For Research Use Only.

Not for use in diagnostic procedures.

KAPA HyperPETE Somatic Tissue DNA Workflow v1.0

Instructions for Use

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 Tissue DNA Preparation enables primer extension targeted enrichment of custom-defined regions of the genome for somatic DNA applications. Proprietary design algorithms improve capture uniformity and reduce the amount of sequencing needed to efficiently identify sequence variants. The KAPA HyperPETE is intended for capture of gDNA primary target regions up to 250kb.

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 dialog.roche.com, or upon request from the local Roche office.

Changes to Previous Version

New version.

Ordering Information

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

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Contact and Support

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Preface

Regulatory Disclaimer

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Contact Information

Technical Support

If you have questions, contact your local Roche Technical Support. Go to sequencing.roche.com/support.html 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	Identifies a resource in a different area of this manual or on a web site.
Italic type	Identifies the external general resources or names
Bold type	Identifies names of paragraphs, sections or emphasized words

Chapter 1. Before You Begin

These Instructions for Use describes the process for enrichment of individual FFPET and non-formalin compromised DNA shotgun libraries using KAPA HyperPETE Panels. Specifically, this Instructions for Use provides a protocol for the workflow outlined in *Figure 1* using the KAPA NGS DNA Extraction Kit, KAPA NGS FFPE DNA QC Kit, KAPA FFPE DNA Polishing Kit, KAPA HyperPlus and KAPA HyperPETE Kits. The output of this protocol are enriched FFPET and non-formalin compromised DNA 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 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 HyperPlus Kit, or KAPA RNA HyperPrep Kit
    - KAPA HyperPure Beads, KAPA HyperCapture Bead Kit, and KAPA HyperPETE Reagent Kit
    - KAPA NGS DNA Extraction Kit, KAPA NGS FFPE DNA QC Kit and KAPA FFPE DNA Polishing Kit
- Catalog panels as well as customizable content through the HyperDesign portal and a team of expert designers.
- Compatible with NAVIFY Mutation Caller to provide an end-to-end solution including fast and easy analysis and detection.

Overview of the KAPA HyperPETE Somatic Tissue DNA Preparation Workflow

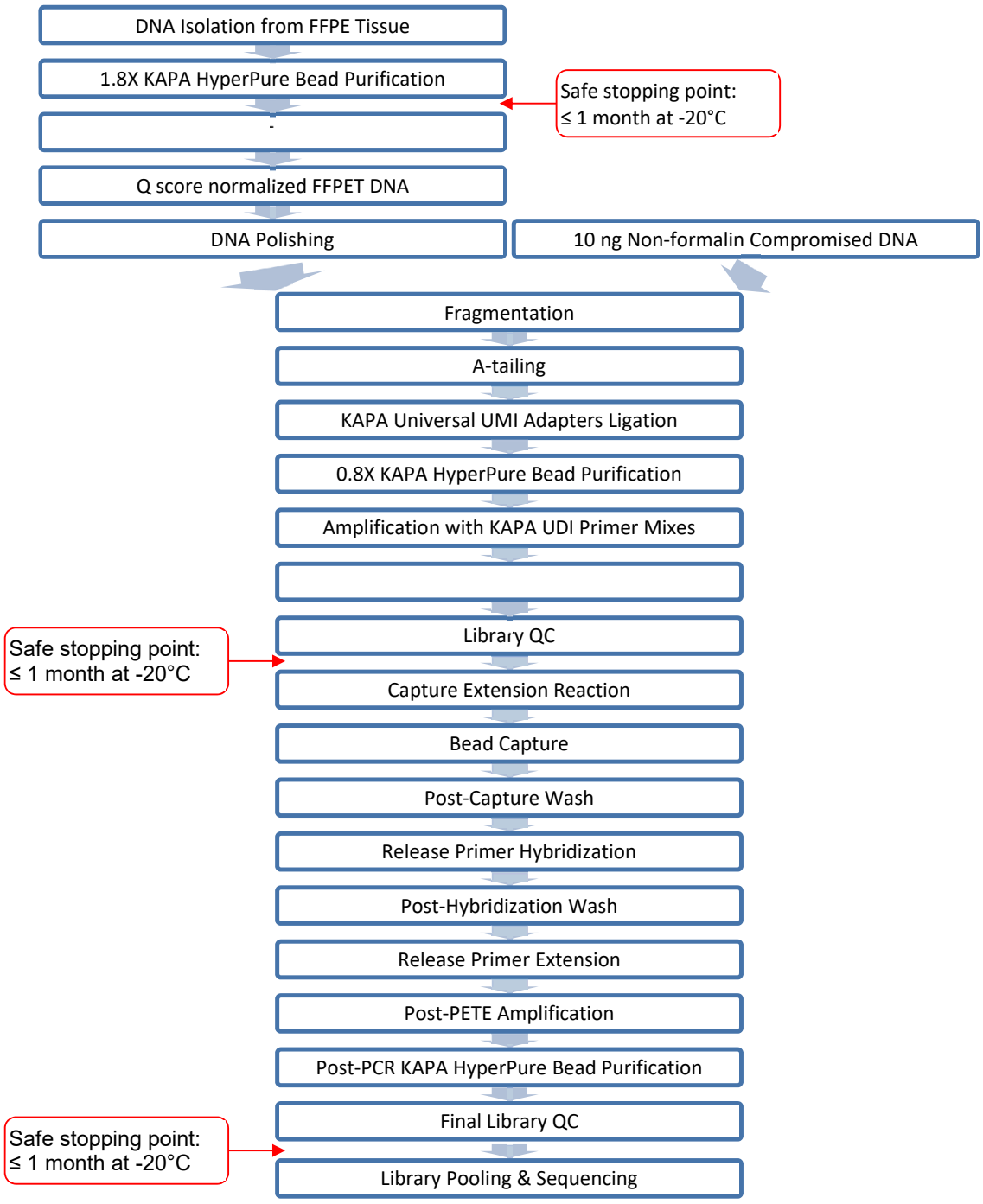


Fig. 1 Somatic Tissue DNA HyperPETE Workflow

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 release primers provided by Roche to perform target enrichment.

Sample Library: The initial shotgun library generated from genomic DNA by fragmentation 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 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 Library: The completed library ready for pooling and sequencing

UDI primer: Unique Dual-Indexed primer

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

- Thermomixer should be programmed with the following:
  - DNA Isolation Incubation program (*Chapter 3*, DNA Isolation from FFPE Tissue, Step 1)
- qPCR machine should be programmed with the following:
  - QC PCR program (*Chapter 3*, DNA Isolation from FFPE Tissue, Step 3)
- Thermocyclers should be programmed with the following:
  - Library Preparation
    - DNA Polishing program (*Chapter 4*, Library Preparation from DNA, Step 1)

- Fragmentation program (*Chapter 4*, Library Preparation from DNA, Step 2)
- End Repair and A-Tailing Program (*Chapter 4*, Library Preparation from DNA, Step 3)
- Adapter Ligation program(*Chapter 4*, Library Preparation from DNA, Step 4)
- Amplification with KAPA UDI Primer Mixes program (*Chapter 4*, Library Preparation from DNA, Step 6)

- Primer Extension Target Enrichment
  - Capture Primer Extension program (*Chapter 5*, Primer Extension Target Enrichment, Step 2)
  - Release Primer Hybridization program (*Chapter 5*, Primer Extension Target Enrichment, Step 6)
  - Release Primer Extension program (*Chapter 5*, Primer Extension Target Enrichment, Step 9)
  - Post-PETE Amplification program (*Chapter 5*, Primer Extension Target Enrichment, 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*, 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 capable of 20,000 x g for 1.5 mL tubes	Multiple Vendors	N/A
Microcentrifuge for 0.2 mL, 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
384-well quantitative (real-time) PCR machine capable of SYBR Green I dye detection (Recommended: LightCycler® 480 Instrument II, 384-well, Roche, catalog number 05015243001).	Multiple Vendors	N/A
Plate centrifuge	Multiple Vendors	N/A
Vortex mixer	Multiple Vendors	N/A
MS 3 Vortexer	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
Thermomixer (Recommended: Eppendorf ThermoMixer)	Multiple Vendors	N/A
NextSeq 500/550 or MiSeq	Illumina	Multiple models
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.

Component	Package Size/Contents	Catalog No.
KAPA HyperPlus Kit	8 reactions 24 reactions 96 reactions	07 962 380 001 07 962 401 001 07 962 428 001
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	96 reactions	09 134 336 001
KAPA Universal UMI Adapter	96 reactions 4 X 96 reactions*	09 329 862 001 09 329 889 001
KAPA NGS DNA Extraction Kit	24 reactions 96 reactions	09 189 823 001 09 190 023 001
KAPA NGS FFPE DNA QC Kit	24 reactions 4 X 24 reactions*	09 217 193 001 09 217 207 001
KAPA NGS FFPE DNA Polishing Kit	24 reactions 96 reactions	09 217 215 001 09 217 223 001
KAPA HyperPETE Reagent Kit	24 reactions 96 reactions	09 211 624 001 09 211 683 001
KAPA HyperPETE Panel	Pan Cancer 24 reactions Pan Cancer 96 reactions Pan Cancer 384 reactions Hot Spot 24 reactions Hot Spot 96 reactions Hot Spot 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	09 329 161 001 09 329 196 001 09 329 226 001 09 329 234 001 09 329 277 001 09 329 307 001 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

	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	09 418 911 001 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
LC480 Multiwell Plate 384 (recommended)	50 plates with foils	04 729 749 001

* 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: § 0.2 mL PCR tubes § 0.2 mL PCR strip tubes § 1.5 mL DNA low bind microcentrifuge tubes	Multiple Vendors	N/A	N/A
Water, PCR Grade	Multiple Vendors	N/A	N/A
TapeStation High Sensitivity D1000 Reagents	Agilent	1 kit	5067-5585
TapeStation High Sensitivity D1000 ScreenTape	Agilent	1 box of 7 tapes	5067-5584
TapeStation Parts and Accessories	Agilent	N/A	N/A
NextSeq 500/550 High Output Kit v2.5 (300 Cycles), NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles), or MiSeq Reagent Kit v3 (600-cycle)	Illumina	1 kit	Multiple catalogue numbers

Chapter 2. Prepare and Store the Reagents

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

- KAPA NGS DNA Extraction Kit
- KAPA NGS FFPE DNA QC Kit
- KAPA FFPE DNA Polishing Kit
- KAPA HyperPlus 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 NGS DNA Extraction Kit	-15°C to -25°C
KAPA NGS FFPE DNA QC Kit	-15°C to -25°C
KAPA FFPE DNA Polishing Kit	-15°C to -25°C
KAPA HyperPlus Kit	-15°C to -25°C
KAPA HyperPure Beads	+2°C to +8°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 HyperPETE Reagent Kit	-15°C to -25°C
KAPA HyperPETE Panel	-15°C to -25°C

The HyperCapture Bead kit and 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 # 09134336001.

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 µL 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 tubes or strip tubes for storage.

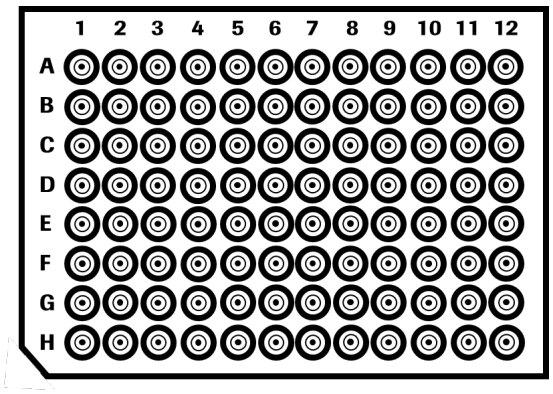


Fig. 2 KAPA UDI Primer Mixes plate layout

Chapter 3. DNA Isolation from FFPE Tissue

This chapter describes the recommended protocols for DNA isolation using the KAPA NGS DNA Extraction Kit and sample quality assessment using the KAPA NGS FFPE DNA QC Kit. A normalized quality score (Q-score) is calculated and used to determine the quality of the DNA and optimal input into library preparation.


If starting with non-formalin compromised gDNA, proceed to *Chapter 4, step 2.*


If starting with extracted FFPET DNA, proceed to *Chapter 3, step 3*


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.

Components from the following kits are required:

- KAPA NGS DNA Extraction Kit
- KAPA NGS FFPE DNA QC Kit
- KAPA HyperPure Beads

Ensure the following is available:

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

Step 1. Preparation of FFPE Tissue and DNA Isolation


The recommended input for FFPET DNA isolation is one to two 10 micron curl(s) in one tube. If necessary, additional extractions from the same sample can be combined to obtain a sufficient amount of DNA.

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

Component	Thawing procedure
KAPA NGS DNA Extraction Kit	Room temperature then place on ice
KAPA NGS FFPE DNA QC Kit	Room temperature then place on ice
KAPA HyperPure Beads	Room temperature

2. Ensure FFPET curls are placed in 1.5 mL tubes.
3. Prepare the Extraction Master Mix, as described below:

Component	Volume per Individual Sample
Nuclease-free Water	88 µL
Extraction Buffer (10x)	10 µL
Extraction Enzyme	2 µL
Total	**100 µL**

4. Vortex and spin the master mix, and then add 100 µL of the master mix to each tube containing FFPET curl(s).
5. If the curl is not near the bottom of the tube, tap the tube or use a pipette tip to get the tissue as submersed in liquid as possible.
6. Incubate the tubes in a thermomixer at 75°C and shake at 2000 RPM with the lid on for 1 hour.
7. Following the 1 hour incubation, spin the tubes at 20,000 x g for 5 minutes to pellet the remaining cellular debris. A visible and/or solid wax layer may form after the spin.
8. Poke through the wax layer with a pipette tip and transfer the liquid to strip tubes.
    a. Carefully transfer each sample to a unique position in the strip tube
    b. Transfer as much liquid as possible while avoiding cellular debris/wax carryover

Note that moderate wax carryover may leave the solution cloudy even after bead purification, but this will not impact downstream applications.

If 2 different samples are accidentally added to the same tube, no usable data will be obtained from these samples.

Step 2. Post-Extraction 1.8X 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 it to room temperature. Vortex Beads until thoroughly resuspended right before use.
2. Add 180 µL of KAPA HyperPure Beads to each sample.
3. Mix thoroughly by carefully pipetting to avoid spill over.

The total volume will be 280 µL in strip tubes. Care should be taken to ensure no spillover during mixing if using0.2 mL tubes.

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 for a second wash (steps 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 tubes from the magnet and resuspend the beads in 40 µL of 10 mM Tris-HCl, pH 8.0.
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 40 µL of the eluate to a new strip tube. The eluate contains the extracted DNA.

    Avoid mixing up samples by ensuring tubes are labelled when processing multiple samples. Samples are indexed in *Chapter 4 Step 6* and sample confusion will lead to error.
17. Quantify the concentration of individual samples with the Qubit® dsDNA HS Assay kit.
18. Freeze at -20°C for up to 1 month, or proceed to the next step.

Step 3. QC of FFPET-extracted DNA

Quantitative PCR (qPCR) will be used to determine input DNA quality based on the Normalized Q score. Each sample should be processed with three technical replicates along with the QC PCR DNA Standard and water as NTC (no template control).

1. Dilute the input DNA samples and QC PCR DNA Standard in water to a 500-fold final dilution.

   To increase accuracy and precision, it is recommended to split the 500-fold dilution into 2 steps: first, 100-fold dilution, and then 5-fold dilution.

   The QC PCR DNA Standard must be included in each qPCR run
2. Prepare the following master mix for each QC PCR Primer Mix separately (66 bp and 191 bp):

   Each sample should be processed with 3 technical replicates along with the QC PCR DNA Standard and water as NTC (No Template Control).

Component	Volume per Individual Sample
QC PCR Reaction Mix (2X)	5 µL
QC PCR Primer Mix (66bp) or QC PCR Primer Mix (191bp)	1 µL
Total	**6 µL**

3. Vortex and spin the master mix and add 6 µL of the appropriate master mix to each well to be used in a 384 well plate.

4. Add 4 µL of the diluted sample, the diluted Standard, and the water (NTC) to the applicable wells.
5. Seal the plate well and centrifuge briefly.
6. Perform qPCR as follows, using a qPCR machine that can detect SYBR green:

QC PCR is validated on the LightCycler® 480 Instrument II. As a result, Crossing Point (Cp) values are used as an example to calculate Q-score.

Step	Temperature	Hold Time at Temperature	Number of Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	10 sec	40
Primer Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Cooling	40°C	30 sec	1

7. Use the 3 replicates of Cp values per amplicon to calculate the Q scores for the sample(s) and the QC PCR DNA standard by using the following equation:

$$\text{Q Score} = 2^{(\text{average(Cp66)-average(Cp191)})}$$

8. Obtain a normalized Q score for each sample through the following equation:

Normalized Q Score = sample Q score / QC PCR DNA standard Q score

Please note that it is recommended that samples with normalized Q score of less than 0.04 be avoided as results will not be optimal.

Step 4. Using the Normalized Q score to Determine DNA Input

Primer capture and sequencing performance will vary greatly depending on DNA quality. For best performance, we recommend assessing DNA quality and following the Normalized Q score DNA input procedure described below. For more information, please proceed to the troubleshooting guide of this Instructions for Use.

1. Utilize the Normalized Q score to determine the recommended input mass for each sample as follows:

Input mass in ng = (10/(normalized Q Score)) + 10

Use the normalized Q score of 1 in the formula if it is greater than 1.

2. Use the Qubit® dsDNA HS Assay kit to quantify the DNA concentration and calculate the recommended DNA input volume as follows:
3. Input volume in µL = Input mass in ng/Qubit concentration in ng/µL

The maximum sample input volume for this workflow is 30.5 µL.

The number of unique DNA molecules sequenced is directly correlated to the number of amplifiable input DNA molecules into the workflow. However, when Normalized Q score adjusted input DNA is unavailable, use the entire available DNA down to 10 ng.

Chapter 4. Library Preparation from DNA

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

Components from the following kits are required:

- KAPA NGS FFPE DNA Polishing Kit
- KAPA HyperPlus 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 water, PCR Grade
- Freshly prepared 80% Ethanol

Sample Requirements

This workflow was validated using Normalized Q score adjusted DNA input for FFPET DNA of various quality. Without Normalized Q score adjustment, FFPET DNA input down to 10 ng can be used for pre-capture input library preparation, but performance is not guaranteed. To ensure compatibility with downstream reagents, it is recommended to use the KAPA NGS DNA Extraction Kit and the KAPA NGS FFPET DNA QC Kit following the instructions in *Chapter 3*.

This workflow was also validated using 10 ng non-formalin compromised gDNA input, such as cell line gDNA. Non-formalin compromised gDNA should be quantified by using the Qubit dsDNA HS Assay Kit. Non-formalin compromised gDNA input down to 1 ng can be used for pre-capture input library preparation, but performance is not guaranteed.

Preparation of pre-capture input libraries can be performed with FFPET DNA isolated using other methods. When alternative methods are used, it is necessary to elute the DNA with an EDTA-free elution buffer. The enzymatic fragmentation reaction is very sensitive to the presence of EDTA, which must be removed prior to fragmentation. Where DNA is eluted in an EDTA-containing buffer, a buffer exchange should be performed and DNA should be resuspended in 10mM Tris-HCl, pH 8.0. This may be achieved by means of a 3X bead-based purification with KAPA HyperPure beads. Please refer to the relevant Technical Data Sheet (KR1705 or KR1245) for a detailed DNA purification protocol.

Please note the Conditioning Solution, HyperPrep ERAT Enzyme Mix and Library Amplification Primer Mix provided in the KAPA HyperPlus Kit will not be used in the library preparation protocol.

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

DNA polishing is not necessary for non-formalin compromised gDNA. Proceed to *Step 2. Enzymatic Fragmentation* with the non-formalin compromised gDNA sample adjusted to 35 µL using nuclease-free water.

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.

Step 1. DNA Polishing

If starting with non-formalin compromised gDNA, add 10 ng of DNA into a 0.2 mL PCR tube. If needed, adjust to 35 µL using nuclease-free water and proceed to *Chapter 4, step 2.*

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

Component	Thawing procedure
KAPA NGS FFPE DNA Polishing Kit	Room temperature then place on ice
KAPA HyperPlus Kit	Room temperature then place on ice
KAPA HyperPure Beads	Room temperature
KAPA Universal UMI Adapters	Room temperature then place on ice
KAPA UDI Primer Mixes	Room temperature then place on ice

1. Add extracted DNA into each tube, using the recommended amount of DNA, as described in the previous section.
2. If needed, adjust the volume in each tube to 30.5 µL using nuclease-free water.
3. Dilute the DNA Polishing Enzyme (100 U/ µL stock) 50-fold using nuclease-free water right before use. Use 1 µL of diluted DNA Polishing Enzyme per sample. Discard the rest of the diluted enzyme after use.

Do not store diluted DNA Polishing Enzyme for long-term use.

4. Prepare the DNA Polishing master mix:

Component	Volume per Individual Sample
Diluted DNA Polishing Enzyme	1 µL
KAPA Frag Buffer (10X)	3.5 µL
Total	**4.5 µL**

5. Add 4.5 µL of DNA Polishing master mix to each sample, for a total volume of 35 µL.
6. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
7. Incubate on a thermocycler:

Temperature	Duration
37°C	30 min
4°C	∞

Set the thermocycler lid to 50°C.

8. Proceed immediately to the next step.

Step 2. Enzymatic Fragmentation

The enzymatic fragmentation reaction is very sensitive to the presence of EDTA, which must be removed prior to fragmentation.

1. Place tubes on ice while setting up the fragmentation reaction.
2. Prepare the appropriate fragmentation master mix on ice or cold block:

For FFPET samples

Component	Volume per Individual Sample
KAPA Frag Buffer (10X)	1.5 µL
KAPA Frag Enzyme	10 µL
Nuclease-free Water	3.5 µL
Total	**15 µL**

For non-formalin compromised gDNA

Component	Volume per Individual Sample
KAPA Frag Buffer (10X)	5 µL
KAPA Frag Enzyme	10 µL
Total	**15 µL**

3. Add 15 µL of the fragmentation master mix to each sample on ice or cold block, for a total volume of 50 µL
4. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
5. Incubate on a thermocycler pre-cooled to 4°C:

Temperature	Duration
37°C	30 min
4°C	∞

Set the thermocycler lid to 50°C.

6. Proceed immediately to the next step. It is not necessary to wait for the thermocycler to reach 4°C before moving on to the next step.

Make sure to keep the samples on ice between Fragmentation and End Repair and A-Tailing reaction and adjust thermocycler lid idle temperature to minimize waiting time between running the Fragmentation and End Repair and A-tailing programs. It is very important not to let Fragmentation continue for longer than 30 minutes.

Step 3. End Repair and A-Tailing

1. Remove samples from the thermocycler and place on ice or cold block.
2. Prepare the End Repair and A-tailing master mix:

Component	Volume
KAPA End Repair & A-tailing Buffer	7 µL
KAPA HyperPlus End Repair & A-tailing Enzyme Mix	3 µL
Total	**10 µL**

Ensure the HyperPlus End Repair & A-tailing Enzyme Mix is used for best performance.

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.

3. Add 10 µL of the End Repair and A-tailing master mix to each sample on ice for a final volume of 60 µL.
4. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
5. Incubate on a thermocycler:

Temperature	Duration
65°C	30 min
4°C	∞

Set the thermocycler lid to 85°C.

6. Proceed immediately to the next step.

Step 4. Adapter Ligation

1. Prepare the Ligation Master Mix:

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 Universal UMI Adapters to the 60 µL of fragmented and A-tailed products. Mix thoroughly and briefly spin. The KAPA Universal UMI Adapter must be added to each well individually prior to the addition of the Ligation Master Mix.

Addition of the KAPA Universal UMI 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 on 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 5. Post-Ligation 0.8X 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 it 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 min and quickly spin down the liquid without pelleting the beads.

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

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 for a second wash (steps 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 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 µLof 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 6 and sample confusion will lead to error.

17. Immediately proceed to the next step.

Step 6. Amplification with KAPA UDI Primer Mixes

For guidance on color balancing indices for low-plex pooling of post-capture sample multiplexing, please refer to the KAPA UDI Primer Mixes Instructions for Use, catalog # 09134336001.

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

1. Retrieve 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 Mixes to the 20 µL of adapter-ligated sample.
5. Add 25 µL of the KAPA HiFi HotStart ReadyMix to the 25 µL of purified ligated sample and KAPA UDI Primers Mixes, bringing the total volume to 50 µL
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. If using the KAPA UDI Primer Mixes in a plate, apply a new adhesive foil seal provided in the kit.

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

7. Amplify on 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	Cyle number depends

Primer Annealing	60°C	30 sec	on DNA input amount and quality. See table below
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
Hold	4°C	∞	1

Set the thermocycler lid to 105°C.

Sample Type	DNA Input	Number of PCR Cycles
FFPET	Q-score Input	8
	50 ng	9
	10 ng	11
Non-formalin compromised gDNA	10 ng	8
	1 ng	11

PCR cycle conditions are recommendations and can be adjusted to achieve the yield requirements in step 8.3.

8. Proceed immediately to the next step.

Step 7. 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 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 for a second wash (steps 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 µL 10mM 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.

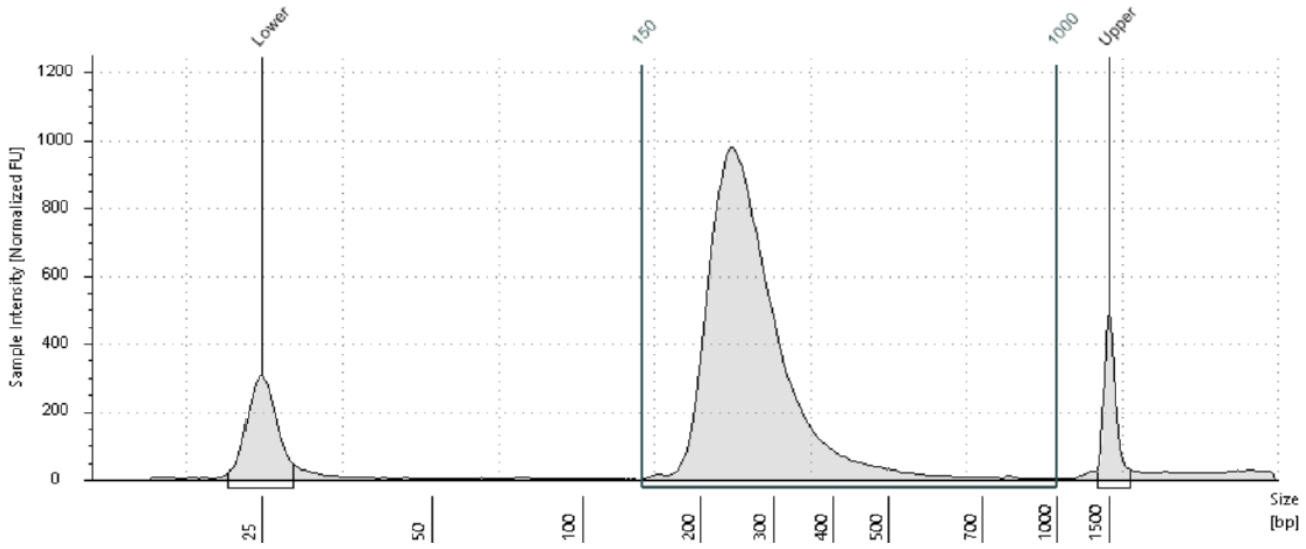
    Visually confirm that the beads are pelleted.

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

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

Step 8. 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 ScreenTape Assay D1000 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 ≥ 35000pg/µL or contain ≥ 875 ng of total DNA in 25 µL 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 an average fragment size distribution between 250-350bp. Figure 3 is an example pre-capture input library prepared from FFPET DNA with a mean fragment size of ~295 bp. 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.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
150	1000	277	2910	17300	94.47		

Fig. 3 Example Tapestation trace for pre-capture input library prepared from FFPET DNA

Freeze at -20°C for up to 1 month, or proceed to the next step.

Chapter 5. Primer Extension Target Enrichment (PETE)

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

This chapter requires the use of components from the following kits:

- 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 HyperPlus Kit for tissue DNA library preparation following the instructions in *Chapter 4*.

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 0.2 mL 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	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 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.

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

Step 2. Capture Extension Reaction

1. Ensure that 10-15 µL of pre-capture input library contains 500-3000 ng of library.

Exact ng input amount can vary between the indicated ranges to keep 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 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 input library volume.

3. Add 35-40 µL of the Capture Extension Reaction Master Mix to the pre-capture input libraries for a final volume of 50 µL.
4. 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
5. Incubate in a thermocycler programmed as outlined below:

Step	Temperature	Ramp Rate to Temperature	Hold Time at Temperature
HOLD	20°C	100 %	N/A
Load Samples			
Denaturation	95°C	100 %	2 min
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. 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).

Lid temperature should be set to 105°C.

6. During the primer annealing and extension incubation proceed to *Step 3. Prepare the Capture Beads.*
7. 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 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 and 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 captures use 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.

     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 µL 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 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 *Step 6.1*.
4. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.
5. 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.**

1. Briefly spin down samples.
2. Place samples on a magnet for 1 minute or until the supernatant clears.
3. Remove and discard 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 bead washes, proceed immediately to the next step.


Do not allow beads to dry out at this step.

Step 6. Release Primer Hybridization

1. Prepare the Release Primer Hybridization Master Mix:

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


The Release Primer Hybridization Master Mix should be made during *Step 4.3*.


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

2. Resuspend the sample beads 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	N/A
Load Samples		
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 described in Step 9.1 and the PCR Master Mix described in Step 10.1.
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 (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 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 sample.
5. Vortex samples on an IKA MS 3 Vortexer set to 2400 rpm for 1 minute.
6. Briefly spin down samples.
7. Place samples on a magnet for 1 minute or until the supernatant clears.
8. Remove and discard 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 and 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 the beads to dry out at this step

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

Step 9. Release Primer Extension

1. Prepare the following 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**

The Release Primer Extension Master Mix should be made during *Step 6.5*.

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

2. Resuspend the sample beads 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	N/A
Load Samples		
Primer Extension	50°C	2 min
HOLD	4°C	N/A

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. Prepare the following PCR Master Mix:

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

   The PCR Master Mix should be made during *Step 6.5*.

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	N/A	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 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 for a second wash (steps 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 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 previously prepared and aliquoted in *Step 1.3*. Do not use the same 1X Wash & Resuspension Buffer as the bead washes to avoid contamination.

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.

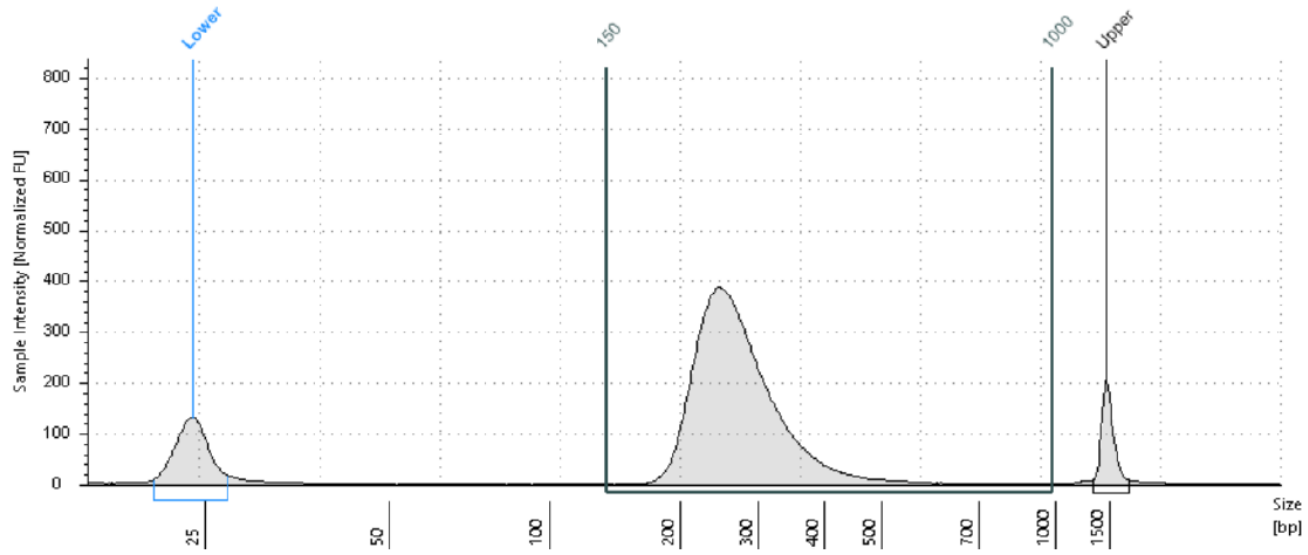
    Visually confirm that the beads are pelleted.

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.

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 ≥4nM or ≥ 4000 pmol/L in the region of 150-1000 bp on the Tapestation. If the Primer Extension Target Enrichment library contains <4nM of total DNA, please refer to the troubleshooting section for guidance.
3. Primer Extension Target Enrichment libraries should have a mean fragment size of between 250 and 400 bp depending on the quality of the input DNA. Figure 4 is an example Primer Extension Target Enrichment library prepared from a FFPET DNA pre-capture input library with a mean fragment size of ~290 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.



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
150	1000	290	2910	16200	96.97		

Fig. 4 Example Tapestation trace for Primer Extension Target Enrichment library prepared from FFPET DNA

Step 13. Pooling and Sequencing

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 # 09134336001.

1. Prepare a pool of Primer Extension Target Enrichment libraries using the nM concentrations measured in step 12. Any number of Primer Extension Target Enrichment libraries can be pooled together as long as the total paired-end read requirement of the entire pool (sum of read requirements for all pooled Primer Extension Target Enrichment libraries and any added PhiX library) does not exceed the total output of the sequencing platform used. The volume to pool from a Primer Extension Target Enrichment library should be between 2µL and 20µL. Some libraries may need to be diluted in order to have a pooling volume within 2µL and 20µL. The final library pool should have a concentration ≥4nM.
    a. To pool libraries with equal read requirements, add an equal number of molecules from each library into the library pool.
    b. To pool libraries with unequal read requirements, including libraries generated from different HyperPETE IFUs, determine the ratio of reads required between libraries and add a direct proportion of molecules from each library into the library pool. For example, if library A requires 20 million reads and library B requires 10 million reads, the calculated ratio would be 2:1, therefore the number of molecules added from library A would be 2:1 compared to the number of molecules from library B.

When pooling low read requirement Primer Extension Target Enrichment libraries, any contamination during processing can cause significant decreases in performance. It is critical that all precautions are taken to prevent contamination including using clean gloves, using new tips, avoiding the splashing of liquids, and avoiding contact with the interior of tube caps.

HyperPETE Panel	Panel Capture Target Size (kb)	Application	Total Paired-End Reads Required per Library
Hot Spot	37	Somatic Tissue	7,500,000
Pan Cancer	301	Somatic Tissue	55,000,000
Custom	Panel capture target size varies depending on the panel. Use the Estimated Coverage size given by HyperDesign and not the primary target size.	Somatic Tissue	200 reads per capture target base (200 x Estimated Coverage bp)

The read requirement calculation given for custom panels is a suggestion. Satisfactory results may be achievable with fewer reads.

2. Sequence pools following the Illumina protocol on an appropriate Illumina instrument.

Illumina Platform	Read Lengths	Index
NextSeq High output	2x151	2x8
NextSeq Mid output	2x151	2x8
MiSeq	2x151	2x8

If using a NextSeq High Output Kit, at least 2 indexed Primer Extension Target Enrichment libraries must be sequenced for compatibility with the downstream NAVIFY Mutation Caller pipeline.

Step 14. Library Pool Storage

1. Store Primer Extension Target Enrichment libraries at -15°C to -25°C for up to 1 month.

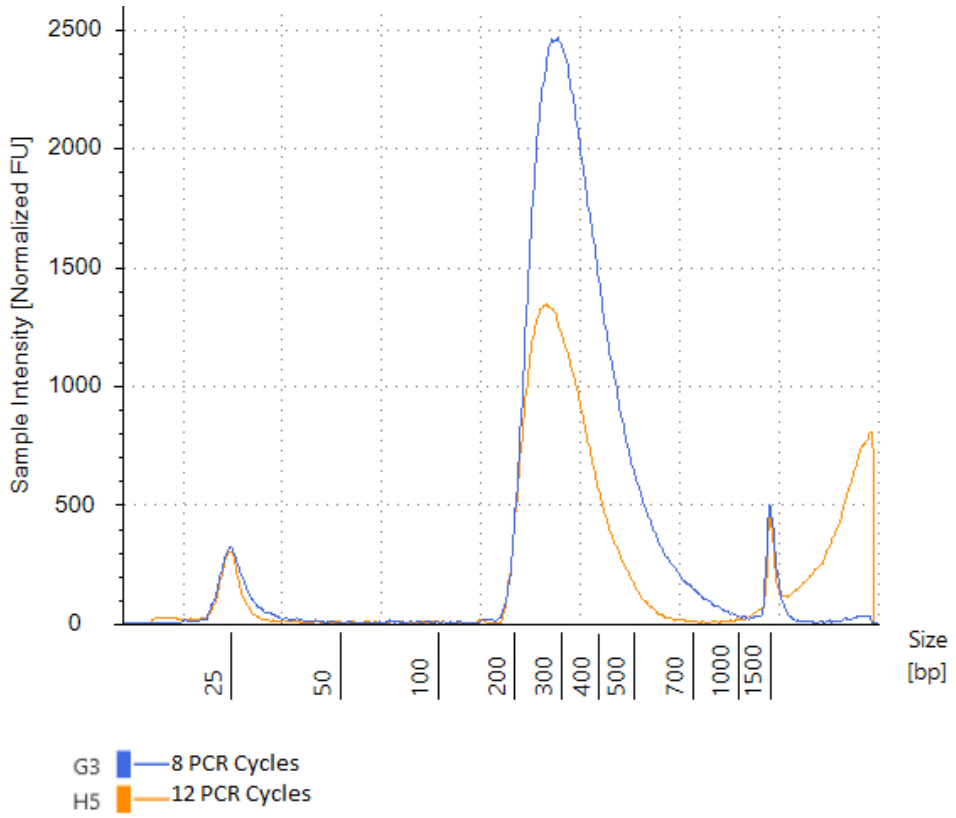
Appendix A. Troubleshooting

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)
DNA Isolation from FFPE Tissue	
No or low yield from DNA isolation	Poor tissue quality ● Ensure that a sufficient sized sample is used. Forgot to add Extraction Enzyme to extraction master mix OR Incorrect incubation conditions (temperature, incubation times, etc.): ● Ensure proper volumes and incubation times are used during DNA isolation.
Library Preparation	
Less than normalized Q score adjusted DNA input is available for library preparation.	Without normalized Q score adjustment, libraries generated using down to 10ng of input DNA can produce capture results; however pre-capture input library yield may be low and the following sequencing metrics may have poor performance: ● Coverage Uniformity ○ Percent Panel Exon Region >= 300X ○ ● Dedup Depth
Using Q < 0.04 for FFPET DNA	Usage of FFPET DNA samples with a normalized Q score < 0.04 is not recommended and can result in low pre-capture input library yield. The following sequencing metrics may have low performance: ● Coverage Uniformity ○ Percent Panel Exon Region >= 300X ● Dedup Depth
Pre-capture input library yield is <35ng/µL for Primer Extension Target Enrichment.	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: ● Use normalized Q score adjusted DNA input amount if available ● Increase the number of PCR cycles during library preparation amplification by 1– 3 cycles until yield is >= 35ng/µL Poor ligation efficiency: ● Ensure that the proper amount of input DNA and KAPA Universal UMI 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 >= 35ng/µL Sample loss: ● Ensure that KAPA HyperPure Bead purification steps are performed properly ● Do not let KAPA HyperPure Beads over dry
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 Fragmentation Master Mix is made and added to samples on ice. ● Ensure that the Fragmentation step does not proceed past 30 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. ● Ensure that the DNA Polishing Enzyme is diluted 50-fold using nuclease-free water before use
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 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.

	 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 input library after the first PCR	Insufficient DNA or poor quality DNA used in the assay: ● Ensure proper quantification of the input DNA. ● Use normalized Q score adjusted DNA input amount if available Poor ligation efficiency: ● Ensure that the proper amount of input DNA and Universal UMI Adapters are used. ● Ensure adapter and Ligation Master Mix 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. 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 <4nM	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 >= 4nM ● Repeat with a DNA sample that was previously processed with success. Incorrect washes. ● Ensure the washes are performed according to the user guide.

	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 >= 4nM 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 5 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 purificationsteps are performed properly. ● Do not let KAPA HyperPure Beads over dry.
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. See Appendix B. Insufficient DNA or poor quality DNA used ● Ensure proper quantification of the input DNA. ● Follow the recommended DNA amount for Library Preparation. Use Q score adjusted DNA input if available.
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 < 30kb. 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 or poor quality DNA used ● Ensure proper quantification and/or QC 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 or poor quality DNA used ● Ensure proper quantification and/or qualification of the input DNA. ● Follow the recommended DNA input amount for Library Preparation and Primer Extension Target Enrichment 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 2µL and 20µL
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 error rate for FFPET	Poor DNA polishing efficiency ● Ensure that the DNA Polishing Enzyme is diluted fresh before each use and DNA Polishing is performed according to the user guide for FFPET DNA samples
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. NAVIFY Mutation Caller Analysis & Performance Considerations

1. MSI Loci

MSI loci in the Pan Cancer panel and the inclusion of the MSI module in custom panels will result in lower reported uniformity and depth of coverage from the NAVIFY Mutation Caller pipeline compared to panels that do not include the MSI module. Smaller panels will be impacted to a larger degree. This is due to the impact of the stringent mapping quality filter applied in the analysis pipeline on the homopolymer stretches found in the MSI loci. This will not have an impact on the ability to call MSI status or the CNV, SNV, and InDel variant detection capabilities of the pipeline.

2. 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.

3. CNV Calling for Custom Panels

For any new custom panel, in order to make use of the CNV calling capabilities of the NAVIFY Mutation Caller pipeline, a set of non-tumor/normal samples needs to be processed and used to generate background files. This is required for new custom panels that do not already have these background files generated. Please see the latest available version of the NAVIFY Mutation Caller User Assistance for more details

Appendix C.

1. Limited Warranty

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

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