INSTRUCTIONS FOR USE OF

KAPA HyperPETE Germline DNA Evolved Workflow v2.0

INSTRUCTIONS FOR USE WITH:

KAPA HYPERPETE HEREDITARY ONCOLOGY PANEL, KAPA HYPERPETE NEWBORN SCREENING* PANEL, KAPA HYPERPETE CHOICE, AND KAPA HYPERPETE EXPLORE PRIMER PANELS

* Not available for sale in the United States. Contact the local Roche affiliate for availability in other regions.

For Research Use Only. Not for use in diagnostic procedures.

February 2024, Version 2.0

Storage and Stability

Products are stable at the temperatures listed in ***Chapter 2 Step 1. Store the Reagents*** until the expiration date printed on the label.

Application

The KAPA HyperPETE Workflow Germline DNA Evolved Instructions for Use enables primer extension targeted enrichment of Roche-defined (catalog panels) and customer-defined regions of the genome for germline applications. Proprietary design algorithms improve capture uniformity and reduce the amount of sequencing needed to efficiently identify sequence variants. The ***KAPA HyperPETE kit*** is intended for capture of DNA primary target regions up to 250 kb.

The KAPA HyperPETE Hereditary Oncology Panel is a 200 Kb capture target panel focusing on hereditary breast and ovarian cancer research (including BRCA1 and BRCA2) as well as hereditary colorectal cancer research. It covers 47 related genes in these cancer types and has been optimized to deliver high uniformity and specificity in germline variant research.

The KAPA HyperPETE Newborn Screening Panel* is a 290 Kb capture target panel covering 89 related genes. It is optimized to deliver high uniformity and specificity in germline variant research.

KAPA HyperPETE Choice Panels and KAPA HyperPETE Explore Panels enable custom designs up to 250Kb for DNA inputs and 50Kb for RNA inputs.

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Warnings and Precautions

- Handle all samples as if potentially infectious, using safe laboratory procedures. As the sensitivity and titer of potential pathogens in the sample material can vary, the operator must optimize pathogen inactivation and follow appropriate measures according to local safety regulations.
- 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.
- Wear protective disposable gloves, laboratory coats, and eye protection, when handling samples and kit reagents.
- Wash hands thoroughly after handling samples and reagents.
- In the event of a spill, clean up the solution with absorbent pads, allow it to dry, and dispose of pads. Observe all national, regional, and local regulations for waste disposal and management.

Waste Handling

- Discard unused reagents and waste in accordance with country, federal, state and local regulations.
- Safety Data Sheets (SDS) are available online on ***elabdoc-prod.roche.com***, or upon request from the local Roche office.

Changes to Previous Version

Removed reference to KAPA HyperPlus kit in library preparation as this is now captured in a separate method document. Library preparation section replaced with KAPA EvoPlus V2 kit information and workflow.

Ordering Information

For a complete overview of Roche Sequencing products, including those used in KAPA HyperPETE Evolved Workflow go to ***sequencing.roche.com/products***.

Trademarks

Contact and Support

If you have product related questions, please contact your local Roche Technical Support. Go to ***sequencing.roche.com/support*** for contact information.

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For patent license limitations for individual products, refer to: ***sequencing.roche.com/support***.

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Preface

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.

Manufacturer and Distribution

Manufacturer	Roche Sequencing Solutions, Inc. Santa Clara, CA USA
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 website.
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 enrichment of individual or multiplexed genomic DNA (gDNA) shotgun libraries using KAPA HyperPETE Panels. Specifically, this Instructions for Use provides a protocol for the workflow outlined in ***Figure 1*** using the KAPA EvoPlus V2 and KAPA HyperPETE Kits. The output of this protocol are enriched gDNA libraries that can be directly sequenced using an Illumina sequencing instrument.

The *KAPA HyperPETE Kit* and Evolved Workflow provides:

- A fast and easy capture workflow based on primer extension reactions that can generate sequencing ready enriched libraries in under 10 hours when starting from purified nucleic acid as input
  - The enrichment procedure following library preparation can be completed in approximately 4 hours
  - The enrichment workflow includes simple room temperature washes with a single wash buffer for ease of use
  - Performance output is comparable to hybridization capture workflows which make use of an overnight hybridization step
- Single vendor service and support for NGS sample preparation including but not limited to
  - KAPA EvoPlus Kit V2
  - KAPA HyperPure Beads, KAPA HyperCapture Bead Kit, and KAPA HyperPETE Reagent Kit
- Catalog panels as well as customizable content through the ***HyperDesign Tool*** and a team of expert designers

Overview of the KAPA HyperPETE Germline DNA Evolved Workflow

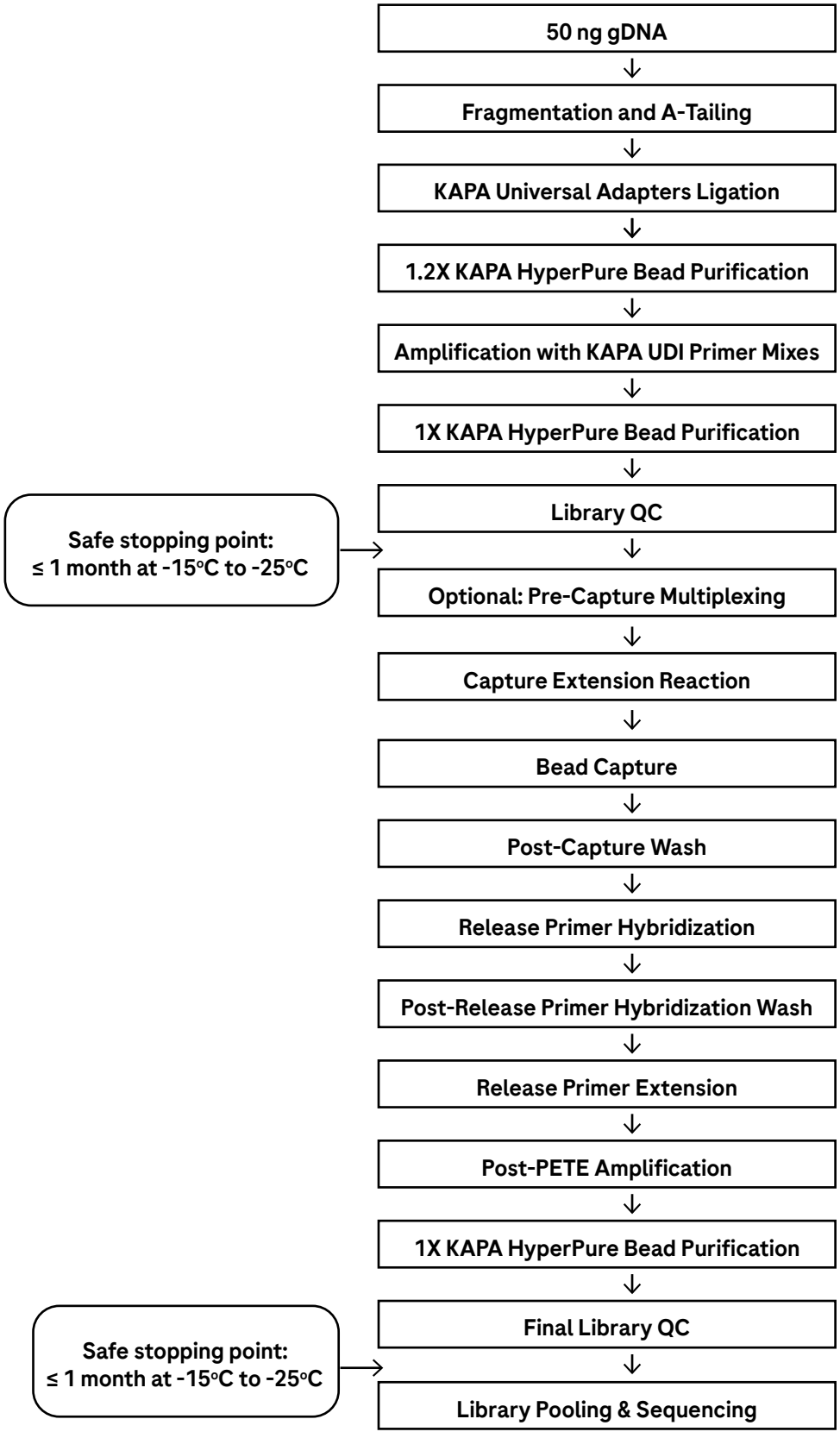


Fig. 1 KAPA HyperPETE Germline DNA Evolved Workflow v2.0

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Clean work area thoroughly before and after all lab procedures.
- Vortex all reagents <2 mL and invert mix all reagents >2 mL before use.
- 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 either vortexing for 10 seconds or pipetting up and down 10 times.
- If liquid has collected in a tube's cap after mixing, gently tap or briefly spin the sample to collect the liquid into the tube's bottom, ensuring that the mixture remains homogeneous before progressing to the next step.
- It is recommended to perform thermocycler incubations using a thermocycler with a programmable heated lid set to the provided temperature for incubations.
- Clearly label tubes at the required steps to minimize sample mix-up.

Terminology

Target Enrichment (or Capture): The process of selecting targeted regions from genomic DNA. In the context of this document, the hybridization and extension of the KAPA HyperPETE Capture Panel to the amplified input library and subsequent washing steps.

KAPA HyperPETE Panels: The complete set of biotinylated oligonucleotide Capture Primers and non-biotinylated Release Primers (KAPA HyperPETE catalog panels, or custom content with KAPA HyperPETE Choice and KAPA HyperPETE Explore panels) provided by Roche for target enrichment.

Catalog Panels: A set of Roche pre-designed panels targeting a specific region(s); e.g. ***KAPA HyperPETE Hereditary Oncology,*** and ***KAPA HyperPETE Newborn Screening**** panels.

* Not available for sale in the United States. Contact the local Roche affiliate for availability in other regions.

Sample Library: The initial shotgun library generated from genomic DNA by fragmentation and adapter ligation. In the context of this document, this is the sample before amplification and prior to capture.

Pre-capture Input Library: The initial shotgun library generated from genomic DNA by fragmentation and ligation. In the context of this document, this is the input library prior to capture.

Enriched Library: The input library after the Capture Extension reaction prior to amplification.

Primer Extension Target Enrichment (PETE) Library: The completed enriched library ready for pooling and sequencing.

UDI Primer: Unique Dual-Indexed Primer. Acts as a unique Sample Identifier.

UMI Adapter: Universal sample adapter containing a double stranded Unique Molecular Index (UMI).

Primary Target: Regions against which primer pairs are designed. Regions with no primer pairs selected are excluded from the Primary Target region.

Capture Target: Regions covered directly by one or more primer pairs. This can include flanking regions outside of the Primary Target leading to larger Capture Target regions than Primary Target regions.

Prepare the Following Reagents and Equipment

- Thermocyclers should be programmed with the following:
  - Library Preparation
    - Fragmentation and A-Tailing program (***Chapter 3, Library Preparation from DNA, Step 1***)
    - Adapter Ligation program (***Chapter 3, Library Preparation from DNA, Step 3***)
    - Amplification with KAPA UDI Primer Mixes program (***Chapter 3, Library Preparation from DNA, Step 5***)
  - Primer Extension Target Enrichment
    - Capture Primer Extension program (***Chapter 4, Primer Extension Target Enrichment, Step 3***)
    - Release Primer Hybridization program (***Chapter 4, Primer Extension Target Enrichment, Step 7***)
    - Release Primer Extension program (***Chapter 4, Primer Extension Target Enrichment, Step 10***)
    - Post-PETE Amplification program (***Chapter 4, Primer Extension Target Enrichment, Step 11***)

It is recommended to use a thermocycler with a programmable heated lid. For guidance on lid temperatures, please follow the recommended lid temperatures in this Instructions for Use. If further guidance is needed, please contact ***Roche Technical Support***.

- The following steps should be taken before beginning the workflow:
  - Resuspend the KAPA UDI Primer Mixes (***Chapter 2, Prepare and Store the Reagents, Step 2***)

To verify you are using the most up-to-date version of this Instructions for Use to process your captures, go to ***sequencing.roche.com/support.html***.

Required Equipment, Labware & Consumables

Roche does not assume any responsibility with the use of equipment, labware, and consumables described below. These protocols are designed for use with the specified labware, consumables and calibrated equipment.

Laboratory Equipment

Equipment	Supplier	Catalog No.
Microcentrifuge for 1.5 mL, 0.2 mL tubes and 0.2 mL strip tubes	Multiple Vendors	N/A
Qubit Fluorometer	ThermoFisher	Multiple models
TapeStation	Agilent	Multiple models
Thermocycler with programmable heated lid and adjustable ramp rate (Recommended: Veriti™ Dx 96-well Thermal Cycler, 0.2 mL, Thermo Fisher, catalog number 4452300)	Multiple Vendors	N/A
Plate centrifuge	Multiple Vendors	N/A
Vortex mixer	Multiple Vendors	N/A
MS 3 Vortexer with PCR plate adapter	IKA	4674100
Magnetic Separation Rack or Plate for 1.5 mL tubes	Multiple Vendors	N/A
Magnetic Separation Rack or Plate for 0.2 mL strip tubes (Recommended: 0.2 mL PCR Strip Magnetic Separator, Permagen, catalog number MSR812)	Multiple Vendors	N/A
Pipettes	Multiple Vendors	N/A
Plate Roller	Multiple vendors	N/A

Consumables Available from Roche

For additional information including kit components, please refer to the individual product Instructions for Use.

Description	Package Size/Contents	Catalog No.
KAPA EvoPlus V2 Kit	24 reactions 96 reactions 96 reactions (plated format) 384 reactions	09 420 037 001 09 420 053 001 09 420 339 001 09 420 428 001
KAPA HyperPure Beads	5 mL 30 mL 60 mL 4 x 60 mL 450 mL	08 963 835 001 08 963 843 001 08 963 851 001 08 963 878 001 08 963 860 001
KAPA UDI Primer Mixes, 1-96 KAPA UDI Primer Mixes, 97-192 KAPA UDI Primer Mixes, 193-288 KAPA UDI Primer Mixes, 289-384	96 reactions 96 reactions 96 reactions 96 reactions	09 134 336 001 09 329 838 001 09 329 846 001 09 329 854 001
KAPA Universal Adapter	96 reactions 384 reactions*	09 063 781 001 09 063 790 001
KAPA HyperPETE Reagent Kit	24 reactions 96 reactions	09 211 624 001 09 211 683 001
KAPA HyperPETE Panel	Hereditary Onco, 24 reactions Hereditary Onco, 96 reactions Hereditary Onco, 384 reactions Newborn Screening**, 24 reactions Newborn Screening**, 96 reactions Newborn Screening**, 384 reactions Choice 75Kb, 96 reactions Choice 75Kb, 384 reactions Choice 75Kb, 1536 reactions Choice 75Kb, 10000 reactions Choice 150Kb, 96 reactions Choice 150Kb, 384 reactions Choice 150Kb, 1536 reactions Choice 150Kb, 10000 reactions Choice 250Kb, 96 reactions Choice 250Kb, 384 reactions Choice 250Kb, 1536 reactions Choice 250Kb, 10000 reactions Explore 75Kb, 96 reactions Explore 75Kb, 384 reactions Explore 75Kb, 1536 reactions Explore 75Kb, 10000 reactions Explore 150Kb, 96 reactions Explore 150Kb, 384 reactions Explore 150Kb, 1536 reactions Explore 150Kb, 10000 reactions Explore 250Kb, 96 reactions Explore 250Kb, 384 reactions Explore 250Kb, 1536 reactions Explore 250Kb, 10000 reactions	09 329 315 001 09 329 340 001 09 329 374 001 09 329 382 001 09 329 439 001 09 329 463 001 09 418 741 001 09 418 776 001 09 418 784 001 09 418 792 001 09 418 806 001 09 418 849 001 09 418 857 001 09 418 865 001 09 418 873 001 09 418 903 001 09 418 911 001 09 418 920 001 09 419 004 001 09 419 047 001 09 419 055 001 09 419 063 001 09 419 071 001 09 419 101 001 09 419 110 001 09 419 128 001 09 419 136 001 09 419 179 001 09 419 187 001 09 419 195 001
KAPA HyperCapture Bead Kit	24 reactions 96 reactions	09 075 780 001 09 075 798 001

* Virtual kits
** Not available for sale in the United States. Contact the local Roche affiliate for availability in other regions.

Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
10 mM Tris-HCl, pH 8.0	Multiple Vendors	N/A	N/A
Ethanol, 200 proof (absolute), for molecular biology	Multiple Vendors	N/A	N/A
Qubit dsDNA HS Assay Kit	ThermoFisher	1 kit	Q32851
Qubit Assay Tubes	ThermoFisher	1 package of 500 tubes	Q32856
Tubes: ▪ 0.2 mL PCR tubes ▪ 0.2mL PCR strip tubes ▪ 1.5 mL low bind microcentrifuge tubes	Multiple Vendors	N/A	N/A
Nuclease-free, PCR Grade Water	Multiple Vendors	N/A	N/A
TapeStation High Sensitivity D1000 Reagents	Agilent	1 kit	5067-5585
TapeStation High Sensitivity D1000 ScreenTape	Agilent	7 tapes	5067-5584
TapeStation Parts and Accessories	Agilent	N/A	N/A

Chapter 2.

Prepare and Store the Reagents

This chapter describes the preparation and storage conditions for the following kits:

- KAPA EvoPlus V2 Kit
- KAPA Universal Adapter
- KAPA UDI Primer Mixes
- KAPA HyperPure Beads
- KAPA HyperCapture Bead Kit
- KAPA HyperPETE Reagent Kit
- KAPA HyperPETE Panel

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA EvoPlus V2 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 HyperPure Beads	+2°C to +8°C
KAPA HyperCapture Bead Kit	+2°C to +8°C
KAPA HyperPETE Reagent Kit	-15°C to -25°C
KAPA HyperPETE Panel	-15°C to -25°C

The KAPA HyperCapture Bead Kit and KAPA HyperPure Beads must not be frozen to ensure the highest performance.

Step 2. Resuspension of the KAPA UDI Primer Mixes

For additional information including plate layout and sequencing indexes, please refer to the KAPA UDI Primer Mixes Instructions for Use, catalog # *09 134 336 001*.

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. Spin the KAPA UDI Primer Mixes plate at 280 x ***g*** for 1 minute to ensure the contents are at the bottom of the wells.
3. Before removing the foil cover, please 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 nuclease-free, PCR Grade water.

   A new pipette tip should 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. Visually confirm that 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 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. Spin the plate at 280 *x g* for 30 seconds to ensure the liquid is at the bottom of the well.
9. Thoroughly vortex the plate on an IKA MS 3 Vortexer set to 2000 rpm for 1 minute ensuring all wells are mixed well.
10. Spin the plate at 280 *x g* for 1 minute 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 pre-capture PCR 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 (correctly labelled) tubes or strip tubes for storage.

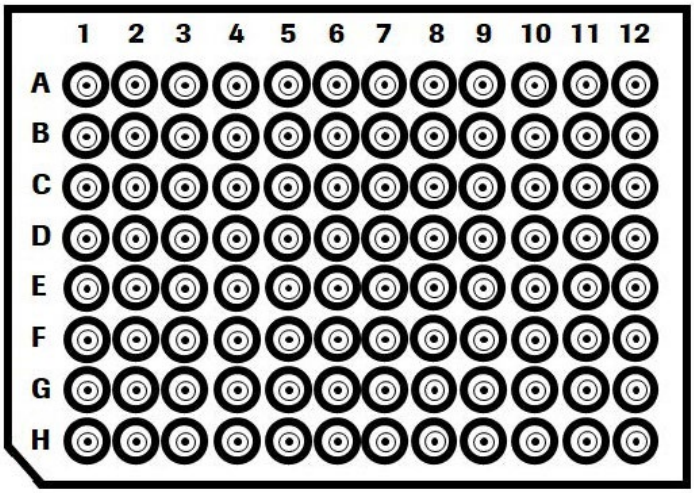


Fig. 2 KAPA UDI Primer Mixes plate layout

Chapter 3.

Library Preparation from DNA

This chapter describes the sample preparation method to generate pre-capture input libraries from high quality gDNA.

Components from the following kits are required:

- KAPA EvoPlus V2 Kit
- KAPA HyperPure Beads
- KAPA Universal Adapters
- KAPA UDI Primer Mixes

Ensure the following is available:

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

Sample Requirements

This workflow was validated with 50 ng of high quality gDNA for input library preparation. The gDNA should be quantified by using the Qubit dsDNA HS Assay Kit. Lower input amounts may not yield equivalent results.

When assembling a master mix for processing samples, always prepare a 10% excess.

It is recommended to always have tubes/plates well labelled throughout this procedure to ensure samples are not lost due to error.

Unless otherwise specified, all 0.2 mL PCR strip tube vortexing steps in this chapter should be performed using the IKA Vortex set to 2400 rpm for 10 seconds or until thoroughly mixed.

Step 1. Enzymatic Fragmentation and A-Tailing

1. Remove the appropriate reagents from storage and allow any frozen reagents to thaw at room temperature.

Component	Thawing Procedure
KAPA EvoPlus V2 Kit	Room temperature then place on ice
KAPA HyperPure Beads	Equilibrate from +2°C to +8°C to room temperature
KAPA Universal Adapters	Room temperature then place on ice
KAPA UDI Primer Mixes	Room temperature then place on ice

2. Add 50 ng of gDNA into a 0.2 mL PCR tube.
3. If needed, adjust the volume in each tube to 35 µL using nuclease-free water or 10mM Tris-HCl, pH 8.0.
4. Place tubes on ice while setting up the Fragmentation and A-Tailing Reaction.
5. Vortex the FragTail ReadyMix briefly. Assemble each Fragmentation and A-Tailing Reaction on ice as follows:

Component	Volume per Individual Sample
50 ng gDNA	35 µL
KAPA FragTail ReadyMix	25 µL
Total	**60 µL**

The KAPA FragTail ReadyMix may contain white precipitates when thawed. Ensure the buffer is thoroughly vortexed at room temperature until the precipitate has been resuspended.

6. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
7. Incubate in a thermocycler pre-cooled to 4°C:

Step	Temperature	Duration
Fragmentation	37°C	18 min
A-Tailing	55°C	30 min
Hold	4°C	∞

Set the thermocycler lid to 65°C.

8. Proceed immediately to the next step.

Step 2. Adapter Ligation

1. Assemble each Ligation Reaction on ice by adding the following directly to the Fragmentation and A-Tailing Reaction in the order shown:

Component	Volume Per Individual Sample
Fragmentation and A-Tailing Reaction	60 µL
KAPA Universal Adapter	5 µL
KAPA DNA Ligation ReadyMix	10 µL
Total	**75 µL**

⚠ The KAPA Ligation ReadyMix contains a high concentration of a crowding agent and is very viscous. Small droplets of the crowding agent may be visible when thawed and require special attention during pipetting. Ensure the ReadyMix is thoroughly vortexed.

⚠ The KAPA Universal Adapter must be added to each well individually prior to the addition of the KAPA Ligation ReadyMix.

2. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
3. Incubate in a thermocycler:

Step	Temperature	Duration
Ligation	20°C	15 min
Hold	4°C	∞

⚠ Set the thermocycler lid to 50°C.

4. Following the incubation, proceed immediately to the next step.

Step 3. Post-Ligation 1.2X Purification using KAPA HyperPure Beads

⚠ Beads are light sensitive and should be protected from light when not in use. Excess light exposure may degrade the buffer and lead to loss of sample.

1. Remove the KAPA HyperPure Beads from cold storage at least 30 minutes prior to use to bring them to room temperature. Vortex beads until thoroughly resuspended right before use.
2. Add 90 µL of KAPA HyperPure Beads to each sample.
3. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and quickly spin down the liquid without pelleting the beads.
4. Incubate at room temperature for 10 minutes.
5. Place the tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6. Carefully remove and discard the supernatant.
7. Keeping the tube(s) on the magnet, wash the pellet by adding 200 µL of 80% ethanol. Incubate at room temperature for ≥ 30 seconds.
8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash (points 7-8).
10. Spin the tube(s) down quickly to bring residual ethanol to the bottom.
11. Place tube(s) on the magnet and remove residual ethanol using a P20 pipette without disturbing the beads.
12. Leave the tube(s) open on the magnet to dry the beads for 3-5 minutes or until all of the ethanol has evaporated.

Do not over dry. Over drying the beads may lead to reduction in yield. Beads are dry when they are not shiny in appearance. Avoid over drying the bead pellet by resuspending before the pellet begins to crack.

13. Remove the tubes from the magnet and resuspend the beads in 20 µL 10 mM Tris-HCl, pH 8.0, by vortexing the samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.
14. Incubate for 2 minutes at room temperature.
15. Quickly spin down the samples, place the tube(s) on a magnet to capture the beads, and incubate until the liquid is clear.

Visually confirm that the beads are pelleted.

16. Transfer 20 µL of the eluate into a new strip tube. The eluate contains the adapter-ligated DNA sample library.

Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples. Samples are indexed in Step 4 and sample confusion will lead to error.

17. Immediately proceed to the next step.

Step 4. Amplification with KAPA UDI Primer Mixes

For guidance on color balancing indices for low-plex pooling of post-capture samples, please refer to the KAPA UDI Primer Mixes Instructions for Use, catalog # *09 134 336 001*.

Each sample must receive a unique Sample Identifier (Primer Mix). Make sure to record the well position of the KAPA UDI Primer Mix used for each sample.

1. Retrieve and thaw (if stored) the KAPA UDI Primer Mixes prepared in *Chapter 2 Step 2*.
2. Spin the plate at 280 *x g* for 30 seconds to collect the contents to the bottom of the wells.
3. If using the KAPA UDI Primer Mixes in a plate, peel off or pierce the foil seal for the appropriate number of wells needed.

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

4. Add 5 µL of a KAPA UDI Primer Mix to the 20 µL of adapter-ligated sample.
5. Add 25 µL of the KAPA HiFi HotStart ReadyMix to the 25 µL of adapter-ligated sample and KAPA UDI Primer Mix.
6. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom of the tube.

If only using a subset of the KAPA UDI Primer Mixes, remove and discard residual primers from the used wells/tubes. Apply a new adhesive foil seal provided in the kit as necessary for plated adapters.

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

7. Apply in a thermocycler with the following conditions:

Step	Temperature	Hold Time at Temperature	Number of Cycles
Initial Denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	8
Primer Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
Hold	4°C	∞	1

Set the thermocycler lid to 105°C.

PCR cycle conditions are recommendations and can be adjusted to achieve the yield requirements in ***Step 7.3***.

8. Proceed immediately to the next step.

Step 5. Post-amplification 1X Purification using KAPA HyperPure Beads

Beads are light sensitive and should be protected from light when not in use. Excess light exposure may degrade the buffer and lead to loss of sample.

1. Remove the KAPA HyperPure Beads from cold storage at least 30 minutes prior to use to bring them to room temperature. Vortex beads until thoroughly resuspended right before use.
2. Add 50 µL of KAPA HyperPure Beads to the PCR product.
3. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and quickly spin down the liquid without pelleting the beads.
4. Incubate at room temperature for 5 minutes.
5. Place the tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6. Carefully remove and discard the supernatant.
7. Keeping the tube(s) on the magnet, wash the pellet by adding 200 µL of 80% ethanol. Incubate at room temperature for ≥ 30 seconds.

80% Ethanol should be prepared fresh daily.

8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash (points 7-8).
10. Spin the tube(s) down quickly to bring residual ethanol to the bottom.
11. Place tube(s) on the magnet and remove residual ethanol using a P20 pipette without disturbing the beads.
12. Leave the tube(s) open on the magnet to dry the beads for 3-5 minutes or until all of the ethanol has evaporated.

Do not over dry. Over drying the beads may lead to reduction in yield. Beads are dry when they are not shiny in appearance. Avoid over drying the bead pellet by resuspending before the pellet begins to crack.

13. Remove the tube(s) from the magnet and resuspend the beads in 25 µL 10 mM Tris-HCl, pH 8.0, by vortexing samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.
14. Incubate for 2 minutes at room temperature.
15. Quickly spin down the samples, place the tube(s) on a magnet to capture the beads, and incubate until the liquid is clear.
16. Transfer the eluate into a new tube. The eluate contains the pre-capture input library.

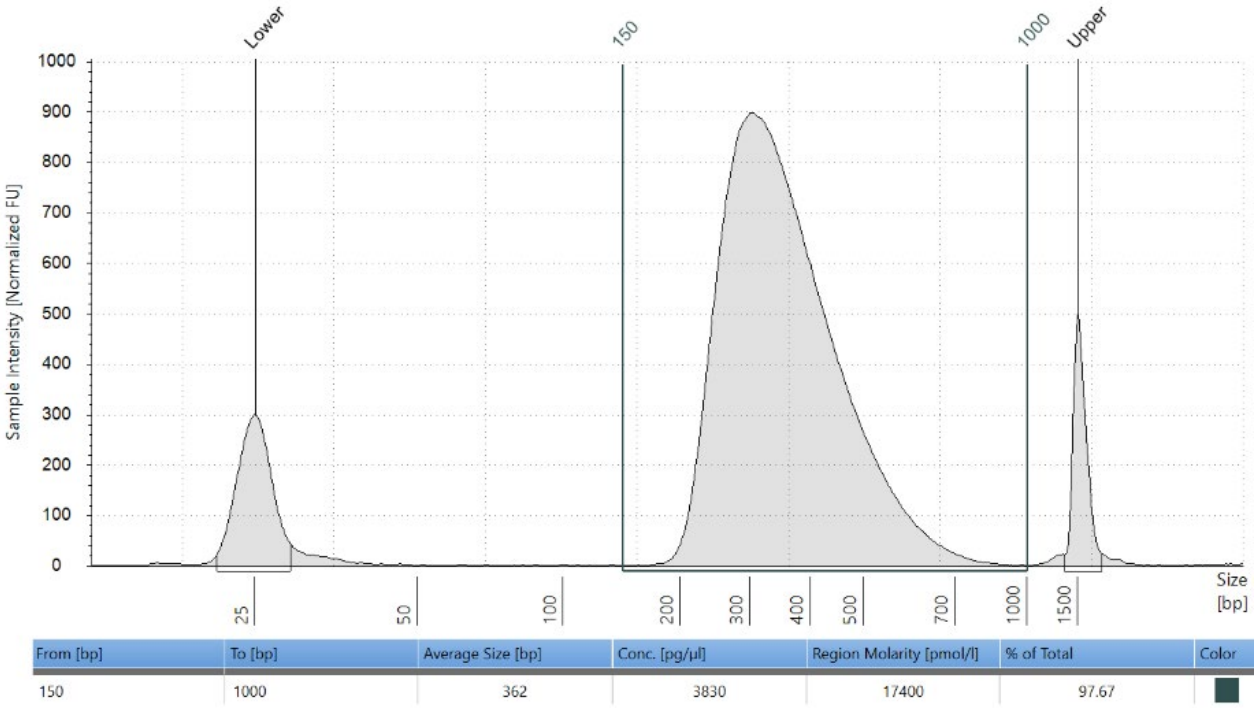
Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

Step 6. Library QC

1. Make a 1:40 dilution of the pre-capture input library by combining 2 µL of library with 78 µL of nuclease-free, PCR Grade water.
2. Use an Agilent TapeStation DNA High Sensitivity D1000 ScreenTape Assay to analyse the appropriate volume of the diluted amplified pre-capture input library (and any controls) as per manufacturer's instructions.
3. The undiluted amplified pre-capture input library should have a concentration of ≥ 35 ng/µL or contain ≥ 875 ng of total DNA in 25 µL in the region of 150-1000 bp on the TapeStation. If the input library contains < 875 ng of total DNA, please refer to the troubleshooting section for guidance.

Multiple pre-capture input libraries can be pooled together into a Multiplex DNA Input Library Pool prior to capture in an optional step, ***Chapter 4 Step 2***. Each Multiplex DNA Input Library Pool must have a final concentration of ≥ 67ng/µL.

4. Pre-capture input libraries should have an average fragment size distribution at ~ 300 bp. *Figure 3* is an example pre-capture input library prepared from gDNA. Sharp peaks may be visible in the region < 150 bp. These peaks correspond to unincorporated primers, primer-dimers or carryover adapter dimers and will not interfere with the capture process.



From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Color
150	1000	362	3830	17400	97.67	

Figure 3. Example TapeStation trace for pre-capture input library prepared from gDNA

5. Store the pre-capture input library at -15°C to -25°C for up to 1 month, or proceed to the next step.

Chapter 4.

Primer Extension Target Enrichment (PETE)

This chapter describes the protocol for target enrichment of the prepared pre-capture input library by primer extension with the KAPA HyperPETE Panels.

Components from the following kits are required:

- KAPA HyperCapture Bead Kit
- KAPA HyperPETE Reagent Kit
- KAPA HyperPETE Catalog or Custom Panels

Ensure the following is available:

- Nuclease-free, PCR Grade water
- Freshly prepared 80% Ethanol

Sample Requirements

This workflow was validated for single-plex primer extension target enrichment and for up to 8-plex primer extension target enrichment. To ensure compatibility with downstream reagents, it is recommended to use the KAPA EvoPlus V2 Kit for gDNA library preparation following the instructions in ***Chapter 3***.

Avoid processing different panels at the same time.

When assembling a master mix for processing samples, always prepare a 10% excess.

Prior to starting the Primer Extension Target Enrichment workflow, retrieve the KAPA HyperCapture Bead Kit and Wash Buffers from storage and allow the reagents to equilibrate to room temperature.

It is recommended to always have tubes/plates well labelled throughout this procedure to ensure samples are not lost due to error.

Unless otherwise specified, all 0.2mL PCR strip tube vortexing steps in this chapter should be performed using the 1 minute preset on an IKA Vortex set to 2400 rpm.

Multiple (up to 8) pre-capture input libraries can be pooled together into a Multiplex DNA Input Library Pool prior to capture in an optional step. Each Multiplex DNA Input Library Pool must have a final concentration of ≥ 67 ng/µL.

Ensure that tube lids are properly closed before each IKA vortexing step.

Step 1. Preparing for Primer Extension Target Enrichment

1. Remove the appropriate reagents from storage and allow any frozen reagents to thaw at room temperature.

Component	Thawing Procedure
KAPA HyperCapture Bead Kit	Equilibrate from +2°C to +8°C to room temperature
KAPA HyperPETE Reagent Kit	Room temperature then place on ice
KAPA HyperPETE Catalog or Custom Panels	Room temperature then place on ice

2. Dilute the Bead Binding Buffer (2.5X) and the Wash & Resuspension Buffer (10X) from the KAPA HyperPETE Reagent Kit to create 1X working solutions. Volumes listed below are sufficient for the processing of one capture sample. Scale up appropriately for more samples.
    a. Prepare the 1X Bead Binding Buffer:

Component	Volume Per Capture Sample
2.5X Bead Binding Buffer	220 µL
Nuclease-free, PCR Grade water	330 µL
Total	**550 µL**

The 2.5X Bead Binding Buffer may contain crystal precipitates when thawed. Ensure the buffer is thoroughly vortexed at room temperature until the precipitate has been resuspended.

    b. Prepare the 1X Wash & Resuspension Buffer:

Component	Volume Per Capture Sample
10X Wash & Resuspension Buffer	70 µL
Nuclease-free, PCR Grade water	630 µL
Total	**700 µL**

Excess buffer volume has already been accounted for in the tables provided.

3. Aliquot 85 µL of the prepared 1X Wash & Resuspension Buffer per capture sample (i.e. for one capture sample use 85 µL and for four capture samples use 340 µL, etc.) to be used in ***Step 7.1 Release Primer Hybridization Master Mix***, ***Step 10.1 Release Extension Master Mix***, and ***Step 12.13 Final Elution***.

   Separation of 1X Wash & Resuspension Buffer for use in the preparation of Master Mixes and Elution from that used in routine wash steps is crucial for ensuring optimal results.

4. Set the 1X working solutions aside at room temperature and proceed to the next step.

Step 2. Prepare the Multiplex DNA Input Library Pool(s) (Optional)

Each Multiplex DNA Input Library Pool must have a final concentration of ≥ 67 ng/µL.

Up to 8 pre-capture input libraries can be pooled into one Multiplex DNA Input Library Pool.

1. If necessary, thaw the indexed pre-capture input libraries (generated in ***Chapter 3***) that will be included in the multiplex capture experiment on ice.
2. Prepare Multiplex DNA Input Library Pool(s) by doing the following:
   a. Combine equal amounts (by mass) of up to 8 uniquely indexed pre-capture input libraries (generated in ***Chapter 3***) to obtain a single pool with a combined minimum volume of 20 µL. This mixture will subsequently be referred to as a 'Multiplex DNA Input Library Pool'.

      Accurate quantification and pipetting are critical to ensure uniquely indexed pre-capture input libraries are pooled in equal amounts. It is important to have equal pooling of pre-capture input libraries in order to obtain an equal number of sequencing reads per library.

   b. Quantify the Multiplex DNA Input Library Pool(s) using Qubit dsDNA HS Assay. The Multiplex DNA Input Library Pool(s) should have a concentration of ≥ 67 ng/µL. If the Multiplex DNA Input Library Pool(s) concentration is < 67 ng/µL, please refer to the ***troubleshooting section*** for guidance.

Step 3. Capture Extension Reaction

1. For capture from individual dual indexed amplified pre-capture input libraries, ensure that 10-15 µL of pre-capture input library contains 500-3000 ng of library. For capture from Multiplex DNA Input Library Pool(s), ensure that 10-15 µL of the library pool contains 1000-1500 ng of library.

   Exact ng input amount can vary between the indicated ranges to keep capture input volume constant when processing multiple samples at the same time.

2. Prepare the Capture Extension Reaction Master Mix:

Component	Volume Per Capture Sample
Capture Extension Reagent (5X)	10 µL
Universal Enhancing Oligo	10 µL
COT Human DNA	10 µL
Capture Panel	5 µL
Nuclease-free, PCR Grade Water	0-5 µL*
Total	**35-40 µL**

* Adjust the volume of water according to the pre-capture input library volume.

3. Add 35-40 µL of the Capture Extension Reaction Master Mix to the pre-capture input libraries or Multiplex DNA Input Library Pool(s) for a final volume of 50 µL.
4. Vortex on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and briefly spin down the tubes to settle the liquid to the bottom of the tube.

5. Incubate in a thermocycler programmed as outlined below:

Step	Temperature	Ramp Rate to Temperature	Hold Time at Temperature
HOLD	20°C	100%	∞
Load Samples and Skip to Next Step			
Denaturation	95°C	100%	2 min
Capture Primer Annealing and Extension	80°C	100%	1 sec
	60°C	2% *	10 min
	65°C	100%	2 min
HOLD	4°C	100%	∞

* The ramp rate will differ on different thermocyclers. Please work with local support to identify alternative protocols. A suitable ramp rate is one which results in a total Capture Extension Reaction time of ~25 minutes (this corresponds with a time of ~10 minutes for the temperature change from 80-60°C, and then a 10 minute incubation at 60°C).

⚠ Set lid temperature to 105°C.

6. During the Capture Primer Annealing and Extension Reaction proceed to ***Step 4. Prepare the Capture Beads***.
7. After the Capture Extension Reaction is completed, proceed to ***Step 5. Bind Capture Extension Reaction to the Capture Beads***.

Step 4. Prepare the Capture Beads

1. Retrieve the Capture Beads from the KAPA HyperCapture Bead Kit at least 30 minutes prior to use to bring them to room temperature.
2. Vortex the Capture Beads for 15 seconds before immediate use to ensure a homogenous mixture.
3. Aliquot 100 µL of beads per capture reaction into a 0.2 mL or a 1.5 mL tube (i.e. for one capture use 100 µL and for four captures use 400 µL, etc.). Beads for one capture can be prepared in a single 0.2 mL tube or up to seven captures can be prepared in a single 1.5 mL tube.
4. Place aliquoted Capture Beads on a magnet to collect the beads. Incubate until the liquid is clear.
5. Remove and discard the supernatant being careful not to disturb the beads.
6. Keeping the tube(s) on the magnet, add 2X the initial volume of beads of 1X Binding Buffer (e.g. for one capture use 200 µL of buffer and for four captures use 800 µL of buffer, etc.).
7. Remove the tube(s) from the magnet and mix thoroughly by vortexing for 15 seconds, follow with a quick spin.
8. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
9. Remove and discard the supernatant being careful not to disturb the beads.
10. Keeping the tube(s) on the magnet, add 2X the initial volume of beads of 1X Binding Buffer (e.g. for one capture use 200 µL of buffer and for four captures use 800 µL of buffer, etc.), for a total of two washes.
11. Remove the tube(s) from the magnet and mix thoroughly by vortexing for 15 seconds, follow with a quick spin.
12. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
13. Remove and discard the supernatant being careful not to disturb the beads.
14. Add half the initial volume of beads of 1X Binding Buffer (e.g. for one capture use 50 µL of buffer and for four capturesuse 200 µL of buffer, etc) to the tube.
15. Remove tube(s) from the magnet and mix thoroughly by vortexing for 15 seconds, follow with a quick spin.
16. If multiple tubes of Capture Beads were prepared, combine all the prepared Capture Beads in one tube, and vortex beads thoroughly for 15 seconds.
17. Aliquot 50 µL of resuspended beads into new PCR strip tubes for each capture.

    Label the tubes with a unique sample ID.

18. Set the prepared Capture Beads aside and proceed to the next step.

Step 5. Bind Capture Extension Reaction to the Capture Beads

1. Transfer 50 µL of Capture Extension Reaction samples into the tubes with the prepared Capture Beads from ***Step 4***.

   Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

2. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.

   Ensure that tube lids are properly closed before each IKA vortexing step.

3. Incubate samples for 10 minutes at room temperature.

   During the incubation, prepare the Release Primer Hybridization Master Mix described below:

Component	Volume Per Capture Sample
Release Hybridization Buffer (5X)	10 µL
Release Panel	10 µL
1X Wash & Resuspension Buffer	30 µL
Total	**50 µL**

   Set aside for use in ***Step 7.1*** and continue with the protocol from ***Step 5.4.***

   Use 1X Wash & Resuspension Buffer prepared and aliquoted in ***Step 1.3***. Do not use the same 1X Wash & Resuspension Buffer as the bead washes to avoid contamination.

   Use new tips when aspirating 1X Wash & Resuspension Buffer to prevent contamination of the 1X Wash & Resuspension Buffer.

4. Vortex samples on the IKA MS 3 Vortexer set to 2400 rpm for 1 minute.

   Ensure that tube lids are properly closed before each IKA vortexing step.

5. Following the incubation, proceed to the next step.

Step 6. Post-Capture Wash

   Use new tips when aspirating 1X Wash & Resuspension Buffer to prevent contamination of the 1X Wash & Resuspension Buffer.

1. Briefly spin down samples.
2. Place samples on a magnet for 1 minute or until the supernatant clears.
3. Remove and discard the supernatant.

   Take care to remove as much of the supernatant as possible without disturbing the beads.

4. Add 120 µL of 1X Wash & Resuspension Buffer to each sample.
5. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.
6. Briefly spin down.
7. Place samples on a magnet for 1 minute or until the supernatant clears.
8. Remove and discard the supernatant.

   Take care to remove as much of the supernatant as possible without disturbing the beads.

9. For a total of two washes, add 120 µL of 1X Wash & Resuspension Buffer to each sample.
10. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute. While the vortexer is running, change gloves.
11. Briefly spin down.
12. Place samples on a magnet for 1 minute or until the supernatant clears.
13. Remove and discard the supernatant.

    Take care to remove as much of the supernatant as possible without disturbing the beads.

14. Following the Capture and Post-Capture Washes, proceed immediately to the next step.

⚠ Do not allow beads to dry out at this step.

Step 7. Release Primer Hybridization

⚠ Keep all Master Mixes on ice.

1. Retrieve the Release Primer Hybridization Master Mix prepared during ***Step 5.3***.
2. Resuspend the capture beads with bound sample(s) in 50 µL of the Release Primer Hybridization Master Mix.
3. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and spin down briefly..

⚠ Ensure that tube lids are properly closed before each IKA vortexing step.

4. Incubate in a thermocycler programmed as outlined below:

Step	Temperature	Hold Time at Temperature
HOLD	55°C	∞
Load Samples		
Release Primer Hybridization	55°C	30 min
HOLD	55°C	∞

⚠ Set the thermocycler lid to 105°C.

5. During the incubation, prepare the Release Primer Extension Master Mix to be used in ***Step 10.1*** and the PCR Master Mix to be used in ***Step 11.1*** with composition as used in the tables below.

Release Primer Extension Master Mix:

Component	Volume Per Capture Sample
Release Extension Reagent (4X)	5 µL
1X Wash & Resuspension Buffer	15 µL
Total	**20 µL**

⚠ Use 1X Wash & Resuspension Buffer prepared and aliquoted in ***Step 1.3***. Do not use the same 1X Wash & Resuspension Buffer as the bead washes to avoid contamination.

⚠ Use new tips when aspirating 1X Wash & Resuspension Buffer to prevent contamination of the 1X Wash & Resuspension Buffer.

PCR Master Mix:

Component	Volume Per Capture Sample
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Universal Illumina Primers (10X)	5 µL
Total	**30 µL**

6. Following the incubation, proceed to the next step.

Step 8. Tube Transfer

1. Prepare and label new PCR tubes.
2. Retrieve the samples from the thermocycler following the completion of the Release Primer Hybridization.
3. Briefly spin down samples.
4. Pipette mix at least 10 times to thoroughly resuspend the beads.

   Do not vortex. Heat from the thermocycler can cause caps to become loose and open during vortexing resulting in sample loss.

5. Transfer the Release Primer Hybridization reaction capture (capture beads+supernatant) to the new tubes.

   Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

Step 9. Post-Release Primer Hybridization Wash

Use new tips when aspirating 1X Wash & Resuspension Buffer to prevent contamination of the 1X Wash & Resuspension Buffer.

1. Briefly spin down samples.
2. Place the new tubes containing the Release Primer Hybridization reaction on a magnet for 1 minute or until the supernatant clears.
3. Remove and discard the supernatant.

   Take care to remove as much of the supernatant as possible without disturbing the beads.

4. Add 120 µL 1X Wash & Resuspension Buffer to each tube.
5. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.

   Ensure that tube lids are properly closed before each IKA vortexing step.

6. Briefly spin down the samples.
7. Place tubes on a magnet for 1 minute or until the supernatant clears.
8. Remove and discard the supernatant.

   Take care to remove as much of the supernatant as possible without disturbing the beads.

9. For a total of two washes, add 120 µL of 1X Wash & Resuspension Buffer to each sample.
10. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute. While the vortexer is running, change gloves.
11. Briefly spin down the samples.
12. Place samples on a magnet for 1 minute or until the supernatant clears.
13. Remove and discard the supernatant.

    Take care to remove as much of the supernatant as possible without disturbing the beads.

14. Proceed immediately to the next step.

    Do not allow beads to dry out at this step.

    Discard any unused 1X Wash & Resuspension Buffer used for washes.

Step 10. Release Primer Extension

1. Retrieve the Release Primer Extension Master Mix prepared during ***Step 7.5***.
2. Resuspend the capture beads with bound sample(s) in 20 µL of the Release Primer Extension Master Mix.
3. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and spin down briefly.

4. Incubate in a thermocycler programmed as outlined below:

Step	Temperature	Hold Time at Temperature
HOLD	50°C	∞
Load Samples		
Release Primer Extension	50°C	2 min
HOLD	4°C	∞

Set the thermocycler lid to 105°C.

5. Briefly spin down the samples.
6. Place samples on a magnet for 1 minute or until supernatant clears.

Do not discard the supernatant. The supernatant contains the enriched library.

7. Proceed immediately to the next step.

Step 11. Post-PETE Amplification

1. Retrieve the PCR Master Mix prepared during ***Step 7.5***.
2. Aliquot 30 µL of the PCR Master Mix into new PCR tubes.

Keep these tubes on ice until required.

3. Transfer 20 µL of the supernatant of the Release Primer Extension reaction to the new tubes containing the prepared PCR Master Mix.

Do not discard the supernatant. The supernatant contains the enriched library.

Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

Take care to remove the supernatant without disturbing the beads.

4. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and spin down briefly.
5. Incubate in a thermocycler programmed as outlined below:

Step	Temperature	Hold Time at Temperature	Number of Cycles
HOLD	20°C	∞	1
Load Samples			
Initial Denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	Cycle number depends on panel size. See Table below.
Primer Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
HOLD	4°C	∞	1

Set the thermocycler lid to 105°C.

PCR cycle numbers based on panel size are to be used as suggestions only. Optimizations may be needed to achieve the yield requirements in ***Step 13.2***.

Panel Capture Target Size	Number of Cycles
~5-10 kb	19
10-30 kb	18
30-100 kb	17
100-150 kb	16
150-200 kb	15
> 200 kb	14

PCR cycle numbers have been verified for the following panels:

Panel	Panel Capture Target Size (kb)	Number of Cycles
Hereditary Onco	203	14
Newborn Screening *	294	14

* Not available for sale in the United States. Contact the local Roche affiliate for availability in other regions.

6. Proceed immediately to the next step.

Step 12. Post-amplification 1X Purification with KAPA HyperPure Beads

Beads are light sensitive and should be protected from light when not in use. Excess light exposure may degrade the buffer and lead to loss of sample.

1. Remove the KAPA HyperPure Beads from cold storage at least 30 minutes prior to use to bring them to room temperature. Vortex beads until thoroughly resuspended right before use.
2. Add 50 µL of KAPA HyperPure Beads to the PCR reaction.
3. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute and quickly spin down the liquid without pelleting the beads.

   Ensure that tube lids are properly closed before each IKA vortexing step.

4. Incubate at room temperature for 5 minutes.
5. Place the tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.
6. Carefully remove and discard the supernatant.
7. Keeping the tube(s) on the magnet, wash the pellet by adding 200 µL of 80% ethanol. Incubate at room temperature for ≥ 30 seconds.

   80% Ethanol should be prepared fresh daily.

8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash (points 7-8).
10. Spin the tube(s) down quickly to bring residual ethanol to the bottom.
11. Place tube(s) on the magnet and remove residual ethanol using a P20 pipette without disturbing the beads.
12. Leave the tube(s) open on the magnet to dry the beads for 3-5 minutes or until all of the ethanol has evaporated.

    Do not over dry. Over drying the beads may lead to reduction in yield. Beads are dry when they are not shiny in appearance. Avoid over drying the bead pellet by resuspending before the pellet begins to crack.

13. Remove the tube(s) from the magnet and resuspend the beads in 25 µL of 1X Wash & Resuspension Buffer by vortexing samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.

    Use 1X Wash & Resuspension Buffer prepared and aliquoted in ***Step 1.3***. Do not use the same 1X Wash & Resuspension Buffer as the bead washes to avoid contamination.

    Use new tips when aspirating 1X Wash & Resuspension Buffer to prevent contamination of the 1X Wash & Resuspension Buffer.

14. Incubate for 2 minutes at room temperature.
15. Quickly spin down the samples, place the tube(s) on a magnet to capture the beads, and incubate until the liquid is clear.

    Visually confirm that the beads are pelleted.

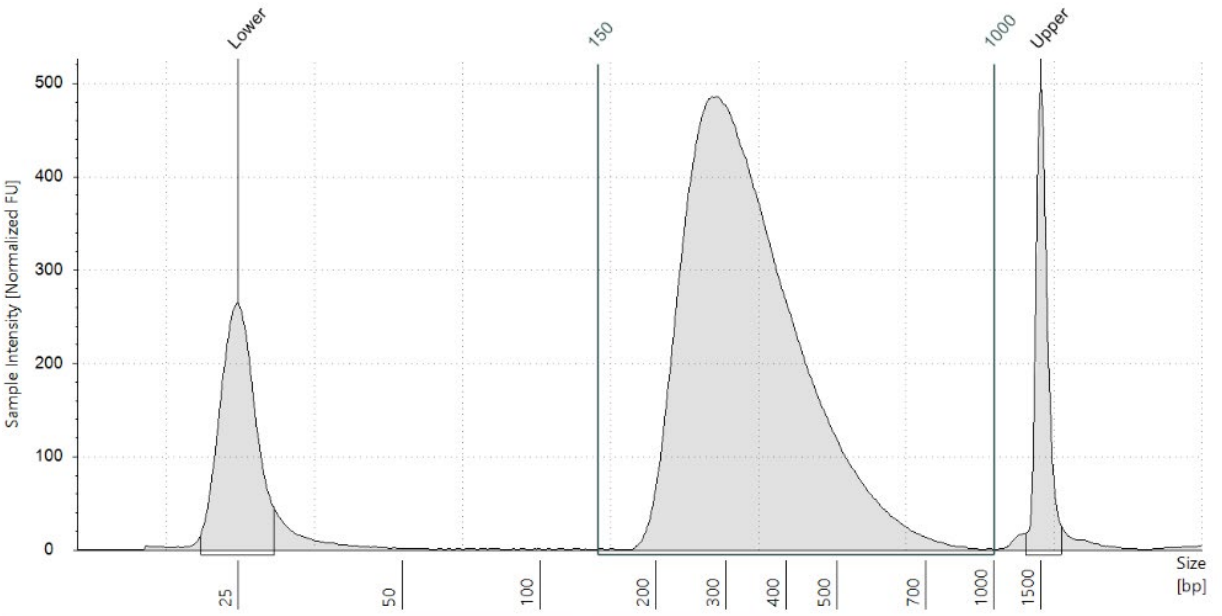
16. Transfer the eluate into a new strip tube. The eluate contains the Primer Extension Target Enrichment library.

    Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

17. Primer Extension Target Enrichment library is ready for sequencing or can be stored at -15°C to -25°C for up to 1 month.

Step 13. Library QC

1. Use an Agilent TapeStation DNA High Sensitivity D1000 ScreenTape Assay to analyse the appropriate volume of the Primer Extension Target Enrichment libraries (and any controls) as per manufacturer's instructions.
2. Primer Extension Target Enrichment libraries should have a region molarity of ≥ 4 nM or 4000 pmol/L in the region of150-1000 bp on the TapeStation. If the Primer Extension Target Enrichment library contains < 4 nM of total DNA, please refer to the ***troubleshooting section*** for guidance.
3. Primer Extension Target Enrichment libraries should have a mean fragment size between 300 and 500 bp. ***Figure 4*** is an example Primer Extension Target Enrichment library prepared from a gDNA pre-capture input library with a mean fragment size of ~348 bp. Sharp peaks may be visible in the region < 150 bp. These peaks correspond to unincorporated primers and primer-dimers and will not interfere with sequencing.



From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Color
150	1000	346	2310	11000	96.27	

Figure 4. Example Primer Extension Target Enrichment library prepared from a gDNA pre-capture input library.

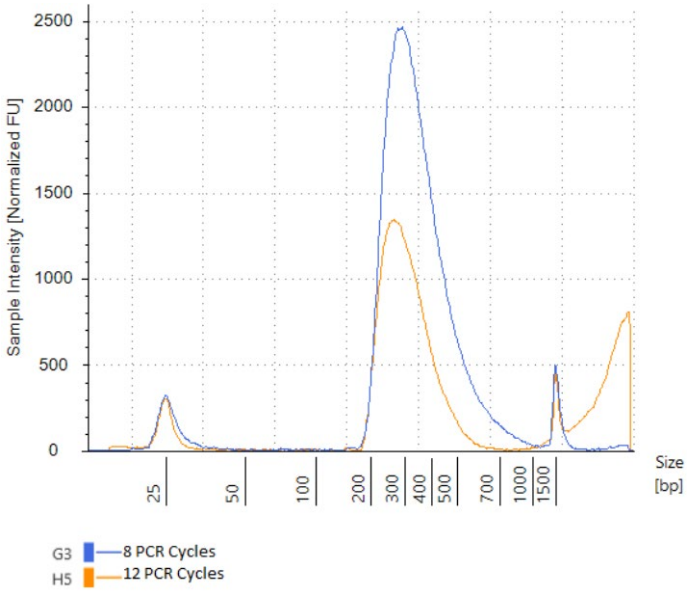
Appendices

Appendix A. 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.html*** for contact information.

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

Observation	Cause(s) / Recommendation(s)
Library Preparation	
Pre-capture input library yield is lower than required for Primer Extension Target Enrichment (Sufficient yield for a single pre-capture input library is is ≥ 35 ng/µL. Sufficient yield for a pre-capture multiplex DNA input library pool is ≥ 67 ng/µL.)	Possible error occurred during library preparation or compromised reagents were used: ▪ Use a previously processed DNA sample as a positive control for library construction and or an evaluated sample library as a positive control for PCR reagents. Poor quality input used or low input used: ▪ Increase the number of PCR cycles during library preparation amplification by 1–3 cycles until yield is sufficient. Poor ligation efficiency: ▪ Ensure that the proper amount of input DNA and KAPA Universal Adapters are used. ▪ Ensure proper ligation incubation time and temperature are used. Poor PCR amplification: ▪ High adapter dimers can inhibit the PCR reaction. Follow proper post-ligation purification steps before PCR. ▪ Ensure that the KAPA UDI Primer Mixes are fully resuspended by carefully following ***Chapter 2 Step 2***. ▪ Ensure that the first PCR reaction is set up properly. ▪ Increase the number of PCR cycles during library preparation amplification by 1–3 cycles until yield is sufficient. Sample loss: ▪ Ensure that KAPA HyperPure Bead purification steps are performed properly. ▪ Do not let KAPA HyperPure Beads overdry.

Fragment distribution (analyzed using the Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000) shows that the average amplified fragment size is not within the size range of 150 to 1000 bp.	Poor fragmentation occurred: ▪ Repeat library preparation. Over-fragmentation: ▪ Ensure that the KAPA FragTail ReadyMix is made and added to samples on ice. ▪ Ensure that the Fragmentation step does not proceed past 18 minutes. Under-fragmentation: ▪ EDTA is present in the sample. Ensure no EDTA is present in the input DNA sample. ▪ Ensure that the Fragmentation Buffer is fully thawed and resuspended. ▪ Ensure proper mixing of viscous Fragmentation Enzyme in the master mix and in the fragmentation reaction with the samples.
Fragment distribution (analyzed using the Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000) is bimodal, with a larger set of fragments observed in addition to the expected set of fragments (Figure A)	Primer depletion due to over-amplification of the pre-capture 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 the Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000 than their actual length in base pairs. The artifact can be resolved by reducing cycle number in the PCR reaction, however the products themselves are perfectly acceptable for use in capture and sequencing, and this artifact will not affect capture performance. The Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000 traces shown in Fig. A show the result of Amplification of the same gDNA pre-capture input library following Amplification with KAPA UDI Primer Mixes for 8 and 12 cycles, respectively. Over-amplification is present in the 12 PCR cycles sample and can be seen as the peak to the right of 1500 bp. The same artifact can appear in Post-PETE PCR amplification.  Figure A. Example TapeStation trace for bimodal fragment distribution.

The Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000 indicates one or more visible sharp peaks that are <150 bp in size.	These peaks, which represent primers, primer-dimers or adapter dimers will not interfere with the capture process.
High adapter dimers in the pre-capture library after the first PCR	Insufficient DNA or poor quality DNA used in the assay: ▪ Ensure proper quantification of the input DNA. ▪ Use the recommended 50 ng DNA input amount if available. Poor ligation efficiency: ▪ Ensure that the proper amount of input DNA and KAPA Universal Adapters are used. ▪ Ensure adapter and Ligation Master Mix is added separately. ▪ Ensure proper ligation incubation time and temperature are used. Poor KAPA HyperPure Bead purification: ▪ Ensure proper volumes are used in the reaction. ▪ Clean the sample again, maintaining the sample to beads ratio from the last cleanup. Note that this additional KAPA HyperPure Bead purification may result in some sample loss and lower Unique Depth.
Primer Extension Target Enrichment	
Primer Extension Target Enrichment library yield is < 4 nM	Low pre-capture input library yield: ▪ Pre-Capture PCR yield should be ≥ 35 ng/µL. See low pre-capture input library yield ▪ Increase the number of PCR cycles during post-PETE amplification by 1–3 cycles until yield is ≥ 4 nM ▪ Repeat with a DNA sample that was previously processed with success. Incorrect washes: ▪ Ensure the washes are performed according to the Instructions for Use. PCR cycle condition not optimized: ▪ Ensure the correct number of cycles are used ▪ Increase the number of PCR cycles during post-PETE amplification by 1–3 cycles until yield is ≥ 4 nM Poor binding with the Capture Beads: ▪ Ensure that the proper beads were used. ▪ Ensure the Capture Panel was used for Capture Extension and the Release Panel was used for Release Hybridization. ▪ Ensure thorough washing and preparation of the capture beads by carefully following *Chapter 4 Primer Extension Target Enrichment Step 3*. Sample loss: ▪ Ensure that the DNA is not accidentally discarded during the enrichment procedure. ▪ Ensure that the Release Extension reaction supernatant was transferred to the PCR Master Mix. ▪ Ensure that KAPA HyperPure Bead purification steps are performed properly. ▪ Do not let KAPA HyperPure Beads overdry.

Sequencing Performance Metrics	
Low Uniformity	Challenging HyperPETE Panel target regions: ▪ Very high or very low GC panel target regions. ▪ CNV or MSI present in panel target regions. Insufficient DNA or poor quality DNA used: ▪ Ensure proper quantification of the input DNA. ▪ Follow the recommended DNA amount for Library Preparation.
Low On Target Rate	Contamination of pre-capture input library or Primer Extension Target Enrichment library: ▪ Ensure a separate UDI Primer Mix is used for each sample. ▪ Ensure samples are kept separate. ▪ Process only one HyperPETE Panel at one time. ▪ Ensure the washes (number of washes and vortexing steps) are performed according to the user guide. ▪ Ensure a clean aliquot of 1x Wash and Resuspension Buffer is used for Master Mixes and elution. ▪ Ensure glove change is performed during both Post Capture and Post Release Primer Hybridization washes. ▪ Ensure new tips are used for every aspiration. Challenging HyperPETE Panel target region: ▪ Highly repetitive target regions. ▪ Target region < 30 kb. See ***Appendix B.*** Primer Extension Target Enrichment not performed correctly: ▪ Ensure that COT DNA or Enhancing Oligo is added to the Capture Extension Reaction. ▪ Ensure washes are performed correctly. It is critical that the correct number of washes are employed and the supernatant is completely removed every time. Incorrect washing can result in higher than expected Primer Extension Target Enrichment library yields in addition to low on target rate. ▪ Ensure no Capture Beads are carried over into PCR. ▪ Ensure the Release Extension supernatant was transferred to the PCR Master Mix and not discarded. ▪ Ensure the Release Hybridization and Release Extension Reactions were performed at the correct temperatures.
Low dedup depth	Insufficient DNA used: ▪ Ensure proper quantification and/or qualification of the input DNA ▪ Follow the recommended DNA amount for Library Preparation and Primer Extension Target Enrichment Insufficient sequencing reads per sample: ▪ Ensure each sample receives the recommended sequencing reads required

High fold 80	Insufficient DNA or poor quality DNA used: ▪ Ensure proper quantification and/or qualification of the input DNA. ▪ Follow the recommended DNA amount for Library. Preparation and Primer Extension Target Enrichment. Insufficient sequencing reads per sample: ▪ Ensure each sample receives the recommended sequencing reads required.
Lower or higher number of sequencing reads than expected	Insufficient DNA used: ▪ Ensure proper quantification of the input DNA. ▪ Follow the recommended DNA input amount for Library Preparation and Primer Extension Target Enrichment. Use the recommended 50 ng of DNA as input if available Insufficient sequencing reads per sample: ▪ Ensure each sample receives the recommended sequencing reads required Improper pooling: ▪ Ensure the ratio of pooling volumes matches the ratio of read requirements between pooling samples ▪ Ensure pooling volumes are between 2 µL and 20 µL
Incorrect variant calling in control samples	Contamination of the pre-capture input library: ▪ Ensure new tips are used for every aspiration ▪ Ensure a separate UDI Primer Mix is used for each sample ▪ Ensure samples are kept separate
High Duplicate rates	Reduction in Pre-Capture and/or Post-Capture PCR cycles may reduce duplicate rates. Take the following points into consideration when altering cycle numbers: ▪ Ensure the recommended amount of input material is used for Library Preparation. Higher input amounts lead to lower duplicate rates. ▪ Ensure the recommended amount of pre-capture input library is used for Primer Extension Target Enrichment. Higher input amounts lead to lower duplicate rates.

Appendix B. Panel Size & Performance Considerations

1. Small Panels

The use of small DNA panels (< 30 kb) may result in more variable and lower on-target rate performance (< 60%) compared to panels larger than 30 kb. This is due to the added level of enrichment needed to achieve higher on-target rates for these smaller panels.

Appendix C. Limited Warranty and Further Liability Limitation

1. Limited Warranty

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B. Under no circumstances shall Roche's liability to Customer exceed the amount paid by Customer for the Services and Products to Roche. Roche will bear all reasonable shipping costs if service is re-performed at Roche or the Products are replaced. This warranty does not apply to any defect or nonconformance caused by (i) the failure by Customer to provide a suitable storage, use, or operating environment for the Materials or Customer's submission of substandard quality Materials or contaminated or degraded Materials to Roche, (ii) Customer's use of non-recommended reagents, (iii) Customer's use of the Products, Materials or Data for a purpose or in a manner other than that for which they were designed, (iv) the failure by Customer to follow Roche's published protocols; or (v) as a result of any other abuse, misuse or neglect of the Products, Materials or Data by Customer. This warranty applies only to Customer and not to third parties.

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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.

2. Further Liability Limitation

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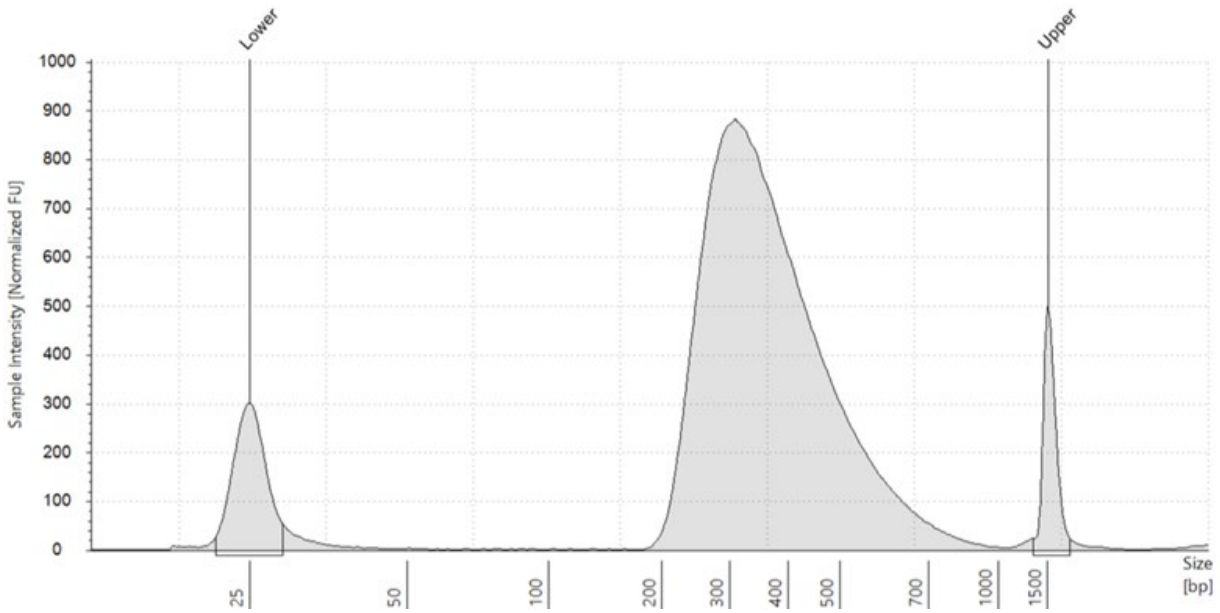
Go to ***sequencing.roche.com/support*** for contact information.

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

Appendix D. Comparative Electrophoretic Device Images (Agilent BioAnalyzer 2100 and Agilent TapeStation 4200)

Pre-Capture Electrophoretic Images

A.



B.

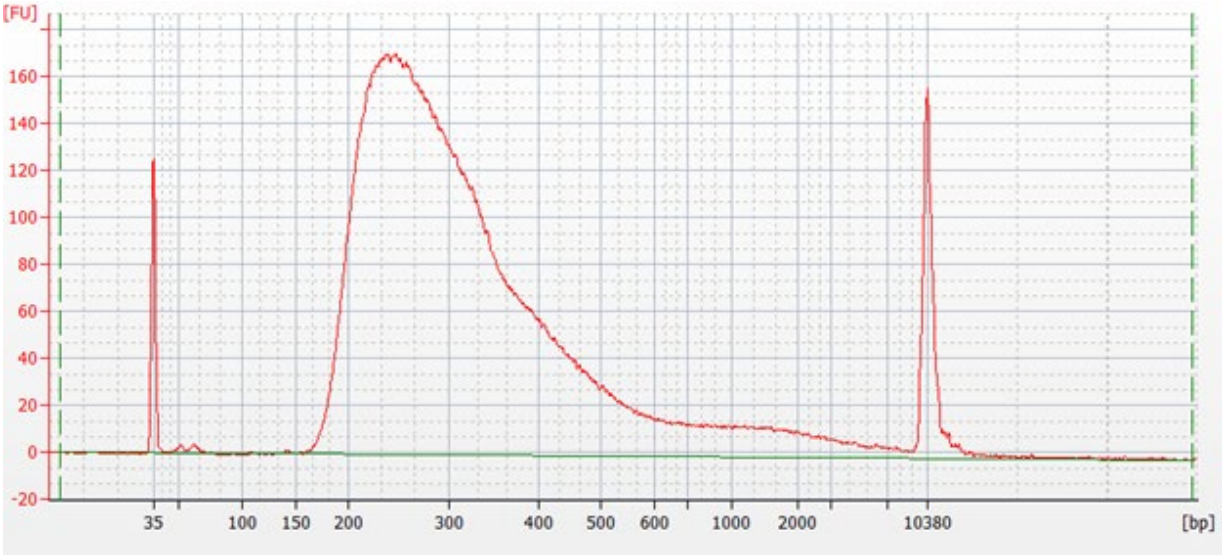
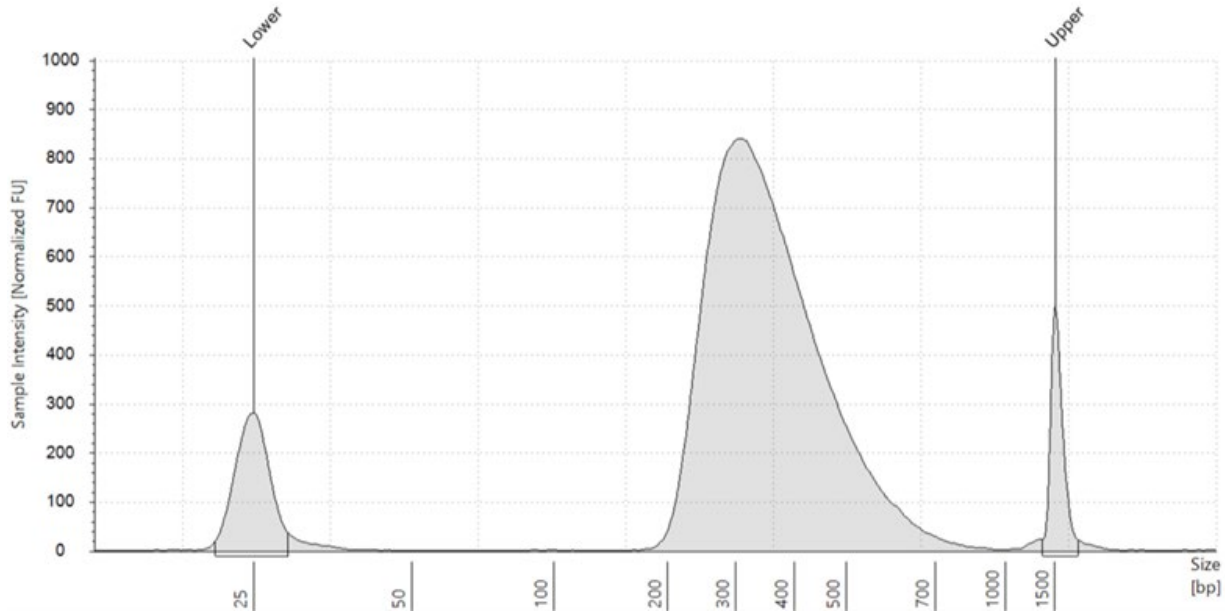


Figure 1: A) Example of an amplified pre-capture sample library analyzed using the Agilent TapeStation 4200. B) Example of an amplified pre-capture sample library analyzed using the Agilent BioAnalyzer 2100.

Appendix D. Comparative Electrophoretic Device Images (Agilent BioAnalyzer 2100 and Agilent TapeStation 4200)

Post-Capture Electrophoretic Images

A.



B.

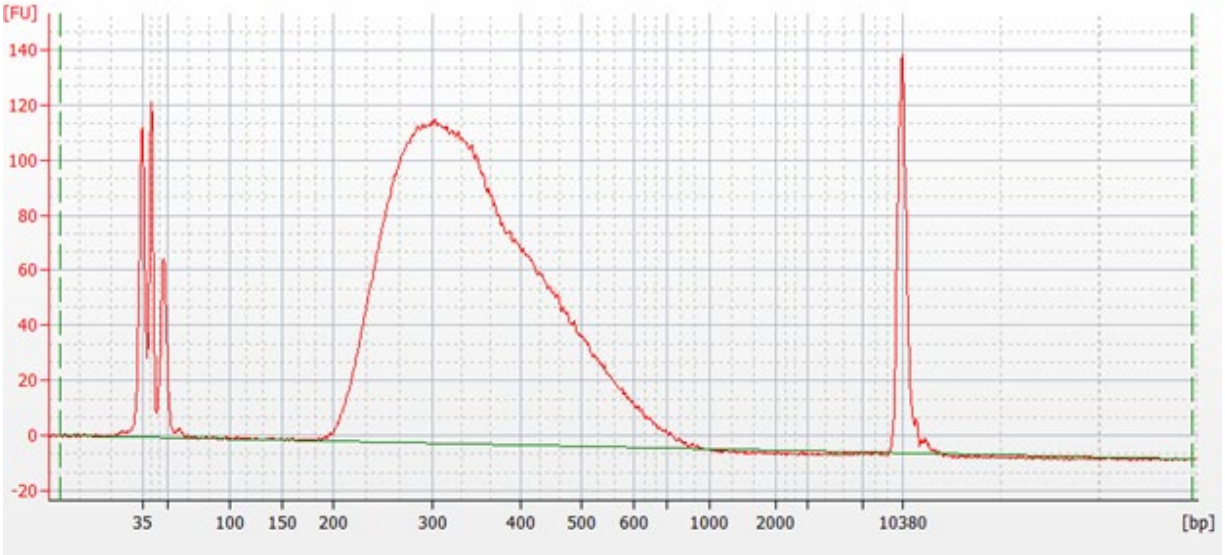


Figure 2: A) Example of an amplified post-capture sample library analyzed using the Agilent TapeStation 4200. B) Example of an amplified post-capture sample library analyzed using the Agilent BioAnalyzer 2100.

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