TECH NOTE

Using Covaris microTUBE-15 to shear samples for SMARTer ThruPLEX library preparation

Tech note developed in collaboration with: Hamid Khoja and Guillaume Durin, Covaris, Inc., Woburn, Massachusetts, United States

Introduction

Robust library preparation methods that ensure high-quality libraries from a wide range of samples are critical for research applications as next-generation sequencing progresses to the clinic. In translational genomics where NGS is used extensively with oncological samples, the emphasis is on compatibility with a wide range of input types, quantities, and quality. Also important is the ability to accommodate various levels of sample degradation, especially with FFPE tissue, and overall simplicity of the workflow. In this application note, we describe a library preparation workflow that combines the SMARTer ThruPLEX DNA-seq Kit and the Covaris truSHEAR microTUBE-15 for a fully optimized low DNA input solution.

The Covaris truSHEAR mechanical shearing technology is recognized as the gold standard for high-quality, robust, and highly reproducible fragmentation of nucleic acids. It employs patented Adaptive Focused Acoustics (AFA) technology for the controlled and easily tunable shearing of nucleic acids. Compared to enzymatic-based fragmentation or tagmentation, AFA hydrodynamic shearing generates a tight and completely random fragmentation fundamental to obtaining uniform genome coverage (Van Nieuwerburgh et al. 2014; Lan et al. 2015) and high-complexity libraries (Samorodnitsky et al. 2015).

AFA-based hydrodynamic shearing is highly versatile, with the unique ability to deliver the same level of performance across a broad combination of DNA input sources, concentrations, and volumes. The AFA microTUBE-15 is optimized for a 15-µl sample volume and is ideal for the SMARTer ThruPLEX DNA-seq input requirements. The microTUBE-15 incorporates AFA-Beads that enable fully controllable, easy-to-use, and highly reproducible DNA shearing in very low sample volumes with high recovery.

The SMARTer ThruPLEX DNA-seq library preparation kit uses a patented stem-loop technology to repair DNA, reduce background, and generate high-complexity libraries. The SMARTer ThruPLEX DNA-seq Kit can generate DNA libraries from as little as 50 pg of DNA while providing up to 96 indexes for multiplexing. This kit can be used with fragmented DNA from any sample source—biofluids such as cell-free DNA, DNA from FFPE materials, and cDNA (Murtaza et al. 2013). The entire SMARTer ThruPLEX DNA-seq Kit workflow is performed in a single tube or well in about 2.5 hours and requires no intermediate purification steps or sample transfers. It can be used in a variety of applications, including DNA-seq, RNA-seq, and ChIP-seq, and offers robust target enrichment performance with all of the leading platforms.

The combination of AFA-based DNA mechanical shearing and library preparation with SMARTer ThruPLEX DNA-seq enables a uniquely simple, fast, and robust workflow optimized not only for routine use, but also for precious and rare samples, since DNA input can be as low as 50 pg. The total processing time of the present workflow is less than 3 hours (Figure 1).

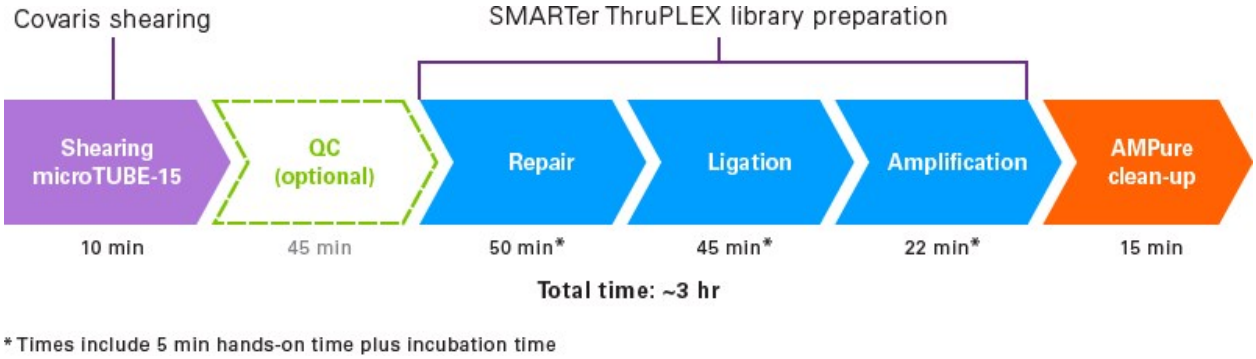

Figure 1. Complete DNA library preparation workflow beginning with shearing in the Covaris microTUBE-15 followed by Takara Bio's single tube,

low-volume, SMARTer ThruPLEX repair, extension, and amplification workflow. The total workflow time is less than 3 hours. *Each step represents total time including 5 min hands-on plus incubation time.

Results

The main purpose was to create a straightforward workflow combining the shearing and library preparation steps without need for additional sample manipulation or concentration adjustment while maintaining comparable results to more tedious protocols.

A summary of the fragment sizes and tubes is indicated in Table I. For the shearing step, each sample was prepared in duplicate with a total of 24 samples. The library preparation step was then completed in triplicate for each sample.

Input amount for different average fragment sizes			
		Fragment size	
Input amount		150 bp	350 bp
	50 pg (5 pg/µl)	15 µl microTUBE-15 130 µl standard volume	15 µl microTUBE-15 130 µl standard volume
	1 ng (0.1 ng/µl)	15 µl microTUBE-15 130 µl standard volume	15 µl microTUBE-15 130 µl standard volume
	10 ng (1 ng/µl)	15 µl microTUBE-15 130 µl standard volume	15 µl microTUBE-15 130 µl standard volume

Table I. Three different DNA input amounts in two different average sizes were prepared for downstream library generation.

Sheared DNA was analyzed on a Bioanalyzer (Agilent Technologies) to measure the size and reproducibility of shearing. Examples of each category are shown below (Figure 2).

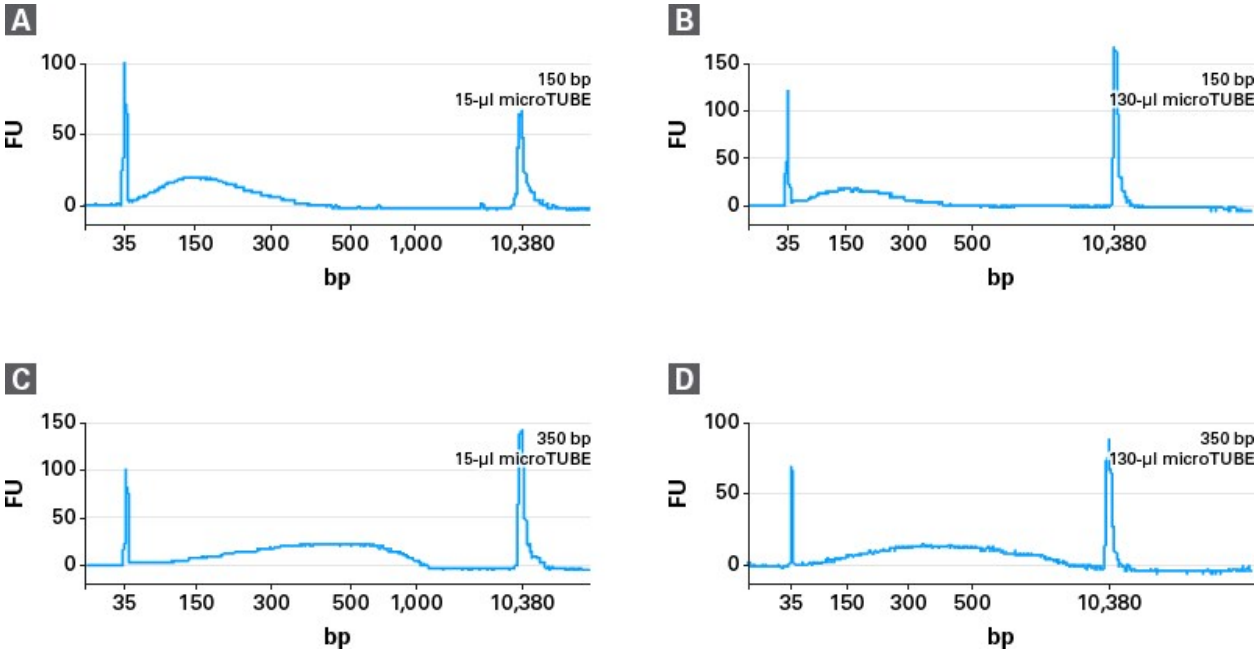

Figure 2. Examples of 1 ng sheared DNA. Panel A. 150 bp in a 15-µl microTUBE. Panel B. 150 bp in a 130-µl microTUBE. Panel C. 350 bp in a 15-µl microTUBE. Panel D. 350 bp in a 130-µl microTUBE. Peaks at 35 bp and 10,380 bp are BioAnalyzer High Sensitivity DNA Markers.

All 24 DNA samples were used to prepare libraries with the SMARTer ThruPLEX DNA-seq Kit. Libraries were then analyzed on the Bioanalyzer. Examples are shown in Figure 3.

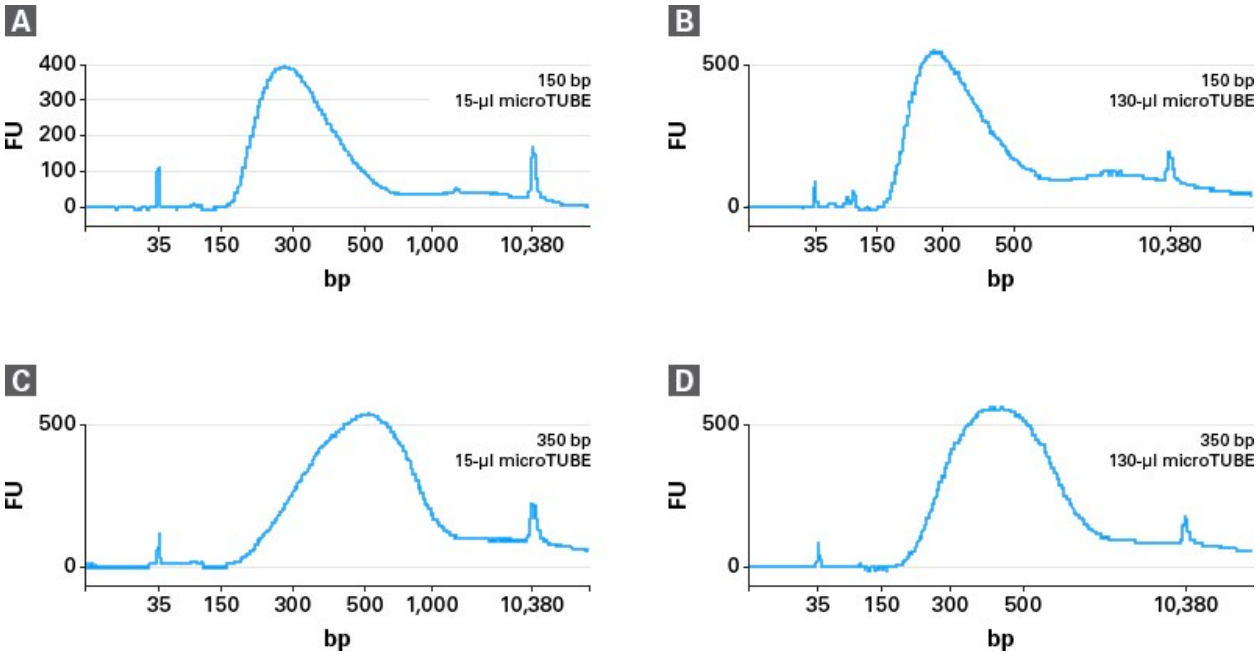

Figure 3. Examples of libraries with 2 insert sizes and different tubes. SMARTer ThruPLEX DNA-seq library preparation adds approximately 140 bases to the DNA molecules. Panel A. 150 bp in a 15 µl-microTUBE. Panel B. 150 bp in a 130-µl microTUBE. Panel C. 350 bp in a 15-µl microTUBE. Panel D. 350 bp in a 130-µl microTUBE.

After quantification, a fraction of each library was pooled, loaded on an Illumina® MiSeq® instrument, and sequenced following a paired-end 2 x 75-bp

protocol. Data was collected and down-sampled to 156K read pairs for analysis. A comparative summary of some key metrics is shown in Table II and Figure 4.

Sequencing metrics comparing input amounts, fragment size, and shearing volume												
Input	50 pg				1 ng				10 ng			
Size	150 bp		350 bp		150 bp		350 bp		150 bp		350 bp	
Shearing volume	130 µl	15 µl	130 µl	15 µl	130 µl	15 µl	130 µl	15 µl	130 µl	15 µl	130 µl	15 µl
Estimated library size	2.62×10^7	1.65×10^7	8.31×10^6	7.64×10^6	3.12×10^8	2.05×10^8	2.46×10^8	1.16×10^8	1.39×10^9	1.16×10^9	1.07×10^9	9.97×10^8
% Duplicates	0.60	1.00	1.92	2.17	0.06	0.08	0.08	0.22	0.02	0.02	0.03	0.03
% Unmapped reads	0.75	0.97	0.85	0.89	0.30	0.29	0.35	0.33	0.27	0.28	0.34	0.33

Table II: Key sequence metrics of different libraries calculated by the Picard pipeline. Numbers are average values of duplicates for each sample. First line: Estimated library size reflects the diversity of each library. Percent duplicate: corresponds to the identical reads generated by PCR. Percent unmapped reads: a quality metric that shows the absence of contamination in the human DNA prep.

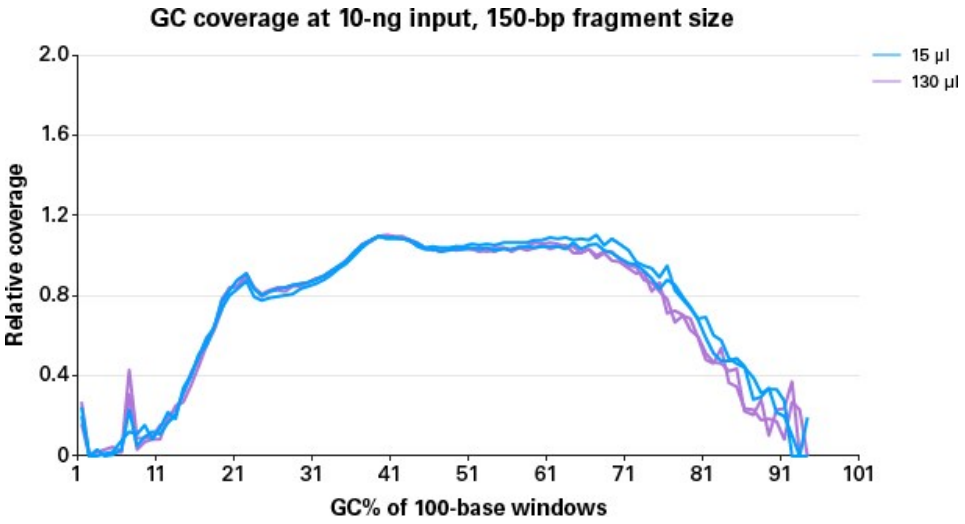

Figure 4. An example of the GC bias calculated over a window of 100-bp reads. Each curve represents the average distribution of two replicates for each sample.

We also evaluated if shearing volume, input amounts, or fragment size had any impact on the sequencing data metrics. To do so, we plotted the estimated library size as a function of read pairs by differentially pooling our data set (Figures 5 and 6).

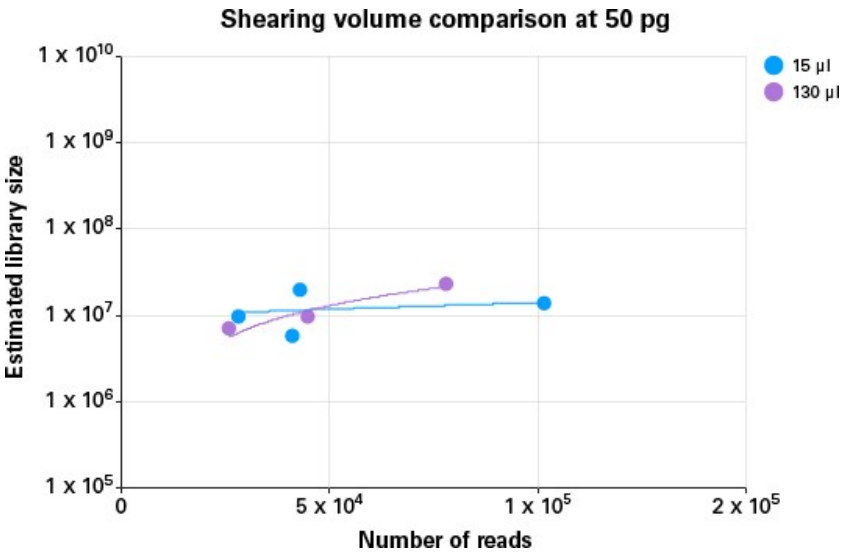

Figure 5. Estimated library size comparison between samples sheared in the micoTUBE-15 and standard 130-µl tube. The plot shown for 50 pg is identical to the plots for both 1 ng and 10 ng (data not shown).

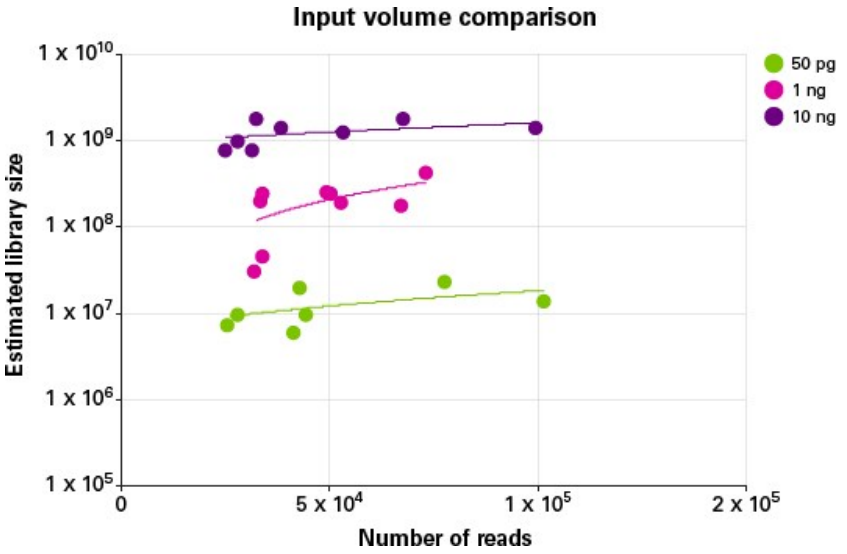

Figure 6. Comparison of estimated library sizes using a pool of both shearing volumes (standard vol. and microTUBE-15) and various input amounts (50 pg, 1 ng, and 10 ng).

The superimposed lines show equivalency between the two types of tubes used to shear the DNA, indicating that shearing volume does not affect the estimated library size (Figure 5). As expected, varying the input amount results in a difference in library size, indicating that libraries are more complex as the input amounts increase, as indicated by the comparable flat slopes (Figure 6). Together, Figures 5 and 6 strongly suggest that in this range of read pairs, the estimated library size is stable regardless of the shearing volumes used.

In consideration of these data, it is clear that the use of the microTUBE-15 in combination with the SMARTer ThruPLEX DNA-seq Kit presents a much simpler and more practical low-sample volume workflow for preparing DNA libraries, without compromising library quality, quantity, or complexity. The workflow described here is fully functional for any application requiring sheared DNA for Illumina library preparation and is especially useful for applications where the DNA is of limited amount and subject to loss and/or contamination.

Methods

DNA mechanical shearing

Human genomic DNA NA12878 (Coriell Institute for Medical Research) was quantified by Qubit using the dsDNA HS Assay Kit (Thermo Fisher Scientific) and diluted to 5 pg/µl, 100 pg/µl, and 1 ng/µl. A 15-µl aliquot of each diluted DNA was loaded into a Covaris microTUBE-15 AFA Beads (PN 520145) or 130 µl was loaded into a control Covaris microTUBE AFA Fiber Snap-Cap (PN 520045). All mechanical shearing was performed in the Covaris model M220 Focused-ultrasonicator with Holder XTU (PN 500414) using the appropriate insert for each type of shearing volume: M220 Holder XTU Insert microTUBE 130 µl (PN 500489) or M220 Holder XTU Insert microTUBE 15 µl (PN 500420). DNA was sheared to 150 bp and 350 bp in duplicate for each concentration. For processing, preprogrammed settings in the Covaris SonoLab 7.2 software were used.

DNA quality control

After shearing, 1 µl of each 1 ng/µl sample was visualized on the Agilent Bioanalyzer using the High Sensitivity DNA Analysis kit (lower concentrations were below the limit of detection of the Bioanalyzer).

Library preparation and sequencing

For library preparation, 10 µl of each sheared sample was used as input for the SMARTer ThruPLEX DNA-seq Kit, resulting in starting gDNA inputs of 50 pg, 1 ng, and 10 ng. After library purification, SMARTer ThruPLEX DNA-seq libraries were quantified by Qubit and qPCR, analyzed for size distribution on the Bioanalyzer, and sequenced using Illumina MiSeq V3 flow cell and reagents (paired end 2 x 75 base reads).

Data analysis

Data was analyzed in DNANexus. Briefly, reads were mapped to the human genome reference, hg19, using the Burrows-Wheeler Alignment algorithm (Li and Durbin 2009) by BWA-MEM to generate BAM files for each sample. Duplicates were marked and removed using Picard Mark Duplicates* and the quality of the libraries was further analyzed using Picard Tools and BEDTools.

*Picard - A set of tools (in Java) for working with next-generation sequencing data in the BAM format. http://broadinstitute.github.io/picard.

References

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Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754–1760 (2009).

Murtaza, M. *et al.* Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 497, 108–112 (2013).

Van Nieuwerburgh, F. *et al.* Illumina sequencing of 15 deafness genes using fragmented amplicons. *BMC Res. Notes* 7, 509 (2014).

Samorodnitsky, E. *et al.* Comparison of Custom Capture for Targeted Next-Generation DNA Sequencing. *J. Mol. Diagnostics* 17, 64–75 (2015).

Related Products

Cat. #	Product	Size	License	Details
R400406	SMARTer® ThruPLEX® DNA-seq 48D Kit	48 Rxns		
R400427	SMARTer® ThruPLEX® DNA-seq 48S Kit	48 Rxns		
R400407	SMARTer® ThruPLEX® DNA-seq 96D Kit	96 Rxns		
R400428	ThruPLEX® DNA-seq 12S (48) Kit	48 Rxns		

Takara Bio USA, Inc.
United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999