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Not for use in diagnostic procedures.

Instructions for Use
KAPA EvoPlus Kit

August 2022

Contents

The KAPA EvoPlus Kits contain:

Kit Code	Description		Material Number
09420037001 09420045001*	**KAPA EvoPlus Kit (24 rxn)**		
	FragTail ReadyMix	(600 µL)	09420606001
	Ligation ReadyMix	(240 µL)	09420665001
	KAPA HiFi HotStart ReadyMix (2X)	(690 µL)	08203075001
09420053001 09420304001*	**KAPA EvoPlus Kit (96 rxn)**		
	KAPA FragTail ReadyMix	(3.0 mL)	09420614001
	KAPA Ligation ReadyMix	(1.2 mL)	09420681001
	KAPA HiFi HotStart ReadyMix (2X)	(3.0 mL)	08203008001
09420339001 09420371001*	**KAPA EvoPlus Kit (384 rxn)**		
	KAPA FragTail ReadyMix	(12 mL)	09420649001
	KAPA Ligation ReadyMix	(4.8 mL)	09420690001
	KAPA HiFi HotStart ReadyMix (2X)	(9.6 mL)	09420711001
09420428001 09420436001*	**KAPA EvoPlus Kit (96-well plate)**		
	KAPA FragTail ReadyMix	(96 x 25 µL)	09420657001
	KAPA Ligation ReadyMix	(96 x 10 µL)	09420703001
	KAPA HiFi HotStart ReadyMix (2X)	(96 x 25 µL)	09420720001
	1 x Replacement (pierceable and peelable) seal per plate		

* Available for PCR-free workflows, and do not contain any library amplification reagents.

** 10% overage +5 µL is provided

Note: KAPA Library Amplification Primers are available separately in tube (384 rxns) and plated (96 rxns) format.

Shipping, Storage and Stability

- KAPA EvoPlus 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 -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 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 (10 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 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

Warnings and Precautions

- Wear the appropriate personal protective equipment, such as gloves and safety glasses, to avoid direct contact while handling the reagents.
- Use good laboratory practices to avoid contamination when working with the reagents.
- 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.

Ordering Information

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

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Changes to Previous Versions

Formatting changes and correction of typographical errors

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Table of Contents

Preface

Regulatory Disclaimer

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Contact Information

Technical Support

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

Manufacturer and Distribution

Manufacturer	Roche Dia 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

Symbol	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 Kit.

The KAPA EvoPlus 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 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

Figure 1 - KAPA EvoPlus Quick Guide

dsDNA sample (35 µL)

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

Add 25 µL of FragTail ReadyMix

5 - 30 min at 37°C
30 min at 55°C
Transfer to 4°C

Application	Sample type	Input
WGS	High quality gDNA	10 - 500 ng
	Low quality FFPET-derived DNA	≥ 100 ng **
WGS (PCR-free)	High quality gDNA	≥75 ng (no SS)* 500 ng (with SS)*
Targeted Sequencing	High quality gDNA	100 ng
	Low quality FFPET-derived DNA	≥ 100 ng

* 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.
** Reach out to Technical Support for possible workflow modifications when using this sample type.

Adapter Ligation (75 µL)

Add 5 µL of Adapter stock
Add 10 µL of Ligation ReadyMix

15 min at 20°C

Input DNA	Adapter stock concentration	Adapter:insert molar ratio*
500 ng	15 µM	20:1
100 ng	15 µM	100:1
75 ng	15 µM	135:1
50 ng	15 µM	200:1
10 ng	6 µM	400:1

*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. The lower adapter:insert molar ratios recommended for inputs >100 ng represent a fair compromise between library construction efficiency and cost; higher library yields will be achieved if a higher adapter concentration is used.

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 incorporated at this point. If applicable, resuspend in 55 µL elution buffer and proceed to *Appendix A - Double-sided Size Selection*.

Post-ligation quantification
KAPA Library Quantification Kit (recommended; dilution range between 1/500 to 1/10,000) or Qubit Fluorometer

Optional
Library Amplification (50 µL)

Add 25 µL of 2X HiFi HotStart ReadyMix
Add 5 µL of 10X Illumina Library Amplification Primer Mix
(or appropriate compatible indexes/primer sets)

Input Amount	Number of amplification cycles for WGS to achieve 4 nM* (if using full length adapters)
75 ng - 500 ng	0 cycles (PCR-free workflow)
50 ng	1 - 2 cycles
10 ng	5 - 7 cycles

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

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 and proceed to *Appendix A - Double-sided Size Selection*.

Electrophoretic profile of amplified libraries

Post-amplification quantification
KAPA Library Quantification Kit (recommended: dilution range between 1/10,000 to 1/200,000) or Qubit Fluorometer

Target Capture or Sequencing

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15 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
- Agilent 2100 Bioanalyzer Instrument Manual
- Agilent Bioanalyzer DNA Kits Guide

Terminology

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

Amplified Sample Library: The sample library after amplification by adapter ligation-mediated PCR.

KAPA UDI Adapter: KAPA Unique Dual-Indexed Adapter.

Prepare the Following Equipment and Reagents

- 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 2)

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	Catalog No.
DynaMag-96 Side Magnet	Thermo Fisher	12331D
Microcentrifuge (16,000 x *g* capability)	Multiple Vendors	
Qubit Fluorometer	ThermoFisher	Multiple models
Bioanalyzer 2100	Agilent	
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	384 reactions (1.92 mL)	09420410001
KAPA Library Amplification Primer Mix 96-well plate	96 x 5 µL	09420479001

Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
Agilent High Sensitivity DNA Kit (recommended)	Agilent	1 kit	5067-4626
10 mM Tris-HCl, pH 8.0	Multiple Vendors		
Ethanol, 200 proof (absolute), for molecular biology	Multiple Vendors	500 mL	
Qubit dsDNA HS Assay Kit	ThermoFisher	1 kit	Q32851
Qubit Assay Tubes	ThermoFisher		Q32856
Low binding Tubes: ▪ 0.2 mL PCR tubes ▪ 1.5 mL microcentrifuge tubes (optional)	Multiple Vendors	1 package of 500 tubes	
Nuclease-free, PCR Grade water	Multiple Vendors		

Chapter 2. Prepare and Store the Reagents

This chapter describes the storage conditions for the following kits:

- KAPA EvoPlus Kit
- KAPA HyperPure Beads
- KAPA Unique Dual-Indexed (UDI) Adapter Kit

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA EvoPlus Kit	-15 to -25°C
KAPA HyperPure Beads*	+2 to +8°C
KAPA UDI Adapter Kit	-15 to -25°C

*The KAPA HyperPure Beads kit must not be frozen.

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

1. Retrieve the KAPA UDI Adapter plate from storage (-15 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 Technical Data Sheet (KR1736) 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.

Input DNA (high quality and/or low quality)	Adapter stock concentration	Adapter:insert molar ratio*
500 ng	15 µM	20:1
100 ng	15 µM	100:1
75 ng	15 µM	135:1
50 ng	15 µM	200:1
10 ng	6 µM	400:1

*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.

Chapter 3. Prepare the Sample Library

In this chapter the KAPA EvoPlus 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 Kit
- KAPA Unique Dual-Indexed Adapter Kit*
- 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

*KAPA Unique Dual-Indexed (UDI) Adapters are recommended for use with the KAPA EvoPlus Kit. However, the kit is also compatible with other full-length adapter designs. If considering a truncated adapter design (e.g. such as KAPA Universal Adapter and KAPA UDI Primer Mixes) several modifications to the workflow will be necessary. Please contact *sequencing.roche.com/support* for guidance.
**KAPA HyperPure Beads are recommended for use with the KAPA EvoPlus Kit. However, the kit is also compatible with KAPA Pure Beads. Conditions for DNA binding and size selection may differ if other beads are used.

Sample Requirements

This workflow was validated with 10 - 500 ng of high quality gDNA, and is compatible with ≥100 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 guidance on lower input amounts or sample quality, please see the **table below** or contact *sequencing.roche.com/support*.

Application	Sample type	Input
WGS	High quality gDNA	10 - 500 ng
	Low quality FFPET-derived DNA	≥ 100 ng **
WGS (PCR-free)	High quality gDNA	≥75 ng (no SS)* 500 ng (with SS)*
Targeted Sequencing	High quality gDNA	100 ng
	Low quality FFPET-derived DNA	≥ 100 ng

* 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.
** Reach out to Technical Support for possible workflow modifications when using this sample type.

It is important to the success of the library preparation workflow to ensure that KAPA EvoPlus Kit components have been fully thawed and thoroughly mixed before use. Specifically, the **FragTail ReadyMix** contains a high concentration of PEG 6000 and glycerol, and is **very viscous** so ensure this solution is sufficiently mixed. 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 buffer is thoroughly vortexed until the droplets have been resuspended.

Both the **FragTail ReadyMix** and **Ligation ReadyMix** components are **very viscous** and require special attention during pipetting.

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

If using the plated format, remove plates 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.

Make sure 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 10 - 500 ng of DNA with 10 mM Tris-HCl, pH 8.0 (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 follows:

Component	Volume Per Individual Sample
10 - 500 ng DNA	35 µL
FragTail ReadyMix	25 µL
Total	**60 µL**

4. Mix the Fragmentation and A-tailing reaction **thoroughly** and centrifuge briefly. Return the plate/tube(s) on 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*	Fragmentation time at 37°C
550 - 640 bp	5 min
330 - 410 bp	10 min
240 - 320 bp	15 min
200 - 260 bp	20 min
170 - 230 bp	25 min
140 - 210 bp	30 min

*Insert sizes (without adapter) observed upon fragmentation of 50, 250 and 500 ng of NA12878 (Coriell Institute of Biomedical Research). Size variation may be observed, depending on DNA type, DNA input and DNA elution buffers. 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

1. Transfer the reaction on ice and proceed to set up the Adapter Ligation reaction on ice.
2. Vortex the Ligation ReadyMix well and centrifuge briefly.
   a. Add 5 µL of a unique KAPA UDI Adapter to each tube/well containing sample from the previous step.

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

   b. Add 10 µL of the Ligation ReadyMix to each tube/well containing 60 µL sample and 5 µL KAPA UDI Adapter, resulting in a total volume of 75 µL.

Component	Volume Per Individual Sample
FragTail product	60 µL
KAPA UDI Adapter	5 µL
Ligation ReadyMix	10 µL
Total	**75 µL**

   c. Mix the ligation reaction **thoroughly** and centrifuge.
   d. Incubate the ligation reaction at +20°C on a thermocycler for 15 minutes.
   e. Following the incubation, proceed immediately to the next step.

Step 3. Purify the Sample Library using KAPA HyperPure Beads

1. To each ligation reaction, add 60 µL of room temperature KAPA HyperPure Beads that have been thoroughly resuspended.

Component	Volume Per Individual Sample
Ligation reaction product	75 µL
KAPA HyperPure Beads	60 µL
Total	**135 µL**

2. Once added, mix thoroughly and centrifuge briefly.

It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous. 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.

Caution: Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance on the surface of the beads. The surface of the beads 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.

If proceeding with a 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 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 eluate to a fresh tube/well.

If proceeding with Library Amplification, 20 µL would be required and the remaining 5 µL can be used for quality control purposes e.g. quantification using the KAPA Library Quantification Kit. If proceeding with double-sided size selection, transfer 50 µL of the eluate to a fresh tube/well and refer to Appendix A for the double-sided size selection guidelines. The remaining 5 µL can be used for quality control purposes e.g. quantification using the KAPA Library Quantification Kit.

18. Proceed immediately to *Chapter 4*. Amplify The Sample Library (optional for sample inputs of ≥75 ng) or *Chapter 5*. Quality Control, if performing a PCR-free workflow.

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 DNA 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, library using KAPA HiFi HotStart ReadyMix and KAPA Library Amplification Primer Mix (10X) (ordered separately).

Ensure that the following is available:

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

Step 1. Prepare the Library Amplification Reaction

Make sure 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, 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 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. Place on ice until use.

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

1. Assemble each library amplification reaction as follows:

Component	Volume per Individual Library
2X HiFi Hotstart ReadyMix*	25 µL
10X Illumina Library Amplification Primer Mix*	5 µL
Adapter-ligated library	20 µL
Total	**50 µL**

*The ReadyMix and Primer Mix should preferably be premixed and added in a single pipetting step.

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 library using the following Library Amplification program with the lid temperature set to +105°C:
    a. Step 1: 45 seconds at +98°C
    b. Step 2: 15 seconds at +98°C
    c. Step 3: 30 seconds at +60°C
    d. Step 4: 30 seconds at +72°C
    e. Step 5: Go to Step 2, Variable (see table below for recommendations)
    f. Step 6: 1 minute at +72°C
    g. Step 7: Hold at +4°C

Recommended number of amplification cycles per DNA input for KAPA EvoPlus Kit

Input Amount	Number of amplification cycles* for WGS to achieve 4 nM** (if using full length adapters)
50 ng	1 - 2 cycles
10 ng	5 - 7 cycles

*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.

Note: When using incomplete, or truncated, adapters, a minimum number of amplification cycles (4) may be required to complete adapter sequences for the next step in the process (target capture or sequencing), irrespective of whether 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.

Based on sequencing recommendations, 4 nM is the minimum starting concentration to proceed with sequencing. For input amounts ≥75 ng, PCR amplification should not be required to achieve the ~4 nM requirement for sequencing. 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

1. Add 50 µL 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.

It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous. Insufficient mixing may compromise recovery.

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 of the ethanol to evaporate.

Caution: Over-drying the beads may result in dramatic yield loss. Over-drying is indicated by a dry, cracked appearance on the surface of the beads. The surface of the beads 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.

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 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 20 µL of the eluate to a new tube/well or if proceeding with double-sided size selection, transfer 50 µL of the eluate to a fresh tube/well and refer to Appendix A for the double-sided size selection guidelines. The remaining 5 µL can be used for quality control purposes e.g. quantification using the KAPA Library Quantification Kit.
18. Purified, amplified sample libraries can be stored at +2 to +8°C for 1 - 2 weeks or at -15 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.

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 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 Kit are given in Figure 2.

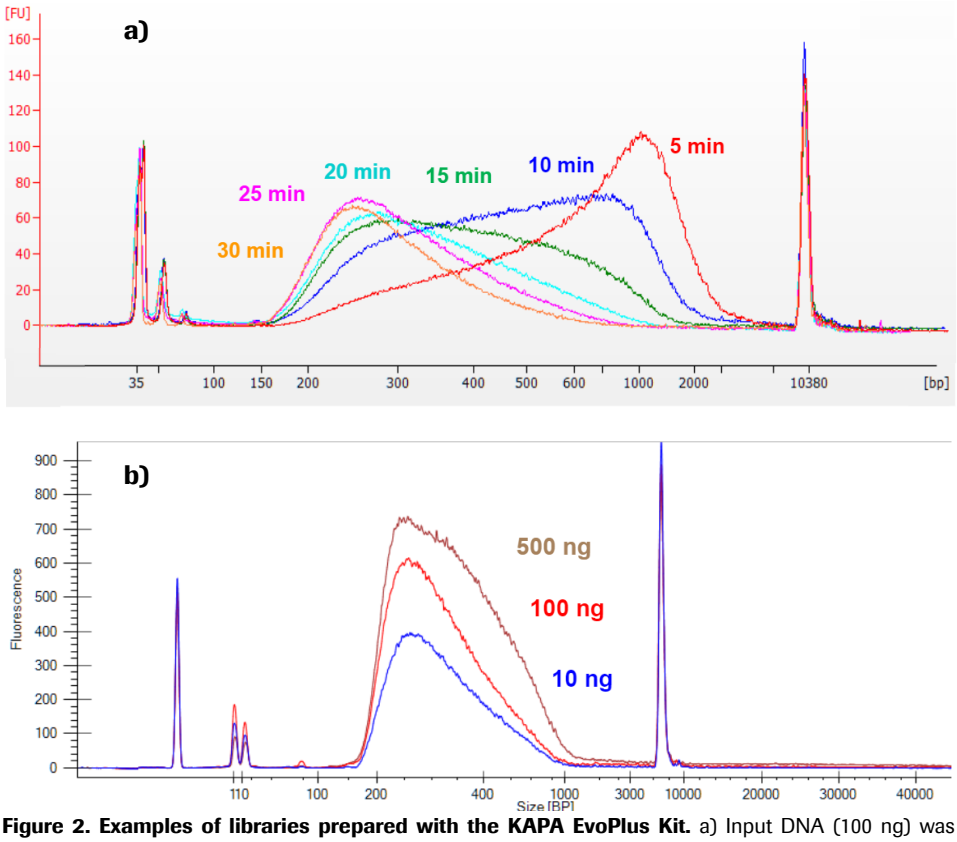


Figure 2. Examples of libraries prepared with the KAPA EvoPlus Kit. a) Input DNA (100 ng) was fragmented for 5 - 30 minutes. b) Various input DNA (10 ng - blue trace, 100 ng - red trace, and 500 ng - brown trace) were fragmented for 20 minutes.

Libraries were prepared as described in the Library Construction Protocol (Chapter 3 & 4), using high-quality human genomic DNA and KAPA UDI Adapters with the recommended adapter:insert molar ratio. Libraries were amplified for limited cycles to enable visualization. Electropherograms were generated with a Bioanalyzer 2100 High Sensitivity DNA Kit or LabChip GX Touch NGS 3K Assay.

Please note that libraries prepared with "forked" 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 vs. 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).

Quantification

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

Appendix A. Double-sided Size Selection

Size selection requirements vary widely for different sequencing applications. For sequencing on 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 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.

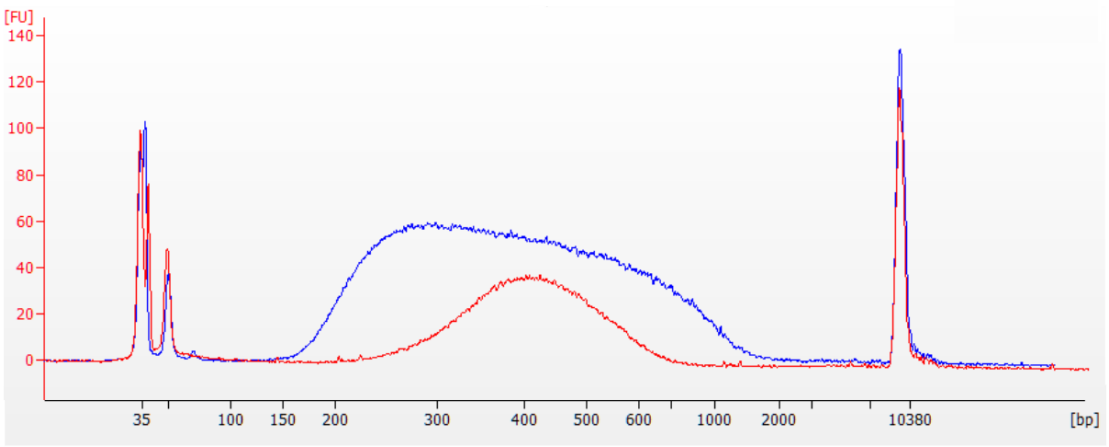


Figure 3: Example library subjected to double-sided size selection. Size selection inevitably leads to a loss of sample material, and can be dramatic (>80%). Blue: library before double-sided size selection. Red: library after double-sided size selection.
Input DNA (100 ng high-quality human genomic DNA) was fragmented for 15 minutes. Libraries were prepared using KAPA UDI Adapters and subjected to 0.6X - 0.8X double-sided size selection using KAPA HyperPure Beads (libraries were amplified for visualization). Electropherograms were generated with a Bioanalyzer 2100 High Sensitivity DNA Kit.

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

Please note that 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.

Appendix A. Double-sided Size Selection

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 adapter) in the range of 300 – 600 bp if fragmenting high quality DNA for ~15 minutes using FragTail ReadyMix. 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)

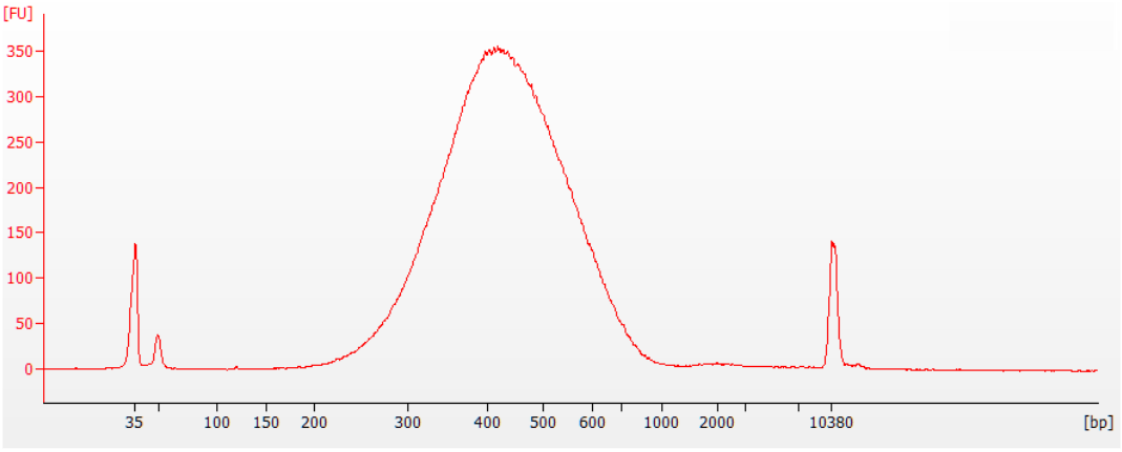


Figure 4. Examples of KAPA EvoPlus libraries subjected to double-sided size selection. Input DNA was fragmented for 15 minutes and subjected to a 0.5X - 0.7X double-sided size selection after the post-ligation cleanup. Electropherograms were generated with a Bioanalyzer 2100 High Sensitivity DNA Kit.

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	**75 µL**

2. Mix the sample library and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets.

It is important at this step to ensure that the solution is thoroughly mixed and appears homogeneous. Insufficient mixing may compromise recovery.

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 plate/tube. It is critical that no beads are transferred with the supernatant. Discard the plate/tube(s) 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	**80 µL**

7. Mix the supernatant from the first size cut and KAPA HyperPure Beads thoroughly and centrifuge briefly to collect all droplets.
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.
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.

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.

17. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

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

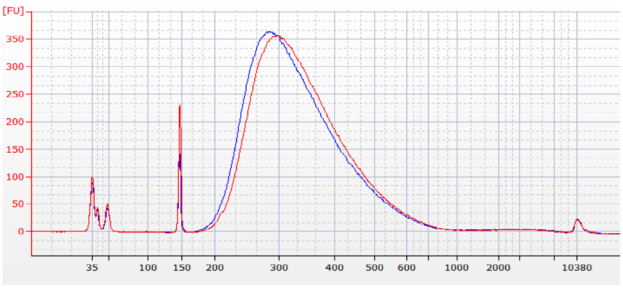
18. Remove the sample from the magnet.
19. Thoroughly resuspend the beads in 25 µL of 10 mM Tris-HCl, pH 8.0. Centrifuge briefly to collect all droplets.
20. Incubate the sample at room temperature for 2 minutes to allow the sample library to elute off the beads.
21. Place the sample on the magnet to 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 at +2 to +8°C for 1 - 2 weeks or at -15 to -25°C for up to 3 months.

Appendix B. Troubleshooting

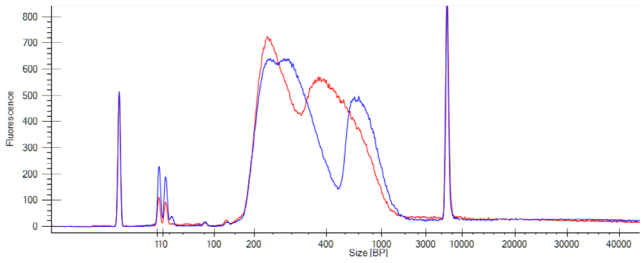
This appendix provides guidance for interpreting unexpected results and recommendations for implementing corrective action if problems occur. For technical questions, contact your local Roche Technical Support. Go to *sequencing.roche.com/support* for contact information.

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

Observation	Cause(s) / Recommendation(s)
Sample Library Preparation	
Low library yields	Input DNA was not quantified correctly. If less DNA is added to the reaction, expected yields will be impacted.
	Quality of input DNA too low to support library preparation. Assess the quality of FFPE material using the KAPA hgDNA Quantification and QC Kit prior to library preparation. Samples with Q129/Q41 ratio of >0.4 meet sequence quality requirements and can be processed in standard library construction pipelines. Low quality DNA (Q129/Q41 ratio of <0.4) 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 overdry 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 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 HS DNA chip) 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 sequencing 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 overdry beads.
	Incorrect bead:sample ratio used. This will result in retention of adapter dimers instead of removal.
	Insufficient mixing performed. The Kapa EvoPlus 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 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 NGS 3K Assay) is bimodal, with a larger set of fragments observed in addition to the expected set of fragments



A. 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 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 an Agilent HS DNA chip 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.

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

Evidence of original purchase is required. It is important to save your sales receipt or packaging slip to verify purchase.

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