Twist FlexPrep UHT Library Preparation Kit Datasheet

INTRODUCTION

Advancements in next-generation sequencing (NGS) continue to decrease sequencing costs, which has enabled population-level genomic experiments that help study and diagnose genetic disorders. Despite sequencer advancements, library preparation remains a labor-intensive and expensive bottleneck. There are numerous library construction methods available, but they are all limited mainly by time and throughput.

The Twist FlexPrep UHT Library Preparation and Target Enrichment Workflows are designed to address these limitations by introducing two technologies: normalization-by-ligation and inline read barcodes. These two technologies offer a solution to reduce the burden of DNA normalization and reaction footprint by generating 12 libraries in a single well. This datasheet describes general performance expectations of various library preparation parameters when using these workflows and details the versatility of these kits. Additionally, we provide guidance for achieving desirable performance from these workflows.

METHODS

Twist FlexPrep UHT Library Preparation Kit was used following the manufacturer protocol[1]. Human genomic DNA (gDNA) NA12878 (41% GC) was obtained from Coriell Institute and quantified with Qubit™ dsDNA Broad Range Quantification Assay before use as substrates for library preparation. Microbial genomes of *Bordetella pertussis* (68% GC), *Clostridium difficile* (29% GC), and *Escherichia coli* (50% GC) were obtained from ATCC. Formalin-compromised DNA were obtained from Horizon and classified as Mild: DNA Integrity Number (DIN) ≥5.1, Moderate: DIN between 2.5 - 5.0, or Severe: DIN ≤2.0. Unless otherwise specified, fragmentation was performed at 32°C for 22 minutes.

For the ligation and PCR steps, 4 µl of Twist FlexPrep Normalization Adapters and 10 µl of Twist Unique Dual Indexed (UDI) - TruSeq Compatible primers were used, respectively. 2x Twist Library Amp Mix was used to amplify post-ligation pooling. gDNA library pools post-elution were quality checked for sizing with a DNA 7500 Kit on the Bioanalyzer and quantified with Qubit dsDNA Broad Range Quantification Assay.

After library preparation, samples were either sequenced at low depth for WGS or captured following Twist FlexPrep Target Enrichment Protocol[2]. When comparing exome performance, comparator samples were prepared using Twist Library Preparation Enzymatic Fragmentation (EF) Kit 2.0 and captured with Twist Target Enrichment Standard Hybridization v2 protocol. Library pools were sequenced 2x74 paired-end with at least 75x coverage on a NextSeq 550 unless otherwise specified. For all figures in the Results section, error bars or error distributions are standard deviations derived from N=2 samples unless otherwise specified.

RESULTS

FRAGMENTATION WITH VARIOUS DNA TYPES

The Twist FlexPrep UHT Library Preparation Kit leverages an enzymatic fragmentation that minimizes bias in coverage across genomes regardless of size or GC content. To demonstrate this, we prepared sequencing libraries from various microbial genomes with varying GC representation (*Bordetella pertussis*, *Clostridium difficile*, and *Escherichia coli*) and compared them against human NA12878 samples (**Figure 1**). The libraries produced with this kit exhibited highly uniform fragmentation, showing minimal bias and consistent insert size performance.

When working with formalin-compromised or damaged samples, it is important to note that the degraded nature may result in smaller fragment sizes and lower yields (**Figure 2**). While the molecular biology for fragmentation remains functional, we recommend optimizing fragmentation time and PCR cycles to achieve the best results. Such adjustments will compensate for the degraded input and ensure more consistent library preparation performance.

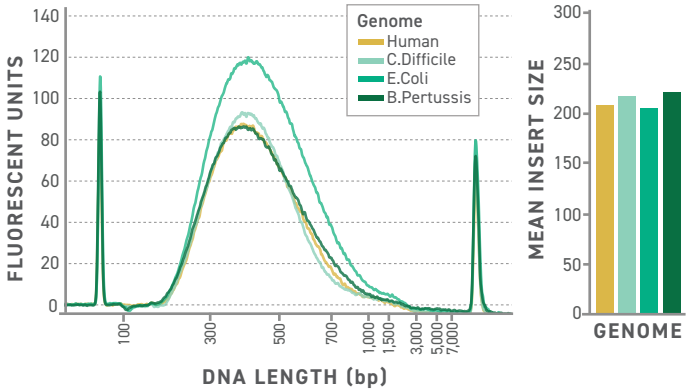

Figure 1. NGS Library Length profiles with various microbial genomes. (Left) Representative gel electropherograms for human and microbial NGS library pools produced from 60 ng of input DNA with fragmentation for 22 minutes at 32°C. (Right) Mean insert sizes as reported from Picard metrics post-sequencing alignment.

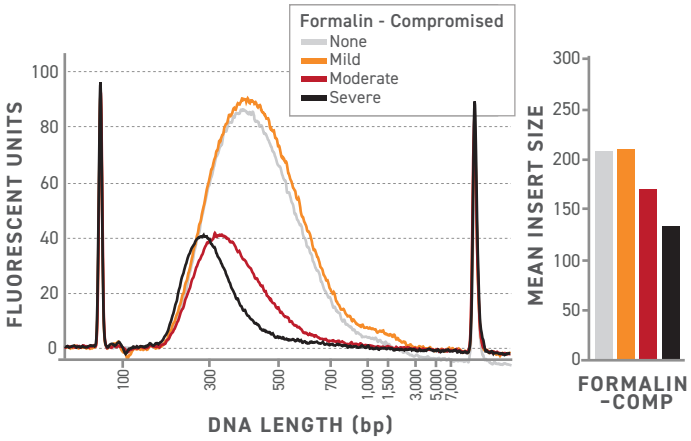

Figure 2. NGS Library Length profiles with various formalin-compromised DNA. (Left) Representative gel electropherograms for Human gDNA and various formalin compromised standards (Mild: DIN ≥5.1, Moderate: DIN between 2.5 - 5.0, Severe: DIN ≤2.0). NGS library pools produced from 60 ng of input with fragmentation for 22 minutes at 32°C. (Right) Mean insert sizes as reported from Picard metrics post-sequencing alignment.

FRAGMENTATION WITH VARIOUS DNA MASSES AND INCUBATION TIMES

The Twist FlexPrep Library Preparation Kit uses an enzymatic fragmentation process that allows for tuning of fragmentation time at 32°C to achieve different insert sizes. Fragmentation performance can be evaluated by performing quality control sizing evaluations at the 12-library pool level or the single-library level. Figures 3 and 4 and Table 1 show the observed fragment sizes and library yields after library preparation and insert sizes post-alignment as a reference in support of fragmentation time optimization guidance listed in the Twist FlexPrep Library Preparation kit protocol (Appendix C)[1].

This data also shows the potential pitfalls of working with sample inputs (5 ng or 600 ng) that are outside of the protocol's recommended 30-300 ng range. The effect of underloading mass can be inferred from the significantly lower library yield as presented.

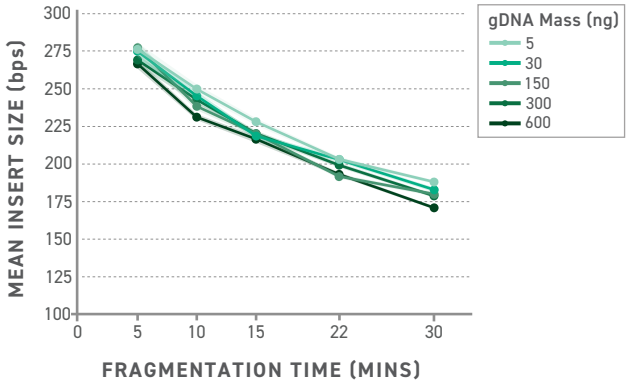

Figure 3. Mean Insert Sizes of libraries with various input gDNA masses and fragmentation time. Three hundred gDNA libraries prepared with 5, 30, 150, 300, and 600 ng of NA12878 template gDNA (Coriell Institute) with fragmentation incubation times of 5, 10, 15, 22, and 30 minutes at 32°C (N=12 for each condition). Mean insert sizes as reported from Picard metrics post-sequencing alignment.

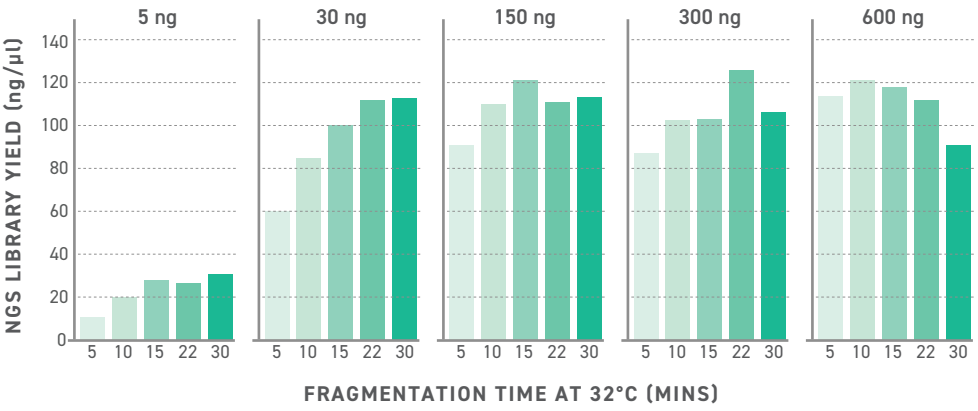

Figure 4. NGS Library Yield of libraries with various input gDNA masses and fragmentation time. gDNA library pools as described in Figure 3 were quantified post-PCR SPRI bead clean-up. NGS library yield is reported as dsDNA concentration (ng/µl) post-elution.

			12-LIBRARY POOLS	LIBRARY
MASS (ng)	TIME (MINS)	AVERAGE FRAGMENT SIZE (bp)	TOTAL YIELD (ng)	MEAN INSERT SIZE (bp)
5	5	554	240	276 ± 8
5	10	502	440	250 ± 6
5	15	458	607	228 ± 4
5	22	412	585	203 ± 3
5	30	392	673	188 ± 2
30	5	549	1320	275 ± 8
30	10	491	1866	245 ± 5
30	15	444	2200	219 ± 4
30	22	420	2464	203 ± 3
30	30	392	2486	183 ± 3
150	5	561	1993	277 ± 8
150	10	484	2420	238 ± 5
150	15	454	2662	220 ± 4
150	22	410	2442	192 ± 3
150	30	390	2486	180 ± 3
300	5	559	1914	269 ± 6
300	10	496	2266	243 ± 5
300	15	460	2266	220 ± 4
300	22	422	2772	199 ± 3
300	30	394	2332	179 ± 2
600	5	602	2508	267 ± 5
600	10	520	2662	231 ± 4
600	15	481	2596	217 ± 4
600	22	435	2464	193 ± 3
600	30	390	1980	171 ± 2

Table 1. Average Fragment Size, Total NGS Library Yield (ng), and Mean Insert Size after using the Twist FlexPrep UHT Library Preparation Kit with various input gDNA masses and fragmentation time. Average fragment sizes are measured by the Bioanalyzer (Agilent) with smear analysis between 150-1000 bp. Average fragment sizes and total yield are measured at the 12-library pool level as quality control steps after ligation and bead cleanup. Mean insert sizes are calculated at the single-library level after in silico demultiplexing. Green colors indicate mass within range and red colors (bold) indicate mass outside of recommended input range.

FRAGMENTATION CONSISTENCY

The Twist FlexPrep UHT Library Preparation Kit has undergone rigorous testing to ensure robustness, particularly in fragmentation performance. We demonstrate consistent, uniform fragmentation across three separate builds using different enzyme lots (**Figure 5**). This level of reliability ensures reproducible results across multiple experiments.

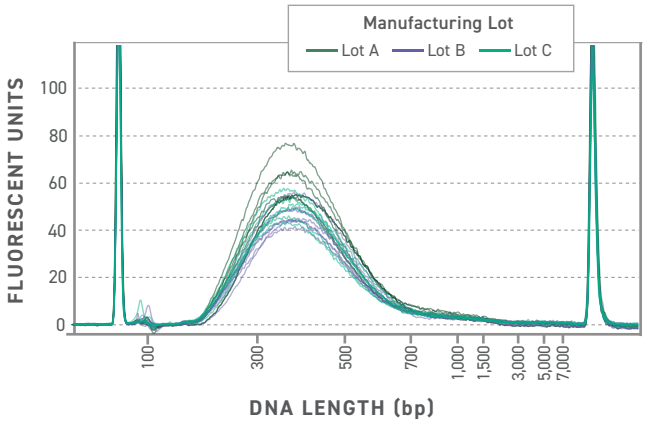

Figure 5. NGS Library Length profiles with separate manufacturing lots of enzymes and buffers. Three manufacturing lots of enzymes and buffers were built for robustness testing of fragmentation consistency. Ninety-six libraries were made with each manufacturing lot for a total of eight library pools per lot. Libraries were diluted 1:5 and ran on an Agilent Bioanalyzer 7500 assay for fragment size evaluation.

ADAPTER SATURATION WITH NORMALIZING LIGATION

gDNA quantification and normalization are essential steps that ensure consistent input for optimal enzyme performance and uniform conversion. This labor-intensive process is typically the rate-limiting step to processing large numbers of samples. The Twist FlexPrep UHT Library Preparation Kit leverages a novel normalization-by-ligation technology (**Figure 6**) that converts a fixed amount of DNA molecules into sequenceable DNA libraries via normalization adapters.

We demonstrate the normalization performance by taking various masses of gDNA (5-600 ng) into the Twist FlexPrep UHT Library Preparation Kit and ligating with either Twist Universal Adapters or Twist FlexPrep Normalization Adapters. Following six cycles of PCR, we present the total NGS library yields as a proxy for DNA conversion (**Figure 7**).

This data shows the uniform but knee-capped conversion of libraries with the Twist FlexPrep Normalization Adapters between 30-300 ng input masses. On the other hand, libraries with Twist Universal Adapters did not have yield limited to <1000 ng. FlexPrep normalization does not affect insert sizes (**Table 2**) but it does limit conversion to levels not suitable for applications involving detecting low-frequency variants.

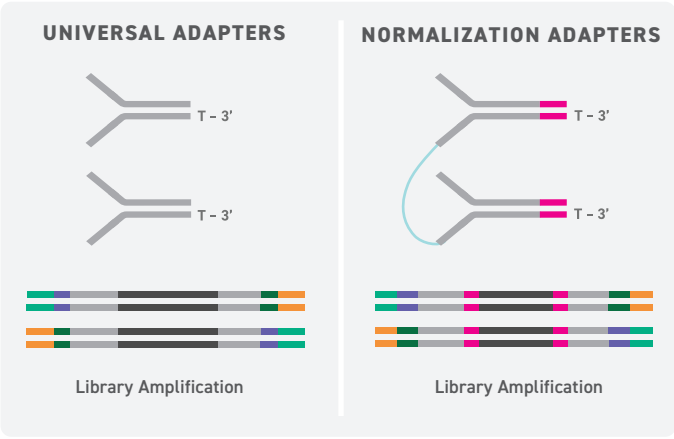

Figure 6. Structure of normalization adapters in comparison to universal adapters. Universal adapters (left) ligate onto insert molecules one side at a time. Both sides need to be ligated independently for successful library amplification. Normalization adapters (right) feature a linked loop and introduce an inline barcode (pink) to allow for multiplexed NGS library pooling for cleanup and PCR. Normalization adapters are compatible with UDIs that are designed for TruSeq libraries.

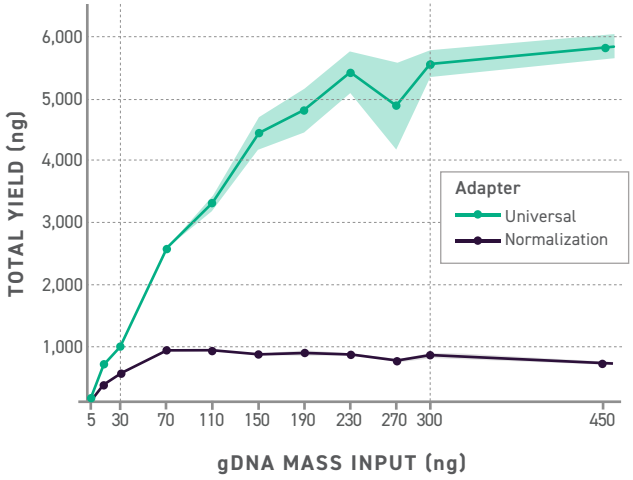

Figure 7. Total NGS Library Yield (ng) after using the Twist FlexPrep UHT Library Preparation Kit with various input gDNA masses. Individual gDNA library pools were prepared with the Twist FlexPrep UHT Library Preparation Kit and ligated with Twist Universal Adapters or Twist FlexPrep Normalization Adapters. After six cycles of PCR, libraries were quantified and total yield was calculated.

	LIBRARY		
MASS (ng)	ADAPTER	AVERAGE FRAGMENT SIZE (bp)	TOTAL YIELD (ng)
5	Twist Universal Adapters	400 ± 11	173 ± 7
15		398 ± 3	700 ± 10
30		393 ± 5	990 ± 10
70		395 ± 4	2580 ± 20
110		396 ± 1	3300 ± 170
150		395 ± 0	4400 ± 370
190		393 ± 2	4810 ± 490
230		393 ± 0	5430 ± 480
270		390 ± 6	4880 ± 990
300		393 ± 1	5600 ± 300
450		394 ± 4	5800 ± 280
600		398 ± 7	6220 ± 150
5	Twist FlexPrep Normalization Adapters	400 ± 13	99 ± 1
15		403 ± 10	380 ± 10
30		401 ± 7	568 ± 5
70		402 ± 6	950 ± 30
110		402 ± 8	939 ± 4
150		400 ± 7	872 ± 10
190		397 ± 4	890 ± 50
230		397 ± 5	862 ± 5
270		397 ± 4	780 ± 10
300		398 ± 8	860 ± 70
450		393 ± 2	729 ± 25
600		394 ± 1	684 ± 1

Table 2. Total NGS Library Yield (ng) and Average Fragment Size after using the Twist FlexPrep UHT Library Preparation Kit with various input gDNA masses. Data from Universal Adapters is on top and from Normalization Adapters is below. Green colors indicate mass within range and red colors (bold) indicate mass outside of recommended input range.

INLINE BARCODES FOR LIBRARY PREPARATION MULTIPLEXING

To reduce the reaction footprint, this kit uses a set of 12 inline barcodes that can be demultiplexed in silico after ligation pooling. This enables accurate assignment of reads back to their original well locations. The inline barcodes are paired and are read as the initial 7-8 cycles of sequencing, inclusive of the base covering the T-tail on the adapter molecule (**Figure 8**). These barcodes are carefully designed to provide uniform representation while maintaining color balancing[3], preventing signal saturation on Illumina platforms, and maintaining data integrity.

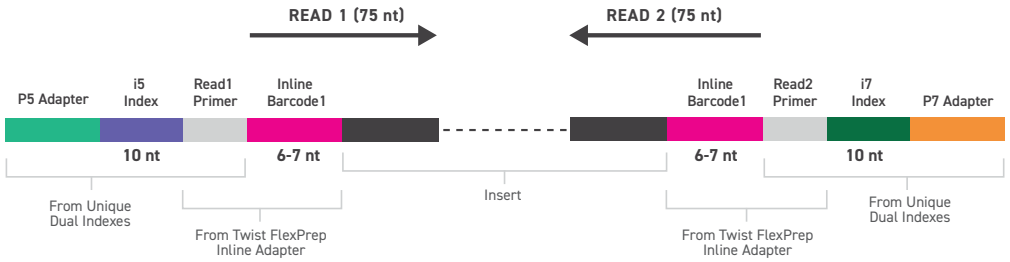

Figure 8. Twist FlexPrep library molecule structure for sequencing. FlexPrep libraries contain an additional paired barcode not present in the traditional NGS library structure that is read immediately at the start of sequencing read 1 and 2. Inline barcodes of two lengths, both 6 nt and 7 nt are used. This was done purposefully to ensure that the T-tail read during sequencing doesn't saturate the signal at one base call and is distributed between two base calls.

Notably, when used with inline barcodes, many ligases have a tendency to introduce bias[4]. However, with the Twist Engineered T4 DNA Ligase[5] and extensively screened barcodes, we observe consistent integration of the inline barcodes. This results in tight uniformity for demultiplexing samples after pooling and sequencing (**Figure 9**).

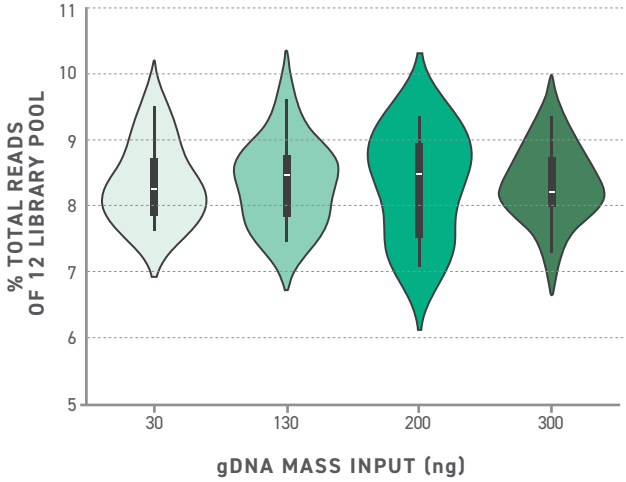

Figure 9. Percentage of NGS Library Reads identified to each inline barcode with constant gDNA mass as input. Sequencing reads are demultiplexed with fgbio and assigned to each identified inline barcode within the library pool of 12.

FLEXIBLE PROTOCOL FOR AUTOMATION

The Twist FlexPrep UHT Library Preparation Kit is designed for automation, featuring inline barcoding, self-normalization, and reduced reaction volumes compatible with 96- and 384-well formats. The standard manufacturer protocol includes specific incubation steps, heat denaturation, and cooling to 4°C, making it ideal for bench-top thermal cyclers. However, recognizing that some automated setups may lack on-deck space for cooling to 4°C, we assessed the impact of cooling to room temperature during the fragmentation and ligation steps. Despite a slight decrease in yield, library size and library yield remained above requirements. Additionally, pre-mixing ligation mix with normalization adapters showed no difference in performance, enabling single-tip use and faster workflows (**Figure 10**).

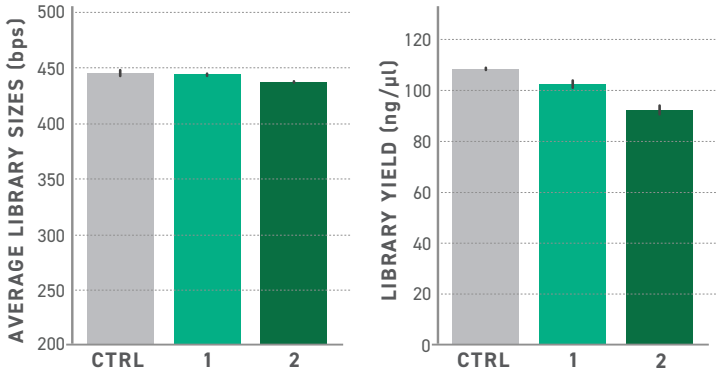

Figure 10. Twist FlexPrep UHT Library Preparation Kit performance with modified protocol conditions. Control reactions followed the standard FlexPrep manufacturer protocol. Condition 1 involved pre-mixing the ligation buffer, enzyme, and adapters before addition to each fragmentation reaction. Condition 2 omitted the 4°C incubation step, with samples instead incubated at room temperature for 15 minutes following the 65°C steps of both fragmentation and ligation. Each condition was tested with 2x12 library pools (replicates).

96-PLEX TARGET ENRICHMENT

To enable ultra-high throughput applications, the Twist FlexPrep Hybridization and Wash Kit allows up to 96 samples to be processed together in one target enrichment reaction. Traditionally, higher plexing has been limited by the number of high-complexity libraries used in target enrichment, with recommendations capped at 8-plex. This new kit overcomes those challenges by employing a ligation normalization chemistry that limits complexity, allowing for higher library plexing without compromising the capture performance. This solution is ideal for applications requiring moderate coverage, such as germline SNP detection or exome sequencing, where ultra-deep sequencing is not required.

Since the Twist Target Enrichment Standard Hybridization v2 protocol recommends 1500 ng library capture input for an 8-plex, we ran a characterization on optimal capture mass input for the 96-plex library capture and measured its performance (**Figure 11**). We see no change in off-target or uniformity with improved complexity when capture mass input is increased. However, when low mass is added into 96-plex library capture, the duplication rate is found to be higher. Based on this data, we recommend loading a total capture mass input of 4-10 µg.

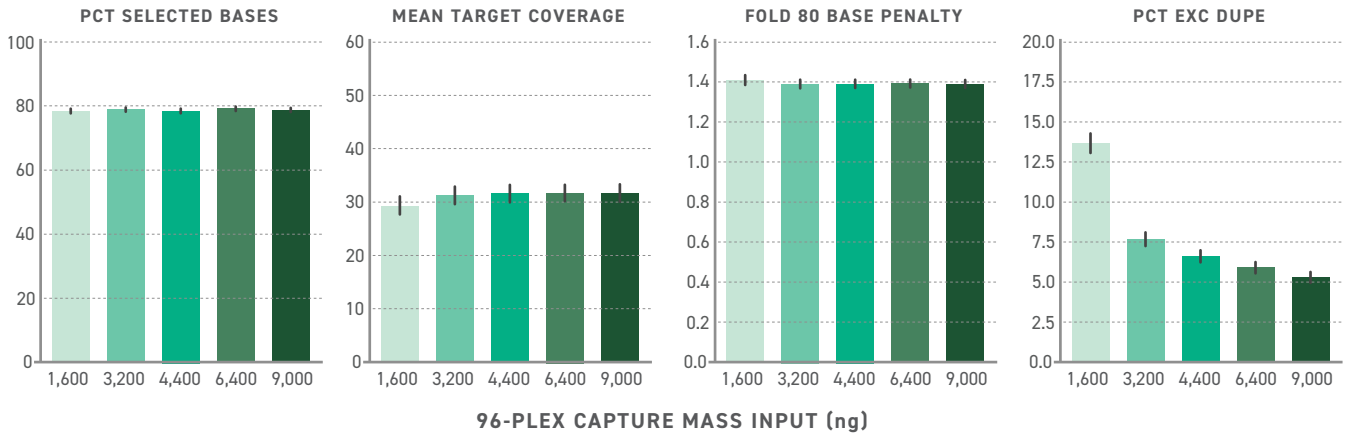

Figure 11. 96-plex target enrichment performance with Twist FlexPrep libraries using different capture mass inputs. Various masses between 30 - 300 ng of gDNA were arrayed out without normalization and loaded into the FlexPrep Library Preparation workflow. The FlexPrep libraries were then pooled into 96-plex pools and processed in the Twist FlexPrep Target Enrichment workflow using an 800 kb panel with various library mass input into capture and sequenced on an Illumina NextSeq 500/550. Analysis was done with bwa-mem and Picard with 75x downsampling.

EXOME PERFORMANCE

While the capture mass input characterization was done with an 800 kb oncology panel, we leveraged this guidance and tested a variety of gDNA mass inputs into library preparation that are combined into pools of 12 followed by a 96-plex exome target enrichment reaction. We observe the workflow produces high-quality results with uniform performance from various masses despite skipping quantification and normalization steps before both the library preparation and the capture protocols (**Figure 12 and Table 3**).

It is important to highlight that the library preparation kit employs normalization adapters, which inherently reduce library conversion. Users should anticipate lower overall performance in terms of estimated library size, an effect from both a lower library conversion and higher capture plexing (96-plex compared to the standard 8-plex). Therefore, we recommend that users carefully assess their specific complexity requirements when selecting workflows. We illustrate this tradeoff by comparing FlexPrep (96-plex) to a high-conversion alternative, the Twist Library Preparation EF Kit 2.0 with Twist Target Enrichment Standard Hybridization v2 (8-plex) (**Figure 13**). Although the FlexPrep kit demonstrates sufficient diversity and coverage to support certain germline applications, it may present limitations for somatic applications.

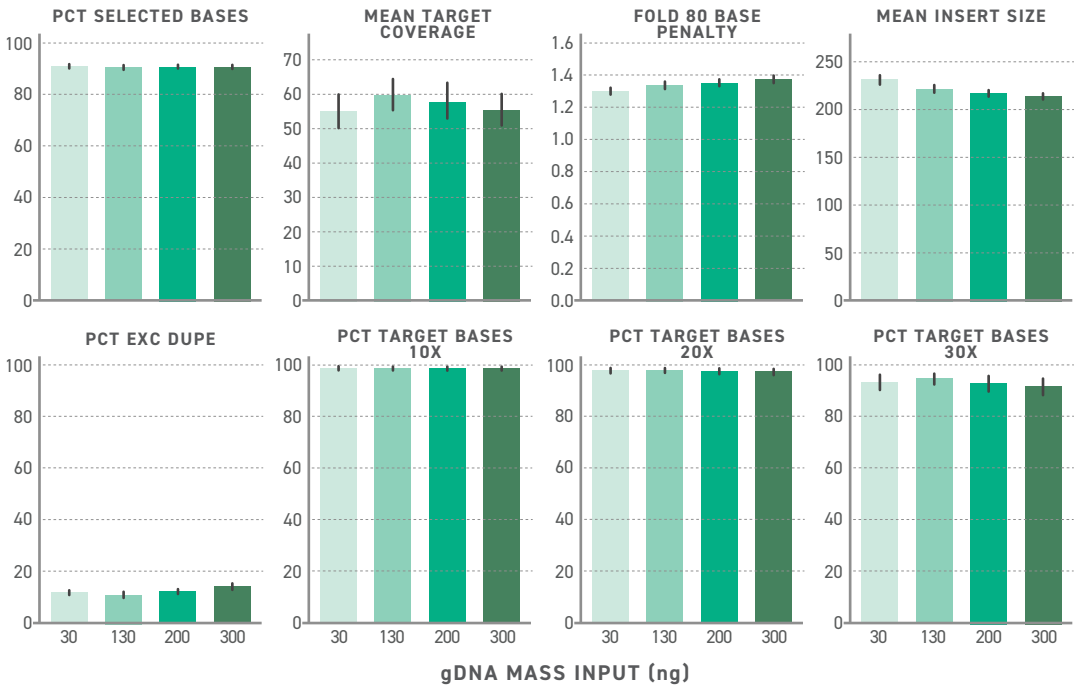

Figure 12. Twist FlexPrep libraries with 96-plex exome capture hybridization selection metrics. Various masses between 30 - 300 ng of gDNA were arrayed out without normalization and processed with the FlexPrep Library Preparation workflow. The FlexPrep libraries were then pooled into a 96-plex reaction and processed in the Twist FlexPrep Target Enrichment Workflow using an Exome panel and sequenced on an Illumina NovaSeq X. Analysis was done with bwa-mem and Picard with 150x downsampling.

MASS (ng)	PCT_SELECTED_BASES	MEAN_TARGET_COVERAGE	FOLD_80_BASE_PENALTY	MEAN_INSERT_SIZE
30	90.9 ± 0.2	55 ± 9	1.30 ± 0.02	231 ± 6
130	90.4 ± 0.4	60 ± 8	1.34 ± 0.02	222 ± 4
200	90.7 ± 0.2	58 ± 10	1.35 ± 0.02	217 ± 3
300	90.6 ± 0.2	55 ± 8	1.37 ± 0.02	214 ± 2
MASS (ng)	**PCT_EXC_DUPE**	**PCT_TARGET_BASES_10X**	**PCT_TARGET_BASES_20X**	**PCT_TARGET_BASES_30X**
30	11.6 ± 0.5	98.6 ± 0.1	97.7 ± 0.7	93.4 ± 4.7
130	10.7 ± 1.1	98.6 ± 0.1	97.8 ± 0.5	94.5 ± 2.8
200	12.0 ± 0.3	98.5 ± 0.1	97.5 ± 0.9	92.9 ± 5.0
300	13.9 ± 1.1	98.5 ± 0.1	97.2 ± 1.0	91.8 ± 5.1

Table 3. Twist FlexPrep 96-Plex Exome Capture Metrics.

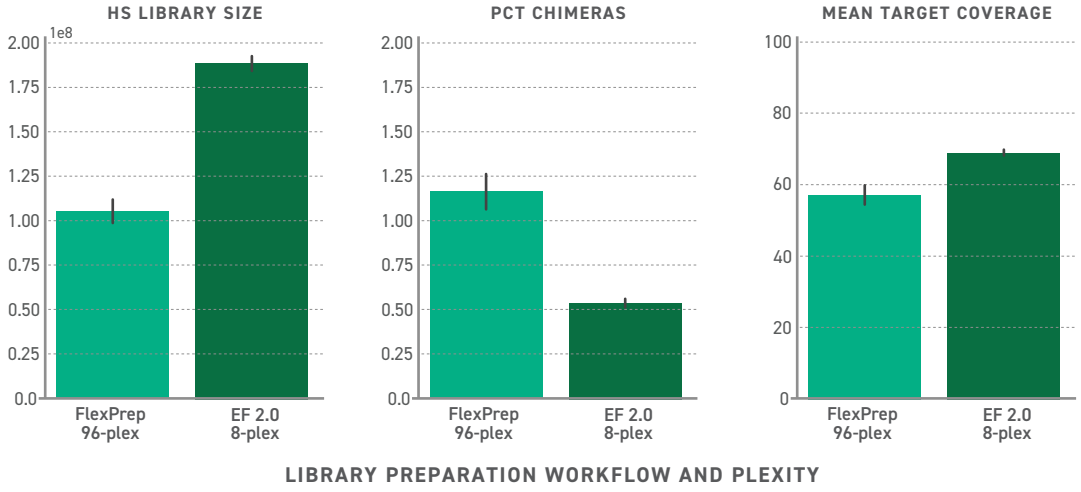

Figure 13. Twist FlexPrep libraries with 96-plex exome capture coverage metrics compared to EF 2.0 with 8-plex. Various masses between 30 - 300 ng of gDNA were arrayed out without normalization and loaded into the FlexPrep Library Preparation workflo w. The FlexPrep libraries were then pooled into 96-plex pools and processed in the Twist FlexPrep Target Enrichment Workflow using the Twist Exome 2.0 panel and sequenced on an Illumina NovaSeq X. The Twist Library Preparation EF Kit 2.0 libraries were prepared from 50 ng samples, pooled into an 8-plex, captured with the Twist Target Enrichment Standard Hybridization v2 workflow using the Twist Exome 2.0 panel, and sequenced 2x100 paired-end on an Illumina NovaSeq X. Analysis was done with bwa-mem and Picard with 150x downsampling.

CONCLUSIONS

Overall, the Twist FlexPrep UHT Library Preparation Kit allows for significant workflow improvement in NGS sample preparation. The kit's workflow skips the initial quantification and dilution of samples required in other standard workflows. The incorporation of inline barcodes during the ligation step allows for early pooling which reduces the number of reactions that need to be processed. In this datasheet, we provided a detailed demonstration highlighting the versatility of this kit with genomes of different GC content and the tunability of the fragmentation step for careful control of insert size selection. We demonstrate that the kit can convert DNA mass inputs within the recommended range (30-300 ng) and we also detail when samples can underperform. This datasheet includes trial methods that are automation-friendly with a minimal decrease in performance. Lastly, we detail 96-plex capture with the FlexPrep UHT Library Preparation Kit, showing the multiplexing performance possible with this kit. In comparison to the Twist Library Preparation EF Kit 2.0, the Twist FlexPrep UHT Library Preparation Kit generates libraries with lower complexity. Nonetheless, the complexity of FlexPrep libraries are adequate for general germline applications.

All charts, figures, and graphs are per Twist internal data, September 2024. Compared to Twist Library Preparation EF Kit 2.0.

REFERENCES

1. Twist Bioscience. Twist FlexPrep UHT Library Preparation Kit with Enzymatic Fragmentation and Twist UDI Primers https://www.twistbioscience.com/resources/protocol/twist-flexprep-uht-library-preparation-kit-enzymatic-fragmentation-and-twist-udi (2024).

2. Twist Bioscience. Twist FlexPrep Target Enrichment Protocol. https://www.twistbioscience.com/resources/protocol/twist-flexprep-target-enrichment-protocol (2024).

3. Twist Bioscience. Twist FlexPrep UHT Library Preparation Kit: Sample Demultiplexing Guide https://www.twistbioscience.com/resources/guideguideline/twist-flexprep-uht-library-preparation-kit-sample-demultiplexing-guide (2024.)

4. Potapov, V. et al. A single-molecule sequencing assay for the comprehensive profiling of T4 DNA ligase fidelity and bias during DNA end-joining. Nucleic Acids Res. 46, e79 (2018). https://pmc.ncbi.nlm.nih.gov/articles/PMC6061786/

5. Twist Bioscience. Twist-Engineered T4 DNA Ligase. https://www.twistbioscience.com/resources/technical-note/twist-engineered-t4-dna-ligase (2024).

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