Genotyping of animal and plant tissue samples using a high-throughput multiplexed NGS library preparation workflow coupled with streamlined DNA extraction

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Abstract

High throughput next-generation sequencing (NGS) workflows are required for population-level genomic research that aid SNP detection and discovery for genetic verification, breeding, and microbiome analysis. The cost and effort required in processing individual samples remain a barrier to efficient NGS library preparation. We present innovative and automation-friendly technologies that reduce cost and time: Normalization by Ligation™ (NBL) and library preparation multiplexing with inline barcodes. We perform experiments using our high-throughput workflow on real-life bovine samples and demonstrate concordance of SNP detection with ground truth datasets.

Genomic DNA (gDNA) isolation remains an obstacle for NGS library preparation kits that require higher purity samples compared to microarray technology. Twist DNA Purification Kit, a part of the Twist FlexPrep™ UHT PureAg DNA Library Preparation kit provides a cost effective and streamlined solution for generation of high quality gDNA using a simple workflow that reduces cost and plastic waste. Importantly, we demonstrate this purification kit is compatible with the high-throughput library preparation for more consistent results across samples.

Enzymatic fragmentation (EF) of samples is the foundation of our ultra-high-throughput method. The EF module allows researchers to adjust the insert size based on sequencing format and to use samples of varying quality and GC content without significant base composition bias. Normalization using NBL produces uniform coverage for samples of 30 ng to 300 ng DNA input, eliminating the need for initial input quantification. NBL integrates inline barcodes to permit multiplexing post-ligation. As a result, the reaction footprint and reagent usage of downstream steps decrease 12-fold, increasing throughput and generating libraries in less time. By controlling conversion from each sample, NBL enables 96-plex in the target enrichment step versus the 8-plex commonly used in other protocols. When paired with automated methods, sample capacity is greatly increased with minimal hands-on time needed.

To demonstrate the utility of our workflow, we extracted genomic DNA (gDNA) from both animal and plant samples using the Twist DNA Purification kit for high-throughput NGS library preparation. We obtain high quality and purity samples from this simple purification process that isolates gDNA in a single spin step without any washing steps. We demonstrate compatibility of this extraction with the FlexPrep UHT (Ultra High Throughput) Library Preparation kit for high-throughput sample processing. We observed great performance in coverage uniformity across non-normalized samples and can call SNPs at regions with various GC content. This high throughput library preparation and target enrichment protocol coupled with Twist DNA Purification allows for a streamlined workflow that saves users time and reduces reagent usage for easy processing of a high volume of samples for NGS.

Twist DNA Purification

Figure 1. Overview of Twist FlexPrep UHT Pure Ag for sample processing. Twist FlexPrep UHT Pure Ag DNA LP Kit enables sample preparation and library preparation, with options for target enrichment.

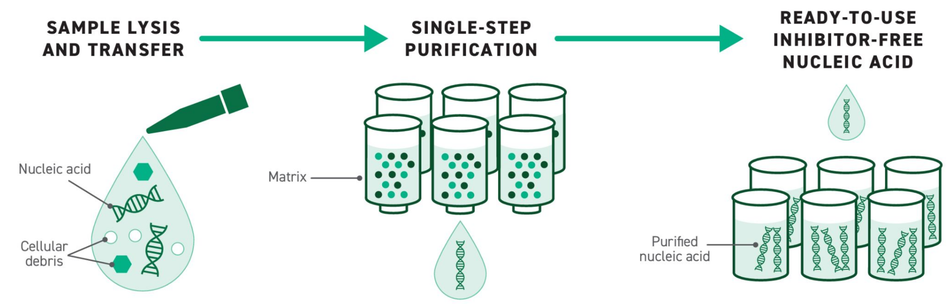

Figure 2. Schematic of Twist DNA Purification Kit workflow. DNA source material first undergoes lysis. The resulting lysate is then passed through a matrix that captures cell debris and contaminants. This one-step purification process requires no additional wash steps nor the use of ethanol. Finally, purified nucleic acid is collected in the flow-through and is ready for further downstream processing.

Twist DNA Purification Kit provides efficient DNA extraction process to generate high quality gDNA in single purification step.

High-Throughput Workflow

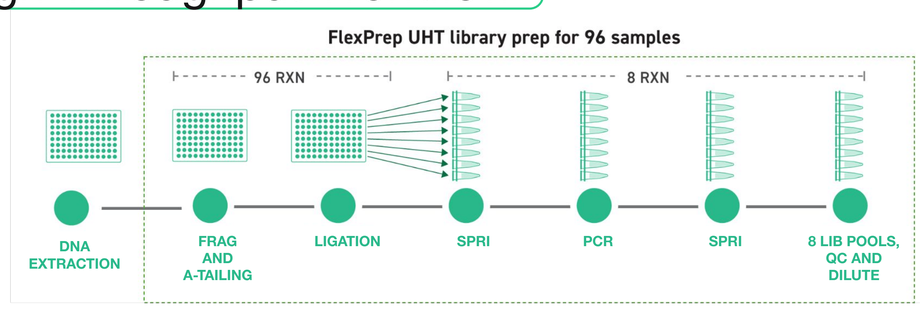

Figure 3. FlexPrep Ultra High-Throughput (UHT) Library Preparation Workflow for 96 Samples. Workflow begins with a fragmentation and A-tailing module in 96-sample format followed by ligation for each reaction individually using self-normalizing and inline barcoded adaptors. Inline barcodes allow 96 samples to be pooled into eight library pools.

Twist FlexPrep UHT Library Preparation Kit provides streamlined workflow that simplifies library preparation and reduces reagent usage.

Inline Barcode Adapters & Normalization

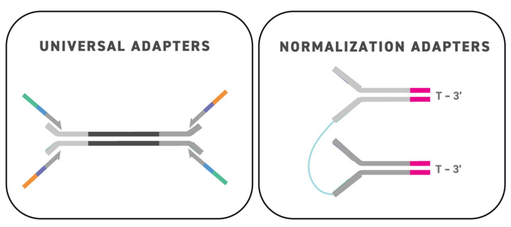

Figure 4. Structure of Normalization Adapters in Comparison to Universal Adapters. Twist FlexPrep Normalization Adapters feature a linked adapters and introduce an inline barcode (pink) to allow for multiplexed NGS library pooling for cleanup and PCR. Twist FlexPrep Normalization Adapters are compatible with Twist UDI primer system.

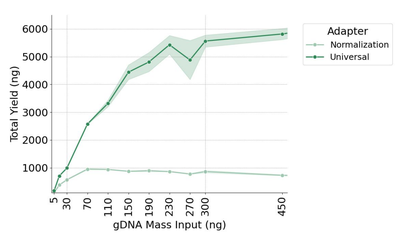

Figure 5. Total NGS Library Yield (ng) After Using the Twist FlexPrep UHT Library Preparation Kit with Various Input gDNA Masses. Individual gDNA library pools were prepared with the Twist FlexPrep UHT Library Preparation Kit and ligated with Twist Universal Adapters or Twist FlexPrep Normalization Adapters. After six cycles of PCR, libraries were quantified and total yield was calculated.

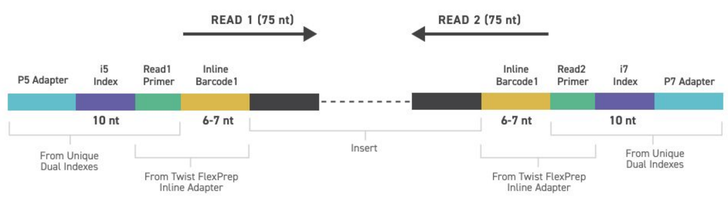

Figure 6. Twist FlexPrep UHT Library Structure. After ligation with Twist FlexPrep Normalization Adapters and indexing with UDI primers by PCR, library molecules will be flanked by hybridization adapters (P5/P7). Molecules will contain standard i5 and i7 indices used for pool-level demultiplexing, and inline with read 1 and read 2 will contain 6-7 bp inline barcodes for sample-level demultiplexing within each pool.

- Inline barcodes on adapters allow for pooling of samples post-ligation.
- Normalization adapters convert independent of insert concentration.

Genomic DNA Extraction and Library Preparation

- Collect tissue punches from bovine ears using Allflex TSU.
- Extract gDNA with Twist DNA Purification kit.

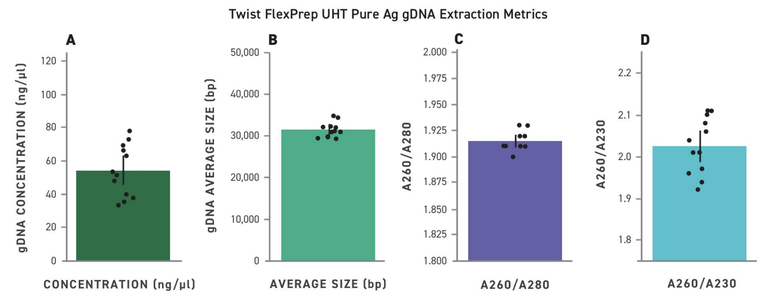

Figure 7. Genomic DNA extraction metrics from twelve samples prepared using Twist DNA Purification Kit. Replicates are n = 12, with individual samples shown as overlain dots. Allflex Tissue Sampling Units (TSUs) were used to collect tissue samples from frozen bovine ears. Lysis was performed with beads for 2 hours at 60°C with shaking every 30 minutes (2000 rpm for 1 minute). (A) gDNA concentration as measured by Broad Range dsDNA Qubit Assay. (B) Average size as measured by Genomic DNA ScreenTape assay; average size of fragments was between 100 bp and 60,000 bp. Nanodrop absorbance ratios of (C) A260/A280 and (D) A260/A230 for extracted gDNA samples.

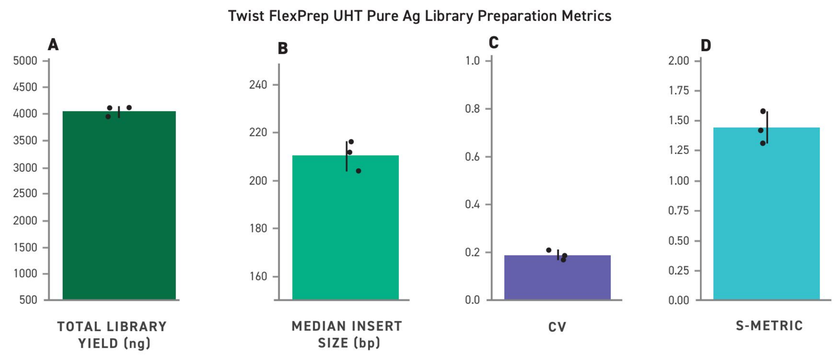

Figure 8. Library preparation and sequencing metrics from three pools of 12 samples prepared using the Twist FlexPrep UHT Pure Ag LP Kit. For each sample, 4 µl of gDNA was used as input into library preparation, skipping DNA normalization. Thirty-six total samples were processed for three total FlexPrep library pools. Library pools were sequenced with Illumina NextSeq 550. (A) Total library yield measured by Broad Range dsDNA Qubit Assay. (B) Median insert size of NGS library from sequencing. (C) CV of read counts from the 12 barcoded libraries in a FlexPrep library pool. (D) Normalization as measured by S-metric. S-metric is the mean of the read counts from each library in a pool of 12 divided by the minimum read count in the pool of 12; it measures how much relative sequencing is needed to catch up the least sequenced library with the mean sequencing depth of the pool.

Twist DNA Purification kit is suitable for gDNA extraction that can be used in Twist FlexPrep UHT Library Preparation.

Genotyping

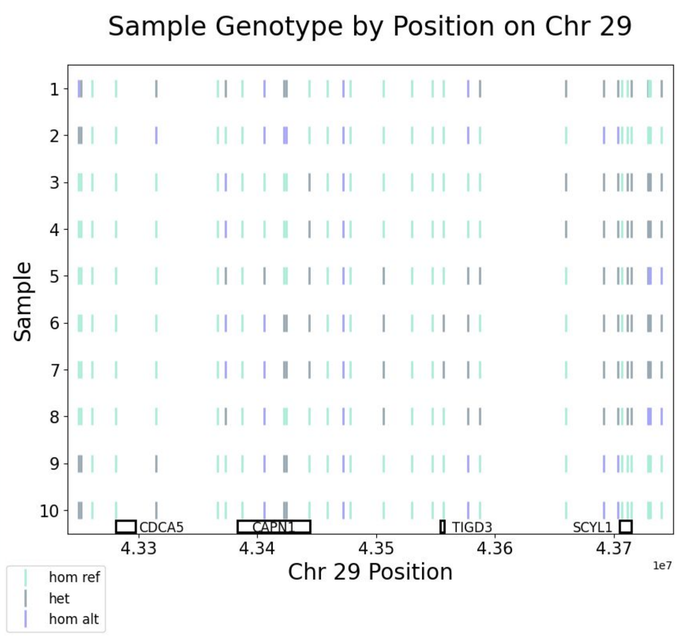

Figure 9. Genotype Calls on Section of Chromosome 29. Genome plot with genotype calls from 10 bovine samples on regions of chromosome 29 from 43.23 Mb to 43.75 Mb. Genotype calls are colored according to the legend above, demonstrating the diversity of calls observed from these individuals at this region. Allele calls: *hom ref* is homozygous reference, *het* is heterozygous, *hom alt* is homozygous alternate.

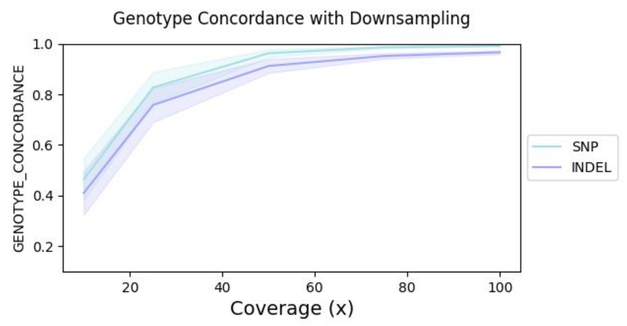

Figure 10. Genotype Concordance with Downsampling. Line plot with standard deviation of genotypes concordance between FlexPrep sample calls and ground truth PCR-free WGS calculated for both SNPs and INDELs. Concordance was calculated using the FlexPrep sample after different levels of downsampling.

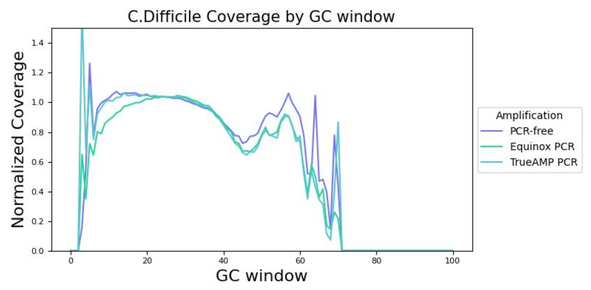

Figure 11. GC window coverage with TrueAmp Polymerase. Line plot with standard deviation of coverage by GC window from C. difficile genome between FlexPrep samples processed with 6-cycle PCR using Equinox or Twist TrueAmp compared to ground truth PCR-free WGS.

- Twist FlexPrep UHT Library Preparation and Target Enrichment can support genotyping.
- Genotype calls show high concordance with ground truth datasets.
- Genotype calls can differ by individual and demonstrate diversity observed in population.
- Twist TrueAmp Polymerase rescues coverage at low GC regions of genome.

Conclusions

We describe the Twist FlexPrep UHT Pure Ag workflow performed on bovine ear punches. Twist Purification Kit enables isolation of high quality gDNA suitable for NGS applications. Utilizing novel normalization adapters with inline barcodes allows the early pooling of samples and self-normalization to reduce the total number of steps, reagents, and consumables needed for the user. Specifically, the multiplexing technology allows for 12 samples to be pooled after ligation and for 96 samples to be pooled into a single target enrichment reaction, which reduces the total number of workflow steps, reagents, and consumables needed. Twist TrueAmp Polymerase Mix is able to recover challenging low GC regions that are lost in other NGS kits. Here we demonstrate the utility of FlexPrep UHT for generating NGS libraries from bovine ear punch samples that can be easily processed for genotyping purposes.

Conflict of Interest Statement

All authors are employees and shareholders of Twist Bioscience.