



Automating the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina on firefly®

Overview

The NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina is optimized for the construction of high quality, high yield libraries using a broad range of DNA input amounts. The library preparation workflow uses NEBNext FS (Fragmentation System) enzymatic fragmentation reagents, eliminating the need to mechanically shear the DNA input. The workflow also uses a single enzyme mix to perform the fragmentation, end repair and dA-tailing, which greatly simplifies the workflow.

Here, we demonstrate how this workflow has been automated on firefly. We provide details on the steps performed in each protocol, together with data to demonstrate the performance of these protocols in generating high quality libraries for sequencing.

Reagents Required

- NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (NEB #E7805 without sample purification beads or NEB #E6177 with sample purification beads)
- NEBNext® Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) Sets 1-5 (NEB # E6440, E6442, E6444, E6446 and E6448)

E7805L and E6440L were used to generate the dataset presented here. See the Technical Note for this application for recommended use of the kits and the reagent volume requirements for each protocol.

Workflow features

- Enzymatic shearing of gDNA
- Fragmentation and End prep (end repair + dA-tailing) are performed in one reaction
- NEBNext adaptors are used – these adaptors have a hairpin loop structure that contains a uracil base
 - Uracil is removed by an enzyme mix (USER) to open the loop and enable PCR
- USER/PCR reagent dispenses and incubations are combined
 - Minimizes the dead volume of USER required by the protocol
 - Reduces the number of operator touch-points in the protocol

firefly protocols

Protocol number	Protocol name
Protocol 1 of 4	1.1-1.2 Fragmentation, End Prep and Adaptor Ligation
Protocol 2 of 4	1.3 Adaptor Clean-up
Protocol 3 of 4	1.4 PCR Enrichment
Protocol 4 of 4	1.5 PCR Clean-up
Optional protocol	2.3 Size Selection for DNA Input ≥ 100 ng

Table 1. firefly protocols used to run the NEBNext Ultra II FS DNA Library Prep Kit for Illumina and are available to download from the firefly community cloud.

NEBNext Ultra II FS DNA Kit on firefly workflow

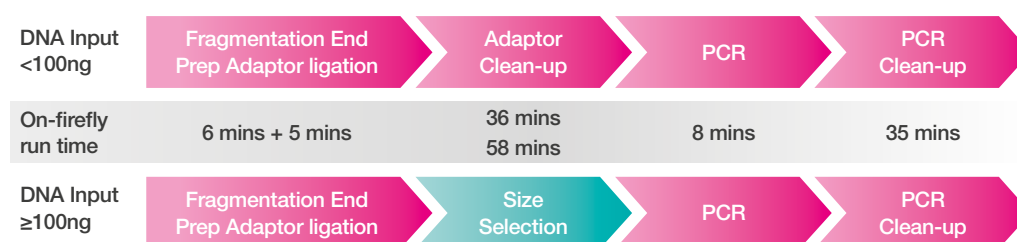


Figure 1. Workflow and firefly run times for the NEBNext Ultra II FS DNA Kit. The PCR reaction setup takes place on firefly. The PCR reaction takes place on an external thermocycler.



Protocol overview

Protocol names reflect the section names and numbering used in the NEB instruction manual for the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (E7805/E6177, v4.0 7/23).

Protocol 1 of 4

1.1-1.2 Fragmentation, End Prep and Adaptor Ligation

Input DNA is transferred to a clean PCR plate using the pipetting head. NEBNext Ultra II FS Reaction Buffer and FS Enzyme Mix are added to the input DNA using the firefly dispense head, with the plate on a chilled thermal block. Reagents are mixed on the shaker.

The user moves the plate to the thermocycler to run the Fragmentation/End Prep program, then moves the plate back to the firefly deck on completion of the program.

NEBNext Ultra II Ligation Master Mix, Ligation Enhancer and Adaptor for Illumina are added to the plate using the firefly dispense head. Reagents are mixed on the shaker.

The user moves the plate to the thermocycler to run the Adaptor Ligation program. This is a safe stopping point where samples can be stored at -20°C if required. See Figure 2.

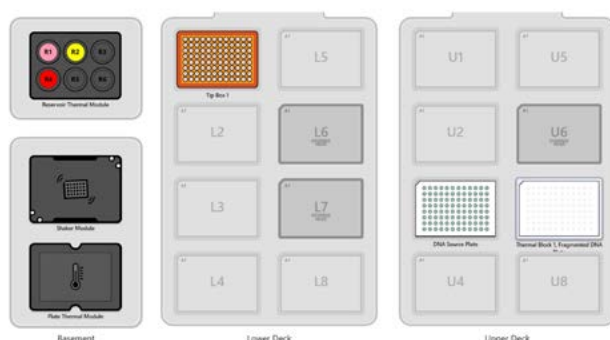


Figure 2. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 1 of 4: 1.1-1.2 Fragmentation, End Prep and Adaptor Ligation. Lower deck: (L1) Tip box 1 – 50 µL pipetting head tips; upper deck: (U3) DNA Source Plate, (U7) empty PCR plate on a thermal block; dispense head reservoirs: (R1) Adaptor - diluted as required, (R2) FS Enzyme + Buffer Mix, (R4) Ligation Master Mix + Enhancer. Example shown for 12 columns of samples.

Protocol 2 of 4

1.3 Adaptor Clean-up

When the Adaptor Ligation program on the thermocycler is complete, the user returns the plate to the firefly deck. The “Fragmented DNA Plate” now contains the ligation reaction product.

The ligation reaction product undergoes a 0.8X bead clean-up, where beads, 80% ethanol and elution buffer are dispensed using the firefly dispense head, and fresh pipetting head tips are used for mixing the beads with the sample, and for all ethanol and supernatant removals. The cleaned-up adaptor ligated libraries are transferred to a fresh plate. This is a safe stopping point where samples can be stored at -20°C if required. See Figure 3 for the starting deck layout for this protocol.

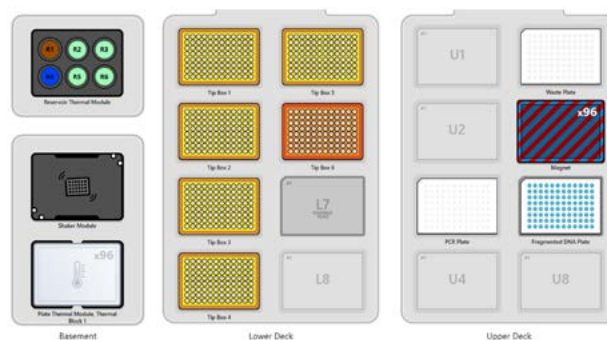


Figure 3. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 2 of 4: 1.3 Adaptor Clean-up. Lower deck: (L1-L5) Tip box 1-5 – 125 µL pipetting head tips, (L6) Tip box 6 – 50 µL pipetting head tips; upper deck: (U3) empty PCR Plate, (U5) Waste Plate, (U6) Alpaqua Magnum FLX magnet, (U7) Fragmented DNA plate containing fragmented, adaptor-ligated DNA, dispense head reservoirs: (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Protocol 3 of 4

1.4 PCR Enrichment

This step deviates from the standard manual protocol. Here, USER is added to the PCR reaction mix and the USER and PCR incubations are combined (see Table 2).

NEBNext Ultra II Q5 Master Mix, USER enzyme and Unique Dual Index Primer Pairs are added to the adaptor-ligated libraries and are mixed on the shaker.

IMPORTANT: In advance of protocol initiation, the seal on primer index plate must be pierced to permit unobstructed access to the wells being used.

On completion of this protocol, the plate is moved to the thermocycler and the USER-PCR program should be run with an appropriate number of cycles – see Table 2 for the cycling conditions. Figure 4 shows the starting deck layout for this protocol.

Step name	Temperature (°C)	Time	Cycles
USER incubation	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4-9*
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	forever	

Table 2. Example of the cycling conditions required for the USER-PCR thermocycler program

**PCR cycle numbers for 1-200 ng input used in this study. Please refer to the NEBNext Ultra II FS DNA manual for cycle number recommendations for other input amounts.*

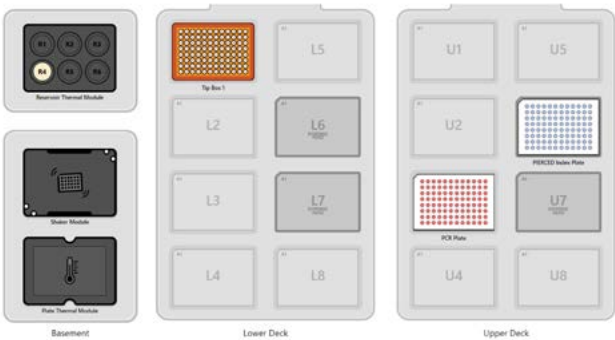


Figure 4. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 3 of 4: 1.4 PCR Enrichment. Lower deck: (L1) Tip box 1 – 50 µL pipetting head tips; upper deck: (U3) PCR Plate, (U6) Index Plate with pre-pierced wells; dispense head reservoirs: (R4) NEBNext Ultra II Q5 Master Mix + USER enzyme. Example shown for 12 columns of samples.

Protocol 4 of 4

1.5 PCR Clean-up

When the USER-PCR program on the thermocycler is complete the user returns the plate to the firefly deck.

A 0.9X bead clean-up is performed on the PCR product, where beads, 80% ethanol and elution buffer are dispensed using the firefly dispense head, and fresh pipetting head tips are used for mixing the beads with the sample, and for all ethanol and supernatant removals. The cleaned-up libraries are moved to a fresh plate. See Figure 5 for the starting deck layout for this protocol.

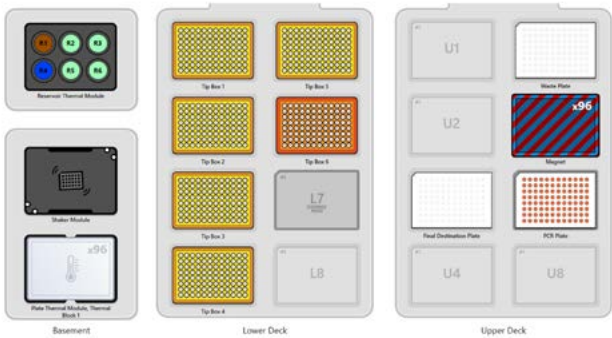


Figure 5. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 4 of 4: 1.5 PCR Clean-up. Lower deck: (L1-L5) Tip box 1-5 – 125 µL pipetting head tips, (L6) Tip box 6 – 50 µL pipetting head tips; upper deck: (U3) empty Final Destination Plate, (U5) Waste Plate, (U6) Alpaqua Magnum FLX magnet, (U7) PCR Plate, dispense head reservoirs (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Optional protocol

2.3 Size Selection for DNA Input ≥ 100 ng

This optional Size Selection protocol can be run instead of protocol 2 of 4 (1.3 Adaptor Clean-up). See Figure 6 for the starting deck layout for this protocol.

The Fragmented DNA plate contains fragmented, adaptor-ligated DNA and starts with 68.5 μ L in each well. 33.5 μ L of 0.1X TE is added to each sample to give a final volume of 102 μ L. A double-sided size selection takes place where the volume of the 1st and 2nd bead addition is set as a variable so the user can modify these volumes as required.

A default bead volume of 40 μ L per well is set for the 1st bead addition. Following the bead addition, tip mixing and incubation on the magnet, the supernatant is transferred to a fresh plate (Intermediate Plate), leaving 4 μ L per well in the source plate.

A default bead volume of 20 μ L per well is set for the 2nd bead addition. Following the bead addition, tip mixing and incubation on the magnet, the supernatant is transferred to a fresh plate (PCR Plate), leaving 2 μ L per well in the source plate.

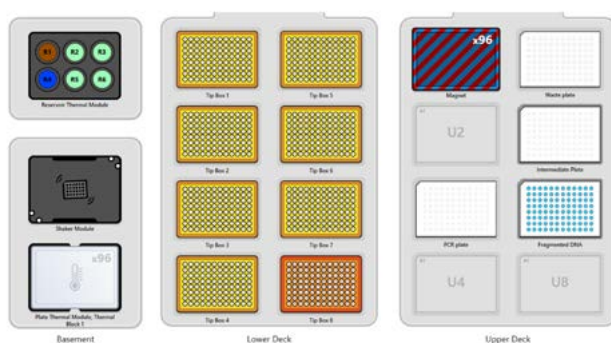


Figure 6. Starting deck layout for firefly NEBNext Ultra II FS DNA Optional protocol: 12.3 Size Selection of Adaptor-ligated DNA for DNA Input ≥ 100 ng. Lower deck: (L1-L7) Tip box 1-7 – 125 μ L pipetting head tips, (L8) Tip box 8 – 50 μ L pipetting head tips; upper deck: (U1) Alpaqua Magnum FLX magnet, (U3) empty PCR Plate, (U5) Waste Plate, (U6) empty Intermediate Plate, (U7) Fragmented DNA plate containing fragmented, adaptor-ligated DNA; dispense head reservoirs: (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Protocol performance

Input titration and size selection

The NEBNext Ultra II FS DNA Kit was run on firefly using human gDNA (Human Mixed, Promega G3041 and NA19240 from Coriell Institute for Medical Research) and run manually using human gDNA from Promega (G3041). gDNA inputs of 1 ng, 50 ng, 100 ng and 200 ng were used, with 8 replicates at each input level. A fragmentation time of 15-minutes was used for all runs. Samples with ≤ 100 ng input underwent a 0.8X adaptor clean-up (using firefly protocol 2 of 4 for the firefly run) whereas 200 ng input samples underwent a size-selection (firefly runs used the default bead volume settings in the “optional” firefly protocol), with the first cut at ~ 0.4 X and second cut at ~ 0.2 X. All samples were processed with the number of cycles of PCR recommended by the NEB protocol, followed by a 0.9X PCR clean-up.

DNA Input	Adaptor dilution	Size-selection	PCR Cycles
1 ng	1 in 25	No	9
50 ng	1 in 10	No	5
100 ng	None	No	4
200 ng	None	Yes	4

Table 3. Run set-up used to compare manual vs firefly performance of the NEBNext Ultra II FS DNA Kit for Illumina.

The resulting libraries were analysed to determine the library concentration using a Lightcycler 480 (Roche, KAPA Library Quantification kit), see Figure 7. Libraries were normalised to 20nM, pooled then sequenced on a Novaseq 6000 in a paired-end 2x76 cycle run, using a SP flow cell. The output data was down-sampled to 9.5 million reads per sample. Reads were mapped using Bowtie2 (version 2.3.2.2) to the GRCh38 reference, and duplicates were marked using Picard MarkDuplicates (version 1.56.0). Library quality metrics were assessed using Picard Alignment Summary Metrics (version 1.56.0). The analysis of these reads for the manual and firefly-automated runs is shown in Figures 8, 9 and 10.

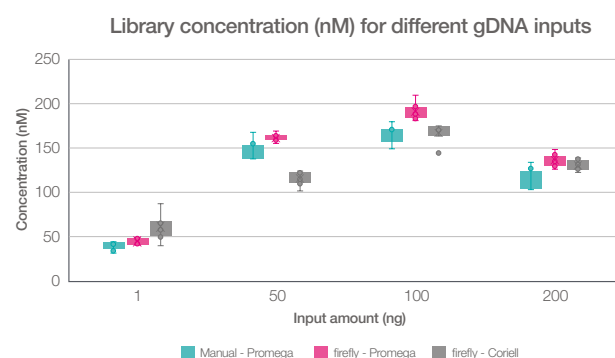


Figure 7. Library concentration determined by qPCR. Each bar represents 8 libraries, prepared manually or on firefly.

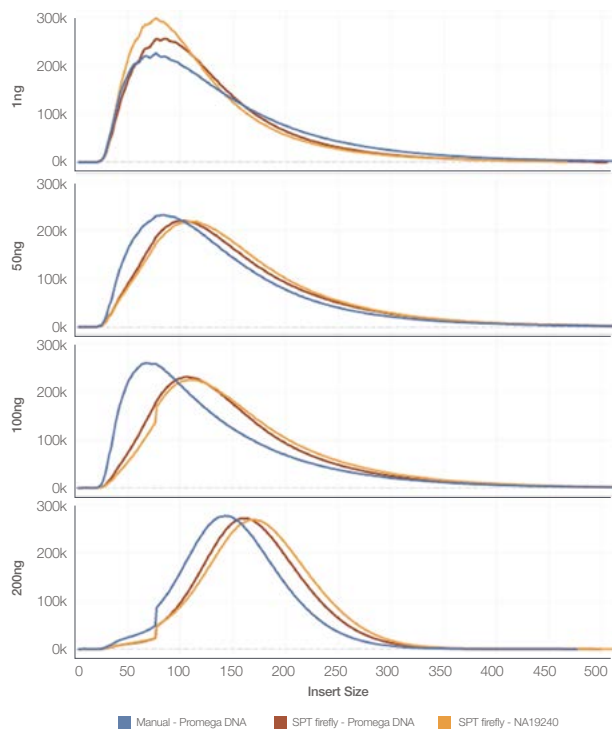


Figure 8. Sequencing analysis showing the average insert size distributions for different gDNA inputs when prepared manually or on firefly. The 1 ng, 50 ng and 100 ng inputs underwent a clean-up without size selection whereas the 200 ng inputs were run through the size selection workflow.

Figure 7 shows that libraries produced manually and on firefly are comparable in concentration.

Figure 8 demonstrates libraries prepared on firefly using the Promega and Coriell gDNA show reproducible insert size distributions, which differ from the manually prepared libraries by ≤ 50 bps. The effect of the size selection used with the 200 ng input can be seen in the slightly higher average insert sizes when compared to the distributions for the 1 ng, 50 ng and 100 ng inputs, where only a single-sided clean-up was used. The fragmentation time can be adjusted from the 15-minute duration used here, to modulate the distribution of insert sizes as required.

Figure 9 compares standard sequencing metrics (adaptor dimers, chimeras, mismatch rate, indel rate, duplicates and error rates) between Libraries prepared manually and on firefly. Libraries from all conditions generated comparable sequencing data, with no discernible differences between the manually prepared libraries and the libraries prepared using firefly.

Figure 10 compares the library coverage uniformity over the genome, represented with respect to GC content. Libraries prepared manually and on firefly exhibit comparable coverage across the genome, suggesting that the automation of this workflow on firefly did not have an impact on library coverage.

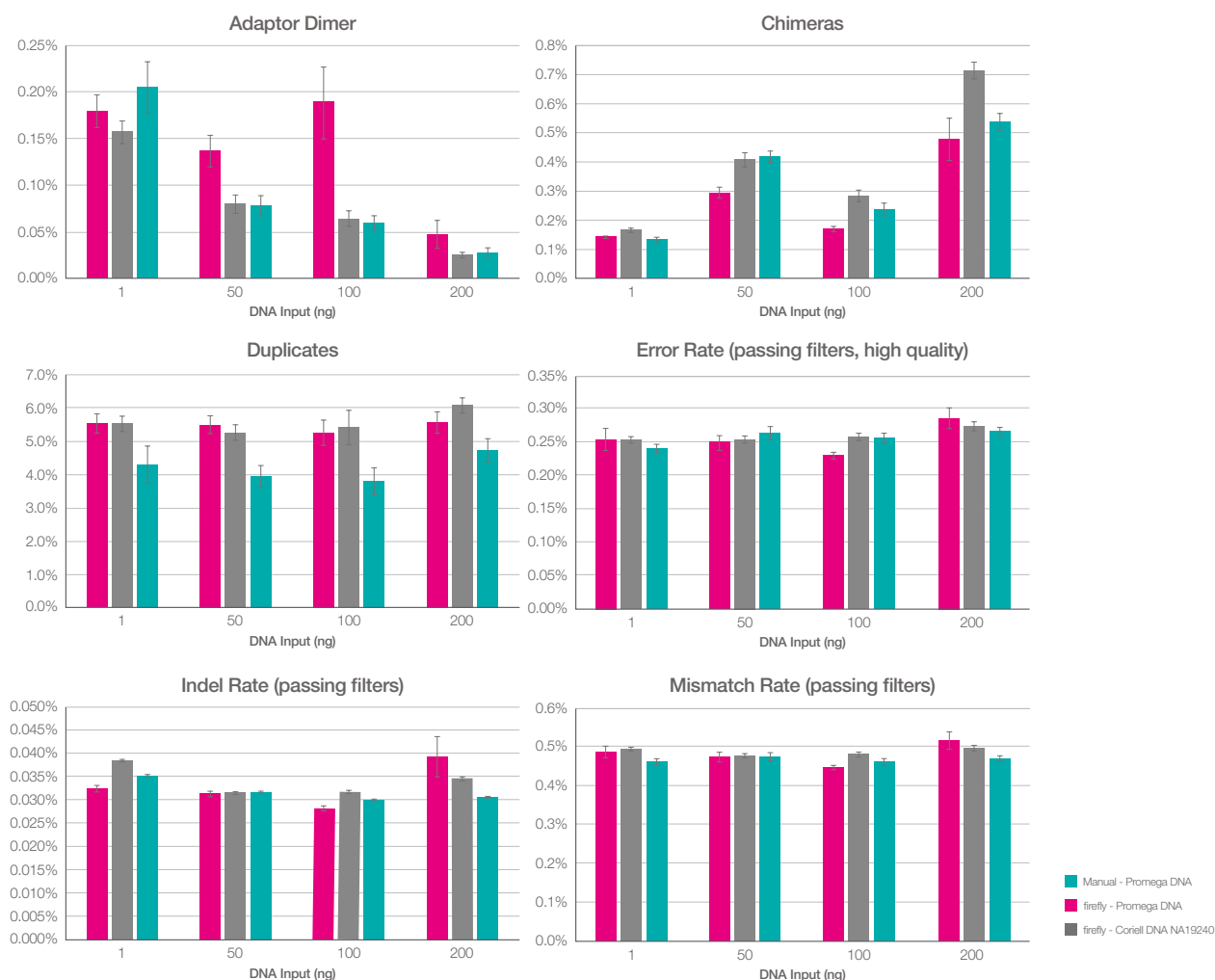


Figure 9. Comparison of sequencing metrics for manual and firefly workflows using Promega gDNA, and for the firefly workflow using Coriell NA19240 gDNA. Each bar represents the data from 8 samples.

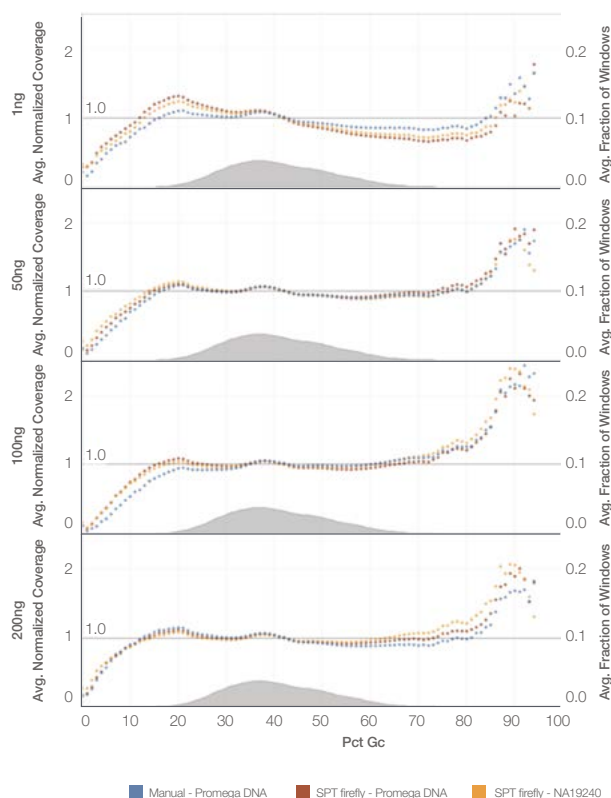


Figure 10. GC coverage plot comparing the genome coverage with respect %GC, for manual and firefly workflows.

High-throughput performance and cross-contamination evaluation

The performance of a high-throughput run and occurrence of well-to-well contamination was evaluated by preparing a total of 96 libraries on firefly; 81 replicates of a 200 ng gDNA input and 15 no template controls (NTCs) interspersed throughout a 96-well plate (Table 4). The workflow was run using a 15-minute fragmentation time, the size-selection protocol with default bead volumes, 4 cycles of PCR and a 0.9X post-amplification clean up.

All libraries were quantified by qPCR, using a LightCycler 480 System (Roche, KAPA Library Quantification Kit). All libraries were also run on the Fragment Analyzer (Agilent, DNF-474 HS NGS Fragment Kit) to determine the fragment size distribution for each library.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200	200	200	200	200	200	200	200	200	200	200	200
B	200	NTC	200	200	200	NTC	200	200	200	NTC	200	200
C	200	200	200	NTC	200	200	200	NTC	200	200	200	200
D	200	NTC	200	200	200	NTC	200	200	200	NTC	200	200
E	200	200	200	NTC	200	200	200	NTC	200	200	200	200
F	200	NTC	200	200	200	NTC	200	200	200	NTC	200	200
G	200	200	200	NTC	200	200	200	NTC	200	200	200	200
H	200	200	200	200	200	200	200	200	200	200	200	200

Table 4. Plate map used for high-throughput and cross-contamination test with sample input (ng) shown for each well position.

	1	2	3	4	5	6	7	8	9	10	11	12
A	117	123	130	129	133	139	127	134	131	130	125	118
B	128	NTC	125	128	113	NTC	112	131	133	NTC	135	135
C	135	128	120	NTC	121	128	134	NTC	131	127	111	101
D	120	NTC	95	119	126	NTC	138	137	131	NTC	131	137
E	114	84	125	NTC	134	130	129	NTC	136	135	122	97
F	131	NTC	122	118	136	NTC	131	130	132	NTC	134	95
G	123	123	125	NTC	120	124	132	NTC	133	126	121	110
H	115	118		123	125	131	135	135	133	130	132	125

Table 5. Concentration (nM) of libraries generated in the high-throughput performance and cross-contamination evaluation. The sample in well H3 was unavailable for analysis.

Table 5 shows the library concentrations from the high-throughput run. The %CV for the concentration of the 80 sample replicates was 8.5%. The NTCs showed no detectable library contamination by qPCR.

Figures 11 and 12 show Fragment Analyzer traces from the high-throughput run and demonstrates the consistency in the fragment size across the 96-well plate and the absence of library contamination in the NTCs. Figure 11 is particularly impressive, given that it overlays the fragment size distribution of 80 libraries. The %CV of the average fragment size was 1.4% (n=80).

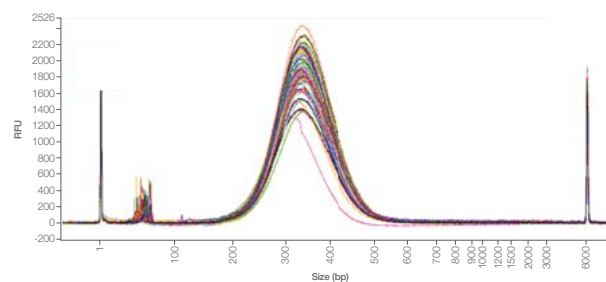


Figure 11. All Fragment Analyzer traces for 200 ng input libraries prepared on firefly for the high-throughput run (n=80).

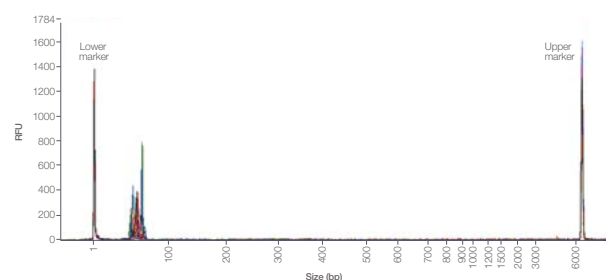


Figure 12. All Fragment Analyzer traces for NTCs prepared on firefly for the high-throughput run (n=15).

Summary

The data presented here demonstrates that high quality libraries can be prepared using firefly and the NEBNext Ultra II FS DNA Kit for Illumina. Libraries prepared using firefly are consistent in both their concentration and fragment size across a 96-well plate, and the sequencing performance of these libraries is comparable to manually prepared libraries.