INSTRUCTIONS FOR USE OF

KAPA EvoPlus V2 Kit

featuring the KAPA EvoT4 DNA Ligase

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

April 2024, Version 4.1

Contents

The KAPA EvoPlus V2 Kits contain:

Kit Material Number	Component Material Number**	Description	Volume
09420037001 09420045001*		**KAPA EvoPlus V2 Kit (24 rxn)**	
	09420606001	KAPA FragTail ReadyMix	600 µL
	10119942001	KAPA Ligation ReadyMix	240 µL
	08203075001	KAPA HiFi HotStart ReadyMix (2X)	690 µL
09420053001 09420304001*		**KAPA EvoPlus V2 Kit (96 rxn)**	
	09420614001	KAPA FragTail ReadyMix	2.4 mL
	10119969001	KAPA Ligation ReadyMix	960 µL
	08203008001	KAPA HiFi HotStart ReadyMix (2X)	3.0 mL
09420339001 09420371001*		**KAPA EvoPlus V2 Kit (384 rxn)**	
	09420649001	KAPA FragTail ReadyMix	9.6 mL
	10119985001	KAPA Ligation ReadyMix	3.84 mL
	09420711001	KAPA HiFi HotStart ReadyMix (2X)	9.6 mL
09420428001 09420436001*		**KAPA EvoPlus V2 Kit (96-well plate***)**	
	09420657001	KAPA FragTail ReadyMix	96 x 25 µL
	10119993001	KAPA Ligation ReadyMix	96 x 10 µL
	09420720001	KAPA HiFi HotStart ReadyMix (2X)	96 x 25 µL
		1 x Replacement (pierceable and peelable) seal per plate	

* Available for PCR-free workflows, and do not contain any library amplification reagents (KAPA HiFi HotStart ReadyMix).
** 10% overage + 5 µL is provided for KAPA EvoPlus V2 Kit (96-well plate).
Note: Accessory kits (KAPA Cleanup Beads, KAPA Universal Adapter & KAPA UDI Primer Mixes, KAPA Unique Dual-Indexed Adapter and KAPA Library Amplification Primer Mix) are sold separately.
*** Ensure that the correct component material number is used for the KAPA EvoPlus V2 workflow.

Shipping, Storage and Stability

- KAPA EvoPlus V2 Kits are shipped on dry ice or ice packs, depending on the destination country.
- The ReadyMixes provided in this kit are temperature sensitive, and should be stored at -15°C to -25°C in a constant-temperature freezer upon receipt.
- When stored under these conditions and handled correctly, the kit components will retain full activity until the expiry date indicated on the kit label.

Applications

KAPA EvoPlus V2 Kits are ideally suited for low- and high-throughput Next-Generation Sequencing (NGS) library construction workflows that require DNA fragmentation, A-tailing, adapter ligation and library amplification (optional). Kits are designed for library construction from a wide range of sample types and inputs (0.1 ng – 500 ng), and are compatible with genomic DNA and low quality DNA such as that extracted from formalin-fixed, paraffin-embedded tissue (FFPET) samples.

This kit is ideally suited for germline as well as somatic mutation detection. It is automation-friendly and may be used for the following workflow applications:

- Whole-genome sequencing (WGS).
- Whole exome (WES) or targeted sequencing, using hybridization capture methods.

⚠ **The KAPA EvoPlus V2 Kits contain an enzymatic fragmentation module that is NOT validated for use in Methyl-Seq applications. The enzymatic fragmentation chemistry involves DNA repair mechanisms that may affect methylation patterns.**

Warnings and Precautions

- Wear the appropriate personal protective equipment, such as gloves and safety glasses, to avoid direct contact while handling the reagents. Wash hands thoroughly after handling samples and reagents.
- Use good laboratory practices to avoid contamination when working with the reagents.
- Do not eat, drink, or smoke in the laboratory area.
- Do not pipette by mouth.
- In the event of a spill, clean up the solution with absorbent pads, allow pads to dry, and dispose of pads.
- Observe all national, regional, and local regulations for waste disposal and management.
- Safety Data Sheets (SDS) are available ***online*** or upon request from the local Roche office.

Ordering Information

For a complete overview of Roche Sequencing products, including KAPA EvoPlus V2 Kits, go to ***sequencing.roche.com/products***.

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Editions

Version 1.0, November 2021; Version 2.0, August 2022; Version 4.1, April 2024

Changes to Previous Versions

Addition of KAPA EvoT4 trademark & typographical updates

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Regulatory Disclaimer

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Not for use in diagnostic procedures.

Contact Information

Technical Support

If you have questions, contact your local Roche Technical Support.

Go to *sequencing.roche.com/support* for contact information.

Manufacturing & Distribution

Manufacturer	Roche Diagnostics Cape Town, South Africa
Distribution	Roche Diagnostics GmbH Mannheim, Germany
Distribution in USA	Roche Diagnostics Corporation Indianapolis, IN USA

Conventions Used in This Manual

Symbols

Symbols	Description
	**Important Note:** Information critical to the success of the procedure or use of the product. Failure to follow these instructions could result in compromised data.
	**Information Note:** Designates a note that provides additional information concerning the current topic or procedure.

Text

Conventions	Description
Numbered listing	Indicates steps in a procedure that must be performed in the order listed.
Italic type, blue	Highlights a resource in a different area of this manual or on a web site.
Italic type	Identifies the external general resources or names.
Bold type	Identifies names of paragraphs, sections or emphasized words.

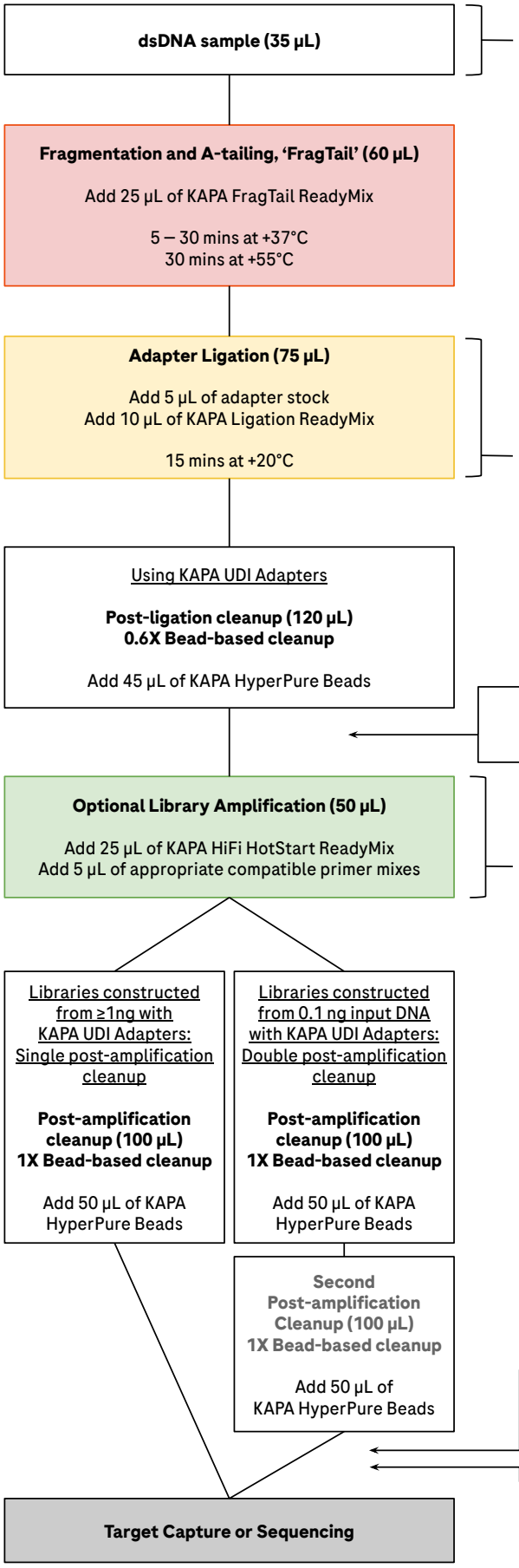
Chapter 1. Before You Begin

These Instructions for Use describe the process for a streamlined DNA fragmentation and library construction protocol for the rapid preparation of libraries for Illumina sequencing. Specifically, these Instructions for Use provide a protocol for the workflow outlined in ***Figure 1*** using the KAPA EvoPlus V2 Kit.

The KAPA EvoPlus V2 Workflow provides:

- Improvements over the KAPA HyperPlus Kit, due to the optimization of reagents and workflow:
  - Ready-to-use and automation-friendly ReadyMixes in tubes, bottles and plated format
  - Enzymatic fragmentation that is insensitive to Ethylenediaminetetraacetic acid (EDTA)
  - Reagents with improved shelf-life and stability
  - Optimized formulations to reduce the occurrence of sequencing artefacts
- An easy to use, streamlined, and automation-friendly workflow with minimal resource requirements.
- Single vendor service when using the following accessory reagents:
  - KAPA HyperPure Beads
  - KAPA Unique Dual-Indexed (UDI) Adapters & KAPA Library Amplification Primer Mix or
  - KAPA Universal Adapter & KAPA UDI Primer Mixes 1 – 384

Figure 1a - KAPA EvoPlus V2 Quick Guide: Library Preparation with KAPA UDI Adapters



Application	Sample Type	Input
WGS	High quality gDNA	0.1 ng – 500 ng
	Low quality FFPET-derived DNA	≥50 ng*
WGS (PCR-free)	High quality gDNA	≥50 ng (no SS)** 500 ng (with SS)**
Targeted Sequencing	High quality gDNA	100 ng
	Low quality FFPET-derived DNA	10 ng – 50 ng

* Reach out to Technical Support for possible workflow modifications when using this sample type.
** SS = double-sided size selection; a requirement when performing WGS on patterned flow cells but may result in sample losses of 60 – 95%, irrespective of whether a bead- or gel-based technique is used. For PCR-free workflows, due to the inherent sample losses, performing double-sided size selection with inputs <500 ng (into library prep) is not recommended.

Input DNA	Adapter stock concentration if using KAPA UDI Adapter
0.1 ng to 1 ng	0.6 µM
10 ng	6 µM
>10 ng to 500 ng	15 µM

Optional double-sided size selection using KAPA HyperPure Beads may be be incorporated at this point. If applicable, resuspend in 55 µL elution buffer and proceed with *Appendix A - Double-sided size selection*

Post-ligation quantification using KAPA Library Quantification Kit (recommended; dilution range between 1/100 to 1/10,000) or Qubit Fluorometer

Input DNA	No. of amplification cycles for WGS to achieve 4 nM* if using KAPA UDI Adapter
0.1 ng	11 – 13
1 ng	7 – 9
10 ng	3 – 5
50 ng to 500 ng	0 (PCR-free)

*The number of cycles needed may change depending on the specific adapter and amplification primer design, as well as input type and quality.

Optional double-sided size selection using KAPA HyperPure Beads may be incorporated at this point. If applicable, resuspend in 55 µL elution buffer and proceed with *Appendix A - Double-sided size selection*

Sample QC:
1. Electrophoretic profile of amplified libraries &
2. Post-amplification quantification using KAPA Library Quantification Kit (recommended; dilution range between 1/10,000 to 1/200,000) or Qubit Fluorometer

Figure 1b - KAPA EvoPlus V2 Quick Guide: Library Preparation with KAPA Universal Adapter

dsDNA sample (35 µL)

Application	Sample Type	Input
WGS	High quality gDNA	0.1 ng – 500 ng
	Low quality FFPET-derived DNA	≥50 ng*
WGS (PCR-free)	High quality gDNA	≥50 ng (no SS)** 500 ng (with SS)**
Targeted Sequencing	High quality gDNA	100 ng
	Low quality FFPET-derived DNA	10 ng – 50 ng

* Reach out to Technical Support for possible workflow modifications when using this sample type.
** SS = double-sided size selection; a requirement when performing WGS on patterned flow cells but may result in sample losses of 60 – 95%, irrespective of whether a bead- or gel-based technique is used. For PCR-free workflows, due to the inherent sample losses, performing double-sided size selection with inputs <500 ng (into library prep) is not recommended.

Fragmentation and A-tailing, 'FragTail' (60 µL)

Add 25 µL of KAPA FragTail ReadyMix

5 – 30 mins at +37 °C
30 mins at +55 °C

Adapter Ligation (75 µL)

Add 5 µL of adapter stock
Add 10 µL of KAPA Ligation ReadyMix

15 mins at +20 °C

Input DNA	Adapter stock concentration if using KAPA Universal Adapter and KAPA UDI Primer Mixes
0.1 ng to 1 ng	0.6 µM
10 ng to 500 ng	15 µM

Using KAPA Universal Adapter & KAPA UDI Primer Mixes

Post-ligation cleanup (135 µL)
0.8X Bead-based cleanup

Add 60 µL of KAPA HyperPure Beads

Optional double-sided size selection using KAPA HyperPure Beads may be be incorporated at this point. If applicable, resuspend in 55 µL elution buffer and proceed with *Appendix A - Double-sided size selection*

Post-ligation quantification using KAPA Library Quantification Kit (recommended; dilution range between 1/100 to 1/10,000) or Qubit Fluorometer

Optional Library Amplification (50 µL)

Add 25 µL of KAPA HiFi HotStart ReadyMix
Add 5 µL of appropriate compatible primer mixes

Input DNA	No. of amplification cycles for WGS to achieve 4 nM* if using KAPA Universal Adapter and KAPA UDI Primer Mixes
0.1 ng	12 - 13
1 ng	8 - 9
10 ng	4 - 5
50 ng to 500 ng	3 - 4

*The number of cycles needed may change depending on the specific adapter and amplification primer design, as well as input type and quality.

Libraries constructed from 0.1 ng – 500 ng input DNA with KAPA Universal Adapter & KAPA UDI Primer Mixes: Double post-amplification cleanup

Post-amplification cleanup (100 µL)
1X Bead-based cleanup

Add 50 µL of KAPA HyperPure Beads

Second Post-amplification Cleanup (100 µL)
1X Bead-based cleanup

Add 50 µL of KAPA HyperPure Beads

Optional double-sided size selection using KAPA HyperPure Beads may be incorporated at this point. If applicable, resuspend in 55 µL elution buffer and proceed with *Appendix A - Double-sided size selection*

Sample QC:
1. Electrophoretic profile of amplified libraries &
2. Post-amplification quantification using KAPA Library Quantification Kit (recommended; dilution range between 1/10,000 to 1/200,000) or Qubit Fluorometer

Target Capture or Sequencing

The FragTail ReadyMix contains a high concentration of PEG 6000 and glycerol, and is very viscous so ensure this solution is sufficiently mixed. Mixing should be performed by vortexing for at least 10 seconds.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15°C to +25°C).
- Unless otherwise specified, all mixing steps are listed as 'mix thoroughly' and indicate that mixing should be performed by vortexing for at least 10 seconds.
- If liquid has collected in a tube's cap after mixing, gently tap or briefly centrifuge the sample to collect the liquid into the tube's bottom, ensuring that the mixture remains homogeneous before progressing to the next step.

References

- Thermocycler Manual
- Qubit Fluorometer Manual
- Qubit dsDNA HS Assay Kit Guide

Terminology

Sample Library: The initial shotgun library generated from DNA by fragmentation and ligation.

Amplified Sample Library: The sample library after amplification by PCR.

KAPA UDI Adapter: KAPA Unique Dual-Indexed Adapter.

KAPA UDI Primer Mixes: KAPA Unique Dual-Indexed Primer Mixes.

KAPA Universal Adapter: Truncated adapter containing a subset of sequencing motifs. Used in conjunction with KAPA UDI Primer Mixes.

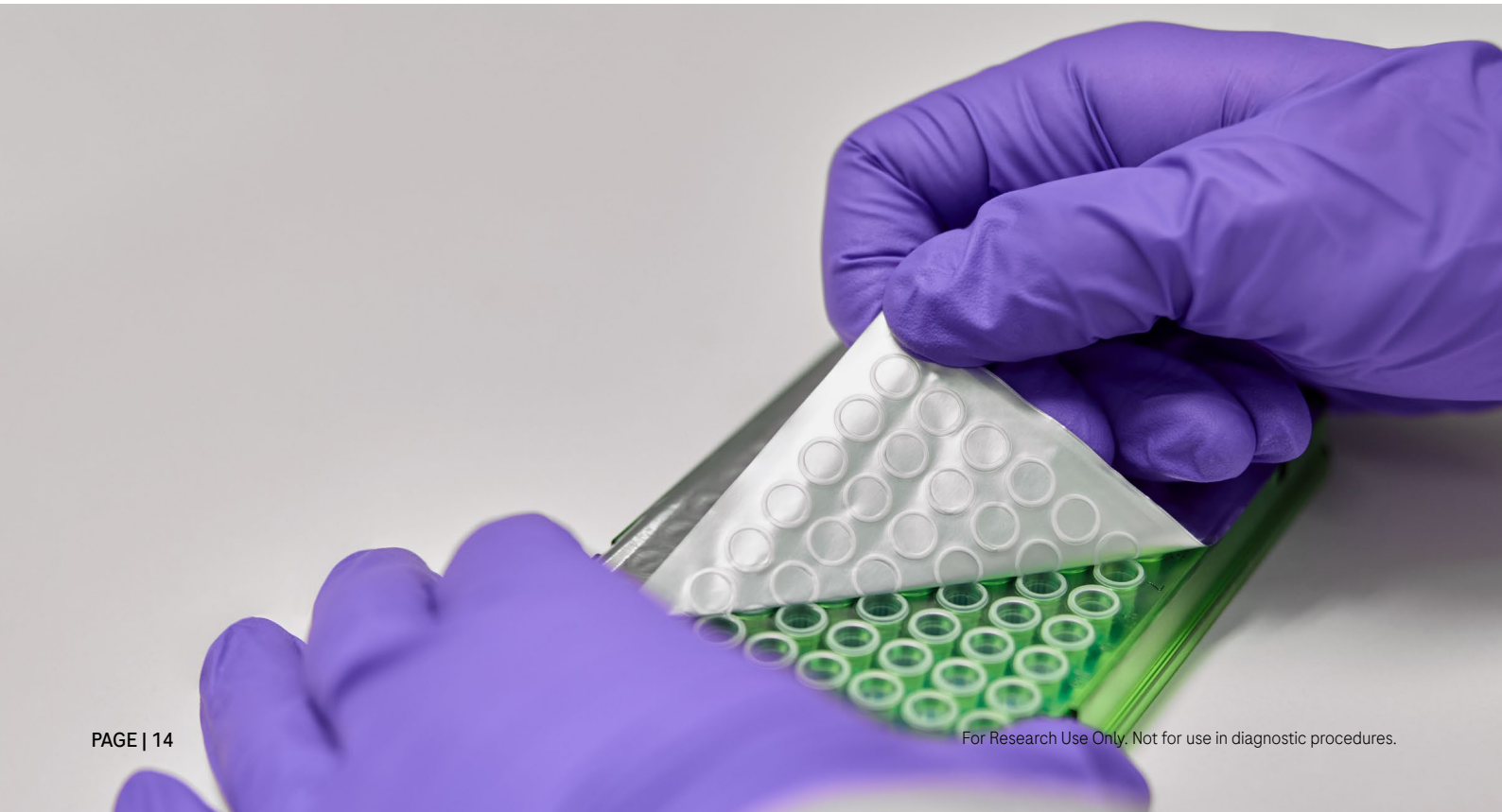
Prepare the Following Equipment

- Thermocyclers should be programmed with the following:
  - Fragmentation & A-tailing program (*Chapter 3, Prepare the Sample Library, Step 1*)
  - Adapter Ligation program (*Chapter 3, Prepare the Sample Library, Step 2*)
  - Library Amplification program (*Chapter 4, Amplify the Sample Library, Step 1*)

It is recommended to use a thermocycler with a programmable heated lid. If not possible, please use the default settings.

- The following steps should be taken before beginning the workflow:

Verify you are using the most up-to-date version of these Instructions for Use, go to *sequencing.roche.com/support*.



Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. This protocol is designed for use with the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier
Tube Magnetic Rack	Multiple Vendors
Microcentrifuge (16,000 x *g* capability)	Multiple Vendors
Qubit Fluorometer	ThermoFisher
Electrophoretic device & associated assays and reagents	Multiple Vendors
Thermocycler	Multiple Vendors
Vortex mixer	Multiple Vendors
Plate Centrifuge (minimum 280 *x g* capability)	Multiple Vendors

Consumables Available from Roche

Description	Package Size	Material Number
KAPA Library Quantification Kit for Illumina platforms	Various options	Various material numbers
KAPA HyperPure Beads	5 mL	08963835001
	30 mL	08963843001
	60 mL	08963851001
	4 x 60 mL	08963878001
	450 mL	08963860001
KAPA Unique Dual-Indexed Adapter Kit	96 x 20 µL	08861919702
KAPA Library Amplification Primer Mix	250 reactions (1.25 mL)	07958994001
KAPA Library Amplification Primer Mix	384 reactions (1.92 mL)	09420410001
KAPA Library Amplification Primer Mix, 96-well plate	96 x 5 µL	09420479001
KAPA Universal Adapter	96 reactions	09063781001
	384 reactions*	09063790001
KAPA UDI Primer Mixes 1 – 96	96 reactions	09134336001
KAPA UDI Primer Mixes 97 – 192	96 reactions	09329838001
KAPA UDI Primer Mixes 193 – 288	96 reactions	09329846001
KAPA UDI Primer Mixes 289 – 384	96 reactions	09329854001

* Virtual kit - consist of 4 x 96 reaction kits

Consumables Purchased from Other Vendors

Component	Supplier
10 mM Tris-HCl, pH 8.0 – 8.5	Multiple Vendors
Ethanol, 200 proof (absolute), for molecular biology	Multiple Vendors
Qubit dsDNA HS Assay Kit	ThermoFisher
Qubit Assay Tubes	ThermoFisher
Low binding Tubes: ▪ 0.2 mL PCR tubes ▪ 1.5 mL microcentrifuge tubes (optional)	Multiple Vendors
Nuclease-free, PCR-grade water	Multiple Vendors

Chapter 2.

Store and Prepare the Reagents

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA EvoPlus V2 Kit	-15°C to -25°C
KAPA HyperPure Beads*	+2°C to +8°C
KAPA UDI Adapter Kit	-15°C to -25°C
KAPA Universal Adapter	-15°C to -25°C
KAPA UDI Primer Mixes or KAPA UDI Primer Mixes (resuspended)	+2°C to +8°C or -15°C to -25°C
KAPA Library Amplification Primer Mix	-15°C to -25°C

*The KAPA HyperPure Beads kit must not be frozen.

Step 2. Prepare KAPA Adapters and KAPA UDI Primer Mixes

For multiplexing guidelines, please refer to either the *KAPA UDI Adapter Technical Data Sheet* or *KAPA UDI Primer Mixes Instructions for Use (*available ***online*** on eLabDoc*)* as applicable.

Step 2a. Dilute the KAPA Unique Dual-Indexed Adapters (if required)

1. Retrieve the KAPA UDI Adapter plate from storage (-15°C to -25°C) and thaw at room temperature.
2. Centrifuge the KAPA UDI Adapter plate at room temperature (280 x *g* for at least 1 min) to ensure the liquid is collected at the bottom of the wells.

   Do not vortex the adapter plate as it could result in cross-contamination of the KAPA UDI Adapters. Pipette-mix individual adapters prior to use.
3. Before removing the foil cover, please ensure the plate is in the correct orientation. Please refer to the *KAPA UDI Adapter Kit Technical Data Sheet* (Document number KR1736, available ***online*** on eLabdoc) for additional handling instructions.
4. Upon first use, carefully remove the foil cover of the plate to avoid cross contamination.

   Discard the original foil cover. Do not reuse.

   Maintain good lab practices when removing the foil seal. E.g, refrain from contact with the underside of the seal. If contact is made, change gloves before handling the adapter or sample plates.

   A new pipette tip must be used for each well to avoid cross contamination. If you are not using the entire contents of the KAPA UDI Adapter plate at this time, apply a new adhesive foil seal provided in the kit. Make sure that the foil is properly aligned and fully covers all 96 wells. Use a roller or other appropriate tool to ensure that the foil is evenly applied.
5. If applicable, dilute adapters to the required concentration, see table below, using the KAPA Adapter Dilution Buffer.

Step 2b. Dilute the KAPA Universal Adapters (if required)

1. Retrieve the KAPA Universal Adapters tube from storage (-15°C to -25°C) and thaw at room temperature or on ice.
2. Mix thoroughly before first use.
3. Centrifuge the KAPA Universal Adapter at room temperature (280 x *g* for at least 1 min) to ensure the liquid is collected at the bottom of the tube.
4. If applicable, dilute KAPA Universal Adapters to the required concentration, see table below, using 10 mM Tris-HCl pH 8.0 – 8.5.

Table 1. Recommended KAPA Adapters concentration

Input DNA (high quality and/or low quality)	KAPA UDI Adapters		KAPA Universal Adapter	
	Adapter stock concentration*	Adapter:insert molar ratio**	Adapter stock concentration*	Adapter:insert molar ratio**
500 ng	15 µM	20:1	15 µM	20:1
100 ng	15 µM	100:1	15 µM	100:1
50 ng	15 µM	200:1	15 µM	200:1
10 ng	6 µM	400:1	15 µM	1000:1
1 ng	0.6 µM	400:1	0.6 µM	400:1
0.1 ng	0.6 µM	4000:1	0.6 µM	4000:1

*Both KAPA UDI Adapters and KAPA Universal Adapter are provided at 15 µM.
**Adapter:insert molar ratio calculations are based on a mode DNA fragment length of 200 bp, and will be higher for longer DNA fragments, or slightly lower for DNA fragmented to a mode size <200 bp.

Step 2c. Preparation of the KAPA UDI Primer Mixes

Before use of the KAPA UDI Primer Mixes, undertake the following steps to resuspend the primers:

1. Retrieve the KAPA UDI Primer Mixes plate from storage (+2°C to +8°C).
2. Centrifuge the KAPA UDI Primer Mixes plate at room temperature (280 x *g* for at least 1 min) to ensure the contents are at the bottom of the wells.
3. Before removing the foil cover, ensure the plate is in the correct orientation before proceeding. In order to have well position A1 on the top left corner, the notched corner must be facing the user on the bottom left, as shown in *Figure 2*.
4. Carefully remove the foil cover on the plate ensuring to avoid cross contamination.

   ⚠ Discard the original foil cover.
5. Using a multichannel pipette, add 10 µL of Nuclease-free, PCR-grade water directly to the bottom of each well and discard tips after dispensing.

   ⚠ A new pipette tip must be used for each well to avoid cross contamination. Be sure to dispense water slowly to the bottom of each well to avoid liquid splash over to adjacent wells.
6. Ensure every well contains 10 µL of Nuclease-free, PCR-grade water and cover the plate with one of the adhesive foil seals provided in the kit.

⚠ Make sure the foil seal is properly aligned and fully covers all 96 wells. Failure to do so can lead to cross contamination of the KAPA UDI Primer Mixes.

7. Use a roller or appropriate tool to ensure the foil seal is evenly applied.
8. Centrifuge the plate at room temperature (280 x *g* for at least 30 seconds) to ensure the dispensed 10 µL are at the bottom of the wells.
9. Thoroughly vortex the plate ensuring all wells are mixed well.

⚠ Make sure wells at the corners of the plate are mixed well by vortexing the corners of the plate. Keep the plate upright.

10. Centrifuge the plate at room temperature (280 x *g* for at least 1 min) to ensure the contents are collected at the bottom of the wells.
11. The KAPA UDI Primer Mixes plate is now ready for use in the ***Library Amplification*** step.
12. Store any unused but already resuspended KAPA UDI Primer Mixes at -15°C to -25°C. To avoid repeated freeze/thaw cycles you may transfer the resuspended primers to separate tubes or tube strips for storage.

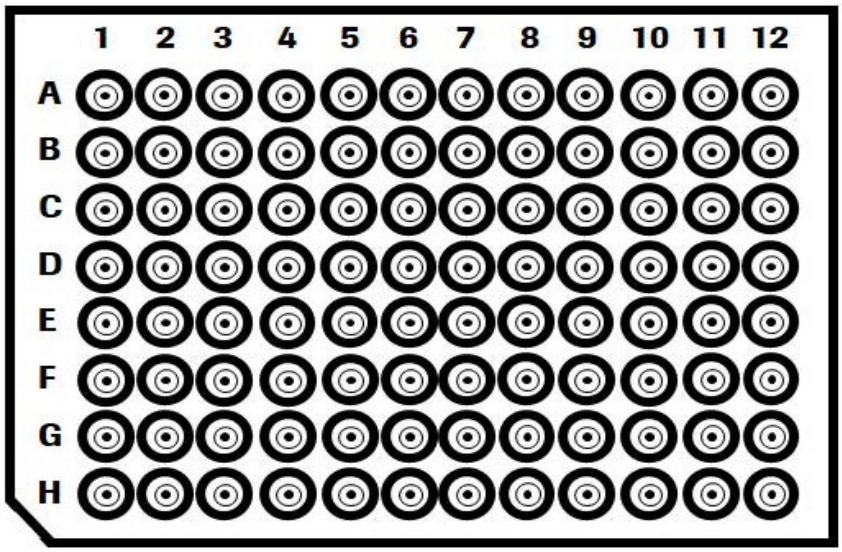


Figure 2: KAPA UDI Primer Mixes plate layout.

Chapter 3.

Prepare the Sample Library

In this chapter the KAPA EvoPlus V2 Kit is used to perform enzymatic fragmentation and indexed libraries are prepared. The workflow requires the use of components from the following kits:

- KAPA EvoPlus V2 Kit
- KAPA Unique Dual-Indexed Adapter Kit & KAPA Library Amplification Primers OR*
- KAPA Universal Adapter** & KAPA UDI Primer Mixes
- KAPA HyperPure Beads***

Ensure that the following are available:

- Nuclease-free, PCR-grade water
- Freshly-prepared 80% ethanol
- 10 mM Tris-HCl, pH 8.0 – 8.5

*KAPA Unique Dual-Indexed (UDI) Adapters are recommended for use with the KAPA EvoPlus V2 Kit. However, the kit is also compatible with KAPA Universal Adapter & KAPA UDI Primer Mixes as well as other full-length or truncated adapter designs from 3rd party suppliers.

The KAPA Universal UMI Adapter (Catalog # 09329862001, and provided at 33 µM), may also be considered for this workflow. The KAPA Universal UMI Adapter with Unique Molecular Identifiers (UMI) allows for molecular barcoding within each sample, enabling proper molecule counting for low-frequency variant detection. However, the KAPA Universal UMI Adapter has only been validated within specific KAPA Target Enrichment workflows and may not confer the same computational benefits for non-target enrichment workflows (such as whole-genome sequencing). Contact your local Roche Technical Support at ***sequencing.roche.com/support* for guidance and important considerations.

***KAPA HyperPure Beads are recommended for use with the KAPA EvoPlus V2 Kit. However, the kit is also compatible with KAPA Pure Beads. Conditions for DNA binding and size selection may differ if other cleanup beads are used.

Sample Requirements

This workflow was validated with 0.1 ng – 500 ng of high quality gDNA, and is compatible with ≥50 ng low quality DNA extracted from FFPET for sample library preparation. The DNA should be quantified using the Qubit dsDNA HS Assay Kit. Lower input amounts and sample quality may not yield equivalent results. For additional guidance on lower input amounts or sample quality, please contact ***sequencing.roche.com/support***.

Table 2. Recommended inputs into library construction

Application	Sample type	Input DNA
WGS	High quality gDNA	0.1 ng – 500 ng
	Low quality FFPET-derived DNA	≥ 50 ng*
WGS (PCR-free)	High quality gDNA	≥50 ng (no SS)** 500 ng (with SS)**
Targeted Sequencing	High quality gDNA	100 ng
	Low quality FFPET-derived DNA	≥10 ng - 50 ng

*Reach out to ***Technical Support*** for possible workflow modifications when using this sample type.
**SS = double-sided size selection; a requirement when performing WGS on patterned flow cells but may result in sample losses of 60 – 95%, irrespective of whether a bead- or gel-based technique is used. For PCR-free workflows, due to the inherent sample losses, performing double-sided size selection with inputs <500 ng is not recommended.

It is important to the success of the library preparation workflow to ensure that KAPA EvoPlus V2 Kit components have been fully thawed and thoroughly mixed before use. Specifically, the FragTail ReadyMix and Ligation ReadyMix contain high concentrations of PEG 6000 and/or glycerol, and are viscous so ensure these solutions are sufficiently mixed and take care when pipetting. Pipette-mix at least 10X or vortex mix for 10 –20 seconds. In some cases there may be small PEG 6000 droplets visible in the FragTail ReadyMix. Ensure the ReadyMix is thoroughly vortexed until the droplets have been resuspended.

Keep all ReadyMixes on ice as long as possible during handling and preparation.

If using the plated format, remove plate from its packaging sleeve and thaw at room temperature or in a suitable cooled reagent block. Once completely thawed, vortex well and centrifuge the plates at room temperature (e.g, for 1 minute at 280 x *g*) to ensure that all liquid is collected in the bottom of wells before the seal is pierced or removed.

Each well of the plate contains sufficient ReadyMix and overage for one (1) reaction.

If you are only using a subset of the 96 reaction plate, pierce the foil of all required wells. Apply part (cut to size) of a new adhesive foil seal (provided in the kit) over the pierced wells after use. Make sure that the foil is properly aligned and fully covers all wells pierced. Use a roller or other appropriate tool to ensure that the foil is evenly applied.

The fragmentation parameters in this Instructions for Use are provided as a starting point and may require optimization for your specific sample type.

Ensure KAPA HyperPure Beads are removed from storage at least 30 minutes prior to starting the workflow and fully equilibrated to room temperature. For best performance, store the beads protected from light when not in use.

Step 1. Fragmentation and A-tailing

1. Dilute 0.1 ng – 500 ng of DNA with 10 mM Tris-HCl, pH 8.0 – 8.5 (recommended) to a total volume of 35 µL in a 0.2 mL tube or well of a PCR plate.
2. Vortex the FragTail ReadyMix well and centrifuge briefly.
3. Assemble each Fragmentation and A-tailing reaction on ice as per the table below:

Component	Volume Per Individual Sample
0.1 ng – 500 ng DNA	35 µL
FragTail ReadyMix	25 µL
Total volume	**60 µL**

4. Mix the Fragmentation and A-tailing reaction thoroughly and centrifuge briefly. Return the plate/tube(s) to ice and proceed immediately to the next step.

   If the Fragmentation and A-tailing reaction is not mixed properly, it can result in increased fragment size.

5. Incubate in a thermocycler, pre-cooled to +4°C and programmed as outlined below. Set the lid temperature to ~ +65°C (if possible):

   a. Pre-cool block: +4°C

   b. Fragmentation: +37°C - See table below

   c. A-tailing: +55°C for 30 minutes

   d. Hold: +4°C

Estimated Insert Size*	Incubation time at +37°C
180 bp	25 - 30 mins
200 bp	20 - 25 mins
250 bp	15 - 20 mins
300 bp	10 - 15 mins
450 bp	5 - 7 mins
500 bp	3 - 5 mins

* Insert sizes (without adapter) generated from fragmentation of 100 ng high quality human DNA (NA12878, Coriell Institute of Biomedical Research). Size variation may be observed, depending on DNA type, DNA input and DNA elution buffer. We recommend optimizing the fragmentation time with a non-precious sample.

⚠ This is not a validated safe stopping point. Proceed directly to ***Step 2: Adapter Ligation***.

Step 2. Adapter Ligation

KAPA UDI Adapter and KAPA Universal Adapter, unless specified, will hereafter be referred to as KAPA Adapters.

⚠ The KAPA Ligation ReadyMix contains a high concentration of PEG 6000 and is very viscous. Ensure the buffer is thoroughly vortexed before use.

⚠ KAPA Adapters must be added to each tube/well individually prior to addition of the Ligation ReadyMix.

⚠ If using KAPA Universal Adapter, sample indexes are incorporated during the Library Amplification step. Precautions should be taken to avoid sample cross contamination.

1. Transfer the reaction from the thermocycler to ice.
2. Vortex the Ligation ReadyMix well and centrifuge briefly.
3. In the same plate/tube(s) in which fragmentation and A-tailing was performed, assemble each Adapter Ligation reaction on ice as per the table below:

Component	Volume Per Individual Sample
FragTail product	60 µL
KAPA Adapters (*Chapter 2*)	5 µL
Ligation ReadyMix	10 µL
Total volume	**75 µL**

4. Mix the Adapter Ligation reaction thoroughly and centrifuge.
5. Incubate the Adapter Ligation reaction at +20°C on a thermocycler for 15 minutes.
6. Following the incubation, proceed immediately to the next step.

Step 3. Purify the Sample Library using KAPA HyperPure Beads

1. To each Adapter Ligation reaction, add the appropriate volume of room temperature KAPA HyperPure Beads that have been thoroughly resuspended.

Component	Volume per individual sample when using KAPA UDI Adapter	Volume per individual sample when using KAPA Universal Adapter
Ligation reaction product	75 µL	75 µL
KAPA HyperPure Beads	45 µL (0.6X)	60 µL (0.8X)
Total volume	**120 µL**	**135 µL**

2. Once added, mix thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.

   It is important to ensure that the solution is thoroughly mixed and appears homogeneous to collect all droplets.

   Insufficient mixing may compromise recovery and result in size selection.

3. Incubate the sample at room temperature for 5 minutes to allow the sample library to bind to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
7. Incubate the sample at room temperature for ≥30 seconds.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
10. Incubate the sample at room temperature for ≥30 seconds.
11. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
12. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads:
    14.1. in 25 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with Library Amplification *(Chapter 4)*, or
    14.2. in 55 µL of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) to proceed with Double-sided Size Selection *(Appendix A)*.
15. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.
16. Place the sample on the magnet to collect the beads. Incubate until the liquid is clear.
17. Transfer an appropriate volume of the clear supernatant/eluate to a fresh tube/well:
    17.1. to proceed with Library Amplification *(Chapter 4)*, transfer 20 µL of supernatant, or
    17.2. to proceed with Double-sided Size Selection *(Appendix A)*, transfer 50 µL of supernatant.

    The remaining 5 µL can be used for quality control purposes e.g., quantification using the KAPA Library Quantification Kit.

18. Proceed to ***Chapter 4. Amplify the Sample Library*** (optional for sample inputs of ≥50 ng but mandatory if using KAPA Universal Adapter) or ***Chapter 5. Quality Control***, if performing a PCR-free workflow (not applicable if using KAPA Universal Adapter).

Safe stopping point – If necessary, this is a safe stopping point. Purified, adapter-ligated library may be stored at +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for ≤1 month before amplification and/or sequencing. To avoid degradation, always store sample library in a buffered solution (10 mM Tris-HCl, pH 8.0 – 8.5) when possible, and minimize the number of freeze-thaw cycles.

Chapter 4.

Amplify the Sample Library

This chapter describes how to amplify the adapter-ligated library, if necessary, using KAPA HiFi HotStart ReadyMix and appropriate compatible indexes or primer sets.

If KAPA UDI Adapters were used for adapter ligation, ensure KAPA Library Amplification Primer Mix is used for library amplification.

If KAPA Universal Adapters were used for adapter ligation, amplification is mandatory. Ensure a unique KAPA UDI Primer Mix is added to each sample library.

Ensure that the following is available:

- Freshly-prepared 80% ethanol
- 10 mM Tris-HCl, pH 8.0 – 8.5
- Nuclease-free, PCR-grade water

Step 1. Prepare the Library Amplification Reaction

Ensure the KAPA HyperPure Beads are removed from storage at least 30 minutes prior to starting the workflow to ensure they are equilibrated to room temperature. For best performance, store the beads protected from light when not in use.

KAPA HiFi HotStart ReadyMix (2X) contains isostabilizers and may not freeze completely, even when stored at -15°C to -25°C. Nevertheless, always ensure that the ReadyMix is **fully thawed** and **thoroughly mixed** before use.

If using the plated format of KAPA HiFi HotStart ReadyMix, remove the plate from its packaging sleeve and thaw at room temperature (<1 hr) or in a suitable cooled reagent block (>1 hr). Once completely thawed, vortex well and centrifuge the plate at room temperature (e.g., for 30 seconds at 280 x *g*) to ensure that all liquid is collected in the bottom of wells before the seal is pierced or removed. Place on ice until use.

If applicable, retrieve and thaw the KAPA UDI Primer Mixes plate prepared in ***Chapter 2, Step 2c.*** Centrifuge the plate at 280 x *g* for 30 seconds to collect the contents to the bottom of the wells and peel off or pierce the foil seal for the appropriate number of wells needed. If only using a subset of the KAPA UDI Primer Mixes from the original plate, remove and discard residual primers from the well and apply a new adhesive foil seal provided in the kit.

If piercing the foil seal, avoid cross contamination by using a new pipette tip for every well.

Proper re-sealing and storage of the KAPA UDI Primer Mixes plate is necessary for unused primer mixes utilization at a later date.

Keep the KAPA HiFi HotStart ReadyMix on ice as long as possible during handling.

1. Assemble each Library Amplification reaction as per table below:

Component	Volume per Individual Sample
KAPA HiFi HotStart ReadyMix (2X)	25 µL
KAPA Library Amplification Primer Mix* OR KAPA UDI Primer Mix**	5 µL
Adapter-ligated library	20 µL
Total volume	**50 µL**

* If KAPA UDI Adapters were used for Adapter Ligation, ensure KAPA Library Amplification Primer Mix is used for library amplification. The KAPA HiFi HotStart ReadyMix and KAPA Library Amplification Primer Mix should preferably be premixed and added in a single pipetting step.

**If KAPA Universal Adapters were used for Adapter Ligation ensure a unique KAPA UDI Primer Mix is added to each sample library.

2. Mix thoroughly and centrifuge briefly. Immediately proceed to the next step.

Step 2. Perform the Library Amplification

1. Place the sample in the thermocycler and amplify the adapter-ligated sample library using the following Library Amplification program with the lid temperature set to +105°C:

Step	Temperature	Duration	Cycles
Initial denaturation	+98°C	45 sec	1
Denaturation	+98°C	15 sec	Variable, see Table below
Annealing	+60°C	30 sec	
Extension	+72°C	30 sec	
Final extension	+72°C	1 min	1
Hold	+4°C	∞	1

Table 2: Recommended number of amplification cycles per DNA input for KAPA EvoPlus V2 Kit

Input DNA	Number of amplification cycles* for WGS to achieve 4 nM** if using KAPA UDI Adapters	Number of amplification cycles* for WGS to achieve 4 nM** if using KAPA Universal Adapter & KAPA UDI Primer Mixes
50 ng – 500 ng	0 (PCR-free workflow)	3 – 4
10 ng	3 - 5	4 - 5
1 ng	7 - 9	8 - 9
0.1 ng	11 - 13	12 - 13

*The number of cycles needed depends on the specific adapter and amplification primer design, as well as input type, quality and whether double-sided size selection is performed. When using incomplete, or truncated, adapters in conjunction with indexed amplification primers (such as KAPA Universal Adapter & KAPA UDI Primer Mixes), a minimum number of amplification cycles (3) are required to complete adapter sequences for the next step in the process (target capture or sequencing), irrespective if a sufficient amount of library is available after ligation. The number of cycles needed depends on the specific adapter, downstream application and amplification primer design. Certain sample types, such as FFPET-derived DNA may require additional cycles of amplification to reach 4 nM threshold. This may also depend on the quality (low vs high) of the FFPET-derived DNA.

**Based on sequencing recommendations, 4 nM is the minimum starting concentration to proceed with sequencing. For input amounts ≥50 ng, PCR amplification should not be required to achieve the ~4 nM requirement for sequencing (unless libraries were constructed using KAPA Universal Adapter). Users requiring concentrations >4 nM can adjust the number of amplification cycles in 2 cycle increments until the target concentration is achieved. This may require optimization. Note: increasing cycle numbers ultimately decreases the library complexity by increasing the duplication rate.

2. Proceed immediately to the next step.

Step 3. Purify the Amplified Sample Library using KAPA HyperPure Beads

If libraries were constructed from >0.1 ng of input DNA using KAPA UDI Adapters – proceed to Step 3a or,

If libraries were constructed from 0.1 ng of input DNA using KAPA UDI Adapters – proceed to Step 3b or,

If libraries were constructed from 0.1 ng - 500 ng using KAPA Universal Adapter and KAPA UDI Primer Mixes – proceed to Step 3b.

Step 3a. Purify the Amplified Sample Library constructed using >0.1 ng of input DNA with KAPA UDI Adapters & KAPA Library Amplification Primer Mix

1. Add 50 µL (1X) of room temperature, thoroughly resuspended, KAPA HyperPure Beads to each amplified sample library.
2. Mix the amplified sample library and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
3. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
7. Incubate the sample at room temperature for ≥30 seconds.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
10. Incubate the sample at room temperature for ≥30 seconds.
11. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
12. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 25 µL (or appropriate volume) of 10 mM Tris-HCl, pH 8.0 – 8.5. Centrifuge briefly to collect all droplets.

    Do NOT allow beads to pellet.

    If proceeding with double-sided size selection, resuspend the beads in 55 µL of elution buffer.

15. Incubate the sample at room temperature for 2 minutes to allow the amplified sample library to elute off the beads.
16. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer an appropriate volume of the clear supernatant/eluate to a fresh tube(s)/well and proceed with double-sided size selection (refer to ***Appendix A***), library QC, target capture or sequencing.
18. Purified, amplified sample libraries can be stored at +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for up to 3 months.

Step 3b. Purify the Amplified Sample Library constructed using 0.1 ng of input DNA with KAPA UDI Adapters OR libraries constructed from 0.1 ng – 500 ng with KAPA Universal Adapters and KAPA UDI Primer Mixes

A second post-amplification cleanup is highly recommended when using KAPA Universal Adapter & KAPA UDI Primer Mixes with KAPA library preparation kits. A second post-amplification cleanup is also recommended when using KAPA UDI Adapter and 0.1 ng DNA input amounts within the KAPA EvoPrep Workflow. Especially if WGS libraries will be sequenced on an Illumina NovaSeq or HiSeq X system (with patterned flow cells). This additional cleanup will serve to remove any indexed primer carryover which may exacerbate index hopping.

1. Add 50 µL (1X) of room temperature, thoroughly resuspended, KAPA HyperPure Beads to each amplified sample library.
2. Mix the amplified sample library and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets.
3. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
4. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
7. Incubate the sample for ≥30 seconds.
8. Carefully remove and discard the ethanol.
9. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
10. Incubate the sample for ≥30 seconds.
11. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
12. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

    Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 50 µL of 10 mM Tris-HCl, pH 8.0 – 8.5. Centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
15. Incubate the sample at room temperature for 2 minutes to allow the amplified sample library to elute off the beads.
16. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
17. Transfer 50 µL of the clear supernatant (amplified adapter-ligated library) to a new tube(s). Any beads carried over from this step will be removed in the following steps.
18. Add 50 µL of room temperature, thoroughly resuspended, KAPA HyperPure Beads to each sample.
19. Mix thoroughly by pipetting or vortexing and centrifuge briefly to collect all droplets.
20. Incubate the sample at room temperature for 5 minutes to allow the amplified sample library to bind to the beads.
21. Place the sample on a magnet to capture the beads. Incubate until the liquid is clear.
22. Carefully remove and discard the supernatant.
23. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.

24. Incubate the sample for ≥30 seconds.
25. Carefully remove and discard the ethanol.
26. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
27. Incubate the sample for ≥30 seconds.
28. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
29. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

30. Remove the sample from the magnet.
31. Thoroughly resuspend the beads in 25 µL (or appropriate volume) of 10 mM Tris-HCl, pH 8.0 – 8.5. Centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.

If proceeding with double-sided size selection (***Appendix A***), resuspend the beads in 55 µL of elution buffer.

32. Incubate the sample at room temperature for 2 minutes to allow the amplified sample library to elute off the beads.
33. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
34. Transfer an appropriate volume of the clear supernatant to a fresh tube(s)/well and proceed with double-sided size selection (refer to ***Appendix A***), library QC, target capture or sequencing.
35. Purified, amplified sample libraries can be stored at +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for up to 3 months.

Chapter 5.

Quality Control

This chapter describes how to determine the concentration and size distribution of the sample library. Your specific library construction workflow should be tailored and optimized to yield a sufficient amount of adapter-ligated molecules of the desired size distribution for the next step in the process (e.g., target capture or sequencing), as well as for library QC and archiving purposes.

Quantification

KAPA Library Quantification Kits for Illumina platforms are recommended for qPCR-based quantification of libraries generated using the KAPA EvoPlus V2 workflow. Libraries may also be quantified using a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit.

Sizing

The size distribution of the final libraries should be confirmed with an electrophoretic method. A LabChip GX, GXII, or GX Touch (PerkinElmer), Bioanalyzer, TapeStation (Agilent Technologies), Fragment Analyzer (Advanced Analytical) system or similar instruments are recommended over conventional gels. KAPA EvoPlus V2 Kit libraries may require dilution prior to electrophoretic assessment. Refer to the respective instrument and assay user manuals for guidance on how to dilute your sample library so as not to exceed the detection limit of the specific assay.

Typical electrophoretic profiles for libraries prepared with the KAPA EvoPlus V2 Kit are given in *Figure 3*.

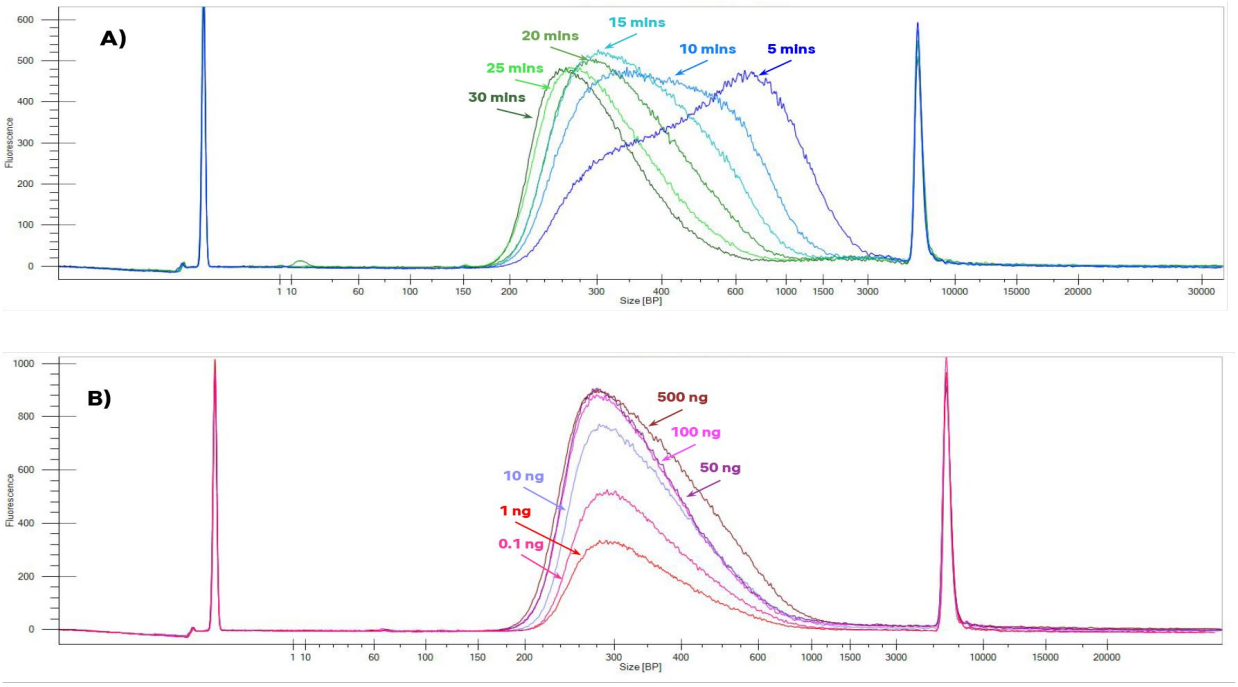


Figure 3: Examples of libraries prepared with the KAPA EvoPlus V2 Kit.
A) 100 ng of high-quality human genomic DNA was fragmented for 5 - 30 minutes and used to prepare libraries with KAPA UDI Adapters at the recommended adapter:insert molar ratio. Libraries were amplified for 3 cycles to enable visualization. Electropherograms were generated with LabChip GX Touch NGS 3K Assay.
B) Various high-quality human genomic DNA inputs (0.1 - 500 ng) were fragmented to ~200 bp and used to prepare libraries with KAPA Universal Adapters at the recommended adapter:insert molar ratios. Libraries were amplified according to DNA input based recommendations and diluted appropriately to fall within the dynamic range of the assay. Electropherograms were generated with LabChip GX Touch NGS 3K Assay.

Please note that libraries prepared with “forked” or “Y” adapters in PCR-free workflows will appear to have a longer than expected mode fragment length, and/or may display a broad or bimodal size distribution when analyzed electrophoretically. The difference in overall appearance and fragment size distribution of an unamplified versus the corresponding amplified library varies, and depends on the adapter design and electrophoretic system used. To accurately determine the size distribution of an unamplified library, an aliquot of the library may be subjected to a few cycles of amplification prior to electrophoretic analysis, to ensure that all adapter-ligated molecules are fully double-stranded (e.g, amplify 1 µL adapter-ligated library for 4 amplification cycles followed by a bead cleanup).

Appendices

Appendix A. Double-sided Size Selection

Size selection requirements vary widely for different sequencing applications. For sequencing on e.g., Illumina HiSeq X and NovaSeq instruments, narrow insert size distributions (in the range of 300 – 650 bp), and sequencing-ready libraries free of short fragments, such as unligated adapter and adapter-dimer, are required. This is essential to ensure optimal cluster generation, mitigate the potential impact of index misassignment, and facilitate data analysis.

If required, any commonly used bead- or gel-based size selection techniques may be integrated in the KAPA EvoPlus V2 workflow.

Size selection may be carried out at different points in the overall workflow, for example after the post-ligation cleanup, or after the library amplification cleanup.

Size selection inevitably leads to a loss of sample material. These losses can be dramatic (60 – 95%), and may significantly increase the number of amplification cycles required to generate sufficient material for the next step in the process (capture or sequencing). The potential advantages of one or more size selection steps in a library construction workflow should be weighed against the potential loss of library complexity, especially when input DNA is limited. A well-optimized fragmentation protocol, especially for shorter insert libraries and/or read lengths, may eliminate the need for size selection, thereby simplifying the library construction process and limiting sample losses.

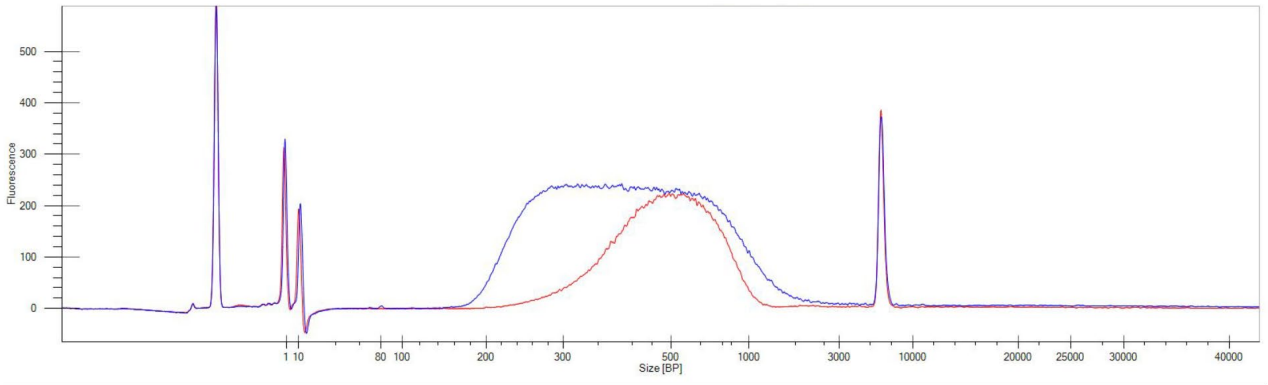


Figure 4: Example of libraries prepared with the KAPA EvoPlus V2 Kit subjected to double-sided size selection.
Size selection inevitably leads to a loss of sample material, and can be dramatic (60 - 95%). Blue trace: library before double-sided size selection. Red trace: library after double-sided size selection. Input DNA (500 ng high quality *E. coli* gDNA) was fragmented for 12 minutes. Libraries were prepared using KAPA UDI Adapters and subjected to 0.5X - 0.7X double-sided size selection post-ligation using KAPA HyperPure Beads (libraries were amplified for visualization). Electropherograms were generated with LabChip GX Touch NGS 3K Assay.

A double-sided size selection consists of a first and second “cut”, performed with different bead-to-sample volume ratios. The first cut determines the upper size limit of the size-selected sample library, whereas the second cut determines the lower size limit.

To increase the upper size limit of the selected fragments, reduce the volume of KAPA HyperPure Beads used for the first cut. To decrease the upper size limit of the selected fragments, increase the volume of KAPA HyperPure Beads used in the first cut.

To increase the lower size limit of the selected fragments, reduce the volume of KAPA HyperPure Beads added in the second cut. To decrease the lower size limit of the size selected fragments, increase the volume of KAPA HyperPure Beads added in the second cut.

⚠ **The volume of KAPA HyperPure Beads needed for the second cut is calculated relative to the volume of the sample library at the start of the size selection procedure, not the volume of the sample containing supernatant transferred after the first cut.**

The second size cut should be performed with at least 0.2 volumes of original input of KAPA HyperPure Beads.

Sample recovery is dramatically reduced if the difference between first and second cuts is less than ~0.2 volumes. To increase the amount of sample recovered, >0.2 volumes of KAPA HyperPure Beads may be used for the second cut, but note that this may result in the recovery of smaller library fragments and/or a broader size distribution.

The double-sided size selection protocol outlined in this appendix (0.5X – 0.7X) is designed for the selection of library molecules (inclusive of a full length adapter such as KAPA UDI Adapter) in the range of 300 – 600 bp if fragmenting high quality DNA for ~15 minutes using FragTail ReadyMix. The protocol may need to be modified if truncated adapters were used for library construction. Contact *Technical Support* for guidance if needed. To obtain a population of shorter or longer molecules, the protocol may be modified as follows:

Upper size limit of captured fragments	Modification	Lower size limit of captured fragments	Modification
Increase	Decrease the ratio of the first cut (e.g, 0.4X or 0.45X)	Increase	Decrease the ratio of the second cut (e.g, 0.6X or 0.65X)
Decrease	Increase the ratio of the first cut (e.g, 0.6X or 0.65X)	Decrease	Increase the ratio of the second cut (e.g, 0.8X or 0.85X)

1. Perform the first (0.5X) size cut (to bind and exclude library molecules larger than ~600 bp) by combining the following:

Component	Volume per Individual Sample
Library to be size selected	50 µL
KAPA HyperPure Beads	25 µL
Total volume	**75 µL**

2. Mix the sample library and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
3. Incubate the sample at room temperature for 5 minutes to allow the library molecules larger than ~600 bp to bind to the beads.
4. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
5. Carefully transfer ~70 µL of supernatant containing library molecules smaller than ~600 bp to a new tube(s)/well. It is critical that no beads are transferred with the supernatant. Discard the tube(s)/well with the beads to which library molecules larger than ~600 bp were bound.
6. Perform the second size cut (0.7X), to retain library molecules >300 bp by combining the following:

Component	Volume per Individual Sample
Supernatant from first size cut	70 µL
KAPA HyperPure Beads	10 µL*
Total volume	**80 µL**

*The volume of KAPA HyperPure Beads needed for the second cut is calculated relative to the volume of the sample library at the start of the size selection procedure, not the volume of the sample containing supernatant transferred after the first cut. A volume of 10 µL of KAPA HyperPure Beads is added during the second cut. **This is not an error.** The supernatant from Step 5 contains PEG/NaCl from the initial 0.5X volume of KAPA HyperPure Beads, and is carried over from the first cut into the second cut. This volume of PEG/NaCl (the crowding reagent) is the critical functional component. The 0.7X ratio required for the second cut is thus a cumulative total ratio. It is the sum of the original 0.5X ratio that is retained from the first cut plus the 0.2X added during the second cut for a total ratio of 0.7X (0.5X + 0.2X).

7. Mix the supernatant from the first size cut and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
8. Incubate the sample at room temperature for 5 minutes to allow the library molecules larger than >300 bp to bind to

the beads.

9. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
10. Carefully remove and discard the supernatant.
11. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
12. Incubate the sample at room temperature for ≥30 seconds.
13. Carefully remove and discard the ethanol.

The low bead volume used for the second cut results in a small bead pellet that is easily disturbed and may also dry out considerably faster than during other reaction cleanups.

14. Keeping the sample on the magnet, add 200 µL of freshly-prepared 80% ethanol.
15. Incubate the sample at room temperature for ≥30 seconds.
16. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
17. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance of the bead pellet. The bead pellet should have a matte appearance when sufficiently dried.

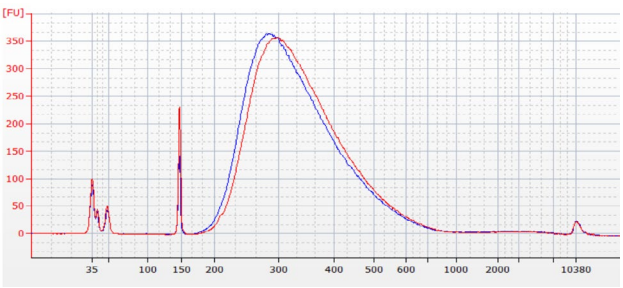
18. Remove the sample from the magnet.
19. Thoroughly resuspend the beads in 25 µL of 10 mM Tris-HCl, pH 8.0 – 8.5. Centrifuge briefly to collect all droplets. Do NOT allow beads to pellet.
20. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.
21. Place the sample on the magnet to capture the beads. Incubate until the liquid is clear.
22. Transfer 20 µL of the eluate to a new tube/well.
23. Purified libraries can be stored as follows:
    - 23.1. post-ligation libraries: +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for up to 1 month.
    - 23.2. post-amplification libraries: +2°C to +8°C for 1 – 2 weeks or at -15°C to -25°C for up to 3 months.

Appendix B. Troubleshooting

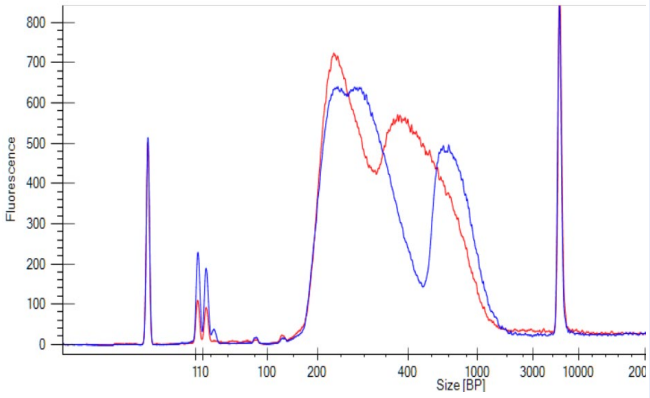
This appendix provides guidance for interpreting unexpected results and recommendations for implementing corrective action if problems occur. For technical questions, contact your local Roche Technical Support.
Go to ***sequencing.roche.com/support*** for contact information.

⚠ The Illumina sequencing workflow is not supported by Roche Technical Support.

Observation	Cause(s) / Recommendation(s)
Low library yields	Input DNA was not quantified correctly. If less DNA is added to the reaction, expected yields will be impacted.
	Low quality DNA (typically with a DIN of <7) may require additional workflow modifications compared to intact DNA, such as increasing the input into library construction.
	Adapter quality was compromised. Ensure high quality adapters are used with minimal freeze/thaw cycles (F/T). E.g, KAPA UDI Adapters are stable up to 10 F/T cycles.
	Adapter concentration too low. Ensure the correct adapter concentration is used.
	Improper bead cleanup practises followed. Ensure best practices are used during bead cleanups. ▪ Equilibrate KAPA HyperPure Beads to room temperature prior to use. ▪ Always prepare fresh 80% ethanol for bead cleanups. Long term storage of 80% ethanol will result in evaporation, resulting in a lower ethanol percentage being used and subsequent sample loss. ▪ Do not freeze/thaw KAPA HyperPure Beads. Beads will be damaged if stored at -20°C. ▪ Protect KAPA HyperPure Beads from light during long term storage. ▪ Do not over-dry beads.
	Double-sided size selection performed. Size selection will result in significant sample loss and should only be performed if absolutely necessary.
	Insufficient mixing performed. The KAPA EvoPlus V2 Kit components are very viscous and insufficient mixing may result in inefficient enzymatic reactions. Pipette-mix at least 10X or vortex for 10 – 20 seconds.
	Libraries stored incorrectly. Libraries may degrade over time if stored incorrectly.

Fragment distribution (analyzed using an Agilent Bioanalyzer 2100 High Sensitivity DNA assay displays a sharp peak at ~150 bp (adapter-dimer) Generally, another 0.8X or 1X bead cleanup will remove the adapter-dimer contamination. A second cleanup is recommended if the adapter-dimer contamination is >5% of the total library concentration. The percentage adapter-dimer can be calculated by performing a smear analysis using e.g, the Bioanalyzer software. Adapter-dimers are to be avoided at all cost if libraries will be sequenced on patterned flow cells such as those utilized by the Illumina NovaSeq and HiSeq X.  	Input DNA was not quantified correctly and the incorrect adapter concentration used as a result.
	Degraded or FFPET-derived DNA may not support efficient ligation, resulting in adapter-dimer formation. Reduce the adapter concentration for degraded DNA. Titrate until a compromise is achieved between yield and adapter-dimer carryover.
	Adapter quality was compromised. Ensure high quality adapters are used with minimal freeze/thaw cycles (F/T). E.g, KAPA UDI Adapters are stable up to 10 F/T cycles.
	Molar ratio of adapter:insert will affect adapter-dimer formation. If the concentration of adapter is too high it may result in adapter-dimer carryover.
	Improper bead cleanup practises followed. Ensure best practises are used during bead cleanups. ▪ KAPA HyperPure Beads need to be equilibrated to room temperature prior to use. ▪ Always prepare fresh 80% ethanol for bead cleanups. Long term storage of 80% ethanol will result in evaporation, resulting in a lower ethanol percentage being used and subsequent sample loss. ▪ Do not freeze/thaw KAPA HyperPure Beads. Beads will be damaged if stored at -20°C. ▪ Protect KAPA HyperPure Beads from light during long term storage. ▪ Do not over-dry beads.
	Incorrect bead:sample ratio used. This will result in retention of adapter dimers instead of removal.
	Insufficient mixing performed. The KAPA EvoPlus V2 Kit components are very viscous and insufficient mixing may result in inefficient enzymatic reactions. Pipette-mix at least 10X or vortex for 10 – 20 seconds.
Fragment distribution shows that the average amplified fragment is not within the expected size range and high molecular artefacts are visible.	Poor fragmentation occurred. Repeat library preparation. Ensure that the correct incubation time and temperature were selected for the desired fragment size. Insufficient mixing performed. The KAPA EvoPlus V2 Kit components are very viscous and insufficient mixing may result in inefficient enzymatic reactions. PEG 6000 droplets should not be visible. Pipette-mix at least 10X or vortex for 10 – 20 seconds.

Fragment distribution (analyzed using a LabChip GX Touch and LabChip NGS 3K Assay) is bimodal, with a larger set of fragments observed in addition to the expected set of fragments



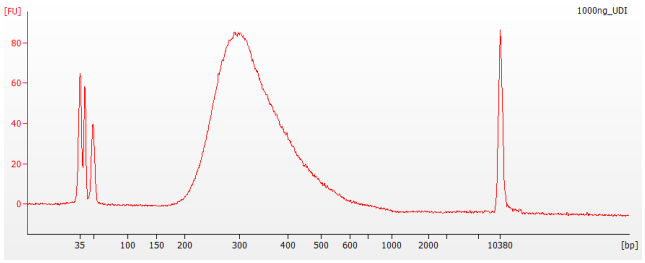
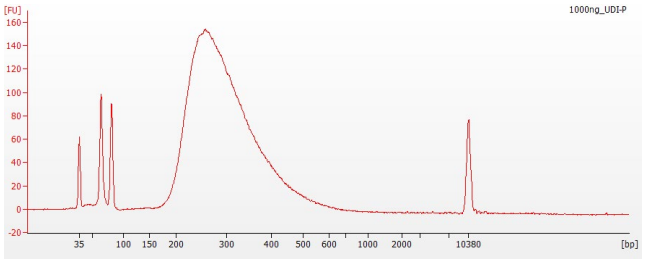
A. PCR-free library was subjected to electrophoretic analysis.

In PCR-free workflows it is difficult to obtain accurate average fragment sizes from electrophoretic systems, as molecules flanked by adapters with long single-stranded terminals migrate anomalously in gel matrices, thereby appearing to be longer than they truly are. Easy workarounds for this problem include the following:

- Use the average length of the fragmented DNA plus the total length of the two adapters (usually ~140 bp) as an estimate for the average library fragment size in concentration calculations. This approach is only feasible if no size selection was performed, or if the size selection parameters were optimized to preserve the size distribution of the fragmented DNA.
- Amplify a small aliquot of the PCR-free library for 2 – 5 cycles (followed by a 1X bead cleanup) prior to electrophoretic analysis. Amplification will render all molecules fully double-stranded, and yield a reliable size determination from the electrophoretic assay.

B. Primer depletion

Primer depletion due to over-amplification of the sample library relative to the amount of primers available in the reaction results in single stranded amplification products. These products can anneal to each other via adapter homology on both ends of the fragments to form heteroduplexes, and migrate as larger products on a fragment analyzer than their actual length in base pairs. The artifact can be resolved by increasing primer concentration or reducing cycle number in the PCR reaction, however the products themselves are perfectly acceptable for use in sequence capture and sequencing.

Fragment distribution (analyzed using an Agilent Bioanalyzer High Sensitivity DNA chip) displays sharp peaks at 44 & 45 bp or ~ 47 & 49 bp (primer carryover)	
  **Top image:** primer carryover for libraries constructed using KAPA UDI Adapter and amplified with KAPA Library Amplification Primer Mix. **Bottom image:** indexed primer carryover for libraries constructed using KAPA Universal Adapter and amplified using KAPA UDI Primer Mixes.	Primer carryover is typically seen in amplified libraries and not completely removed by the post-amplification cleanup. Carryover of standard KAPA Library Amplification Primers (used to amplify libraries constructed using full length, KAPA UDI Adapter) will not interfere with sequencing, and may be safely ignored. However, when using KAPA UDI Primer Mixes (in conjunction with KAPA Universal Adapter), carryover indexed primers may exacerbate index hopping on patterned flow-cells (e.g., Illumina NovaSeq or HiSeq X instruments). Generally another 1X bead cleanup after amplification will remove the primer carryover and is highly recommended when using KAPA UDI Primer Mixes.

Appendix C. Limited Warranty

1. Limited Warranty

A. Products: Roche Sequencing Solutions, Inc. (“Roche”) warrants that its Products conform to its published specifications and are free from defects in material or workmanship. Customer’s sole and exclusive remedy (and Roche’s sole and exclusive liability) under this limited warranty shall be to either (a) replace the defective Products, or (b) provide Customer with a refund, as solely determined by Roche.

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D. Any action by Customer against Roche for Roche’s breach of this warranty must be commenced within 12 months following the date of such breach. Notwithstanding such 12-month period, within twenty (20) days of the delivery of Data and/or Products to Customer, Customer must notify Roche in writing of any nonconformity of the Services and Products, describing the nonconformity in detail; otherwise all Services and Products shall be conclusively deemed accepted without qualification.

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