

## Introduction

Cell-free DNA (cfDNA) sequencing is increasingly essential for applications such as liquid biopsy, minimal residual disease monitoring (MRD), and ultra-low variant detection. These workflows require highly sensitive library preparation methods capable of converting extremely low quantities of fragmented DNA into high-quality sequencing libraries. Achieving high conversion efficiency, uniform coverage, and minimal bias is especially challenging when inputs are limited to the 1 – 10 ng range typical of cfDNA specimens. As laboratories scale their cfDNA sequencing efforts, automation has become critical for maintaining reproducibility, reducing hands-on time, and minimizing user-driven variability. At the same time, reagent lot-to-lot consistency is essential to ensure stable performance over time, particularly in translational and clinical environments where process variability must be tightly controlled.

The Watchmaker DNA Library Prep Kit was engineered to support low-input, highly fragmented templates while delivering uniform, high-complexity libraries suitable for cfDNA analysis. When integrated on the Hamilton Microlab® NGS STAR MOA platform, the workflow enables fully automated, end-to-end library construction with minimal user interaction. This study focuses exclusively on evaluating lot-to-lot reproducibility of the Watchmaker DNA Library Prep Kit for automated low-input cfDNA library preparation. Using four independent manufacturing lots, we assessed library yields, fragment size distributions, sequencing metrics, and concordance with manual preparation. Additionally, no-template controls (NTCs) were incorporated to evaluate contamination risk. Together, these results establish the robustness of the automated MOA workflow for sensitive cfDNA applications where consistency and reliability are essential.

## Automated method design

A single automated library preparation run was performed on the Hamilton Microlab NGS STAR configured with the Multiprobe Head On-Deck Thermocycler Add-On (MOA). The automated script executed all enzymatic steps, SPRI cleanups, and thermocycling operations on deck according to the Watchmaker DNA Library Prep Kit User Guide. The automated method is designed to allow for flexibility at runtime including dynamic SPRI ratios, adapter selection, optional PCR amplification for low-input samples, and integrated safe stopping points.

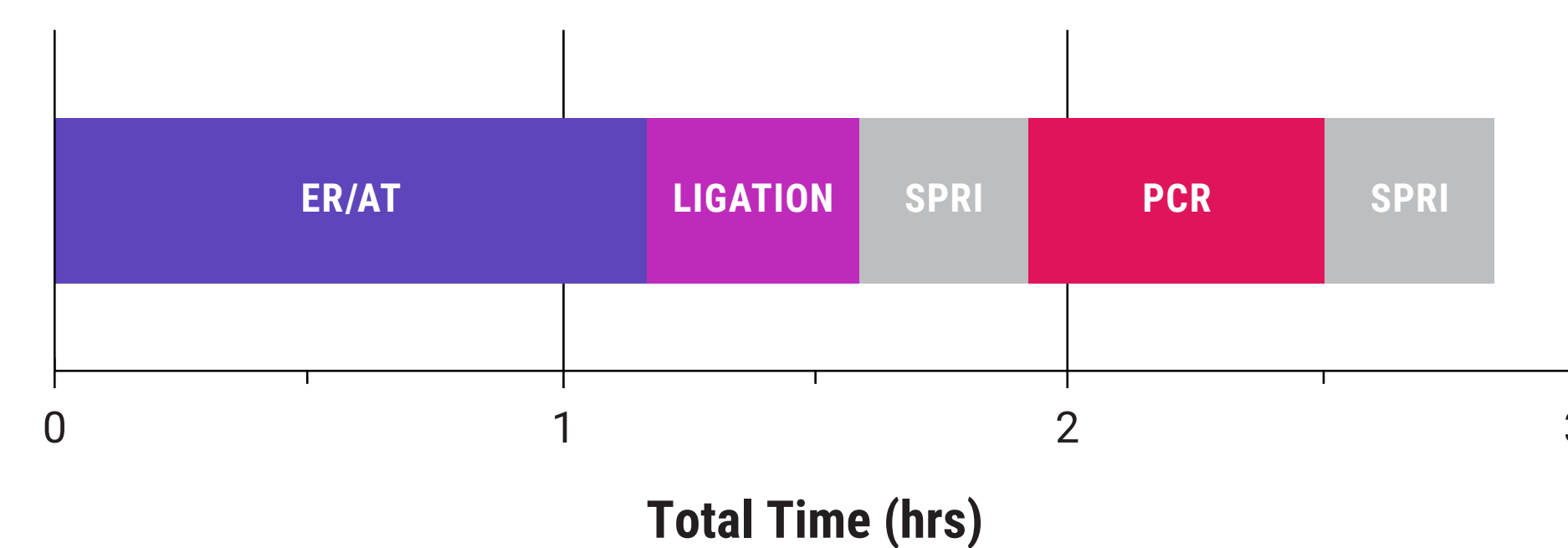


**Figure 1. Dynamic User Options.** (A) The Hamilton Microlab NGS STAR MOA enables flexibility and speed utilizing both its MPH and 8-independent channels. The Watchmaker DNA Library Prep script includes (B) dynamic runtime user options for safe start/stop points, (C) run conditions, (D) PCR cycles, (E) SPRI bead cleanup ratios, and more.

## Experimental design

### Library Preparation Chemistry

The Watchmaker DNA Library Prep Kit was used for all samples and reagent lots.



**Figure 2. Watchmaker DNA Library Prep Kit workflow overview.**

### Sample Inputs and Lot-to-Lot Evaluation

- **cfDNA inputs:** Human-derived cfDNA (IsoPure, Ripple Biosolutions)
  - 1 ng: 10 replicates per reagent lot (total 40 samples)
  - 10 ng: 2 replicates per reagent lot (total 8 samples)
- **Reagent lots:** Four independent manufacturing lots of the Watchmaker DNA Library Prep Kit processed in the same run
- **SPRI ratios:** 0.8X Post-ligation, 1.0X Post-amplification
- **Adapter:** 15 µM IDT xGen Stubby Adapters
- **PCR cycles:** 9 cycles
- **Controls:** 48 no-template controls (NTCs) interspersed across the plate to assess cross-contamination

|   | 1    | 2    | 3    | 4    | 5     | 6     | 7    | 8    | 9    | 10   | 11   | 12   |
|---|------|------|------|------|-------|-------|------|------|------|------|------|------|
| A | 1 ng |      | 1 ng |      | 10 ng |       | 1 ng |      | 1 ng |      | 1 ng |      |
| B |      | 1 ng |      | 1 ng |       | 10 ng |      | 1 ng |      | 1 ng |      | 1 ng |
| C | 1 ng |      | 1 ng |      | 10 ng |       | 1 ng |      | 1 ng |      | 1 ng |      |
| D |      | 1 ng |      | 1 ng |       | 10 ng |      | 1 ng |      | 1 ng |      | 1 ng |
| E | 1 ng |      | 1 ng |      | 10 ng |       | 1 ng |      | 1 ng |      | 1 ng |      |
| F |      | 1 ng |      | 1 ng |       | 10 ng |      | 1 ng |      | 1 ng |      | 1 ng |
| G | 1 ng |      | 1 ng |      | 10 ng |       | 1 ng |      | 1 ng |      | 1 ng |      |
| H |      | 1 ng |      | 1 ng |       | 10 ng |      | 1 ng |      | 1 ng |      | 1 ng |

**Figure 3. Sample plate-layout showing all inputs and NTCs.** For each independent kit lot, libraries were prepared using 1 ng or 10 ng input DNA, with NTC libraries interspersed to test for cross-contamination. Lot 1 was used in rows A and E, lot 2 in rows B and F, lot 3 in rows C and G, and lot 4 in rows D and H.

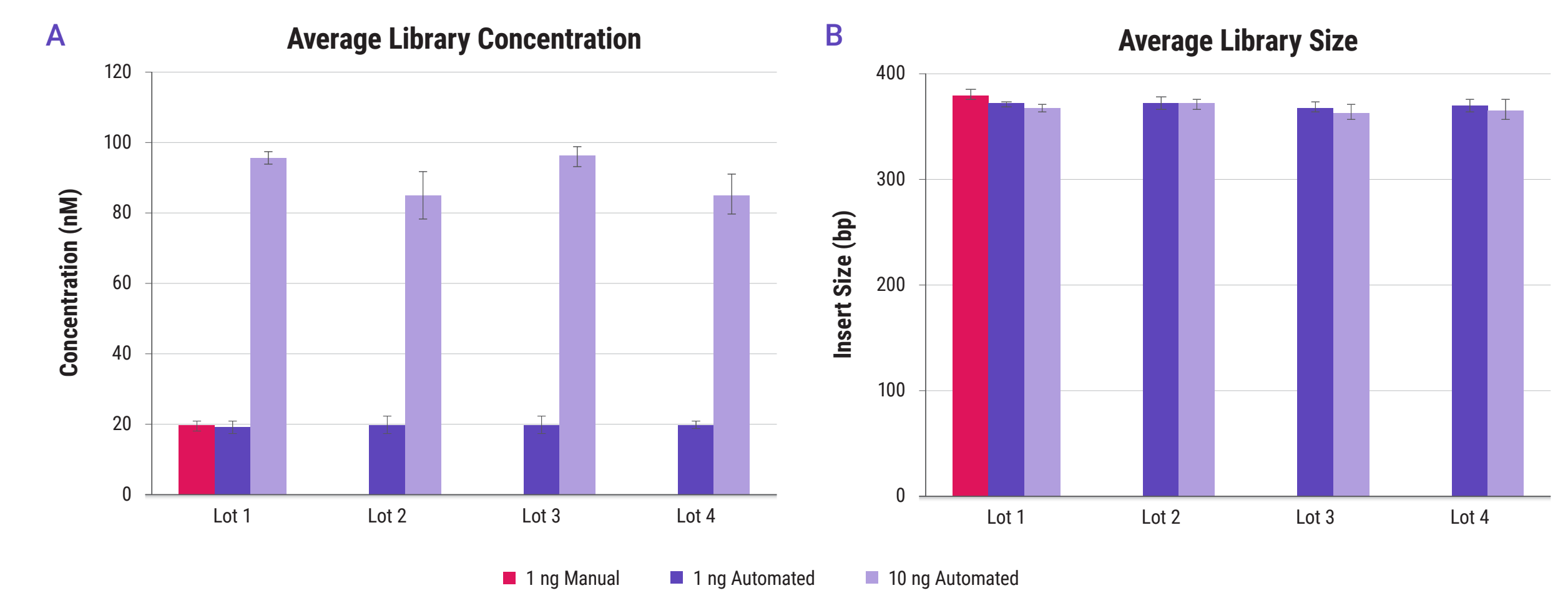
### Quality Control

- **Library yield:** Measured using DeNovix dsDNA Broad Range assay
- **Fragment size:** Evaluated on Agilent TapeStation D1000; electropherograms and average fragment sizes used to assess insert size consistency and lot-to-lot variation

### Sequencing and Data Analysis

- **Platform:** Illumina NextSeq 2000 (P2, 2x150 bp)
- **Sequencing data:** downsampled to 1M reads per sample for all libraries sequenced
- **Metrics evaluated:** Alignment rate, insert size, GC uniformity, and duplication rate

## Results

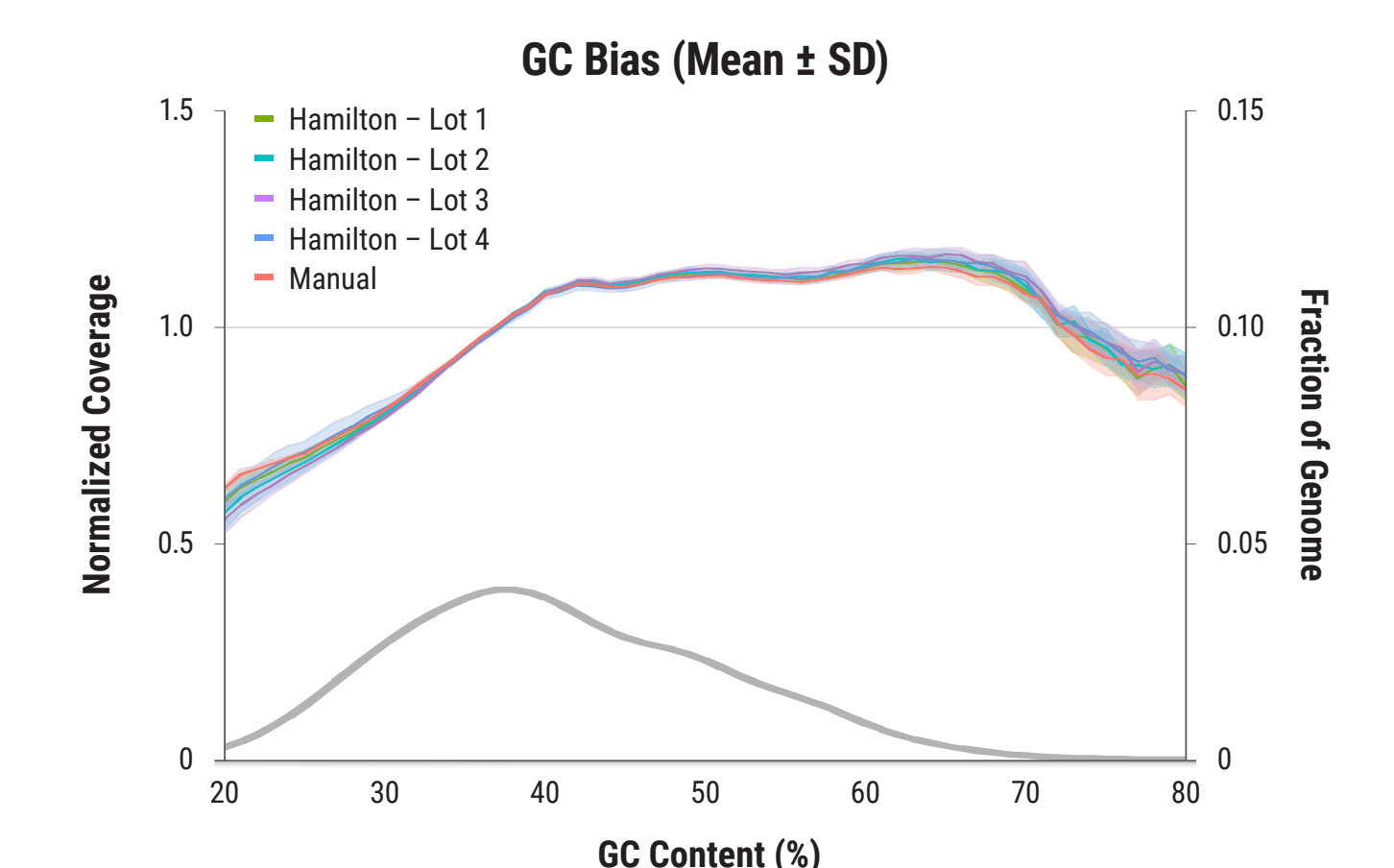


**Figure 4. All lots and inputs show stable, reproducible yield and size performance.** (A) As expected, library concentration varied according to input amount but was constant between kit lots and comparing manual and automated methods. (B) Average library size was not affected by input amount or manual vs. automation preparation method.

**Table 1. Sequencing performance**

| Kit lot | Input (ng) | Prep      | Alignment rate (%) | Insert size (bp) | GC uniformity (%) | Duplication rate (%) |
|---------|------------|-----------|--------------------|------------------|-------------------|----------------------|
| 1       | 1          | Manual    | 99.89              | 171              | 95.0              | 5.5                  |
|         |            | Automated | 99.88              | 170              | 94.7              | 6.4                  |
| 1       | 10         | Automated | 99.92              | 170              | 94.7              | 5.3                  |
|         |            | Automated | 99.88              | 170              | 94.5              | 6.6                  |
| 2       | 10         | Automated | 99.92              | 170              | 94.6              | 5.8                  |
|         |            | Automated | 99.88              | 170              | 94.3              | 6.3                  |
| 3       | 10         | Automated | 99.91              | 170              | 94.2              | 5.2                  |
|         |            | Automated | 99.88              | 170              | 95.0              | 6.0                  |
| 4       | 10         | Automated | 99.91              | 170              | 94.3              | 4.7                  |

Library prep performance was highly consistent across kit lots, input amounts, and preparation methods. All conditions showed >99.8% alignment, ~170 bp insert sizes, stable GC uniformity (~94 – 95%), and low duplication rates, with comparable results between manual and automated workflows.



**Figure 5. Consistent GC coverage across lots and inputs.** Normalized coverage for libraries prepared using four different kit lots using automation and one lot manually.

## Conclusions

Across all samples, inputs, and reagent lots, the automated workflow on the Hamilton Microlab NGS STAR MOA produced highly consistent, high-quality cfDNA libraries. Library yields and insert sizes were reproducible across lots, and the workflow demonstrated strong agreement with manually prepared libraries, confirming robust and scalable automated performance. Together, these results support the suitability of this automated workflow for sensitive cfDNA applications – including liquid biopsy, MRD monitoring, and ultra-low variant detection – where reproducibility, low-input performance, and process consistency are critical.

### Key highlights:

- **Insert size:** Tight distributions centered at ~170 bp across 1 ng and 10 ng cfDNA inputs (Table 1).
- **Sequencing quality:** Alignment rates >99.9% and low duplication (~5 – 7%) (Table 1).
- **GC uniformity:** Excellent coverage (~94 – 95%) across GC-rich and GC-poor regions (Table 1).
- **Reproducibility and cleanliness:** Consistent performance across lots with no detectable cross-contamination in no-template controls.