Real-Time AutoNormalization™ of NGS Libraries: Overcoming Index and Sample Variability with iconPCR™

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Abstract

Index primers play a critical role in Next Generation Sequencing (NGS) workflows by enabling sample multiplexing through the incorporation of unique sequence tags. However, not all index primers perform equally. Variations in sequence composition, concentration, and synthesis quality can significantly impact amplification efficiency and downstream sequencing outcomes. Some indexes are prone to dimer formation or poor annealing, leading to reduced amplification performance. These issues can introduce amplification bias, cause uneven or incomplete coverage, and ultimately compromise data quality.

Using iconPCR, a high-throughput real-time PCR platform with flexible thermal cycling and temperature control, we evaluated the performance of hundreds of index primers under varied reaction conditions. No Template Control (NTC) experiments revealed that while some indexes are highly prone to primer dimer formation, others remained clean even after 25 or more cycles. Comparative analyses further demonstrated that specific index sets can adversely affect amplification profiles, and these effects were consistently dependent on the index rather than the sample.

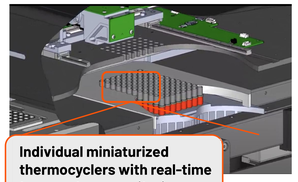
A number of factors affect the performance of index primers. Elevated primer concentrations promoted the formation of non-specific products, while concentration optimization restored ideal amplification curves. Additionally, tuning annealing temperatures and buffer compositions successfully rescued several underperforming index primers, highlighting the importance of reaction context in primer performance.

Our findings underscore that index primer selection is a significant yet often overlooked variable in NGS library preparation. iconPCR not only identifies poorly performing indexes but also enables AutoNorm, a real-time DNA synthesis tracking feature that equalizes amplification outputs across reactions. This capability reduces PCR bias and supports higher-quality, more consistent sequencing results in large-scale studies.

iconPCR with AutoNorm overview

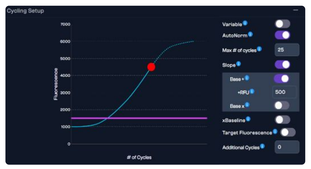
Engineering revolution

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

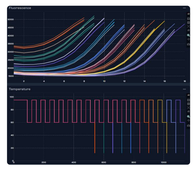


With a fundamental change in hardware design, iconPCR can independently control the temperature and cycling of each well.

Use of AutoNorm for 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.

Not all indexes are created equal

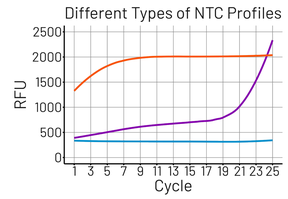
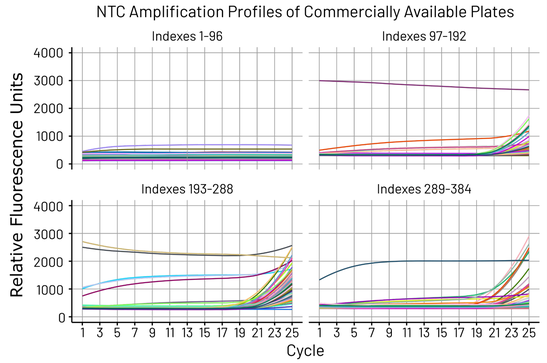


Figure 1. Three different types of NTC profiles. The blue trace reflects an NTC that remains negative over the full 25 cycles. The purple trace highlights a set of indexes that generates dimers (especially after cycle 20). The orange trace is characteristic of a set of indexes where one oligo is disproportionately abundant in comparison with the other.

Figure 2. Testing of NTC in four 96-well plates of indexes. This figure illustrates the differences in quality between different sets of indexes. While Plate 1 displays no dimer amplification over 25 cycles, nearly all sets in plate 3 seem to produce dimer signal by cycle 20. Plates 2, 3 and 4 also display several traces typically associated with high concentration of one of the indexes (above baseline of 1000 RFU).



Index differences can affect library generation

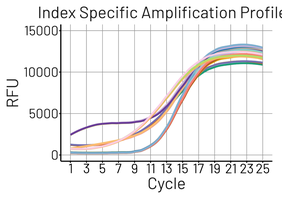
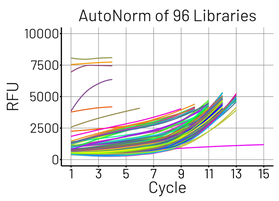


Figure 3. Amplification profiles for the same library amplified with 8 different indexes. Differences in amplification profiles and efficiency are not typically observed during library preparation, since traditional protocols do not add intercalant chemistry during index PCR. It underscores the relevance of iconPCR as a tool not only able to identify these differences, but also normalize libraries so their impact is minimized.

Figure 4. AutoNorm experiment using 96 libraries (Target Fluorescence mode). The samples used here were first round amplicon products provided by Sagan Friant and Christian Herrera, Penn State University.



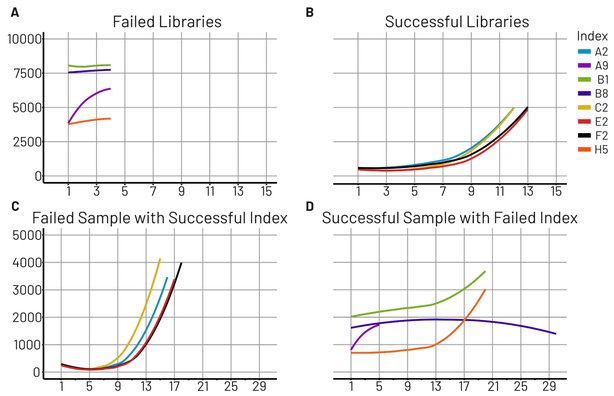


Figure 5a. Examples of premature termination profiles, seen during AutoNorm of samples 1-4 with indexes A9, B1, B8 and H5. **Figure 5b.** Examples of successful AutoNorm using samples 5-8 with indexes A2, C2, E2 and F2. **Figure 5c.** AutoNorm of samples 1-4 with indexes previously known to produce successful libraries (A2, C2, E2 and F2). **Figure 5d.** AutoNorm of samples 5-8 with indexes that led to abnormal profiles (A9, B1, B8 and H5).

Index concentration will influence amplification profile

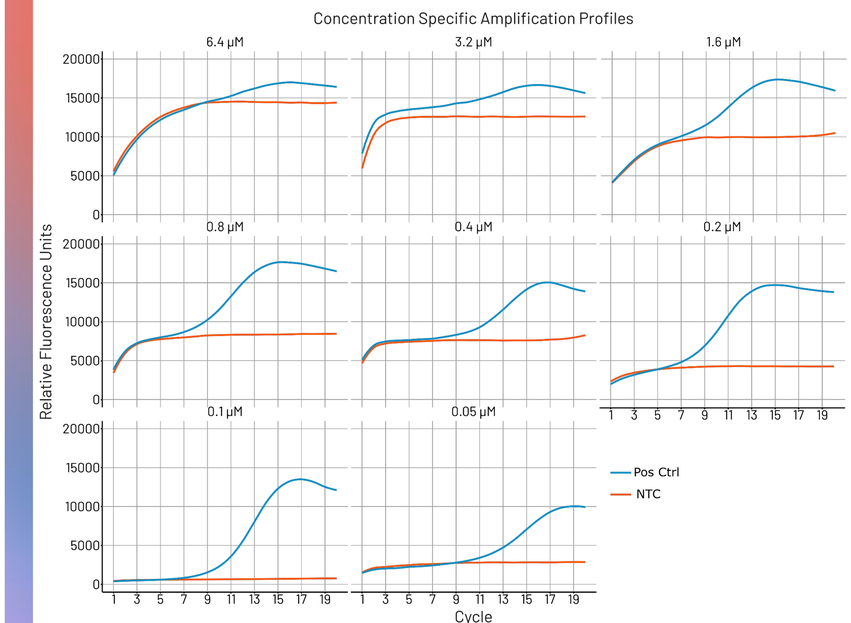


Figure 6. Influence of index concentration on library amplification profile. We observe that decreasing the concentration of indexes in the reaction restores a negative NTC profile (orange trace), while also reestablishing a normal amplification profile for the positive control (blue trace).

Influence of annealing temperature and reaction mix

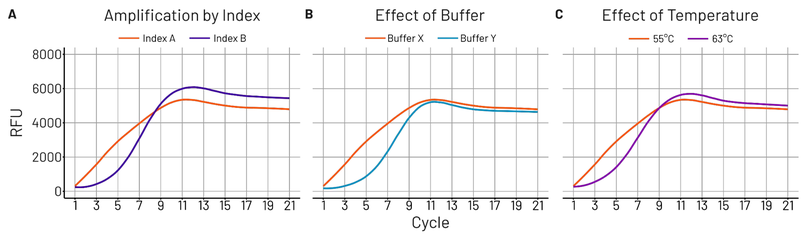


Figure 7a. Comparison of index A (orange) and index B (navy) with similar conditions (55°C, Buffer X). Index A showcases an atypical amplification profile, as opposed to the classical S-shape displayed by index B. **Figure 7b.** Comparison of Buffer X (orange) and Buffer Y (blue) with similar conditions (55°C, index A). The utilization of buffer Y restores the amplification profile of index A to a normal S-shape curve. **Figure 7c.** Comparison of annealing temperatures, 55°C (orange) and 63°C (purple) with similar conditions (Buffer X, index A). The increase of annealing temperature to 63°C restores the amplification profile of index A to a normal S-shape curve.

Conclusion

NGS indexes play a crucial role in multiplexing samples, but can also exhibit performance variations due to differences in primer design and synthesis. Index performance disparities can impact NGS library generation and sequencing results, leading to biased representation and incomplete coverage. Factors such as primer concentration, annealing temperature, and reaction mix conditions can influence index amplification profiles. Optimizing index primer design and PCR conditions is critical for minimizing amplification biases and ensuring reliable, high-quality NGS data.

iconPCR can be used to reveal, but also alleviate differences in index performance. AutoNorm of libraries can circumvent differences in PCR efficiency and allows users to bring all libraries to equivalent levels by monitoring the amount of DNA synthesis (intercalant dye fluorescence), rather than applying the same number of cycles to all libraries.