High-throughput library preparation for whole genome and target panel-based sequencing

Keywords: High-throughput library preparation, sparQ DNA Frag & Library Prep Kit, automation, 384-well format

ABSTRACT

A rapid decrease in overall sequencing costs has led to greater accessibility of next-generation sequencing (NGS) applications. The rise in demand for sequencing has also led to a higher demand for high-throughput library preparation. In this application note, we demonstrate the ability to scale the sparQ DNA Frag & Library Prep kit for a high-throughput 384-well format.

INTRODUCTION

Decades since the initial publication of the Human Genome Project, sequencing continues to provide valuable information about human genetics.[1,2] The advent of next-generation sequencing technologies such as Illumina's sequencing-by-synthesis have further improved the ability to sequence not only human samples but also many other animal, plant, and bacterial species. Further improvements to these technologies have decreased the cost of sequencing, enabling access to even more labs. As sequencing prices continue to decrease on a cost-per-Gb basis, high-throughput sequencing becomes more relevant and allows for a greater number of samples to be processed at a time. In order to efficiently make use of these decreasing costs, many labs are turning to liquid handling platforms and other robotic instrumentation to improve speed and throughput of the library preparation. Implementing and validating library preparation kits in 96-well and 384-well formats will be essential to addressing this need. In this application note, we demonstrated how to enzymatically fragment genomic DNA and prepare libraries in a high-throughput 384-well format using the sparQ DNA Frag & Library Prep Kit (Quantabio).

METHODS

Input DNA

For this study we used human genomic DNA isolated from human buffy coat (11691112001, Roche) without further purification. This protocol is optimized for 50 and 100 ng input DNA.

384-well library preparation

All reactions were performed in a Hard-Shell® 384-Well PCR Plate, thin wall, skirted, clear/clear (#HSP3801, Bio-Rad Laboratories). For automation purposes, Nunc™ 384-Well Polypropylene DeepWell™ Storage Plates were used for liquid waste.

DNA Fragmentation and Polishing

1. The thermal cycler was programmed with the parameters in Table 1 with a heated lid set to 70°C. When the thermal cycler block reached 32°C, the program was paused.

Note: Use of heated lid at >70°C may affect the temperature of the fragmentation reaction.

Step	Temperature (lid 70°C)	Incubation time
1	32°C	8 to 24 min
2	65°C	30 min
3	4°C	Hold

Table 1 **Incubation conditions for the single-step DNA fragmentation and polishing.** Fragmentation time in step 2 depends on input DNA amount, GC-content, and desired fragment size.

2. The DNA Frag and Polishing Master Mix was prepared on ice according to Table 2.

Component	Volume per reaction (µL)
Nuclease-free Water	1
DNA Frag & Polishing Buffer (10X)	1
DNA Frag & Polishing Enzyme Mix (5X)	2
DNA Frag & Polishing Enhancer Solution	1
Total	5

Table 2 **Fragmentation and polishing master mix.** The master mix was prepared including a 10% overage.

3. 5 µL Frag & Polishing Master Mix from Table 2 was added to 5 µL DNA and mixed by pipetting.
4. The samples were immediately transferred to the pre-chilled thermal cycler (4°C). The cycling program from step 1 was resumed.
5. When the thermal cycler program was completed and block temperature returned to 4°C, the sample plate was removed from the block and placed on ice before proceeding to adapter ligation.

Adapter Ligation

Note: Adapters may need to be diluted before use by adding UDI Dilution Buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5 mM EDTA). To achieve optimal adapter ligation efficiency for various input DNA amounts, it is recommended to adjust insert/ adapter molar ratio accordingly. Additional detailed guidance is provided in Appendix C of the sparQ DNA Frag & Library Prep Kit IFU.

6. For this experiment, sparQ UDI adapters (Quantabio) were freshly diluted to a 1 µM working stock solution. To the fragmented DNA from step 5, 2 µL of the diluted sparQ UDI adapter was added, mixed gently by pipetting, then cooled on ice. Use caution when pipetting adapters to avoid cross contamination.
7. A Ligation Master Mix was prepared per Table 3.

Component	Volume per reaction (µL)
Nuclease-free Water	2
DNA Rapid Ligation Buffer (5X)	4
DNA Ligase	2
Total	8

Table 3 Ligation Master Mix. The master mix was prepared including a 10% overage.

8. To the 12 µl fragmented DNA and adapters sample from step 6, 8 µl of the Ligation Master Mix was added. The ligation reaction was then incubated according to Table 4 without enabling the heated lid.

Step	Temperature (lid off)	Incubation time
1	20°C	15 min
2	4°C	Hold

Table 4 Incubation conditions for adapter ligation.

9. After incubation, the samples were removed and proceeded immediately to adapter ligation cleanup using sparQ PureMag Beads (Quantabio).

Adapter Ligation Cleanup

10. The sparQ PureMag Beads were equilibrated to room temperature (RT) for at least 20 min, then the slurry was vortexed thoroughly.
11. To the ligation sample from step 9, 16 µl (0.8X) sparQ PureMag Beads were added and incubated at room temperature (RT) for 5 min. The beads were then pelleted on a magnet and the supernatant removed and discarded.
12. The samples were kept on the magnet and 45 µL freshly prepared 80% ethanol added to wash the beads. The beads were allowed to re-pellet for 30 s before removing and discarding the ethanol. The wash was repeated for a total of two washes.
13. Keeping the tube on the magnet, the bead pellet was dried for 5 mins or until the beads appeared matte and no longer shiny.
14. To elute the samples, samples were removed from the magnetic stand and then the dried beads were resuspended in 11.5 µl of 10 mM Tris-HCl, pH 8.0 and incubated at room temperature for 3 min.
15. The beads were then pelleted on the magnetic stand and 9.4 µL of supernatant was carefully collected.

Library Amplification

16. The PCR Master Mix was prepared by combining the HiFi PCR Master Mix (2X) and Primer Mix per Table 5.

Component	Volume per reaction (µL)
HiFi PCR Master Mix (2X)	10
Primer Mix	0.6
Total	10.6

Table 5 PCR Master Mix. The master mix was prepared including a 10% overage.

17. To the 9.4 µL of DNA sample from step 15, 10.6 µL of the PCR Master Mix from step 16 was added.

18. A thermal cycler was set up with the parameters listed in Table 6. The instrument's heated lid was set to 105°C. When the thermal cycler block reached 98°C, the program was paused, samples were transferred to the instrument, and then the program resumed.

Step	Temperature (lid 105°C)	Incubation time	Cycles
1	98°C	2 min	1
2	98°C	20 sec	5–8
3	60°C	30 sec	
4	72°C	30 sec	
5	72°C	1 min	1
6	4°C	Hold	1

Table 6 **Cycling conditions for PCR amplification.** Cycle numbers for step 2-4 can be adjusted based on initial sample input and quality.

19. When the thermal cycler program is complete and sample block returned to 4°C, samples were removed from the block and then proceeded immediately to post-amplification cleanup using sparQ PureMag Beads.

Post-Amplification Cleanup

20. The sparQ PureMag Beads were equilibrated to room temperature (RT) for at least 20 min, then the slurry was vortexed thoroughly.
21. To the ligation sample from step 24, 16 µL (0.8X) sparQ PureMag Beads were added and incubated at room temperature (RT) for 5 min. The beads were then pelleted on a magnet and the supernatant removed and discarded.
22. The samples were kept on the magnet and 45 µL freshly prepared 80% ethanol added to wash the beads. The beads were allowed to re-pellet for 30 sec before removing and discarding the ethanol. The wash was repeated for a total of two washes.
23. Keeping the tube on the magnet, the bead pellet was dried for 5 mins or until the beads appeared matte and no longer shiny.
24. To elute the samples, the samples were removed from the magnetic stand and then the dried beads were resuspended in 17 µL of 10 mM Tris-HCl, pH 8.0 and incubated at room temperature for 3 min.
25. The beads were then pelleted on the magnetic stand and 15 µL of supernatant was carefully collected.

Library QC

To assess the size of the DNA fragments, 1 µL fragmented and cleaned DNA was run on a D1000 ScreenTape on the 4200 TapeStation® System (Agilent). To determine the size distribution of the final libraries, 1 µL purified library was run on a D5000 ScreenTape (Agilent).

Hybridization and Sequencing

Using 50 ng input and 21 minutes fragmentation time with the modified protocol above, 48 samples were processed. Hybrid capture was performed using the xGen™ Pan-Cancer Hyb Panel (IDT) following the manufacturer's recommended protocol. Enriched libraries were then sequenced on an Illumina® instrument. Sequencing data was analyzed for mapping rate as well as on-target and off-target percentages.

RESULTS

Tunable fragmentation using reduced reaction volumes

Tunable fragmentation allows users to choose the appropriate fragmentation time for their sample type and desired sequencing configuration. Because reaction volumes are more limited in a 384-well format, the reaction kinetics of fragmentation could be impacted. Tunable fragmentation and appropriate fragmentation times needed to be established. First, libraries were generated from 100 ng input with various fragmentation times from 8 to 24 minutes in a reduced 10 µL reaction (Figure 1A). As fragmentation time increased, the average size of the library decreased in a step-wise manner, demonstrating tunable fragmentation even in the smaller reaction volumes. Fragmentation was also tested using 50 ng input to verify that lower inputs also observed the same trend (Figure 1B). In summary, both 100 ng and 50 ng inputs had a linear response to various fragmentation times in reduced reaction volumes (Figure 1C).

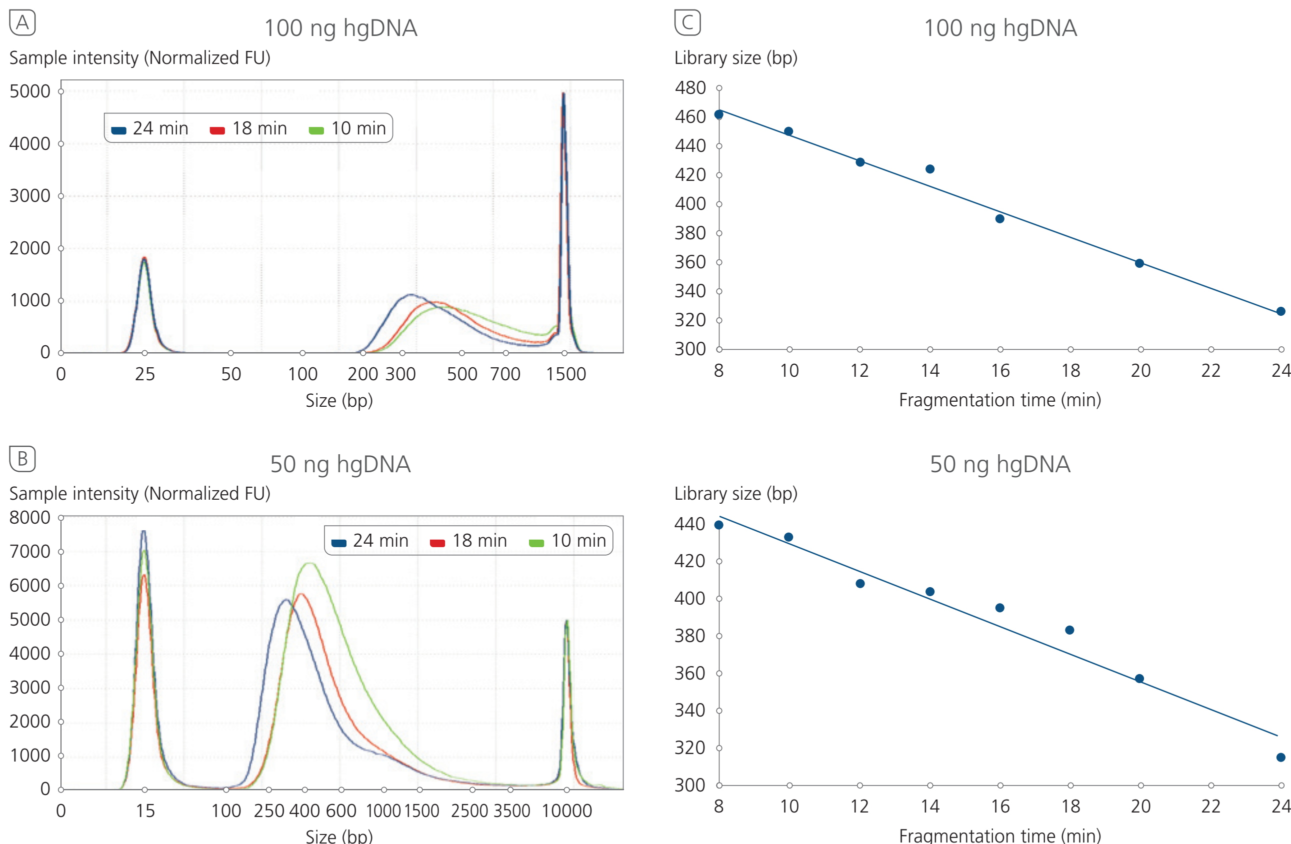
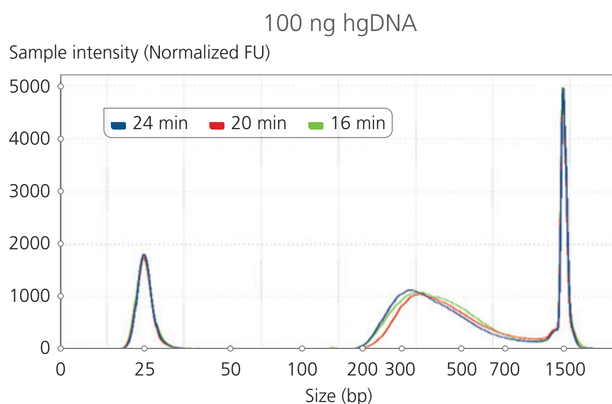


Figure 1 **Tunable fragmentation using reduced reaction volumes.** A Libraries were generated from 100 ng gDNA input with reduced reaction volumes with fragmentation times varying from 8 to 24 min. All samples were PCR amplified using 6 cycles and run on a 4200 TapeStation, resulting in yields of 500-700 ng. The average library size decreased as the fragmentation time increased. B Libraries generated using 50 ng input and 8 PCR cycles in reduced volumes. C Linear response of library size to fragmentation time using 50 ng and 100 ng as input.

Comparable fragmentation patterns between 96-well and 384-well formats

After tunable fragmentation was established using the reduced reaction volumes, a baseline fragmentation time needed to be determined and compared against the original protocol for the 96-well format. For this comparison, the target insert size was approximately 250 bp. Libraries were generated using the 384-well protocol with 100 ng starting input and fragmentation times of 20 and 24 minutes. These libraries were compared against a library generated using 100 ng input and a 16 minute fragmentation time in the volumes specified for the 96-well format (Figure 2). Libraries prepared using the 384 well format protocol with 20 and 24 minutes fragmentation time produced similar yields with 6 cycles of PCR amplification and an average library size of 300 bp as compared to libraries generated with the protocol as originally written for larger reaction volumes and 5 cycles of amplification.



	Format	Input DNA	Frag time	# of PCR Cycles
(blue)	384 well	100 ng	24 min	6
(red)	384 well	100 ng	20 min	6
(green)	96 well	100 ng	16 min	5

Figure 2 **Libraries prepared from 100 ng input using 96-well and 384-well protocol.** Libraries were generated with 100 ng gDNA. Each library showed a comparable fragmentation profile.

Consistent fragmentation when processing many samples

When scaling up sample throughput, a common concern is whether or not all samples perform similarly when processed in high numbers. To assess the consistency of the protocol with larger sample numbers, 48 samples were prepared using the 384-well protocol with 50 ng input DNA and a fragmentation time of 21 minutes. The samples were analyzed post-sequencing for insert size (Figure 3). All 48 libraries had a similar insert size with an average size of 261 bp. This demonstrates fragmentation consistency across a large number of samples.

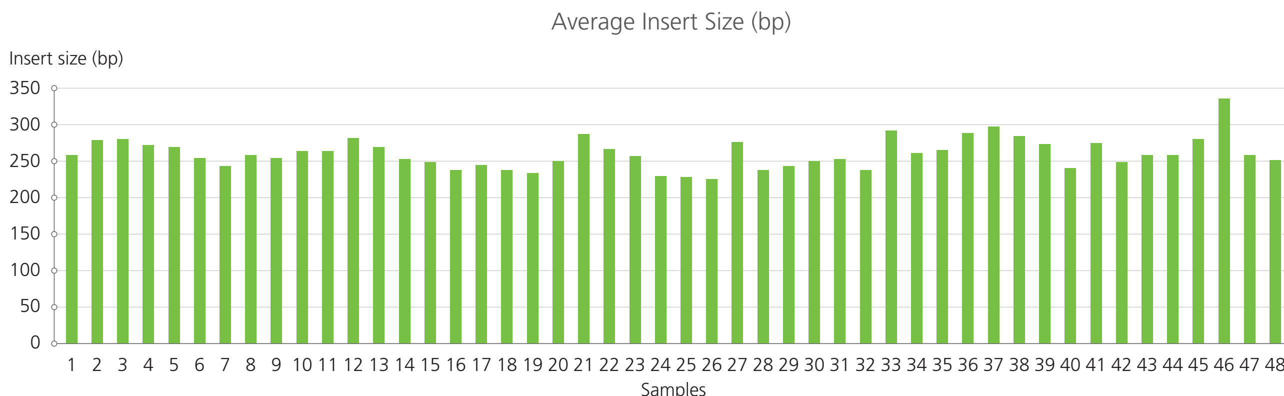


Figure 3 **Insert size from 48 libraries prepared using the 384-well protocol.** Sizing was determined using 4200 TapeStation analysis.

High-quality sequencing data

After library generation, the libraries were sequenced and then evaluated for data quality. A small subset of libraries were analyzed post-sequencing by mapping the sequencing data to the human reference genome and determining the percentage of mapped reads (Figure 4). All libraries had a mapping rate >98%.

The sparQ Frag & Library Prep kit is compatible with hybrid capture methods for enrichment of targeted regions within the genome. Using the 384-well protocol, 48 samples were prepared with 50 ng input DNA and a fragmentation time of 21 minutes. The libraries were then used for hybrid capture using xGen™ Pan-Cancer Hyb Panel (IDT) according to the manufacturer protocol. All 48 libraries performed consistently for all samples with an On Target rate of about 45% with a duplication rate around 10% (Figure 5). This was comparable to data generated previously. Taken together, the sequencing data shows that this 384-well protocol is consistent for high-throughput processing and produces high-quality sequencing data.

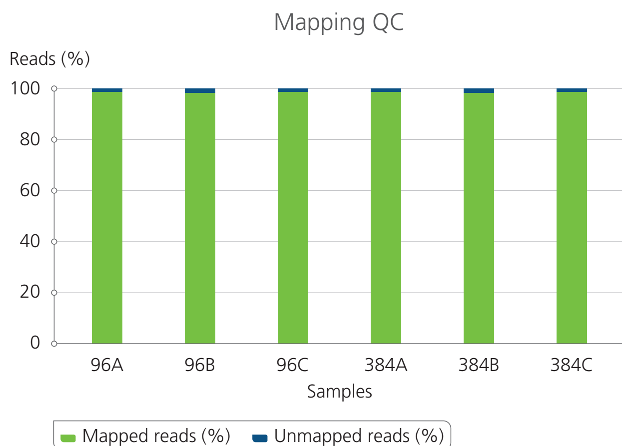


Figure 4 **Mapping rates from a subset of samples. All libraries were mapped to the human reference genome.** Three libraries were prepared using the 384 well format reaction volumes and 18 min fragmentation times (384A, 384B, 384C) and compared against 3 libraries generated using the 96-well volumes and 16 min fragmentation times (96A, 96B, and 96C).

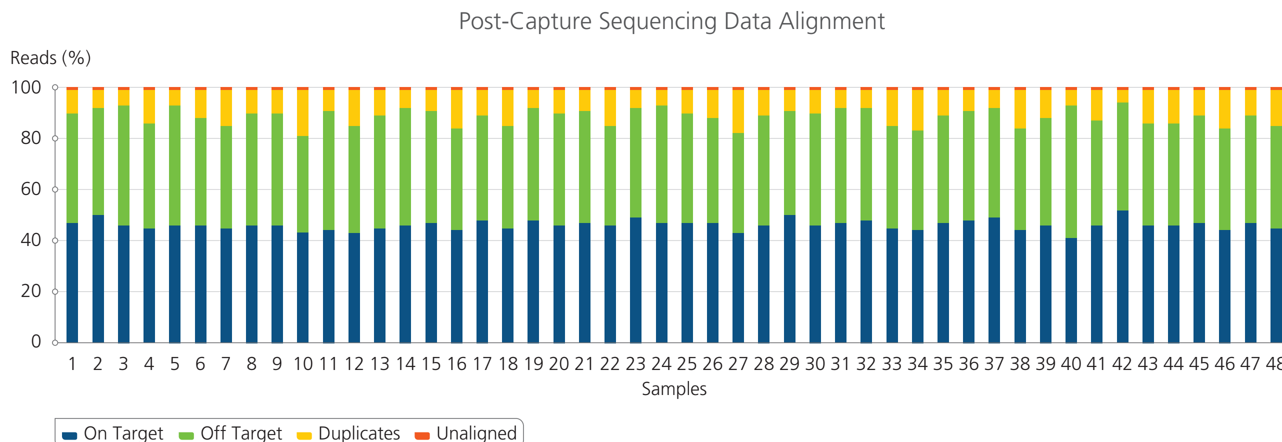


Figure 5 Alignment from hybrid capture library enrichment using the xGen™ Pan-Cancer Hyb Panel (IDT) from 48 samples.

CONCLUSIONS

To date, the sparQ Frag and Library Prep kit has been used for a wide variety of applications including whole genome,[3,4] whole genome amplified single cell,[5] and amplicon sequencing.[6] Here we have demonstrated that the sparQ Frag and Library Prep kit can be adapted for use with 384-well plates, allowing for high-throughput processing. Fragmentation patterns and sequencing metrics using the reduced volume reaction conditions are comparable to the original protocol. Simple modifications to the fragmentation time and reaction volumes allow for reliable high-throughput library preparation compatible with automation, thereby reducing the cost and turnaround time to sequencing for a variety of applications.

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