

INSTRUCTIONS FOR USE OF

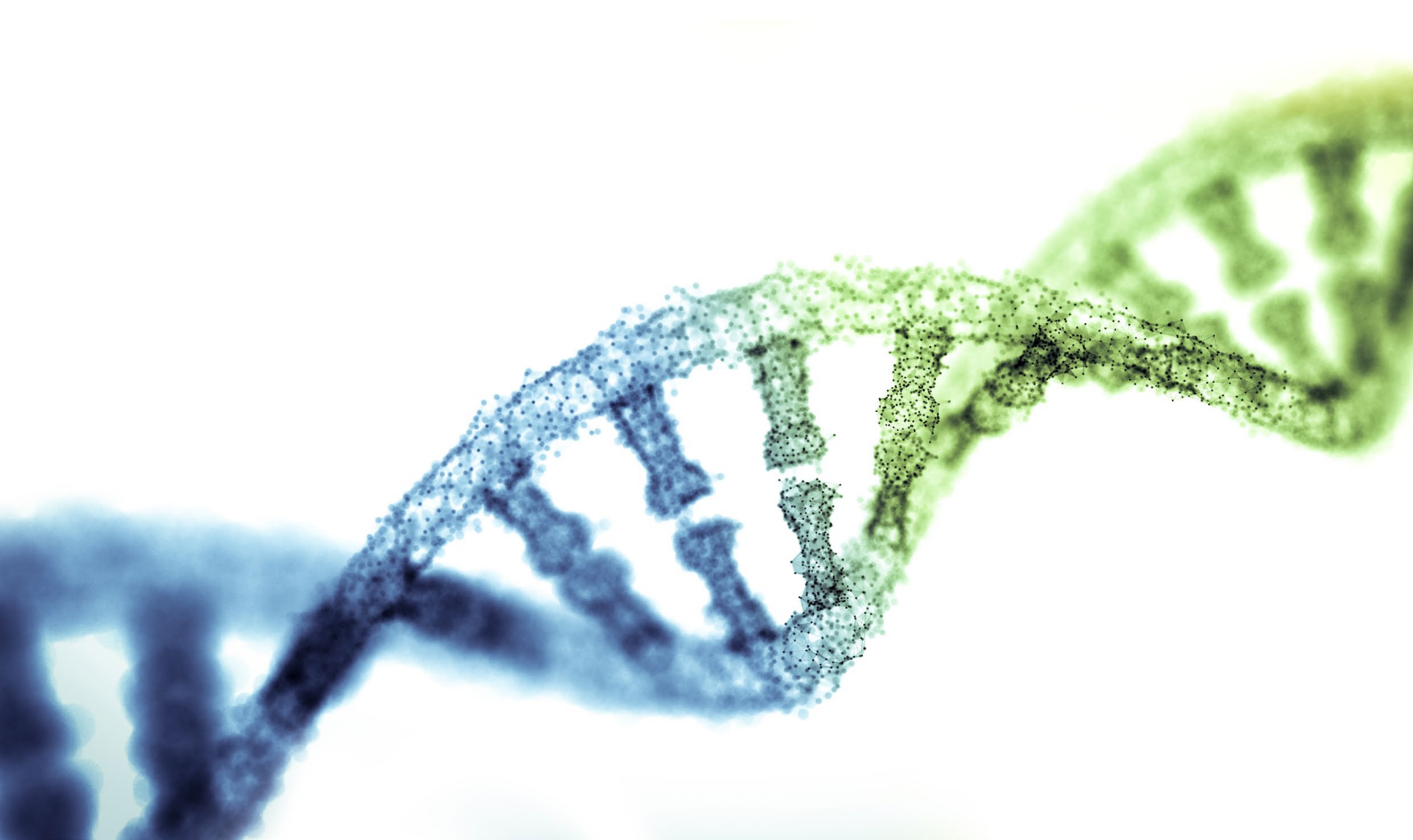
KAPA HyperPETE Somatic Plasma cfDNA Workflow v1.1

INSTRUCTIONS FOR USE WITH:

KAPA HYPERPETE HOT SPOT PANEL, KAPA HYPERPETE PAN
CANCER PANEL, KAPA HYPERPETE CHOICE,
AND KAPA HYPERPETE EXPLORE PRIMER PANELS

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

February 2024, Version 1.1



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 Somatic Plasma cfDNA Preparation enables primer extension targeted enrichment of Roche-defined (catalog panels) and customer defined regions of the genome for somatic cfDNA 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 cfDNA primary target regions up to 250 kb.

The [KAPA HyperPETE Hot Spot Panel](#) is a 40 Kb capture target panel covering 37 targets from the genomic 'hot spot' regions that are frequently mutated in human cancer genes. It is optimized to deliver high uniformity and specificity from low quality, low quantity FFPET DNA and cfDNA samples.

The [KAPA HyperPETE Pan Cancer Panel](#) is a 302 Kb capture target panel covering 1324 target regions, 86 cancer related genes and 190 MSI loci relevant to somatic oncology research applications. The panel has been optimized to deliver high uniformity and specificity from low quality, low quantity FFPET DNA and cfDNA samples.

[KAPA HyperPETE Choice Panels and KAPA HyperPETE Explore Panels](#) enable custom designs up to 250Kb for DNA inputs and 50Kb for RNA inputs.

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

Formatting changes to align with updated Roche branding. Grammatical and typo corrections made. References to Navify Mutation Caller removed as the product was retired. Storage temperature labeling has been updated to reflect ranges as per Roche product labels. Removed reference to specific sequencer instruments and associated reagents.

Ordering Information

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

Trademarks

HYPERDESIGN, KAPA, KAPA HYPERPETE, KAPA HYPERPREP, and KAPA HYPERPURE are trademarks of Roche. All other product names and trademarks are the property of their respective owners.



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.

Copyright

©2015-2024 Roche Sequencing Solutions, Inc. All Rights Reserved.
Roche Sequencing Solutions, Inc.
4300 Hacienda Drive
Pleasanton, CA 94588 USA

Editions

Version 1.0, July 2021; Version 1.1, February 2024

Restrictions and Liabilities

This document is provided “as is” and Roche Sequencing Solutions, Inc. (Roche) assumes no responsibility for any typographical, technical, or other inaccuracies in this document. Roche reserves the right to periodically change information that is contained in this document; however, Roche makes no commitment to provide any such changes, updates, enhancements, or other additions to this document to you in a timely manner or at all.

OTHER THAN THE LIMITED WARRANTY CONTAINED IN THIS USER GUIDE, ROCHE MAKES NO REPRESENTATIONS, WARRANTIES, CONDITIONS OR COVENANTS, EITHER EXPRESS OR IMPLIED (INCLUDING WITHOUT LIMITATION, ANY EXPRESS OR IMPLIED WARRANTIES OR CONDITIONS OF FITNESS FOR A PARTICULAR PURPOSE, NON-INFRINGEMENT, MERCHANTABILITY, DURABILITY, TITLE, OR RELATED TO THE PERFORMANCE OR NON-PERFORMANCE OF ANY PRODUCT REFERENCED HEREIN OR PERFORMANCE OF ANY SERVICES REFERENCED HEREIN).

This document might contain references to third party sources of information, hardware or software, products, or services and/or third party web sites (collectively the “Third-Party Information”). Roche does not control, and is not responsible for, any Third-Party Information, including, without limitation the content, accuracy, copyright compliance, compatibility, performance, trustworthiness, legality, decency, links, or any other aspect of Third-Party Information. The inclusion of Third-Party Information in this document does not imply endorsement by Roche of the Third-Party Information or the third party in any way.

Roche does not in any way guarantee or represent that you will obtain satisfactory results from using Roche products as described herein. The only warranties provided to you are included in the [Limited Warranty](#) enclosed with this guide. You assume all risk in connection with your use of Roche products.

Roche is not responsible nor will be liable in any way for your use of any software or equipment that is not supplied by Roche in connection with your use of Roche products.

Conditions of Use

You are responsible for understanding and performing the protocols described within. Roche does not guarantee any results you may achieve. These protocols are provided as Roche’s recommendations based on its use and experience with Roche products.

Use Restrictions

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

01

Instructions for Use	2
----------------------	---

Storage and Stability	2
Application	2
Warnings and Precautions	2
Waste Handling	2
Changes to Previous Version	2
Ordering Information	2
Trademarks	2
Contact and Support	3
Copyright	3
Editions	3
Restrictions and Liabilities	3
Conditions of Use	3
Use Restrictions	3
Table of Contents	4

02

Preface	7
---------	---

Regulatory Disclaimer	7
Contact Information	7
Conventions Used in This Manual	8

03

Chapter 1. Before You Begin	9
-----------------------------	---

Overview of the KAPA HyperPETE Somatic Plasma cfDNA Workflow v1.1	10
Protocol Information & Safety	11
Terminology	11
Prepare the Following Reagents and Equipment	11
Required Equipment, Labware & Consumables	12

04

Chapter 2. Store and Prepare the Reagents 15

Step 1. Store the Reagent Kits	16
Step 2. Resuspension of the KAPA UDI Primer Mixes	16

05

Chapter 3. Library Preparation from cfDNA 18

Sample Requirements	19
Step 1. End Repair and A-Tailing	19
Step 2. Adapter Ligation	20
Step 3. Post-Ligation 0.8X Purification using KAPA HyperPure Beads	21
Step 4. Amplification with KAPA UDI Primer Mixes	21
Step 5. Post-amplification 1X Purification using KAPA HyperPure Beads	22
Step 6. Library QC	23

06

Chapter 4. Primer Extension Target Enrichment (PETE) 25

Sample Requirements	26
Step 1. Preparing for Primer Extension Target Enrichment	26
Step 2. Capture Extension Reaction	27
Step 3. Prepare the Capture Beads	28
Step 4. Bind Capture Extension Reaction to the Capture Beads	28
Step 5. Post-Capture Wash	29
Step 6. Release Primer Hybridization	30
Step 7. Tube Transfer	31
Step 8. Post-Release Primer Hybridization Wash	31
Step 9. Release Primer Extension	32
Step 10. Post-PETE Amplification	32
Step 11. Post-amplification 1X Purification with KAPA HyperPure Beads	33
Step 12. Library QC	34

07

Appendices	35
------------	----

Appendix A. Troubleshooting	36
Appendix B. Panel Size & Performance Considerations	41
Appendix C. Limited Warranty and Further Liability Limitation	42

Preface

Regulatory Disclaimer

For Research Use Only.

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.
<i>Italic type, blue</i>	Highlights a resource in a different area of this manual or on a website.
<i>Italic type</i>	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 cell-free DNA (cfDNA) shotgun libraries using the KAPA HyperPETE Panels. Specifically, these Instructions for Use provide a protocol for the workflow outlined in [Figure 1](#) using the KAPA HyperPrep and KAPA HyperPETE Kits. The output of this protocol are enriched cfDNA libraries that can be directly sequenced using an Illumina sequencing instrument.

The [KAPA HyperPETE Kit](#) and Workflow provides:

- A fast and easy capture workflow based on primer extension reactions that can generate sequencing ready 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 HyperPrep Kit
 - 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 Somatic Plasma cfDNA Workflow

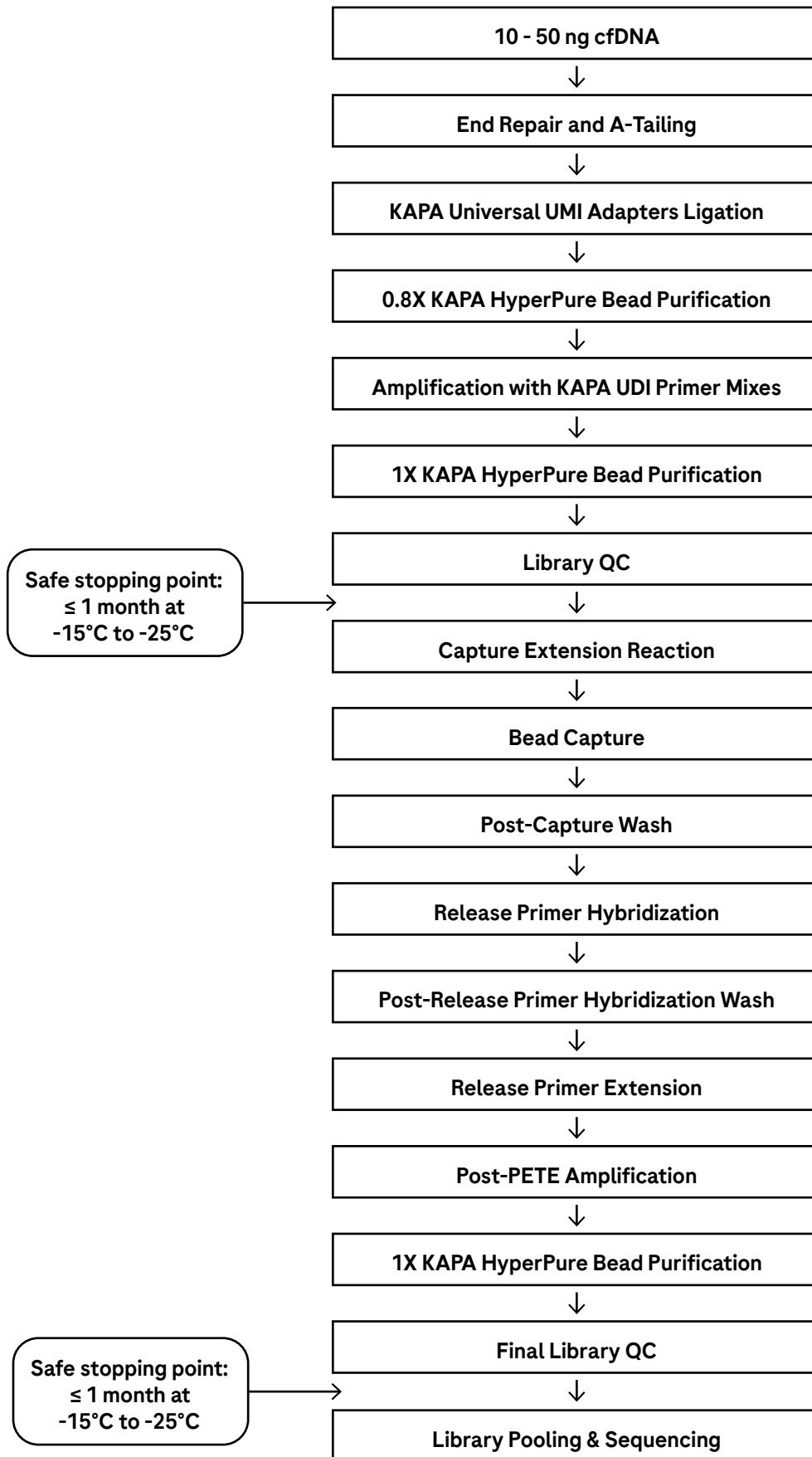


Fig. 1 Somatic Plasma cfDNA HyperPETE Workflow v1.1

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 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 Hot Spot* and *KAPA HyperPETE Pan Cancer* panels.

Sample Library: The initial shotgun library generated from cfDNA by end repair, A-Tailing and 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 cfDNA by end repair, a-tailing 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 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
 - End Repair and A-Tailing Program ([Chapter 3, Step 1](#))
 - Adapter Ligation program ([Chapter 3, Step 2](#))
 - Amplification with KAPA UDI Primer Mixes program ([Chapter 3, Step 4](#))
 - Primer Extension Target Enrichment
 - Capture Primer Extension program ([Chapter 4, Step 2](#))
 - Release Primer Hybridization program ([Chapter 4, Step 6](#))
 - Release Primer Extension program ([Chapter 4, Step 9](#))
 - Post-PETE Amplification program ([Chapter 4, Step 10](#))



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, 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 HyperPrep Kit	8 reactions*	07 962 312 001
	24 reactions	07 962 347 001
	96 reactions	07 962 363 001
KAPA HyperPure Beads	5 mL	08 963 835 001
	30 mL	08 963 843 001
	60 mL	08 963 851 001
	4 x 60 mL	08 963 878 001
	450 mL	08 963 860 001
KAPA UDI Primer Mixes, 1-96	96 reactions	09 134 336 001
KAPA Universal UMI Adapter	96 reactions	09 329 862 001
	384 reactions**	09 329 889 001
KAPA HyperPETE Reagent Kit	24 reactions	09 211 624 001
	96 reactions	09 211 683 001
KAPA HyperPETE Panel	Pan Cancer, 24 reactions	09 329 161 001
	Pan Cancer, 96 reactions	09 329 196 001
	Pan Cancer, 384 reactions	09 329 226 001
	Hot Spot, 24 reactions	09 329 234 001
	Hot Spot, 96 reactions	09 329 277 001
	Hot Spot, 384 reactions	09 329 307 001
	Choice 75KB, 96 reactions	09 418 741 001
	Choice 75KB, 384 reactions	09 418 776 001
	Choice 75KB, 1536 reactions	09 418 784 001
	Choice 75KB, 10000 reactions	09 418 792 001
	Choice 150KB, 96 reactions	09 418 806 001
	Choice 150KB, 384 reactions	09 418 849 001
	Choice 150KB, 1536 reactions	09 418 857 001
	Choice 150KB, 10000 reactions	09 418 865 001
	Choice 250KB, 96 reactions	09 418 873 001
	Choice 250KB, 384 reactions	09 418 903 001
	Choice 250KB, 1536 reactions	09 418 911 001
	Choice 250KB, 10000 reactions	09 418 920 001
	Explore 75KB, 96 reactions	09 419 004 001
	Explore 75KB, 384 reactions	09 419 047 001
	Explore 75KB, 1536 reactions	09 419 055 001
	Explore 75KB, 10000 reactions	09 419 063 001
	Explore 150KB, 96 reactions	09 419 071 001
	Explore 150KB, 384 reactions	09 419 101 001
	Explore 150KB, 1536 reactions	09 419 110 001
	Explore 150KB, 10000 reactions	09 419 128 001
Explore 250KB, 96 reactions	09 419 136 001	
Explore 250KB, 384 reactions	09 419 179 001	
Explore 250KB, 1536 reactions	09 419 187 001	
Explore 250KB, 10000 reactions	09 419 195 001	
KAPA HyperCap Bead Kit	24 reactions	09 075 780 001
	96 reactions	09 075 798 001

* 8 reaction pack sizes are being phased out. Only supported until 31 December 2024.

** Virtual kits

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: <ul style="list-style-type: none"> • 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 HyperPrep Kit
- KAPA Universal UMI 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 HyperPrep Kit	-15°C to -25°C
KAPA Universal UMI 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 HyperCapture Bead Kit	+2°C to +8°C
KAPA HyperPure Beads	+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 dispensed 10 μ L 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.

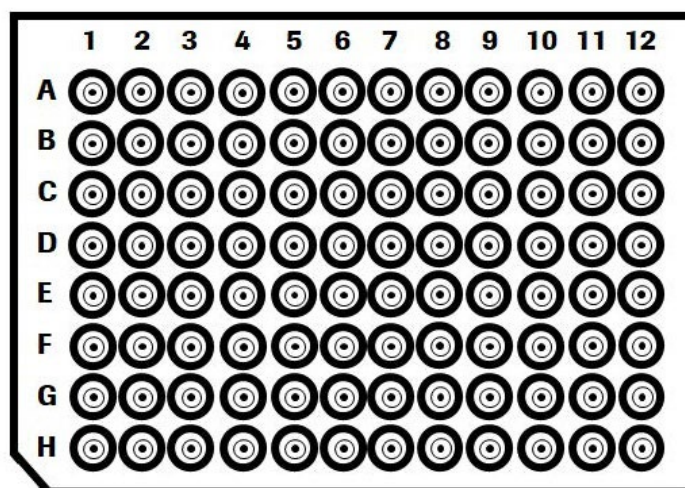


Fig. 2 KAPA UDI Primer Mixes plate layout

Chapter 3.

Library Preparation from cfDNA

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

Components from the following kits are required:







- KAPA HyperPrep Kit
- KAPA HyperPure Beads
- KAPA Universal UMI 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 for pre-capture input library preparation of cfDNA. When available it is recommended to input up to 50 ng of cfDNA into the workflow for best performance. However, if 50 ng is unavailable, use the total available cfDNA down to 10 ng. Performance cannot be guaranteed with lower than 10 ng cfDNA inputs into library preparation. The cfDNA should be quantified by using the Qubit dsDNA HS Assay Kit.

-  Quality of cfDNA requires proper blood collection methods and careful handling of plasma samples. The user is responsible for ensuring that the selected collection tube is used according to its manufacturer's protocol. Ensure that the blood collection tube contains the anticoagulant EDTA. The anticoagulant heparin should not be used as it will interfere with the library preparation. To minimize cellular lysis which releases high molecular weight genomic DNA that can interfere with library generation, separate and collect the plasma soon after blood collection.
-  Please note the Library Amplification Primer Mix provided in the KAPA HyperPrep Kit will not be used in the library preparation protocol.
-  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 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.
-  Ensure that tube lids are properly closed before each IKA vortexing step.


Step 1. End Repair and A-Tailing

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

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

2. Add 10 to 50 ng of cfDNA into a 0.2 mL PCR tube.
3. If needed, adjust the volume in each tube to 50 µL using nuclease-free, PCR Grade water.
4. Place tubes on ice while setting up the End Repair and A-tailing Master Mix.
5. Prepare the End Repair and A-tailing Master Mix:

Component	Volume Per Individual Sample
KAPA End Repair & A-Tailing Buffer	7 µL
KAPA HyperPrep ERAT Enzyme Mix	3 µL
Total	10 µL

-  The KAPA End Repair & A-Tailing Buffer may contain white precipitates when thawed. Ensure the buffer is thoroughly vortexed at room temperature until the precipitate has been resuspended.

6. Add 10 μL of the End Repair and A-Tailing Master Mix to each sample on ice, for a final volume of 60 μL .
7. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
8. Incubate in a thermocycler:

Temperature	Duration
20°C	30 min
65°C	30 min
4°C	∞



Set the thermocycler lid to 85°C.

9. Proceed immediately to the next step.

Step 2. Adapter Ligation

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

Component	Volume Per Individual Sample
KAPA Ligation Buffer	30 μL
KAPA DNA Ligase	10 μL
Total	40 μL



The KAPA Ligation Buffer 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 buffer is thoroughly vortexed at room temperature until the droplets have been resuspended.

2. Add 10 μL of KAPA Universal UMI Adapter to the 60 μL of End Repaired and A-tailed product. Mix thoroughly and briefly spin.



The KAPA Universal Adapter must be added to each well individually prior to the addition of the Ligation master mix. Addition of the KAPA Universal Adapter to the Ligation master mix will cause formation of adapter dimers.

3. Add 40 μL of the Ligation Master Mix to each sample.
4. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
5. Incubate in a thermocycler:

Temperature	Duration
20°C	15 min
4°C	∞



Set the thermocycler lid to 50°C.

6. 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 88 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.



The total volume will be 198 μL . Care should be taken to ensure no spillover during mixing if using 0.2mL tubes.

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.



80% ethanol should be prepared fresh daily.

8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash for a second 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 10mM 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.



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 Mixes 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 $280 \times g$ 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 μ L of a KAPA UDI Primer Mixes to the 20 μ L of adapter-ligated sample.
5. Add 25 μ L of the KAPA HiFi HotStart ReadyMix to the 25 μ L of adapter-ligated sample and KAPA UDI Primers Mixes, bringing the total volume to 50 μ L.
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 use 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	Cycle number depends on cfDNA input amount. 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.

DNA Input	Number of PCR Cycles
50 ng	6
10 ng	9

 PCR cycle conditions are recommendations and can be adjusted to achieve the yield requirements in [Step 6.3](#).

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 μ L 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 μ L of 80% ethanol. Incubate at room temperature for \geq 30 seconds.



80% ethanol should be prepared fresh daily.

8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash for a second wash (points 7-8).
10. Spin the tubes down quickly to bring residual ethanol to the bottom.
11. Place tubes on the magnet and remove residual ethanol using a P20 pipette without disturbing the beads.
12. Leave the tubes 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 25 μ L 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.



Visually confirm that the beads are pelleted.

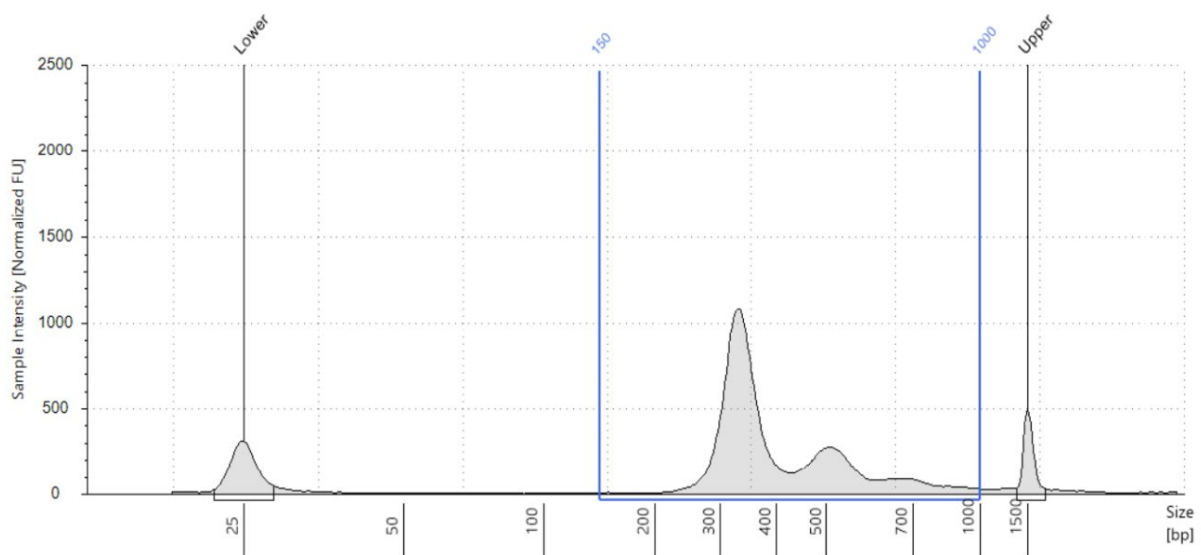
16. Transfer the eluate into a new strip tube. The eluate contains the pre-capture input library.



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 μ L of library with 78 μ L 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 ≥ 35 ng/ μ L or contain ≥ 875 ng of total DNA in 25 μ L in the region of 150-1000 bp on the TapeStation. If the pre-capture input library contains < 875 ng of total DNA, please refer to the troubleshooting section for guidance.
4. Pre-capture input libraries should have a primary peak between 300 and 400 bp with possibly several minor peaks > 400 bp in the region of 150-1000 bp on the TapeStation. [Figure 3](#) is an example pre-capture input library prepared from cfDNA with a mean fragment size of ~ 416 bp and a primary peak between 300-400 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 the capture process.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/ul]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
150	1000	416	2750	11200	95.00		■

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

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. To ensure compatibility with downstream reagents, it is recommended to use the KAPA HyperPrep Kit for cfDNA 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 2400rpm.
-  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.

- 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 6.1 Release Primer Hybridization Master Mix](#), [Step 9.1 Release Extension Master Mix](#); and [Step 11.13 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.

- Set the 1X buffers aside at room temperature and proceed to the next step.

Step 2. Capture Extension Reaction

- For single-plex capture, 10 -15 μL of the pre-capture input library must contain a minimum of 500 ng. Up to 3000 ng may be used for single-plex capture, without exceeding 15 μL of library volume.



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

- Prepare the Capture Extension Reaction Master Mix:

Component	Volume Per Capture Sample
Capture Extension Reagent (5X)	10 μL
Universal Enhancing Oligos	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 library volume.

- Add 35-40 μL of the Capture Extension Reaction Master Mix to the pre-capture libraries for a final volume of 50 μL .
- Vortex samples 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.
- 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			
Denaturing	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).






Lid temperature should be set to 105°C.

- During the primer annealing and extension incubation proceed to [Step 3. Prepare the Capture Beads](#).
- After the Capture Extension incubation is completed, proceed to [Step 4. Bind Capture Extension Reaction to the Capture Beads](#).

Step 3. 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 μ L of beads per capture reaction into a 0.2 mL or a 1.5 mL tube (i.e. for one capture use 100 μ L and for four captures use 400 μ L, 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 μ L of buffer and for four captures use 800 μ L 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 μ L of buffer and for four captures use 800 μ L 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 μ L of buffer and for four captures use 200 μ L 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 μ L of resuspended beads into new PCR strip tubes for each capture.
 -  Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.
18. Set the prepared Capture Beads aside and proceed to the next step.

Step 4. Bind Capture Extension Reaction to the Capture Beads

1. Transfer 50 μ L of Capture Extension Reaction samples from Step 2 into the tubes with the prepared Capture Beads from [Step 3](#).
 -  Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.
2. Place samples on an IKA MS 3 Vortexer set to 2400 rpm and vortex 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 in the next page:

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



Set aside for use in [Step 6.1](#) and continue with the protocol from [Step 4.4](#).



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.

- 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.

- Following the incubation, proceed to the next step.

Step 5. Post-Capture Wash



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

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



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

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



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

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



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

- Following the bead washes, proceed immediately to the next step.



Do not allow beads to dry out at this step.

Step 6. Release Primer Hybridization



Keep all Master Mixes on ice

1. Retrieve the Release Primer Hybridization Master Mix prepared during [Step 4.3](#).
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.
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 9.1](#) and the PCR Master Mix to be used in [Step 10.1](#) with composition as described 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 ReadyMix (2X)	25 μL
Universal Illumina Primers (10X)	5 μL
Total	30 μL

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

Step 7. 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 beads+supernatant) to the new tubes.



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

Step 8. 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 μ L 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 the samples.
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 μ L 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. 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 9. Release Primer Extension

1. Retrieve the Release Primer Extension Master Mix prepared during [Step 6.5](#):
2. Resuspend the capture beads with bound sample(s) in 20 μ L 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		
Primer Extension	50°C	2 min
HOLD	4°C	∞



Lid temperature should be set 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 10. Post-PETE Amplification

1. Retrieve the PCR Master Mix prepared during [Step 6.5](#).
2. Aliquot 30 μ L of the PCR Master Mix into new PCR tubes.



Keep these tubes on ice until required.

3. Transfer 20 μ L 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



Lid temperature should be set 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 12.2](#).

Panel Capture Target Size	Number of Cycles
5-10 kb	20
10-20 kb	19
20-30 kb	18
30-100 kb	17
100-150 kb	16
150-350 kb	15



PCR cycle numbers have been verified for the following panels:

Panel	Panel Capture Target Size (kb)	Number of Cycles
Hot Spot	37	17
Pan Cancer	301	15

6. Proceed immediately to the next step.

Step 11. 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 μ L 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 μ L of 80% ethanol. Incubate at room temperature for \geq 30 seconds.



80% ethanol should be prepared fresh daily.

8. Carefully remove and discard the ethanol.
9. Repeat the ethanol wash for a second wash (points 7-8).
10. Spin the tubes down quickly to bring residual ethanol to the bottom.
11. Place tubes on the magnet and remove residual ethanol using a P20 pipette without disturbing the beads.
12. Leave the tubes 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 25 μ L 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.

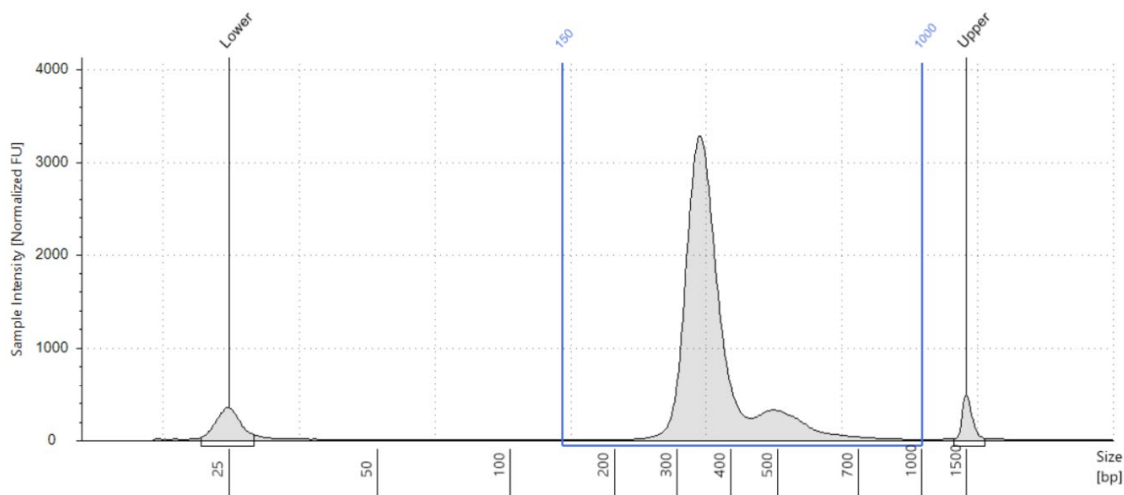


Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples.

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 12. 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 $\geq 4\text{nM}$ or 4000 pmol/L in the region of 150-1000 bp on the TapeStation. If the Primer Extension Target Enrichment library contains $<4\text{nM}$ of total DNA, please refer to the [troubleshooting section](#) for guidance.
3. Primer Extension Target Enrichment libraries should have a primary peak between 300 and 400 bp with possibly several minor peaks >400 bp in the region of 150-1000 bp. [Figure 4](#) is an example Primer Extension Target Enrichment library prepared from a cfDNA pre-capture input library with a mean fragment size of ~ 377 bp and a primary peak between 300 and 400 bp. Several minor peaks at >400 bp and sharp peaks may be visible at <150 bp may be present. Peaks at <150 bp correspond to unincorporated primers and primer-dimers and will not interfere with sequencing.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/ μ l]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
150	1000	377	6010	25400	98.03		■

Fig. 4 Example TapeStation trace for Primer Extension Target Enrichment library prepared from cfDNA

Appendices

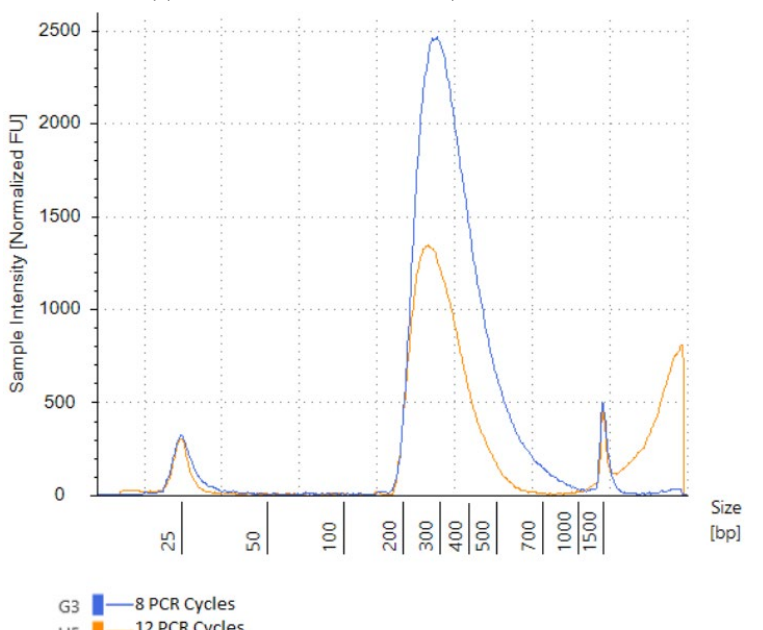
Appendix A. Troubleshooting

- 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)
<p>Library Preparation</p> <p>Less than 50 ng of cfDNA input is available for library preparation.</p>	<p>If less than 50 ng of cfDNA is available, libraries generated using down to 10 ng of input cfDNA can produce capture results; however pre-capture input library yield may be low and the following sequencing metrics may have poor performance:</p> <ul style="list-style-type: none"> ▪ Uniformity <ul style="list-style-type: none"> • Percent Panel Exon Region $\geq 1000X$ • Percent Bases in 2-fold Range ▪ Dedup Depth
<p>Pre-capture input library yield is <35 ng/μL for Primer Extension Target Enrichment.</p>	<p>Possible error occurred during library preparation or compromised reagents were used:</p> <ul style="list-style-type: none"> ▪ Use a previously processed cfDNA sample as a positive control for library construction and or an evaluated sample library as a positive control for PCR reagents <p>Insufficient cfDNA used for Pre-Capture Input Library Preparation:</p> <ul style="list-style-type: none"> ▪ Use the recommended 50 ng of cfDNA as input ▪ Increase the number of PCR cycles during library preparation amplification by 1– 3 cycles until yield is ≥ 35 ng/μL <p>Poor ligation efficiency:</p> <ul style="list-style-type: none"> ▪ Ensure that the proper amount of input cfDNA and KAPA Universal UMI Adapters are used ▪ Ensure proper ligation incubation time and temperature are used <p>Poor PCR amplification:</p> <ul style="list-style-type: none"> ▪ 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 ≥ 35 ng/μL <p>Sample loss:</p> <ul style="list-style-type: none"> ▪ Ensure that KAPA HyperPure Bead purification steps are performed properly ▪ Do not let KAPA HyperPure Beads overdry

<p>Abnormal Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000 profile of the pre-capture input library</p>	<p>High molecular weight DNA present</p> <ul style="list-style-type: none"> Ensure that the input cfDNA into the library prep is free of high molecular weight genomic DNA <p>Contaminating DNA present</p> <ul style="list-style-type: none"> Run a negative control (for example, water) to ensure that there is no product from adapter-only samples
<p>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)</p>	<p>Primer depletion due to over-amplification of the pre-capture input 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 sequence 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.</p>  <p>Figure A. Example TapeStation trace for bimodal fragment distribution.</p>
<p>The Agilent TapeStation DNA High Sensitivity ScreenTape Assay D1000 indicates one or more visible sharp peaks that are <150 bp in size.</p>	<p>These peaks, which represent primers, primer-dimers or adapter dimers will not interfere with the capture process.</p>

<p>High adapter dimers in the pre-capture library after the first PCR</p>	<p>Insufficient cfDNA used in the assay:</p> <ul style="list-style-type: none"> ▪ Ensure proper quantification of the input DNA. ▪ Use the recommended 50 ng cfDNA input amount if available <p>Poor ligation efficiency:</p> <ul style="list-style-type: none"> ▪ Ensure that the proper amount of input cfDNA and KAPA Universal UMI Adapters are used. ▪ Ensure adapter and KAPA Ligation ReadyMix is added separately and on ice. ▪ Ensure proper ligation incubation time and temperature are used. <p>Poor KAPA HyperPure Bead purification:</p> <ul style="list-style-type: none"> ▪ 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	
<p>Primer Extension Target Enrichment library yield is <4 nM</p>	<p>Low pre-capture input library yield:</p> <ul style="list-style-type: none"> ▪ 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 cfDNA sample that was previously processed with success. <p>Incorrect washes:</p> <ul style="list-style-type: none"> ▪ Ensure the washes are performed according to the Instructions for Use. <p>PCR cycle condition not optimized:</p> <ul style="list-style-type: none"> ▪ 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 <p>Poor binding with the Capture Beads:</p> <ul style="list-style-type: none"> ▪ 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 5 Primer Extension Target Enrichment Step 3. <p>Sample loss:</p> <ul style="list-style-type: none"> ▪ Ensure that the cfDNA 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	<p>Challenging HyperPETE Panel target regions</p> <ul style="list-style-type: none"> ▪ Very high or very low GC panel target regions ▪ CNV or MSI present in panel target regions. <p>Insufficient cfDNA or poor quality cfDNA used</p> <ul style="list-style-type: none"> ▪ Ensure proper quantification of the input cfDNA. ▪ Use the recommended 50ng of cfDNA as input if available <p>Improper plasma collection</p> <ul style="list-style-type: none"> ▪ Ensure plasma is separated soon after blood is collected ▪ Ensure a blood collection tube containing the anticoagulant EDTA is used (Recommended: BD k2EDTA) <ul style="list-style-type: none"> • Poor quality cfDNA can result from using the incorrect collection tube. Enrichment libraries made with low quality cfDNA will have lower uniformity metrics and a higher GC content than enrichment libraries made with high quality cfDNA.
Low On Target Rate	<p>Contamination of pre-capture input library or Primer Extension Target Enrichment library:</p> <ul style="list-style-type: none"> ▪ 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 <p>Challenging HyperPETE Panel target region</p> <ul style="list-style-type: none"> ▪ Highly repetitive target regions ▪ Target region < 30kb. See Appendix B <p>Primer Extension Target Enrichment not performed correctly</p> <ul style="list-style-type: none"> ▪ 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	<p>Insufficient cfDNA or poor quality cfDNA used</p> <ul style="list-style-type: none"> ▪ Ensure proper quantification and/or qualification of the input DNA ▪ Follow the recommended DNA amount for Library Preparation and Primer Extension Target Enrichment <p>Insufficient sequencing reads per sample</p> <ul style="list-style-type: none"> ▪ Ensure each sample receives the recommended sequencing reads required
Lower or higher number of sequencing reads than expected	<p>Insufficient DNA or poor quality DNA used</p> <ul style="list-style-type: none"> ▪ Ensure proper quantification of the input cfDNA. ▪ Follow the recommended DNA input amount for Library Preparation and Primer Extension Target Enrichment. Use the recommended 50ng of cfDNA as input if available <p>Insufficient sequencing reads per sample</p> <ul style="list-style-type: none"> ▪ Ensure each sample receives the recommended sequencing reads required <p>Improper pooling</p> <ul style="list-style-type: none"> ▪ 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	<p>Contamination of the pre-capture input library</p> <ul style="list-style-type: none"> ▪ 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	<p>Reduction in Pre-Capture and/or Post-Capture PCR cycles may reduce duplicate rates. Take the following points into consideration when altering cycle numbers.</p> <ul style="list-style-type: none"> ▪ 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.

2. SNV and InDel Calling for Custom Panels

For any new custom panel, a set of healthy donor plasma samples should be processed and used to generate background files, for proper background polishing and variant calling. This is recommended for new custom panels that do not already have these background files generated.



Appendix C. Limited Warranty and Further Liability Limitation

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.

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.

C. TO THE FULLEST EXTENT PERMITTED BY APPLICABLE LAW, ROCHE DISCLAIMS ALL OTHER REPRESENTATIONS, AND WARRANTIES, EXPRESS OR IMPLIED, WITH RESPECT TO THE PRODUCTS, SERVICES AND DATA, INCLUDING BUT NOT LIMITED TO, ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE OR NON-INFRINGEMENT. CUSTOMER’S SOLE REMEDY FOR BREACH OF WARRANTY IS STATED ABOVE.

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

TO THE FULLEST EXTENT PERMITTED UNDER APPLICABLE LAW, ROCHE SHALL NOT HAVE ANY LIABILITY FOR INCIDENTAL, COMPENSATORY, PUNITIVE, CONSEQUENTIAL, INDIRECT, SPECIAL OR OTHER SIMILAR DAMAGES, HOWEVER CAUSED AND REGARDLESS OF FORM OF ACTION WHETHER IN CONTRACT, TORT (INCLUDING NEGLIGENCE), STRICT PRODUCT LIABILITY OR OTHERWISE, EVEN IF ROCHE HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. CUSTOMER UNDERSTANDS THAT ANY RISKS OF LOSS HEREUNDER ARE REFLECTED IN THE PRICE OF THE SERVICES AND PRODUCTS AND THAT THESE TERMS WOULD HAVE BEEN DIFFERENT IF THERE HAD BEEN A DIFFERENT ALLOCATION OF RISK.

If you have any questions concerning service of this product, contact your local Roche Technical Support.

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.



HYPERDESIGN, KAPA, KAPA HYPERPETE, KAPA HYPERPREP, and KAPA HYPERPURE are trademarks of Roche.

All other product names and trademarks are the property of their respective owners.

sequencing.roche.com

© 2015-2024 Roche Sequencing Solutions, Inc.

All rights reserved.

Roche Sequencing Solutions, Inc.

4300 Hacienda Drive

Pleasanton, CA 94588 USA

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