Evaluation of iconPCR™ Technology for Normalization of truCOVER® DNA Libraries from FFPE DNA

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Introduction

The iconPCR technology is a novel library amplification approach designed to normalize DNA library yields by amplifying each library to a predefined threshold. In collaboration with n6, this study evaluated whether n6's independently controlled PCR (iconPCR) technology could simplify normalization of truCOVER DNA library yields derived from DNA of low quality, such as FFPE DNA. Such DNA varies in quality and makes prediction of amplification efficiencies (post-library or during Illumina bridge-amplification) challenging.

A total of 30 DNA libraries were prepared from DNA isolated from HG001 cells as well as FFPE-conserved cells. In addition, libraries from DNA isolated from six different FFPE tissue samples were prepared in parallel. Feasibility of a comprehensive solution from DNA extraction to DNA-Library sequencing has been tested: FFPE DNA extraction, library preparation and PCR-amplification were performed using Covaris's kits and workflows and combined with n6's iconPCR workflow.

Methods

- FFPE DNA was extracted using the truXTRAC® FFPE SMART Solution (Covaris PN 520318) (1).
- DNA libraries were prepared using the truCOVER DNA Library Prep Kit without post-library amplification (Covaris PN 520361) (2). AFA-based shearing was 500 bp for HG001 and 200 bp for all FFPE DNAs. DNA input was 1, 5, and 25 ng for HG001 DNA, 5, and 50 ng for HG001 FFPE DNA and 50 ng for all other FFPE DNAs.
- Library amplification was performed via iconPCR using the truCOVER DNA Amplification kit (Covaris PN 520366) (2).
- Library quality and quantity were assessed by relative fluorescence (mass) and capillary electrophoresis (fragment size distribution).
- Libraries were pooled and sequenced on a NextSeq 2000. Data analysis was via the CLC Genomics Workbench (Qiagen).

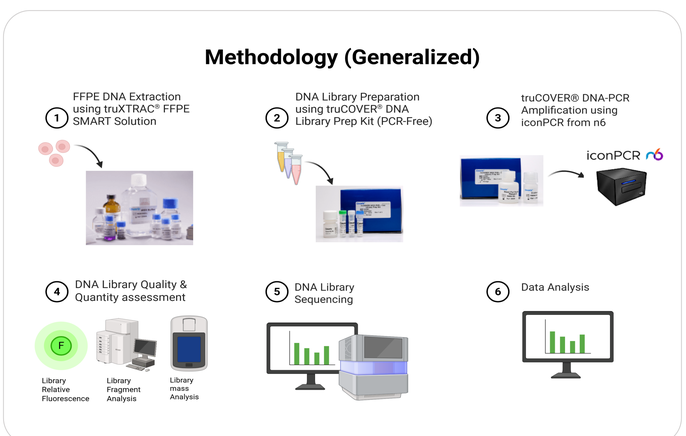


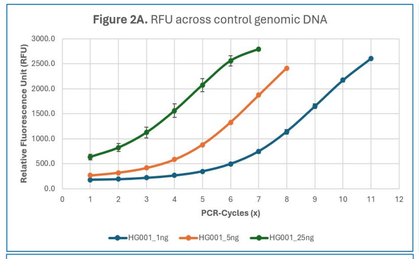
Figure 1. Overview of the workflow: FFPE DNA library preparation, iconPCR amplification, and DNA sequencing analysis workflow.

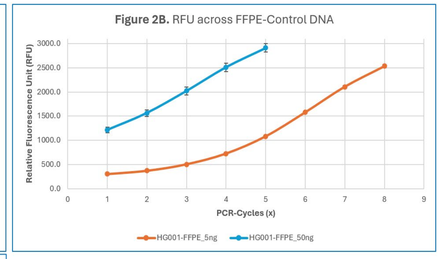
Results and Findings

Relative Fluorescence

Relative Fluorescence Units (RFU) values (mean ± St. Dev.) for each PCR cycle for all input amounts, and sample types were provided by n6 for (HG001 gDNA, N=2; HG001 FFPE and FFPE tissues, N=3 each).

Amplification profiles are depicted across varying input concentrations to assess the effect of input mass on amplification kinetics (***Fig. 2A and 2B***). ***Fig. 2C*** illustrates comparable library amplification behavior for multiple FFPE samples generated using a constant input of 50 ng DNA, despite varying DNA quality. Together, these data enable comparison of amplification consistency across different sample types and input conditions.





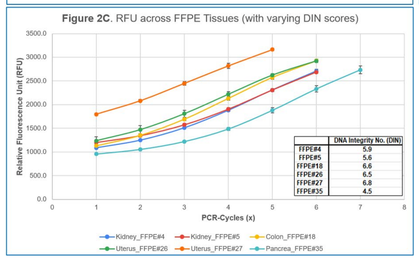
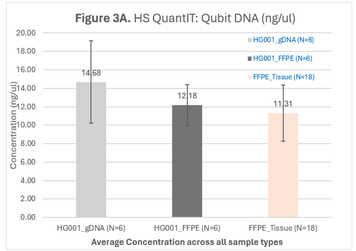


Figure 2. Real-time PCR amplification curves (RFU vs. cycle number) (mean ± St. Dev.). (***A***) control genomic DNA with varying input amounts. (***B***) FFPE control DNA with varying inputs. (***C***) FFPE tissue–derived libraries generated using a constant 50 ng input across a varying range of DNA Integrity Numbers (DIN).

DNA Library Yield

Qubit measurement was performed post-iconPCR, to access the library yields (mean ± St. Dev.) using a High Sensitivity dsDNA kit (Quant-IT Instrument) (***Fig. 3A***); Yield averaged highest for HG001 gDNA (~14.7 ng/µL), followed by HG001 FFPE (~12.2 ng/µL) and tissue samples (~11.3 ng/µL). While tissue-specific DNA quality impacts yield for FFPE libraries (***Fig. 3B***), all samples met the threshold for NGS pooling. Post-PCR quantification remains essential to manage this variability and ensure balanced inputs.



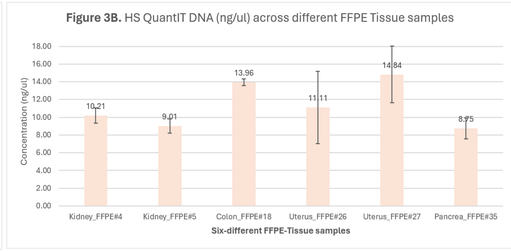
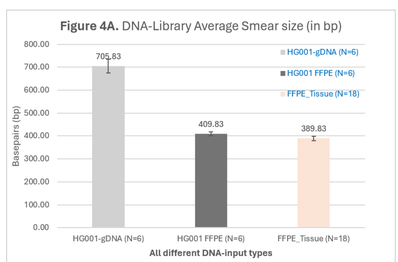


Figure 3. HS Quant-IT library concentrations (mean ± St. Dev.). (***A***) Comparison of control gDNA (light gray), control FFPE (dark gray), and FFPE tissue (orange). (***B***) Individual FFPE tissue samples by DIN score.

DNA Library Fragment Size Analysis

Fragment Analyzer profiles confirmed expected size distributions: ~706 bp for HG001 gDNA and 390–410 bp for FFPE-derived libraries (consistent after initial shearing to 500 and 200 bp, respectively and subsequent adapter ligation).

Uniform sizes across tissue types highlight the robustness of Covaris AFA-Shearing (3). These results indicate that iconPCR via the truCOVER kit and n6-Tec instrument introduces no detectable amplification bias.



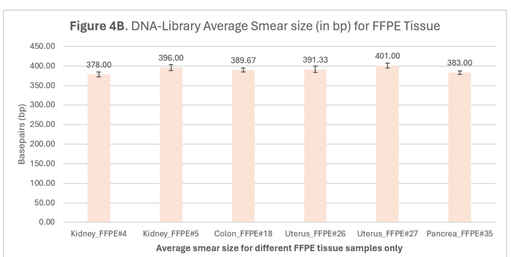
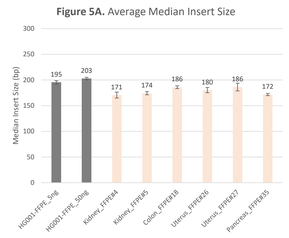


Figure 4. Library fragment size analysis. (***A***) Average fragment sizes for control gDNA (light gray), control FFPE (dark gray), and FFPE tissue (orange). (***B***) Distributions for individual FFPE tissues by DIN score. Size differences reflect protocol-specific shearing (500 bp for gDNA; 200 bp for FFPE). Data presented as mean ± St. Dev.

Next-Generation Sequencing and Analysis



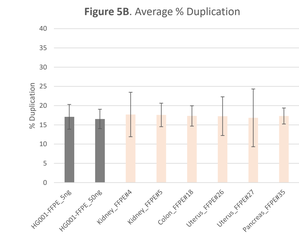


Figure 5. Sequencing metrics from low pass sequencing using a NextSeq 2000. (***A***) Sequenced fragment size across all FFPE libraries (median insert size ± St. Dev.). (***B***) Duplication rates in percent across all FFPE Libraries (Mean% ± St. Dev.).

Discussion and Conclusion

Libraries prepared from FFPE DNA are notoriously difficult to predict in amplifiability, both post-library as well as on flow-cell, resulting in different duplication rates. A-basic sites, chemical base modification and residual crosslinks can impact PCR amplification significantly (4). Thus, it is difficult to predict the necessary amplification cycle number beforehand to generate enough library mass for NGS analysis, and time- and resource-consuming qPCR is frequently used to assess such amplifiability.

The integration of the Covaris truCOVER DNA Library Prep workflow with iconPCR eliminates the need for laborious post-PCR qPCR quantification and manual normalization. The n6-Tec instrument monitors DNA synthesis in real-time to independently terminate each reaction at a required minimum PCR cycle, ensuring uniform library yields regardless of initial DNA integrity (DIN) scores. In Summary, this agnostic approach effectively prevents over-amplification artifacts and results in consistently balanced duplication rates across DNA samples isolated from diverse FFPE tissue types.

References

1. truXTRAC FFPE Total NA Auto 96 Kit | Covaris
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3. Process, Vanessa et al. "Optimization of DNA Fragmentation Techniques to Maximize Coverage Uniformity of Clinically Relevant Genes Using Whole Genome Sequencing." *Diagnostics (Basel, Switzerland)* vol. 15,18 2294. 10 Sep. 2025
4. Steiert, Tim A et al. "A critical spotlight on the paradigms of FFPE-DNA sequencing." *Nucleic Acids Research* vol. 51,14 (2023)

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