Twist cfDNA Pan-cancer Reference Standard Technical Guide

For use with the Twist NGS Workflow

This user guide describes the appropriate methods for preparing and quality checking short read sequencing libraries from the Twist cfDNA Pan-cancer Reference Standard in a next generation sequencing (NGS) protocol. This user guide does not provide bioinformatics help with aligning or calling variant sequences or determining variant allele frequencies.

The Twist cfDNA Pan-cancer Reference Standard is a synthetically-produced, quality-controlled, double-stranded DNA contrived sample intended to mimic plasma-isolated cell-free DNA (cfDNA) containing cancer-associated variant sequences at precise variant allele frequencies. This reference standard is intended for use in analytical limit-of-detection determination for NGS-based and digital PCR (dPCR) assays and ongoing analytical assay performance monitoring.

It is recommended to use either the Mechanical Fragmentation and Twist Universal Adapter System or the Library Preparation with the Twist UMI Adapter System protocol for preparing the Reference Standard for use with NGS-based sequencing. Please refer to twistbioscience.com/products/ngs for the latest protocols for all Twist Library Preparation workflows. **Note that performing library preparation with enzymatic fragmentation on cell-free DNA (cfDNA) samples is not recommended. This product is for research use only. This product is not intended for the diagnosis, prevention, or treatment of a disease or condition. Twist Bioscience assumes no liability regarding the use of the product for applications in which it is not intended.**

LIBRARY PREPARATION PROTOCOL

DOC-001087 Mechanical Fragmentation and Twist Universal Adapter System
DOC-001282 Library Preparation with the Twist UMI Adapter System

REAGENTS COMPONENTS

Required components include those listed in the selected Twist Library Prep protocol and the components listed below. It is recommended to use the Twist Mechanical Fragmentation Library Preparation Kit with the Pan-Cancer Reference Standards and cell-free DNA samples. All user-supplied materials in protocol will be required to use the Twist Pan-Cancer Controls.

Read the product packaging and storage recommendations carefully for each component, and store components as recommended below immediately upon arrival.

PART NUMBER	DESCRIPTION
104549	Twist cfDNA Pan-cancer Reference Standard set, 300 ng per tube*
104563	Twist cfDNA Pan-cancer Reference Standard VAF 0% (WT), 3 µg
104564	Twist cfDNA Pan-cancer Reference Standard VAF 0.1%, 3 µg
104565	Twist cfDNA Pan-cancer Reference Standard VAF 0.25%, 3 µg
104566	Twist cfDNA Pan-cancer Reference Standard VAF 0.5%, 3 µg
104567	Twist cfDNA Pan-cancer Reference Standard VAF 1%, 3 µg
104568	Twist cfDNA Pan-cancer Reference Standard VAF 2%, 3 µg
104569	Twist cfDNA Pan-cancer Reference Standard VAF 5%, 3 µg

**Kit includes 7 VAFs individually: 0% (WT), 0.1%, 0.25%, 0.5%, 1%, 2%, 5% at 300ng each tube*

RECOMMENDED TWIST LIBRARY PREPARATION REAGENTS

Refer to the “Materials Supplied by User section of the selected Twist Library Prep protocol.

PART NUMBER	DESCRIPTION
101280	Twist Library Preparation Kit, Mechanical Fragmentation, 16 Samples
101281	Twist Library Preparation Kit, Mechanical Fragmentation, 96 Samples

PROTOCOL GUIDANCE

Input mass

The reaction size of the Twist cfDNA Pan-cancer Reference Standard is 30 ng of DNA input. This mass of input differs from the mass stated as being optimal in the Twist's Library Preparation protocols. This reaction size reflects the standard isolated sample quantity from a 5 mL plasma DNA isolation from a healthy donor.

Input size profile

The Twist cfDNA Pan-cancer Reference Standard has a DNA Fragment size distribution similar to that of native cfDNA. For example (fig. 1), a Bioanalyzer instrument will detect a sharp, prominent mononucleosomal peak at approximately 167 bp, a secondary lower-intensity dinucleosomal peak at ~334 bp, and possibly additional peaks, though not always. Precise size of peak calls may vary. **The Twist cfDNA Pan-cancer Reference Standard does not require any fragmentation and any further fragmentation would reduce the characteristic size profile of cfDNA expected in the final sequencing library.**

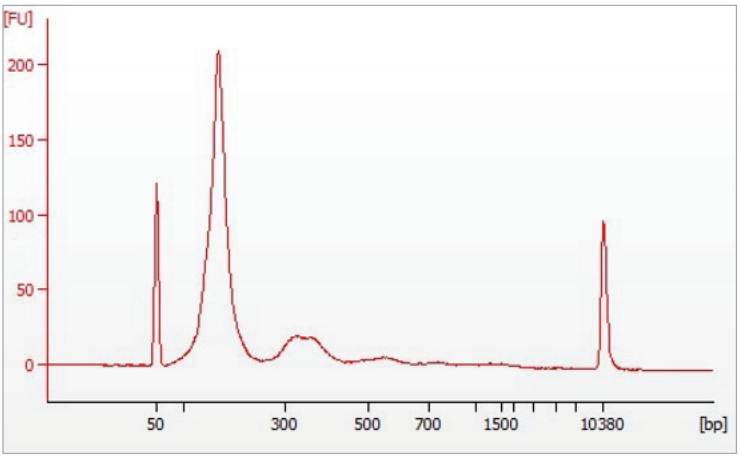

Figure 1: Pan-Cancer Reference Standard size profile. The Pan-Cancer Reference Standard was run on a Bioanalyzer 7500 chip according to the manufacturer's instructions. The size distribution indicates a predominant mononucleosomal peak annotated in this trace at 168 bp, as well as a dinucleosomal peak called in this trace at 331 bp. Additional peaks can sometimes be resolved from baseline signal.

PROTOCOL ALTERATIONS

Minor modifications to the selected Twist library preparation protocol are recommended for optimal results from cfDNA inputs, including the Pan-Cancer Reference Controls.

PARAMETER MODIFICATION	RATIONALE
Reduced input mass to 30 ng	Standard output from a 5 mL plasma DNA isolation from a healthy donor is approximately 30ng, so the input requirement has been lowered to accommodate this amount.
PCR cycles set to 8	The Twist Mechanical Fragmentation Library Prep protocol gives a recommended range of PCR cycles for the thermal cycler and PCR mix. When using the Pan-Cancer Reference Standard 30 ng input, 6 to 8 cycles of library PCR is the recommended range of cycle number and typically gives satisfactory library yield.
Reduce adapter volume to 4 µL	Twist library prep protocols recommend a set volume of adapter, assuming certain ranges of input DNA mass. Because cfDNA typically is available in lower mass aliquots, 4 µL of adapter is the recommended adapter volume to compensate.

OUTPUT DNA

The final amplified indexed libraries should be run on a Bioanalyzer 7500 chip (or equivalent) and quantified for total dsDNA using a Qubit quantification platform (or equivalent), consistent with the Twist Mechanical Fragmentation Library Prep protocol. After 8 cycles of PCR, yield should be greater than 100 ng/µL, though yields outside of that range may be acceptable so long as sufficient material is available for downstream steps such as target enrichment via hybrid capture. Note that too little mass input into hybrid capture may result in low complexity enriched libraries. A Bioanalyzer system should detect a sharp, prominent mononucleosomal peak at ~325 bp, a secondary lower-intensity dinucleosomal peak at ~490 bp (fig 2). Additional cfDNA peaks are usually not present in the prepared libraries.

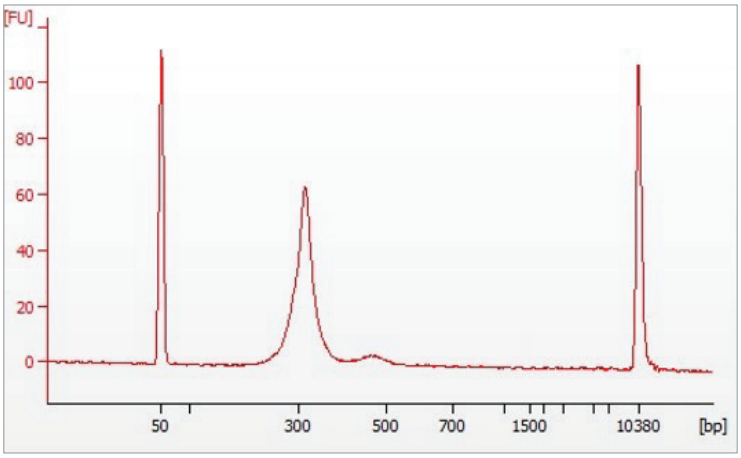

Figure 2: Size profile of prepared Illumina library of Pan-Cancer Reference Standard. A prepared and SPRI-purified Illumina TruSeq library made from the Pan-Cancer Reference Standard was run on a Bioanalyzer 7500 chip according to the manufacturer's instructions. The size distribution indicates a predominant mononucleosomal peak annotated in this trace at 315 bp, as well as a dinucleosomal peak called in this trace at 465 bp. Additional cfDNA peaks are not typically resolved from baseline signal in the library sample.

NEXT STEPS

Once incorporated into a short read library, the Twist cfDNA Pan-cancer Reference Standard can be treated like a cfDNA-derived library. It can be carried into target enrichment. If multiplexing, follow standard Twist multiplexing guidelines (and pool based on mass, like other samples). The Twist cfDNA Pan-cancer Reference Standard can be multiplexed with native cell-free DNA samples. To facilitate analysis, VCF format files of indels and single-nucleotide variants are available on the product website, as well as tables containing the breakpoints for structural variants.