firefly® technical note

KAPA EvoPlus Automated Library Preparation

This technical note provides supporting information for automating the KAPA EvoPlus DNA Library Prep Kit on SPT Labtech's firefly liquid handler. These protocols are available to download from the firefly community. Here we provide information on protocol run times, consumables usage and present data generated using these protocols.

Overview

firefly protocols have been developed to run the KAPA EvoPlus and KAPA EvoPlus PCR-Free library preparation workflows - using the 96-well plate format of the KAPA EvoPlus Kit and the KAPA Unique Dual-Indexed Adapter Kit – see Table 4. The firefly protocols align with steps in the "KAPA EvoPlus Kit Instructions for Use" document. gDNA samples start the workflow in a 96-well PCR plate, 1 to 12 sample columns can be processed at a time.

The KAPA EvoPlus ligation-based next-generation sequencing (NGS) DNA library preparation solution is suitable for low- to high-throughput workflows (with-PCR or PCR-free), and requires as little as 10ng of input DNA. Sample fragmentation is achieved enzymatically, with high tunability of fragment size achieved through varied fragmentation time. The unique KAPA EvoPlus ReadyMix formulations afford a 2-step library prep workflow that requires no master mix setup or inhibitor mitigation steps.

firefly from SPT Labtech is an innovative all-in-one liquid handler that brings together multiple technologies within a single compact design for more efficient library and sample preparation workflows and maximum output. Underpinned by powerful, intuitive software, firefly unlocks the potential of automation for all to accelerate genomic research.

Protocol highlights

- Input gDNA samples ranging from 10ng - 500ng can be processed into Illumina-compatible sequencing libraries with full-length, uniquely indexed adapters.
- Automated KAPA EvoPlus, KAPA EvoPlus PCR-Free and size selection workflows can be run with the flexibility to start from any step in these workflows.
- Protocol variables provide the flexibility to process 1 to 12 sample columns per run and to specify the starting column for the reagent plates (ReadyMix plates and UDI Adapter plates) - enabling multiple low-throughput runs using the same reagent plates and reducing waste.
- KAPA EvoPlus libraries produced on firefly are comparable in yield and fragment size to manually prepared libraries and are uniform in concentration and fragment size across a 96-well plate, with no detectable well-to-well contamination.

firefly protocols

Protocol number	Protocol name	firefly run time (minutes)	Thermocycler run time (minutes)	KAPA EvoPlus PCR-Free workflow	KAPA EvoPlus workflow
1 of 4	3.1 Fragmentation and A-Tailing	6	35-60	✓	✓
2 of 4	3.2 Adapter Ligation	6	15	✓	✓
3 of 4	3.3 Post-Ligation Purification	31-36†	n/a	✓	✓
4 of 4	4.1 - 4.3 Library Amplification and Purification	33-38†	5-25		✓
Optional*	Double-sided Size Selection	46-52†	n/a		

Table 1. Overview of protocols for automating KAPA EvoPlus DNA Library Prep Kit on SPT Labtech firefly liquid handler.

*Run the optional Double-sided Size Selection protocol after protocol 3 or after protocol 4.

†firefly run times vary with the number of columns processed.
There is a safe stopping point after protocol 3.

Input variables

Protocol name	Input Variable	Range	
		Minimum	Maximum
3.1 Fragmentation and A-Tailing	Number of Columns	1	12
	FragTail ReadyMix Starting Column	1	12*
3.2 Adapter Ligation	Number of Columns	1	12
	UDI Adapter Plate Starting Column	1	12*
	Ligation ReadyMix Plate Starting Column	1	12*
3.3 Post-Ligation Purification	Number of Columns	1	12
	Final Elution Volume (µL) †	20	50
4.1 - 4.3 Library Amplification and Purification	Number of Columns	1	12
	HiFi HotStart ReadyMix (2X) Starting Column	1	12*
	Library Amp Primer Mix Starting Column	1	12*
	Final Elution Volume (µL) †	20	50
Double-sided Size Selection	Number of Columns	1	12
	SPRI 1 ratio	0.4	1.4
	SPRI 2 ratio‡	0.6	2.0

Table 2. Variables available for automating KAPA EvoPlus DNA Library Prep Kit on SPT Labtech firefly liquid handler.

*Limited by the "Number of Columns" variable.

†Set this input variable to 20µL if following the standard workflow and 50µL if proceeding to the optional double-sided size-selection protocol
‡SPRI 2 ratio must be at least 0.2 higher than SPRI 1 ratio.

Consumables

Protocol name	125µL filtered strip tip sets required*	Standard dispense head syringes required	Additional plates required
3.1 Fragmentation and A-Tailing	1	0	1 x DNA input plate
3.2 Adapter Ligation	1	0	n/a
3.3 Post-Ligation Purification	6	6	1 x Elution plate 1 x Waste plate
4.1 - 4.3 Library Amplification and Purification	7	6	1 x Elution plate 1 x Waste plate
Double-sided Size Selection	7	6	1 x Elution plate 1 x Intermediate plate 1 x Waste plate

Table 3. Consumables required to run KAPA EvoPlus DNA Library Prep Kit on SPT Labtech firefly liquid handler. Excludes parts provided in the kit.

*Where (number of strip tip sets) x (number of sample columns processed) = number of strip tips needed.
e.g. to process 5 columns of samples through protocol 3 of 4 (3.3 Post-ligation purification): (6 strip tip sets) x (5 columns) = 30 strip tips are required.

Reagents Kit	Catalog #
KAPA EvoPlus Kit, 96 rxn plate or KAPA EvoPlus Kit, 96 rxn plate (PCR-free)	09420428001 or 09420436001
KAPA Library Amp Primer Mix, 96 rxn plate	09420479001
KAPA Unique Dual-Indexed Adapter Kit, (15 µM)	08861919702
KAPA Pure Beads (60 mL)	07983298001
KAPA Library Quantification Kit for Illumina platforms, Complete Kit (Universal)	07960140001

Table 4. Roche reagents required to run KAPA EvoPlus DNA Library Prep Kit on SPT Labtech firefly liquid handler.

Reagent volumes for 80% ethanol, KAPA Pure Beads and 10mM Tris-HCl (pH 8.0) are dependent on user input variables. The required volume of these reagents is shown in the execute section of the firefly software.

Protocol overview

Prior to loading ReadyMix and UDI plates on firefly the wells must be pierced and unobstructed for the columns being run.

Protocol 1 of 4
3.1 Fragmentation and A-Tailing

This protocol adds FragTail ReadyMix to the input DNA plate whilst the latter is on a chilled thermal block. These reagents are then tip-mixed. The user moves the plate to the pre-chilled thermocycler to run the Fragmentation and A-tailing program. See Figure 1.

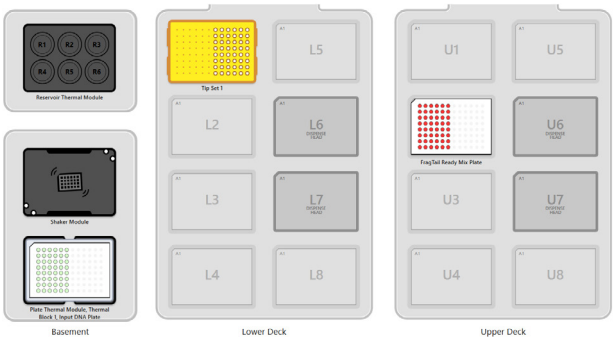

Figure 1. Starting deck layout for Protocol 1 of 4: 3.1 Fragmentation and A-Tailing. Example shown is for 6 columns of samples.

Protocol 2 of 4
3.2 Adapter Ligation

Figure 2 shows the starting deck layout for this protocol, which should be run as soon as the Fragmentation and A-tailing protocol on the thermocycler has completed. The input DNA plate now contains the FragTail product and is loaded onto a chilled thermal plate on firefly. KAPA UDI Adapters and Ligation ReadyMix are added to the FragTail product, and these reagents are tip-mixed. The user moves the input DNA plate to the thermocycler for the ligation reaction (20°C for 15 minutes). Set the deck up for the Post-Ligation Purification once the ligation reaction has been started on the thermocycler.

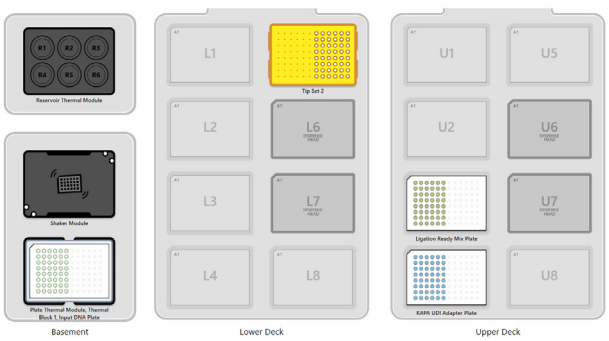

Figure 2. Starting deck layout for Protocol 2 of 4: 3.2 Adapter ligation. Example shown is for 6 columns of samples.

Protocol 3 of 4
3.3 Post-Ligation Purification

This protocol should be started as soon as the Adapter Ligation incubation has completed. In this protocol, the input DNA plate contains the ligation reaction product. The protocol performs a 0.8X bead purification then transfers the cleaned-up adapter ligated libraries to a fresh plate. This a safe stopping point where samples can be stored at 4°C or -20°C if required. See Figure 3 for the starting deck layout for this protocol.

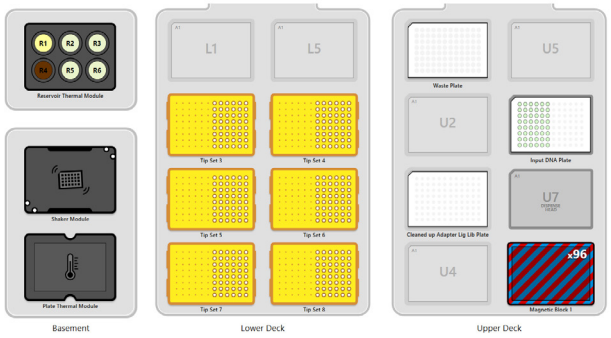

Figure 3. Starting deck layout for Protocol 3 of 4: 3.3 Post Ligation Purification. Example shown is for 6 columns of samples.

Protocol 4 of 4
4.1 - 4.3 Library Amplification and Purification

The Cleaned up Adapter Ligated Library plate contains adapter-ligated library from the Post-Ligation Purification step. This protocol adds Library Amplification Primer Mix and 2X HiFi Hotstart ReadyMix to the adapter-ligated libraries and tip-mixes these reagents. The plate should then be moved to the thermocycler and the relevant KAPA Library Amplification program started – see the "Instructions for Use of KAPA EvoPlus Kit" document. Once the thermocycler program is complete, return the plate to the firefly deck. The protocol then performs a 1.0X bead purification and transfers the cleaned up amplified libraries to a fresh plate. See Figure 4 for the starting deck layout for this protocol.

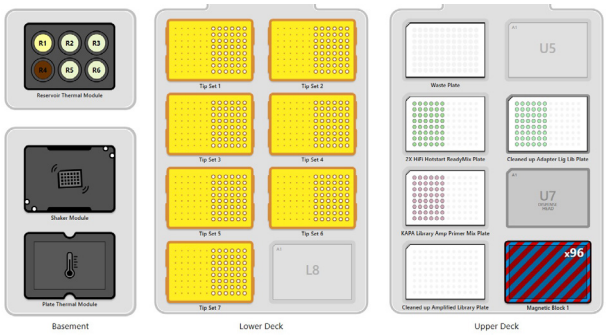

Figure 4. Starting deck layout for Protocol 4 of 4: 4.1 - 4.3 Library Amplification and Purification. Example shown is for 6 columns of samples.

Optional protocol
Double-sided Size Selection

This optional Double-sided Size Selection protocol can be run after protocol 3 of 4 (3.3 Post Ligation Purification), or after protocol 4 of 4 (4.1-4.3 Library Amplification and Purification). The input volume into this protocol is 50µl and output volume is 20µl. This protocol performs a double-sided size selection purification where the user selects the SPRI 1 and SPRI 2 ratios. The Size Selected libraries are then transferred to a fresh plate.

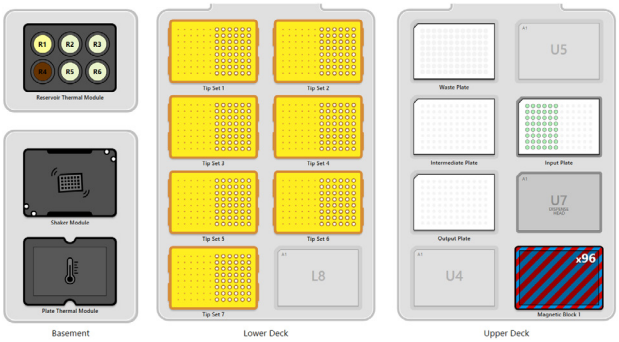

Figure 5. Starting deck layout for Optional protocol: Double-sided Size Selection. Example shown is for 6 columns of samples.

Protocol performance
Input titration and size selection

The KAPA EvoPlus library preparation was run manually and on firefly using human gDNA (Promega) as the input. See Table 5 for the workflows tested. Each run used a gDNA input of 10ng, 100ng or 500ng, with 8 replicates at each input level. All runs used a 20-minute fragmentation time and a 0.8X post-ligation clean up. Samples undergoing the KAPA EvoPlus workflow (with PCR) were processed with 5 cycles of PCR, followed by a 1.0X post-amplification clean up. Samples undergoing the size selection workflow underwent an additional double-sided clean up following the post-ligation clean up, with the first cut at 0.5X and the second cut at 0.7X.

The resulting libraries were analysed to determine their concentration by qPCR and average fragment size - using a LightCycler 480 System (Roche, KAPA Library Quantification kit) and a Fragment Analyzer (Agilent, DNF-474 HS NGS Fragment Kit) respectively.

Workflow	Input	Adapter Conc.	PCR Cycles
KAPA EvoPlus	10 ng	6 µM	5
KAPA EvoPlus PCR-Free	100 ng	15 µM	0*
KAPA EvoPlus PCR-Free	500 ng	15 µM	0*
KAPA EvoPlus PCR-Free with size selection	500 ng	15 µM	0*

Table 5. Run setup used to assess protocol performance.

*5 PCR cycles were required to enable fragment size analysis

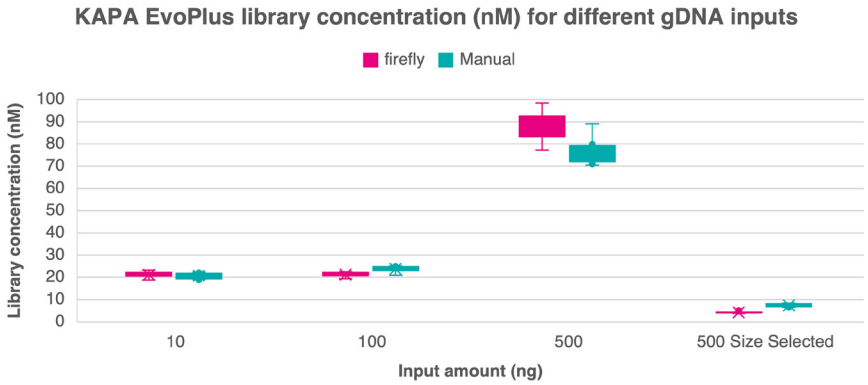

Figure 6. Library concentration (nM) of KAPA EvoPlus libraries measured by qPCR, for gDNA inputs of 10ng, 100ng and 500ng. The 10ng input samples received 5 cycles of PCR. All 100ng and 500ng input samples were run PCR-free. Also shown is the average concentration of KAPA EvoPlus libraries generated with 500ng gDNA input and a size selection (0.5X-0.7X) run after the post-ligation clean up. n=8 for each box plot.

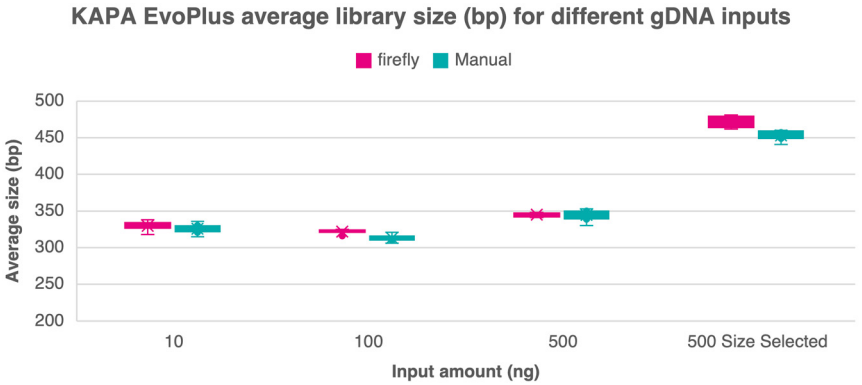

Figure 7. Average size of KAPA EvoPlus libraries generated manually and on firefly for gDNA inputs of 10ng, 100ng and 500ng. The 10ng input samples received 5 cycles of PCR. All 100ng and 500ng input samples were run PCR-free. Also shown is the average size of KAPA EvoPlus libraries generated with 500ng input and a size selection (0.5X-0.7X) run after the post-ligation clean up. n=8 for each box plot.

High-throughput performance and cross contamination evaluation

Two runs were executed to assess the well-to-well contamination and high-throughput performance of the protocols developed for automated KAPA EvoPlus DNA Library Prep Kit on firefly. In each run a total of 96 libraries were prepared on firefly: 81 replicates of a 10ng gDNA input and 15 No Template Controls (NTCs). The KAPA EvoPlus workflow was run using a 20-minute fragmentation time, a 0.8X post-ligation clean up, 5 cycles of PCR and a 1.0X post-amplification clean up.

All libraries were quantified by qPCR, using a LightCycler 480 System (Roche, KAPA Library Quantification kit). A subset of the resulting libraries was analyzed to determine the average library size using a Fragment Analyzer (Agilent, DNF-474 HS NGS Fragment Kit).

Table 6 and Table 7 show the library concentrations for the two high throughput runs. The %CV for the concentration of the 81 sample replicates was 10.8% for run 1 and 11.0% for run 2. The NTCs showed no detectable library contamination by qPCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20.93	21.40	20.80	21.74	20.70	21.45	24.87	22.47	20.05	22.32	21.52	26.10
B	22.85	NTC	19.40	22.86	23.28	NTC	22.94	22.81	27.73	NTC	27.37	25.10
C	25.08	25.06	25.11	NTC	26.67	24.59	26.00	NTC	29.76	24.82	25.76	28.87
D	21.29	NTC	21.08	25.94	29.19	NTC	22.71	26.14	26.54	NTC	28.25	25.58
E	24.19	21.21	24.38	NTC	26.29	24.26	28.53	NTC	27.02	25.90	26.36	25.43
F	23.85	NTC	23.04	24.74	24.19	NTC	26.75	22.95	27.75	NTC	24.83	32.09
G	23.91	24.82	27.11	NTC	22.67	22.33	30.97	NTC	28.33	28.25	24.18	26.67
H	22.10	25.61	26.16	21.61	22.00	22.56	27.19	22.68	27.51	23.19	26.51	29.39

Table 6. Concentration (nM) of libraries generated in run 1 of the high-throughput performance and cross contamination evaluation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	26.75	28.89	28.47	26.92	26.45	26.42	28.84	25.64	25.14	29.40	29.06	28.71
B	28.05	NTC	26.38	6.30	27.10	NTC	27.24	28.46	28.80	NTC	29.92	29.10
C	30.40	27.69	27.61	NTC	28.40	25.42	28.91	NTC	27.70	29.62	29.24	30.45
D	26.05	NTC	25.09	28.36	29.59	NTC	29.82	29.73	27.17	NTC	29.54	30.50
E	34.36	24.21	27.37	NTC	27.66	29.66	27.07	NTC	27.19	28.93	28.87	27.52
F	27.51	NTC	26.37	26.53	26.45	NTC	27.28	28.45	29.75	NTC	29.06	30.26
G	29.05	26.65	27.75	NTC	26.37	27.30	27.96	NTC	30.40	29.02	28.03	29.03
H	24.67	28.70	28.40	28.08	25.75	25.84	26.84	27.36	27.17	27.96	28.17	36.69

Table 7. Concentration (nM) of libraries generated in run 2 of the high-throughput performance and cross contamination evaluation.

Columns 2, 4, 8 and 10 underwent fragment size analysis. For the sample wells in these columns, the %CV of the average fragment size was 1.0% for run 1 and 1.2% for run 2.

Figure 8 and Figure 9 show examples of the Fragment Analyzer traces from high throughput run 1 and demonstrate the consistency in the fragment size across the 96-well plate and the absence of library contamination in the NTCs.

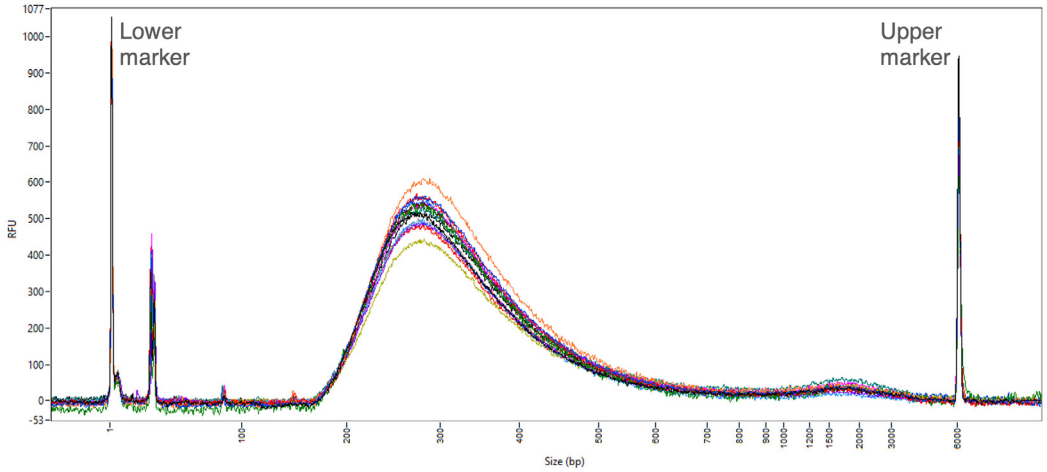

Figure 8. Fragment Analyzer traces for 10ng input libraries prepared on firefly for high-throughput run 1.

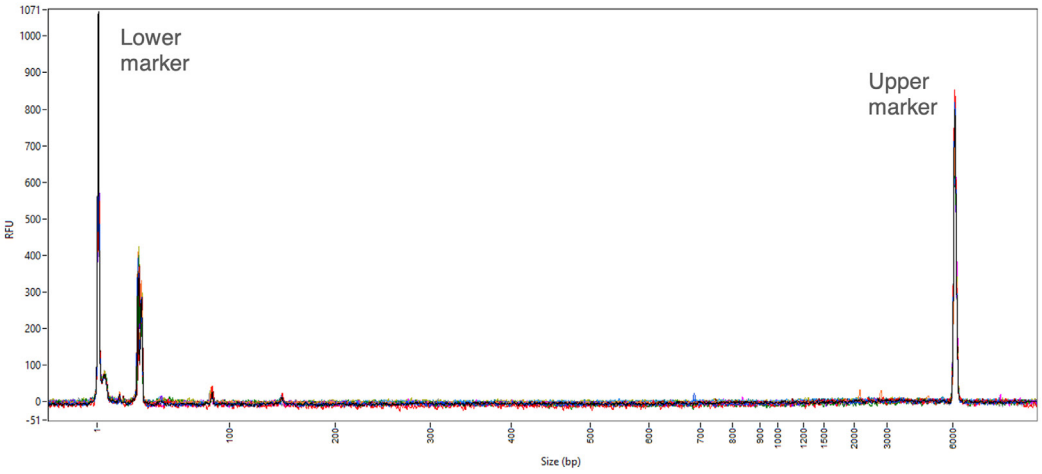

Figure 9. Fragment Analyzer traces for NTCs prepared on firefly for high-throughput run 1.

Figure 10 and Figure 11 compare the library concentration and fragment size for all runs that used a 10ng gDNA input. This data shows that firefly library concentrations and fragment sizes show minimal variation within and between runs and are in line with manually prepared libraries.

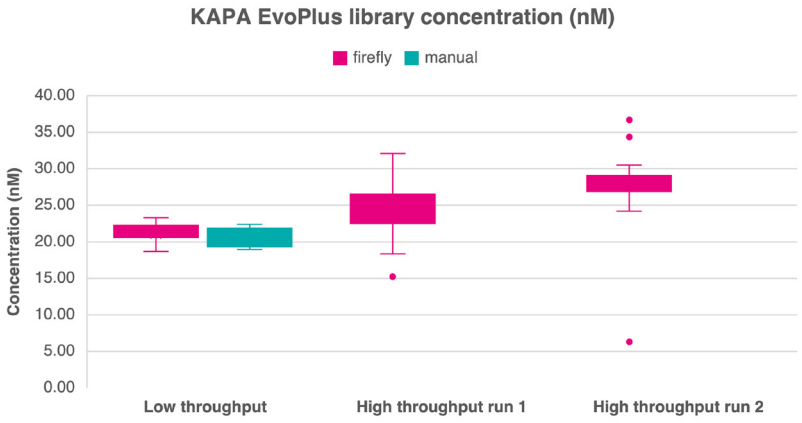

Figure 10. Distribution of library concentration (nM) of KAPA EvoPlus libraries generated on firefly and manually for 10ng input with 5 cycles of PCR. n=8 for each low throughput run. n=81 for each high throughput run.

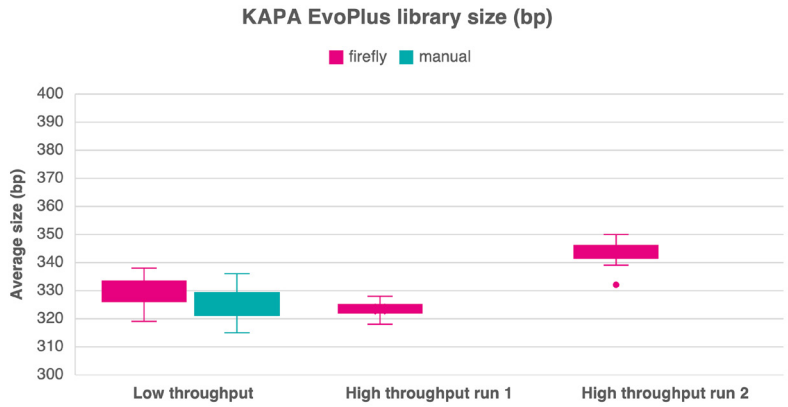

Figure 11. Distribution of average library size (bp) for libraries generated on firefly and manually for 10ng input with 5 cycles of PCR. n=8 for each low throughput run. A subset of samples from columns 2,4,8 and 10 of the high throughput runs are shown. n=19 for high throughput run 1, n=20 for high throughput run 2.

Summary

These results demonstrate that the KAPA EvoPlus and KAPA EvoPlus PCR-free workflows have been successfully automated on firefly to generate Illumina-compatible sequencing libraries.

KAPA EvoPlus sequencing libraries generated on firefly are in line with manually prepared libraries in terms of final library concentration and average fragment sizes.

Libraries prepared on firefly show consistent library concentration and fragment size across a 96-well plate and show no detectable well-to-well contamination.

*Data on file