, requiring three separate thermocycler runs. For iconPCR with AutoNorm, all samples were run simultaneously on a single instrument.

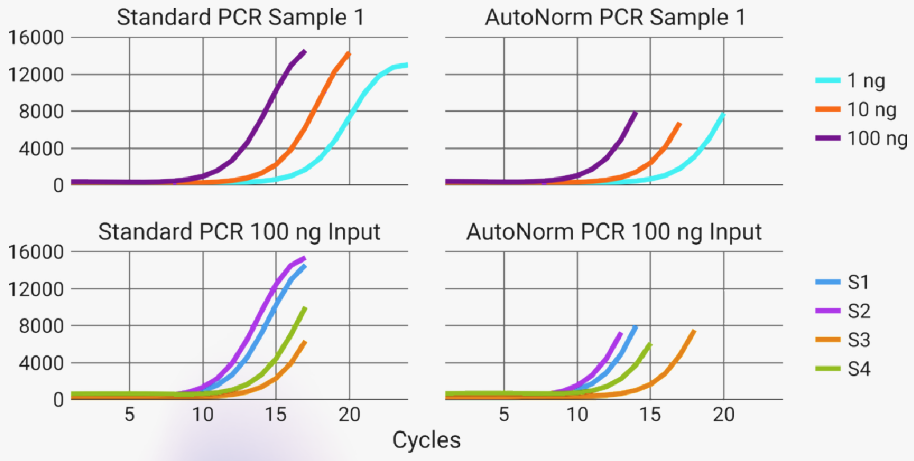

Figure 6. iconPCR allows for simultaneous processing of samples across a range of inputs while ensuring proper amplification is achieved. Different sample inputs require different numbers of PCR cycles to achieve optimal amplification (top), but even across the same input, samples can dramatically differ in the number of required cycles (bottom). iconPCR allows for all samples to be ran simultaneously, while also ensuring that each receive the proper level of amplification.

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Optimized RNA-Seq Workflows, con't.

	1ng		10ng		100ng	
Sample	Standard	AN	Standard	AN	Standard	AN
Sample 1	24	20	20	17	17	14
Sample 2		19		16		13
Sample 3		19		19		18
Sample 4		20		18		15

Table 1. AutoNormaliztion dynamically controls cycle numbers. The table shows the stop cycle for each sample in the study. Use of iconPCR with AutoNorm allows for each sample to stop at a different number of cycles, ensuring that each sample is properly amplified, all within a single PCR run.

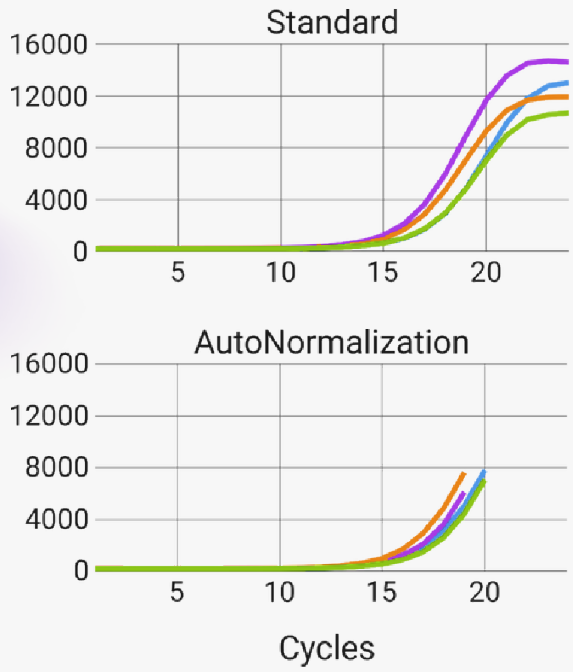

Figure 7. AutoNorm stops overamplification of samples. Using the standard PCR cycling conditions with a 1 ng input, all samples reached the plateau of the amplification curve. AutoNorm prevents samples from reaching the plateau.

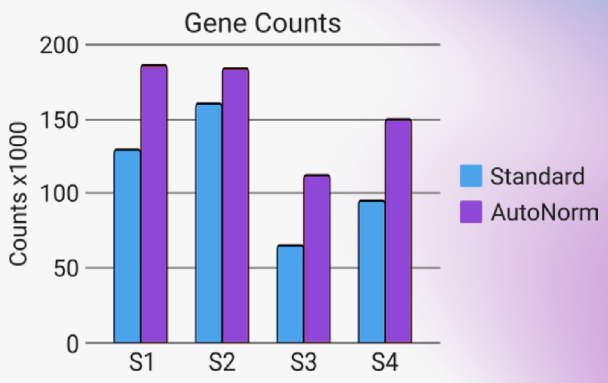

Figure 8. Overamplification results indecreased data quality. Gene counts were lower in the 1 ng input samples from Standard PCR conditions as compared to samples that had undergone AutoNorm where amplification was stopped in the linear phase.

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Application Note

Advantages of iconPCR

Conventional PCR	iconPCR (with AutoNorm)
Fixed cycle count (e.g., 30 cycles)	Real-time fluorescence monitoring
One-size-fits-all amplification	Per-well cycle control based on signal, not guesswork
Under/over-amplification common	Optimal amplification per sample
High chimera rates	Reduced chimera formation
Variable library quality	Uniform library quality across all wells
Manual quant and normalization required	Automated normalization (no post-PCR quant)
More hands-on time	40–60% reduction in hands-on time
Increased reagent waste	Lower reagent waste, fewer failed libraries
Extra QC and rerun costs	Faster turnaround, minimal QC/rescue steps

Conclusion

iconPCR redefines library preparation by eliminating the limitations of traditional PCR workflows. Through its unique per-well AutoNorm mode, iconPCR delivers simplified workflows and improved quality of RNA-Seq libraries by independently amplifying each library to their optimal cycle number.

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