firefly®+ application note

Roche KAPA EvoPlus Boost Kit and KAPA EvoPlus V2 Kit

Overview

Roche KAPA EvoPlus Boost Kits and KAPA EvoPlus V2 Kits are ideally suited for low- and high-throughput Next-Generation Sequencing (NGS) library construction workflows. The KAPA EvoPlus Boost kit uses KAPA EvoAmp ReadyMix powered by KAPA HiFi to construct libraries from input DNA amounts of 0.1 ng to 500 ng. The kits are available in an automation-friendly 96-well plated format.

Full walkaway KAPA EvoPlus Boost (or KAPA EvoPlus V2) protocols have been developed for the SPT Labtech firefly+. This application note provides supporting information for the firefly+ protocols listed below. These protocols are based on the Roche document - "Instructions for Use of KAPA DNA Library Prep Evolved Workflows with enzymatic fragmentation. For use with the KAPA EvoPlus V2 and KAPA EvoPlus Boost kits. Featuring KAPA EvoT4 DNA Ligase and KAPA EvoAmp ReadyMix. (October 2025, Version 5.0)" and are available to download from the firefly community.

Here, we demonstrate how these workflows have 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.

Protocol highlights

- Full walkaway library preparation protocol for KAPA EvoPlus Boost (or KAPA EvoPlus V2)
- Full walkaway library preparation protocol for KAPA EvoPlus Boost PCR-Free
- On-deck thermocycler (ODTC) on firefly+ allows all incubation and thermocycling steps to take place on firefly+
- Flexibility to process 1 to 12 sample columns per run and to specify the starting column for the reagent plates (ReadyMix and UDI Adapters plates) - enabling multiple low-throughput runs using the same reagent plates and reducing waste.
- Additional protocols provide a flexible framework to enable multiple workflow options to be automated, to include:
  - Low DNA input (0.1ng) workflows
  - An optional double-sided size selection can be run either at the post-ligation or post-amplification stage
  - Library Amplification can be run with either a single or double post PCR purification

firefly+ protocols

KAPA EvoPlus Boost or KAPA EvoPlus V2 – firefly+

Protocol name	firefly+ run time
3.1-3.3 Fragmentation and Adapter Ligation	1 hour 56 minutes (8 samples)
3.1-4.3a Fragmentation, Adapter Ligation and Amplification	2 hours 54 minutes (8 samples with 5 cycles PCR) to 3 hours 20 minutes (96 samples with 5 cycles PCR)
Appendix A. Double-sided Size Selection	42 minutes (8 samples)
4.1-4.3a Library Amplification and Single Purification	56 minutes (8 samples with 5 cycles PCR)
4.1-4.3b Library Amplification and Double Purification	1 hour 38 minutes (8 samples with 13 cycles of PCR)

Table 1. firefly+ protocol names and run times.

	firefly+ protocol to run	
Workflow	**3.1-3.3 Fragmentation and Adapter Ligation**	**Appendix A. Double-sided Size Selection**
KAPA EvoPlus Boost PCR-Free	✓	
KAPA EvoPlus Boost PCR-Free with post ligation size selection	✓	✓

Table 2. Summary of the KAPA EvoPlus Boost PCR-Free workflows that can be run and the firefly+ protocols required to run them. The KAPA EvoPlus Boost PCR-Free workflow can be run as a standalone walkaway protocol or with an additional post-ligation size selection.

	firefly+ protocol to run				
Workflow	**3.1-3.3 Fragmentation and Adapter Ligation**	**3.1-4.3a Fragmentation, Adapter Ligation and Amplification**	**Appendix A. Double-sided Size Selection**	**4.1-4.3a Library Amplification and Single Purification**	**4.1-4.3b Library Amplification and Double Purification**
KAPA EvoPlus V2		✓ Using KAPA HiFi HotStart ReadyMix PCR			
KAPA EvoPlus V2 with post ligation size selection	✓		✓	✓ Using KAPA HiFi HotStart ReadyMix PCR	
KAPA EvoPlus V2 with post-amplification size selection		✓ Using KAPA HiFi HotStart ReadyMix PCR	✓		
Low input (0.1 ng) KAPA EvoPlus V2	✓				✓ Using KAPA HiFi HotStart ReadyMix PCR

Table 3. Summary of the KAPA EvoPlus V2 workflows that can be run and the firefly+ protocols required to run them. The KAPA EvoPlus V2 workflow uses the KAPA HiFi HotStart ReadyMix and associated PCR cycling conditions. It can be run standalone or with a post-ligation or post-amplification size selection, or with low input (0.1 ng) by running the relevant combination of protocols.

	firefly+ protocol to run				
Workflow	**3.1-3.3 Fragmentation and Adapter Ligation**	**3.1-4.3a Fragmentation, Adapter Ligation and Amplification**	**Appendix A. Double-sided Size Selection**	**4.1-4.3a Library Amplification and Single Purification**	**4.1-4.3b Library Amplification and Double Purification**
KAPA EvoPlus Boost		✓ Using KAPA EvoAmp ReadyMix PCR			
KAPA EvoPlus Boost with post-ligation size selection	✓		✓	✓ Using KAPA EvoAmp ReadyMix PCR	
KAPA EvoPlus Boost with post-amplification size selection		✓ Using KAPA EvoAmp ReadyMix PCR	✓		
Low input (0.1ng) KAPA EvoPlus Boost	✓				✓ Using KAPA EvoAmp ReadyMix PCR

Table 4. Summary of the KAPA EvoPlus Boost workflows that can be run and the firefly+ protocols required to run them. The KAPA EvoPlus Boost workflow uses the KAPA EvoAmp ReadyMix and associated PCR cycling conditions. It can be run standalone or with a post-ligation or post-amplification size selection or with low input (0.1 ng) by running the relevant combination of protocols.

Protocol overview

SPT Labtech firefly+ protocols have been developed to run the KAPA EvoPlus Boost, KAPA EvoPlus Boost PCR-Free and KAPA EvoPlus V2 workflows – using the 96-well plate format kits and the KAPA Unique Dual-Indexed (UDI) Adapter Kit. The firefly+ protocols run in 96-well plate format and make use of the on-deck thermocycler (ODTC) on firefly+ to maximise walkaway time. Variables allow a user to run from 1 to 12 columns of library preparation. Either the KAPA EvoPlus Boost or KAPA EvoPlus V2 workflow can be run using the same firefly+ protocols.

The automated KAPA EvoPlus V2 workflow should be used with the KAPA EvoPlus V2 Kit (96-well plate). This kit contains a KAPA HiFi HotStart ReadyMix (2X) plate, which needs to be used with the KAPA HiFi HotStart ReadyMix cycling program conditions – by selecting the relevant "KAPA HiFi HotStart ReadyMix cycling program" PCR ODTC protocol when starting the run.

The automated KAPA EvoPlus Boost workflow should be used with the KAPA EvoPlus Boost Kit (96-well plate). This kit contains a KAPA EvoAmp ReadyMix plate, which needs to be used with the KAPA EvoAmp ReadyMix cycling program conditions – by selecting the relevant "KAPA EvoAmp ReadyMix cycling program" PCR ODTC protocol when starting the run.

The KAPA EvoPlus Boost and KAPA EvoPlus V2 workflows on firefly+ cover the following sections of the Instructions For Use document:

Chapter 3. Prepare the Sample Library:

- Step 1. Fragmentation and A-Tailing
- Step 2. Adapter Ligation
- Step 3. Purify the Sample Library using KAPA HyperPure Beads

Chapter 4. Amplify the Sample Library:

- Step 1. Prepare the Library Amplification Reaction
- Step 2. Perform the Library Amplification
- Step 3. Purify the Amplified Sample Library using KAPA HyperPure Beads

Appendix A. Double-sided Size Selection

KAPA EvoPlus Boost PCR-Free workflow

firefly+ protocol - 3.1-3.3 Fragmentation and Adapter Ligation

This full walkaway protocol runs the KAPA EvoPlus Boost PCR-Free workflow on firefly+ following Chapter 3. *Prepare the Sample Library* of the Instructions for Use document.

The protocol adds FragTail ReadyMix to the input DNA and tip mixes these reagents. The input DNA plate is then placed on the On Deck ThermoCycler (ODTC) to run the Fragmentation and A-tailing. On completion of the Fragmentation and A-tailing, the protocol adds KAPA UDI Adapters and Ligation ReadyMix to the FragTail product and tip mixes these reagents. The plate is then incubated at 20ºC for 15 minutes on the ODTC. Following the Adapter Ligation the protocol performs a 0.6X bead purification and then transfers the cleaned-up adapter ligated libraries to a fresh plate.

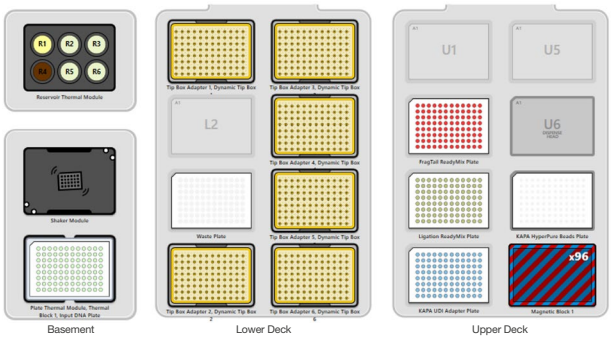


Figure 1. firefly starting deck layout for a 96-sample KAPA EvoPlus Boost PCR-Free 3.1-3.3 Fragmentation and Adapter Ligation library prep run. Deck layout – Basement: Reservoir R1 – Elution Buffer, R2+R3+R5+R6 – 80% Ethanol, R4 – KAPA HyperPure Beads, Plate Thermal Module - 96 sample Thermo Adapter Block and Input DNA Plate. Lower Deck: L1+L4+L5+L6+L7+L8 - 100 µL 96 format ATL tips on ATL 35-125 µL Tip Stands, L3 – Waste Plate. Upper Deck: U2 – FragTail ReadyMix Plate, U3 – Ligation ReadyMix Plate, U4 – KAPA UDI Adapter Plate, U7 – KAPA HyperPure Beads Plate, U8 – Alpaqua Magnum FLX 96 sample Magnetic Block.

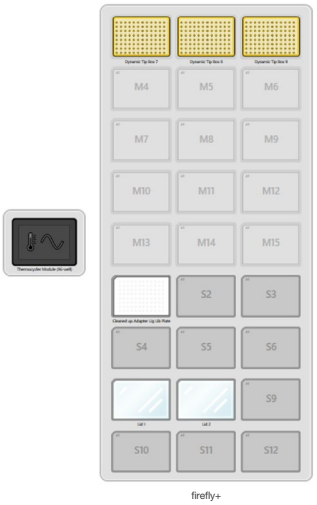


Figure 2. firefly+ starting layout for a 96-sample KAPA EvoPlus Boost PCR-Free 3.1-3.3 Fragmentation and Adapter Ligation library prep run. M1-M3 - 100 µL 96 format ATL tips. S1 – Cleaned up Adapter Ligation Library Plate. S7, S8 – Plate Lids.

KAPA EvoPlus Boost (or KAPA EvoPlus V2) workflow

firefly+ protocol - 3.1-4.3a Fragmentation, Adapter Ligation and Amplification

This full walkaway protocol runs the KAPA EvoPlus Boost (or KAPA EvoPlus V2) workflow on firefly+ following Chapter 3. *Prepare the Sample Library* and Chapter 4 *Amplify the Sample Library* of the Instructions For Use document. This firefly+ protocol runs the same steps as the PCR-Free protocol and then carries on to the amplification and purification steps in Chapter 4 Steps 1-3a *Purification of amplified Sample Library* procedure.

This protocol runs the same initial steps as the PCR-Free protocol and then carries on to the amplification and purification steps.

Following the Adapter Ligation Purification, the protocol adds Library Amplification Primer Mix and KAPA HiFi HotStart ReadyMix (2X) OR KAPA EvoAmp ReadyMix to the Adapter-ligated libraries and tip mixes. The plate is then run on the relevant user defined KAPA Library Amplification program on the ODTC. KAPA EvoPlus Boost workflows use the KAPA EvoAmp plate and cycling conditions. KAPA EvoPlus V2 workflows use the KAPA HiFi HotStart ReadyMix (2X) plate and cycling conditions.

Once the thermocycler program is complete, the plate is returned to the firefly deck. The protocol then performs a 1.0X bead purification and transfers the cleaned-up amplified libraries to a fresh plate.

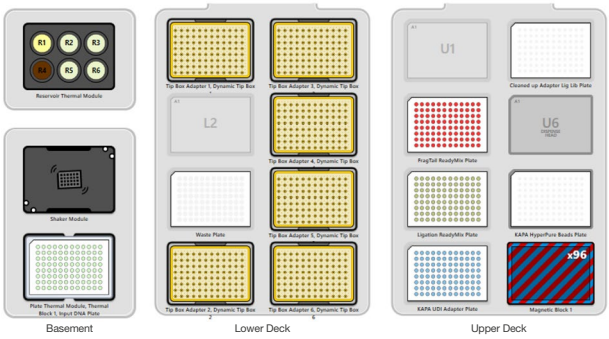
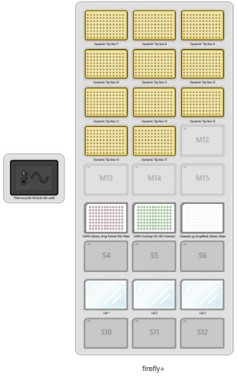


Figure 3. firefly starting deck layout for a 96-sample KAPA EvoPlus Boost (or KAPA EvoPlus V2) 3.1-4.3a Fragmentation, Adapter Ligation and Amplification library prep run. Deck layout – Basement: Reservoir R1 – Elution Buffer, R2+R3+R5+R6 – 80% Ethanol, R4 – KAPA HyperPure Beads, Plate Thermal Module - 96 sample Thermo Adapter Block and Input DNA Plate. Lower Deck: L1+L4+L5+L6+L7 +L8 - 100 µL 96 format ATL tips on ATL 35-125 µL Tip Stands, L3 – Waste Plate. Upper Deck: U2 – FragTail ReadyMix Plate, U3 – Ligation ReadyMix Plate, U4 – KAPA UDI Adapter Plate, U5 – Cleaned Up Adapter Ligation Library Plate, U7 – KAPA HyperPure Beads Plate, U8 – Alpaqua Magnum FLX 96 sample Magnetic Block.

Figure 4. firefly+ starting layout for a 96-sample KAPA EvoPlus Boost (or KAPA EvoPlus V2) 3.1-4.3a Fragmentation, Adapter Ligation and Amplification library prep run. M1-M11 - 100 µL 96 format ATL tips. S1 – KAPA Library Amplification Primer Mix Plate, S2 – KAPA EvoAmp ReadyMix OR KAPA HiFi HotStart ReadyMix (2X) Plate, S3 - Cleaned up Amplified Library Plate, S7-S9 – Plate Lids.



KAPA EvoPlus V2 Double-sided Size Selection workflow

firefly+ protocol - Appendix A. Double-sided Size Selection

This optional Size Selection protocol can be run after either the firefly+ 3.1-3.3 Fragmentation and Adapter Ligation protocol to perform post-ligation sample purification or after the firefly+ 3.1-4.3a Fragmentation, Adapter Ligation and Amplification protocol to perform a post-amplification sample purification. Input volume is 50 µL, and output volume is 20 µL. 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 output plate.

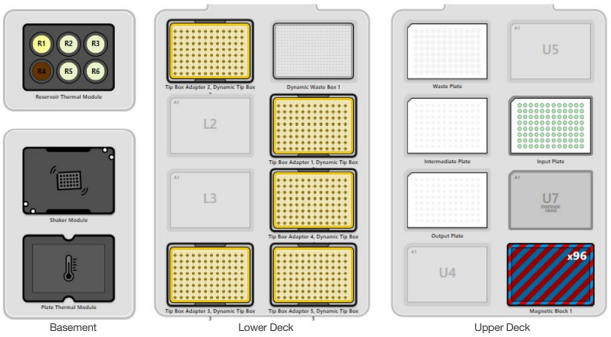


Figure 5. firefly starting deck layout for a 96-sample KAPA EvoPlus V2 Appendix A. Double-sided Size Selection run. Deck layout – Basement: Reservoir R1 – Elution Buffer, R2+R3+R5+R6 – 80% Ethanol, R4 – KAPA HyperPure Beads. Lower Deck: L1+L4+L6+L7+L8 - 100 µL 96 format ATL tips on ATL 35-125 µL Tip Stands, L5 – Empty Waste tip Box. Upper Deck: U1 – Waste Plate, U2 - Intermediate Plate, U3 – Output Plate, U6 – Input Plate, U8 – Alpaqua Magnum FLX 96 sample Magnetic Block.

Figure 6. firefly+ starting layout for a 96-sample KAPA EvoPlus V2 Appendix A. Double-sided Size Selection run. M1 - Empty Waste tip Box, M2-M3 - 100 µL 96 format ATL tips.

[1] *For life science research only. Not for use in diagnostic procedures.*

KAPA EvoPlus Boost (or KAPA EvoPlus V2) Chapter 4. Amplify the Sample Library only

firefly+ protocols -

4.1-4.3a Library Amplification and Single Purification and 4.1-4.3b Library Amplification and Double Purification

These protocols can optionally be run following the 3.1-3.3 Fragmentation and Adapter Ligation protocol to complete the full amplification workflow outlined in Chapter 4. *Amplify the Sample library*, of the Instructions For Use document.

If libraries were constructed from ≥1 ng of input DNA using KAPA UDI Adapters, then protocol 4.1-4.3a Library Amplification and Single Purification should be run. This will run a single 1.0X bead cleanup post-amplification.

If libraries were constructed from 0.1-1 ng of input DNA using KAPA UDI Adapters, then protocol 4.1-4.3b Library Amplification and Double Purification should be run. This will run two 1.0X bead cleanups post-amplification.

Protocol performance

The KAPA EvoPlus Boost library preparation protocols were run on firefly+ and manually. Three tests were performed: Test 1 was a high-throughput run, using 96 samples at 10ng input, including 15 NTCs; Test 2 was a PCR-Free run at was 500 ng input (and then later a Size Selection), and Test 3 was a 0.1 ng low input run. See Table 5 for details. The ODTC was used for thermocycling steps for both the firefly+ and manual runs.

The resulting libraries were analyzed 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. See Table 6 for a summary of the results. Figures 7-10 show a further breakdown of the results. No library was detected in the NTCs – see Figure 9.

Experiment	Input (ng)	firefly+ replicates	Manual replicates	Adapter Conc (µM)	PCR Cycles	Post Amplification cleanup
Test 1 high-throughput	10	96	8	6	5	Chapter 4 Step 3a (Single 1.0X cleanup)
Test 2 (PCR-Free)	500	8	4	15	0*	N/A
Test 3 low input	0.1	8	8	0.6	13	Chapter 4 Step 3b (Double 1.0X cleanup)

Table 5. Experiments run to assess protocol performance of KAPA EvoPlus Boost on firefly+. *5 cycles of PCR run for sizing purposes.

Experiment	Number samples sized	Average Size (bp)	Average Size CV%	Number of Samples quantified	Concentration (nM)	Concentration CV%
Test 1 high-throughput – firefly+	16	351	1.3	80	6.1	13.6
Test 1 manual + ODTC	8	347	2.6	8	7.15	13.4
Test 2 PCR-Free (Post Ligation) – firefly+	8	332	1.6	8	49.78	10.5
Test 2 PCR-Free (Post Ligation) - manual	4	342	3.8	4	33.73	12.0
Test 3 low input – firefly+	8	362	2.1	8	15.67	28.6
Test 3 low input – manual + ODTC	8	394	4.5	8	11.47	27.7

Table 6. Summary of sizing and concentration data for KAPA EvoPlus Boost firefly+ and manual runs.

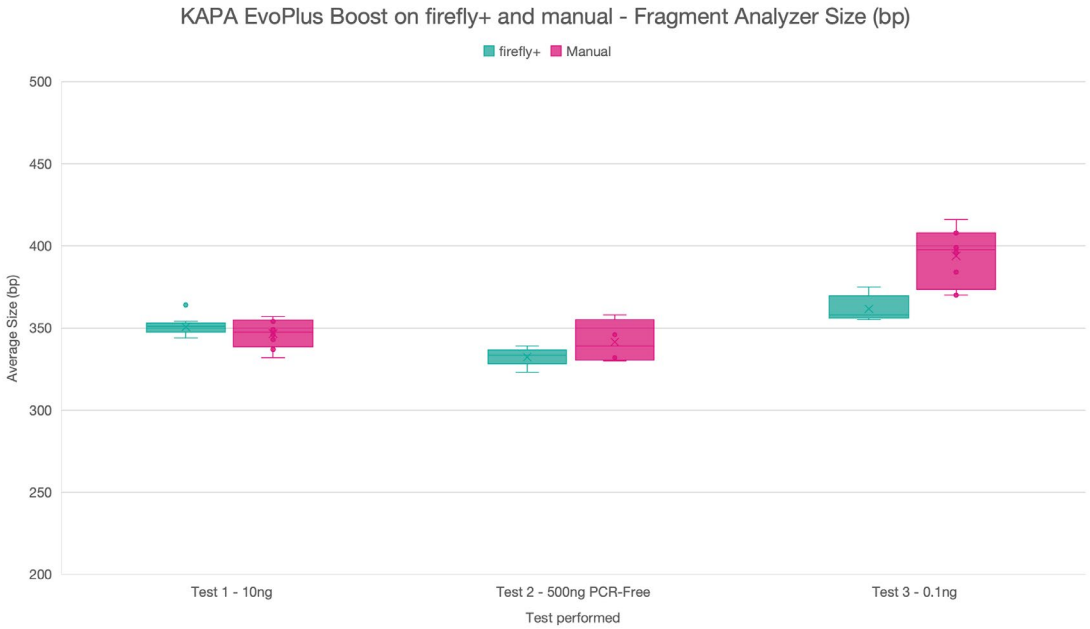


Figure 7. Fragment Analyzer average size (bp) results for KAPA EvoPlus Boost libraries for each of the 3 tests – 10 ng input with 5 cycles of PCR, 500 ng input PCR-Free, and 0.1 ng input with 13 cycles of PCR. The comparison between libraries generated on the firefly+ and manually is shown.

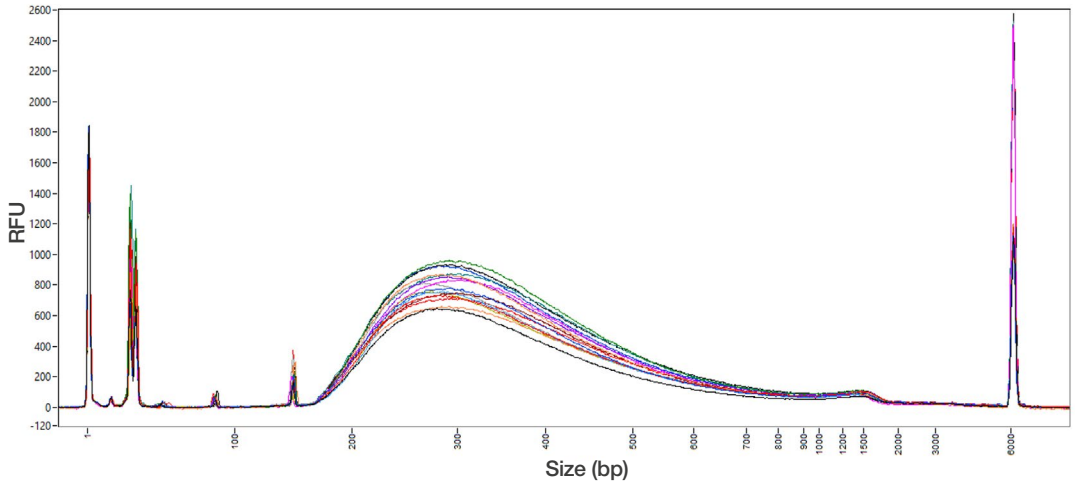


Figure 8. Fragment Analyzer traces for samples run on test 1, 10ng input firefly+ n=16.

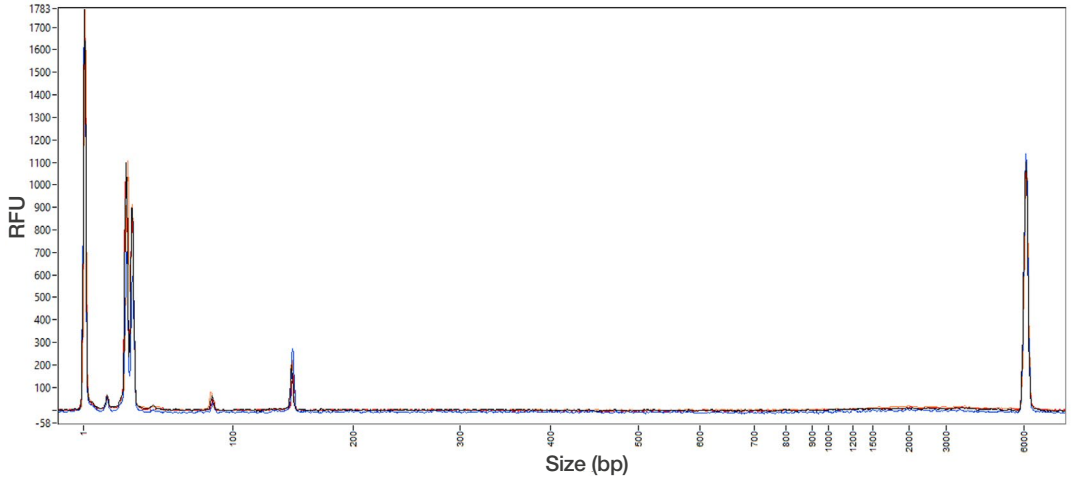


Figure 9. Fragment Analyzer traces for NTCs run on test 1, 10ng input firefly+ n=4.

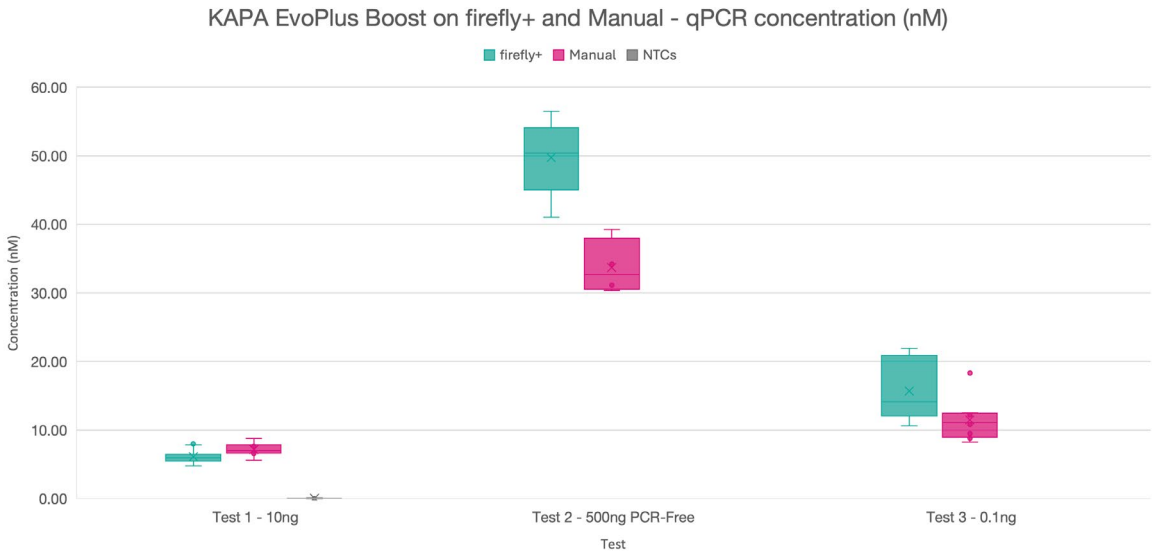


Figure 10. KAPA Library Quantification size-adjusted concentration (nM) results for KAPA EvoPlus Boost libraries for each of the 3 tests – 10 ng input with 5 cycles of PCR, 500 ng input PCR-Free, and 0.1 ng input with 13 cycles of PCR. The comparison between libraries generated on the firefly+ and manually is shown. NTCs run as part of firefly+ test 1 are also shown.

Size Selection

As part of Test 2, the 500 ng input PCR-Free samples underwent 5 cycles of PCR, a post-PCR cleanup, and then an additional double-sided Size Selection cleanup following the post-PCR cleanup, with the first cut at 0.5X and the second cut at 0.7X. Library Size Pre and Post Size Selection was measured for the firefly+ and manual runs using Fragment Analyzer, and the results are shown in Table 7 and Figures 11-12. The results show that a double-sided size selection was able to be run successfully on firefly+ with comparable results to manual size selection.

Run type	Average Size (bp) Pre Size Selection	Average Size (bp) Post Size Selection	Number of Samples
firefly+	368	429	8
Manual	362	408	3

Table 7. Summary of the Fragment Analyzer sizing data for the KAPA EvoPlus Boost libraries undergoing double-sided size selection.

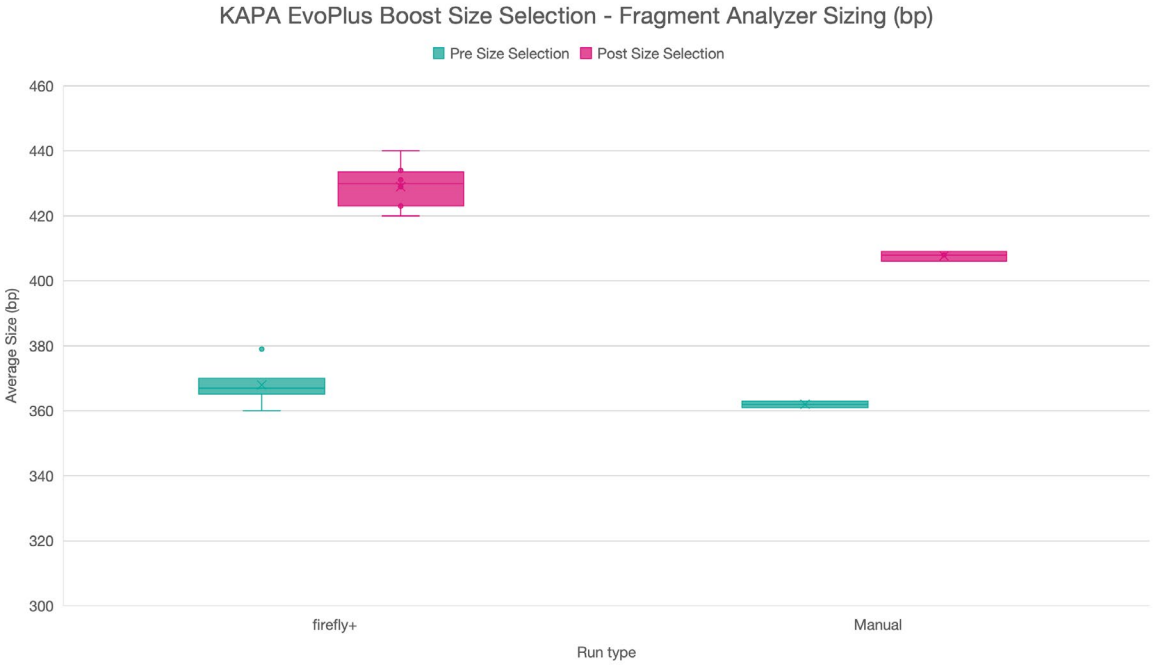


Figure 11. Fragment Analyzer average size (bp) for KAPA EvoPlus Boost libraries undergoing double-sided size selection on firefly+ and run manually.

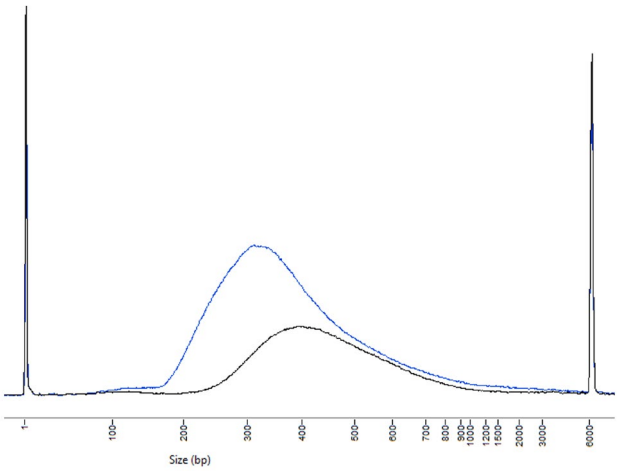


Figure 12. Test 2 Size Selection on firefly+. Fragment Analyzer traces shown are for sample in position A1 pre and post size selection. Size selection was performed following a 5-cycle PCR and cleanup. 1 in 10 dilution run on the fragment analyzer.

Reagents

Kits

Description	Roche Cat No.
KAPA EvoPlus V2 Kit, plated format (96rxn)	09420428001
KAPA EvoPlus Boost Kit, PCR-free, plated format (96rxn)	09420436001
KAPA Library Amp Primer Mix 96-well plate (96rxn)	09420479001
KAPA EvoPlus Boost Kit, plated format (96rxn)	10613726001
KAPA Unique Dual-Indexed Adapter Kit, (15 µM)	08861919702
KAPA Adapter Dilution Buffer (25 mL)	08278539001
KAPA HyperPure Beads Kit (60mL)	8963851001

Table 8. Roche KAPA EvoPlus V2 kits required for firefly+ library prep runs.

Summary

These results demonstrate that the KAPA EvoPlus Boost, KAPA EvoPlus Boost PCR-Free, and KAPA EvoPlus V2 workflows have been successfully automated on firefly+ to generate Illumina-compatible sequencing libraries. Full walkaway firefly+ protocols are available for:

- 3.1-3.3 Fragmentation and Adapter Ligation
- 3.1-4.3a Fragmentation, Adapter Ligation and Amplification

Additional firefly+ protocols are available to perform optional double-sided size selection and alternative versions of the library amplification and cleanup steps. Plate-based reagents and user input variables in the firefly+ protocols allow flexibility to run varying numbers of columns of samples and to start at varying columns in the reagent plates – allowing both high-throughput or multiple low-throughput run approaches from a single kit.

Libraries prepared on firefly+ are in line with manually prepared libraries in terms of average fragment size and concentration. The libraries show consistent fragment sizing and concentration across a 96-well plate and show no detectable well-to-well contamination. The firefly+ size selection workflow also shows expected results consistent with manually prepared libraries.