For Research Use Only.

Not for use in diagnostic procedures.

KAPA HyperPETE Tissue RNA Fusion Transcript 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 Tissue RNA Fusion Transcript Preparation enables primer extension targeted enrichment of custom-defined regions of the transcriptome for somatic RNA 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 RNA primary target regions up to 50 kb.

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 Solutions products, including those used in KAPA HyperPETE Workflow go to *sequencing.roche.com/products*.

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Editions

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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 describe the process for enrichment of individual FFPET and non-formalin compromised RNA shotgun libraries using KAPA HyperPETE Panels. Specifically, this Instructions for Use provides a protocol for the workflow outlined in Figure 1 using the KAPA RNA HyperPrep and KAPA HyperPETE Reagent Kits. The output of this protocol are Primer Extension Target Enrichment 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 Tissue RNA Fusion Transcript Preparation Workflow

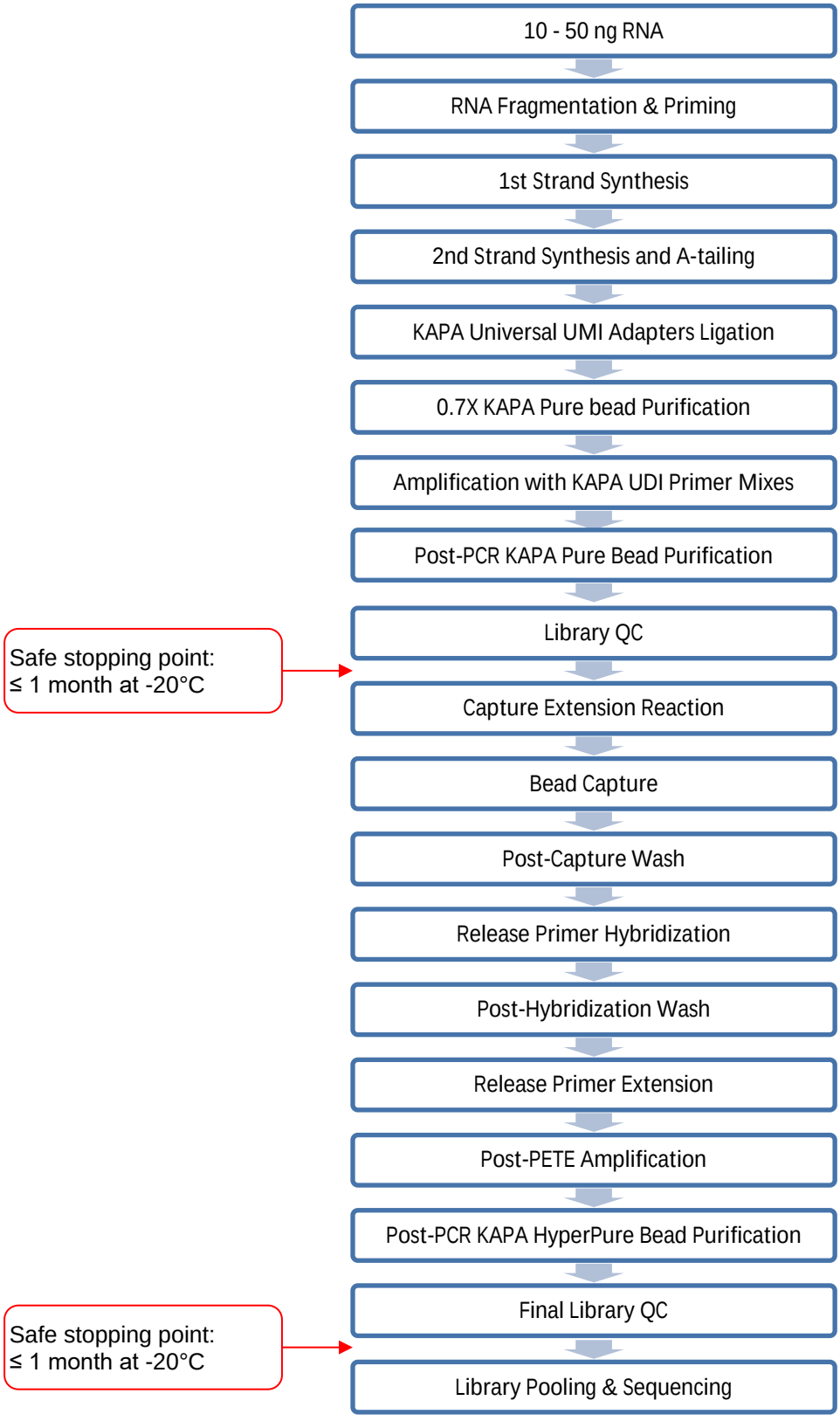


Fig. 1 Tissue RNA Fusion Transcript Preparation HyperPETE Workflow

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 RNA. 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 RNA 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 RNA 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

- Thermocyclers should be programmed with the following:
  - Library Preparation
    - RNA Fragmentation and Priming program (*Chapter 3*, Library Preparation from DNA, Step 1)
    - 1st Strand Synthesis program (*Chapter 3*, Library Preparation from DNA, Step 2)
    - 2nd Strand Synthesis program (*Chapter 3*, Library Preparation from DNA, Step 3)
    - Adapter Ligation program (*Chapter 3*, Library Preparation from DNA, Step 4)
    - Amplification with KAPA UDI Primer Mixes program (*Chapter 3*, Library Preparation from DNA, Step 6)
  - Primer Extension Target Enrichment
    - Capture Primer Extension program (*Chapter 4*, Primer Extension Target Enrichment, Step 2)
    - Release Primer Hybridization program (*Chapter 4*, Primer Extension Target Enrichment, Step 6)

- Release Primer Extension program (*Chapter 4*, Primer Extension Target Enrichment, Step 9)
- Post-PETE Amplification program (*Chapter 4*, 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 for 1.5 mL, 0.2 mL, and 0.2 mL strip tubes	Multiple Vendors	N/A
Qubit Fluorometer	ThermoFisher	Multiple models
TapeStation	Agilent	Multiple Models
Bioanalyzer 2100	Agilent	G2939BA
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
NextSeq 500/550 or MiSeq	Illumina	Multiple models
Pipettes	Multiple Vendors	Multiple Models
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 RNA HyperPrep Kit	24 reactions 96 reactions	08 098 093 702 08 098 107 702

KAPA UDI Primer Mixes, 1-96	96 reactions	09 134 336 001
KAPA Universal UMI Adapters	96 reactions 4 X 96reactions*	09 329 862 001 09 329 889 001
KAPA HyperPETE Reagent Kit	24 reactions 96 reactions	09 211 624 001 09 211 683 001
KAPA HyperPETE Panel	Lung Cancer Fusion, 24 reactions Lung Cancer Fusion, 96 reactions Lung Cancer Fusion, 384 reactions Choice RNA 50KB, 96 reactions Choice RNA 50KB, 384 reactions Choice RNA 50KB, 1536 reactions Choice RNA 50KB, 10000 reactions Explore RNA 50KB, 96 reactions Explore RNA 50KB, 384 reactions Explore RNA 50KB, 1536 reactions Explore RNA 50KB, 10000 reactions	09 329 471 001 09 329 501 001 09 329 536 001 09 418 938 001 09 418 962 001 09 418 989 001 09 418 997 001 09 419 209 001 09 419 233 001 09 419 241 001 09 419 250 001
KAPA HyperCapture Bead Kit	24 reactions 96 reactions	09 075 780 001 09 075 798 001

* 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 RNA HS Assay Kit	ThermoFisher	1 kit	Q32852
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
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	1 box of 7 tapes	5067-5584
TapeStation Parts and Accessories	Agilent	N/A	N/A
Agilent RNA 6000 Pico Kit	Agilent	Reagents and consumables for 25 chips	5067-1513
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. Store and Prepare the Reagents

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

- KAPA RNA HyperPrep Kit
- KAPA Universal UMI Adapters
- KAPA UDI Primer Mixes
- KAPA HyperCapture Bead Kit
- KAPA HyperPETE Reagent Kit
- KAPA HyperPETE Panel

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA RNA HyperPrep Kit	-15°C to -25°C
KAPA HyperCapture Bead Kit	+2°C to +8°C
KAPA Pure Beads*	+2°C to +8°C
KAPA Universal UMI Adapters	-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 HyperPETE Reagent Kit	-15°C to -25°C
KAPA HyperPETE Panel	-15°C to -25°C

The HyperCapture Bead kit and Pure beads provided within the KAPA RNA HyperPrep Kit 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.

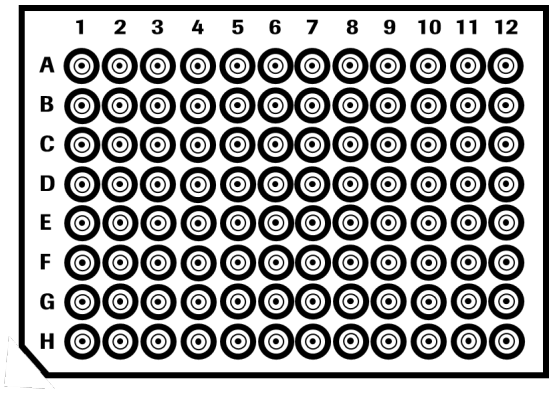


Fig. 2 KAPA UDI Primer Mixes plate layout

Chapter 3. Library Preparation from RNA

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

Components from the following kits are required:

- KAPA RNA HyperPrep Kit
- KAPA Pure Beads*
- KAPA Universal UMI Adapters
- KAPA UDI Primer Mixes

*The KAPA Pure Beads are provided in the KAPA RNA HyperPrep Kit

Ensure the following is available:

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

Sample Requirements

The following workflow has been validated with 10 to 50 ng of total purified FFPET RNA and non-formalin compromised RNA for optimal library preparation. RNA concentrations should be quantified by using the Qubit RNA HS Assay Kit. RNA quality should be assessed using the Agilent RNA 6000 Pico Assay for the BioAnalyzer. Lower input amounts and sample quality may not yield equivalent results.

It is critical that the water used for library preparation is RNase-free PCR grade water.

It is recommended to use an RNAse decontamination solution to clean all surfaces and equipment before starting.

Adjust thermocycler lid idle temperature to minimize waiting time between incubation programs. Samples should be placed on ice while temperatures are being reached.

It is recommended to use pipette mixing for all RNA mixing steps.

Please note the PEG/NaCl solution and Library Amplification Primer Mix provided in the KAPA RNA HyperPrep Kit will not be used in the library preparation protocol.

Unless otherwise noted, when assembling a master mix for processing samples, always prepare a 10% excess.

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

Unless otherwise specified, all 0.2 mL PCR strip tube vortexing steps in this chapter should be performed using the IKA.

Vortex set to 2400rpm for 10 seconds or until thoroughly mixed.

Step 1. RNA Fragmentation and Priming

It is recommended to work primarily on ice to maintain sample integrity throughout this library preparation protocol.

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

Component	Thawing procedure
KAPA RNA HyperPrep Kit	Room temperature then place on ice
KAPA Pure Beads*	Room temperature
KAPA Universal UMI Adapters	Room temperature then place on ice
KAPA UDI Primer Mixes	Room temperature then place on ice

*The KAPA Pure Beads are provided in the KAPA RNA HyperPrep Kit

2. Check RNA quality with the Agilent RNA 6000 Pico kit on the BioAnalyzer. DV200 scores determine if the samples are low quality, mid quality, or high quality (LQ, MQ, or HQ) which is used in step 6.7.

DV200	Quality
30% ≤ DV200 < 50%	LQ
50% ≤ DV200 < 70%	MQ
DV200 ≥ 70%	HQ

RNA samples with DV200 scores of less than 30% should be avoided as results will not be optimal.

3. Check RNA concentrations with the Qubit RNA HS Assay kit.
4. Add 10-50 ng of RNA into a 0.2 mL PCR tube.
5. If needed, adjust the volume in each tube to 10 µL using nuclease-free water.
6. Add 10 µL of Fragment, Prime and Elute Buffer (2X) to the RNA sample while still on ice, for a total of 20 µL.
7. Mix thoroughly by gently pipetting the reaction up and down several times and briefly spin down.

Avoid vortexing the RNA sample to limit RNA sample degradation.

8. Incubate the sample on a thermocycler using one of the following incubation protocols:

For Non-Formalin Compromised RNA

Temperature	Duration
94°C	6 min
4°C	N/A

Set the thermocycler lid to 105°C.

For FFPET RNA

Temperature	Duration
65°C	1 min
4°C	N/A

Set the thermocycler lid to 105°C.

9. After incubation, place tubes on ice and immediately proceed to 1st Strand Synthesis.

Step 2. 1st Strand Synthesis

1. Prepare the 1st Strand Synthesis Master Mix, as described below:

Component	Volume per Individual Sample
1st Strand Synthesis Buffer	11 µL
KAPA Script	1 µL
Total	**12 µL**

Excess reagent master mix has already been accounted for in the tables provided.

The 1st Strand Synthesis Master Mix can be made before starting and placed on ice.

2. Add **10 µL** of the 1st Strand Synthesis Master Mix to each sample on ice, for a total volume of 30 µL.
3. Keeping the tubes on ice, mix thoroughly by gently pipetting the reaction up and down several times.

Avoid vortexing the RNA sample to limit RNA sample degradation.

4. Incubate the sample on a thermocycler with the following protocol:

Temperature	Duration
25°C	10 min
42°C	15 min
70°C	15 min
4°C	N/A

Set the thermocycler lid to 85°C.

5. During the incubation, prepare the 2nd Strand Synthesis Master Mix as described in *Step 3.1*.

6. After incubation, place tubes on ice and immediately proceed to 2nd Strand Synthesis.

Step 3. 2nd Strand Synthesis

1. Prepare the 2nd Strand Synthesis and A-tailing Master Mix, as described below:

Component	Volume per Individual Sample
2nd Strand Marking Buffer	31 µL
2nd Strand Synthesis & A-tailing Enzyme Mix	2 µL
Total	**33 µL**

Excess reagent master mix has already been accounted for in the tables provided.

The 2nd Strand Synthesis Master Mix should be made during *Step 2.4*.

2. Add **30 µL** of the 2nd Strand Synthesis and A-tailing Master Mix to the 1st Strand Synthesis product on ice, for a total volume of 60 µL.
3. Keeping the tubes on ice, mix thoroughly by gently pipetting the reaction up and down several times.
4. Incubate the sample on a thermocycler with the following protocol:

Temperature	Duration
16°C	30 min
62°C	10 min
4°C	∞

Set the thermocycler lid to 85°C.

5. During the incubation, prepare the Diluted KAPA Universal UMI Adapters and Adapter Ligation Master Mix as described in *Step 4.1* and *Step 4.3*.
6. After incubation, place tubes on ice and immediately proceed to Adapter Ligation.

Step 4. Adapter Ligation

1. Dilute the KAPA Universal UMI Adapters, as described below:

Component	Volume per Individual Sample
KAPA Universal UMI Adapters	0.23 µL
10mM Tris-HCl, pH 8.0	4.77 µL
Total	**5 µL**

Please note that only 5 µL of the diluted KAPA Universal UMI Adapters is needed for a single sample library preparation. To avoid pipetting less than 1 µL, it is recommended that dilution should be made for a minimum of 5 samples. Make a fresh dilution of KAPA Universal UMI Adapters each time.

The Diluted KAPA Universal UMI Adapters should be made during *Step 3.5*.

2. Add 5 µL of the diluted KAPA Universal UMI Adapters to the 60 µL 2nd Strand Synthesis product, for a total of 65 µL. Mix thoroughly and briefly spin.

KAPA Universal UMI Adapters must be added to each sample prior to the addition of the Ligation Master Mix. Addition of the KAPA Universal UMI Adapters to the Ligation Master Mix will cause formation of adapter dimers.

3. Prepare Adapter Ligation Master Mix, as described below:

Component	Volume per Individual Sample
Ligation Buffer	40 µL
DNA Ligase	10 µL
Total	**50 µL**

The KAPA HyperPrep 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.

Excess reagent master mix has already been accounted for in the tables provided.

The Adapter Ligation Master Mix should be made during *Step 3.5*.

4. Add **45 µL** of the Ligation Master Mix to each sample, for a total of 110 µL.
5. Mix thoroughly and briefly spin down the tubes to settle the liquid to the bottom.
6. Incubate on a thermocycler:

Temperature	Duration
20°C	15 min
4°C	∞

Set the thermocycler lid to 50°C.

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

Step 5. Post-Ligation 0.7X Purification using KAPA Pure 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 Pure 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.

Do not use KAPA HyperPure Beads

2. Add 77 µL of KAPA Pure 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 187 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 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 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 samples, 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 unused primer mixes for utilization 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	Cycle number depends on RNA input amount and quality. 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.

Sample Type	RNA Input	Number of Cycles
Non-Formalin Compromised RNA	50 ng	12 cycles
	10 ng	14 cycles
HQ and MQ FFPET	50 ng	17 cycles
	10 ng	18 cycles
LQ FFPET	50 ng	18 cycles
	10 ng	19 cycles

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 using KAPA Pure 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 Pure Beads from cold storage at least 30 minutes prior to use to bring them to room temperature. Vortex Beads until thoroughly resuspended well right before use.

Do not use KAPA HyperPure Beads

2. Add 50 µL of KAPA Pure 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.

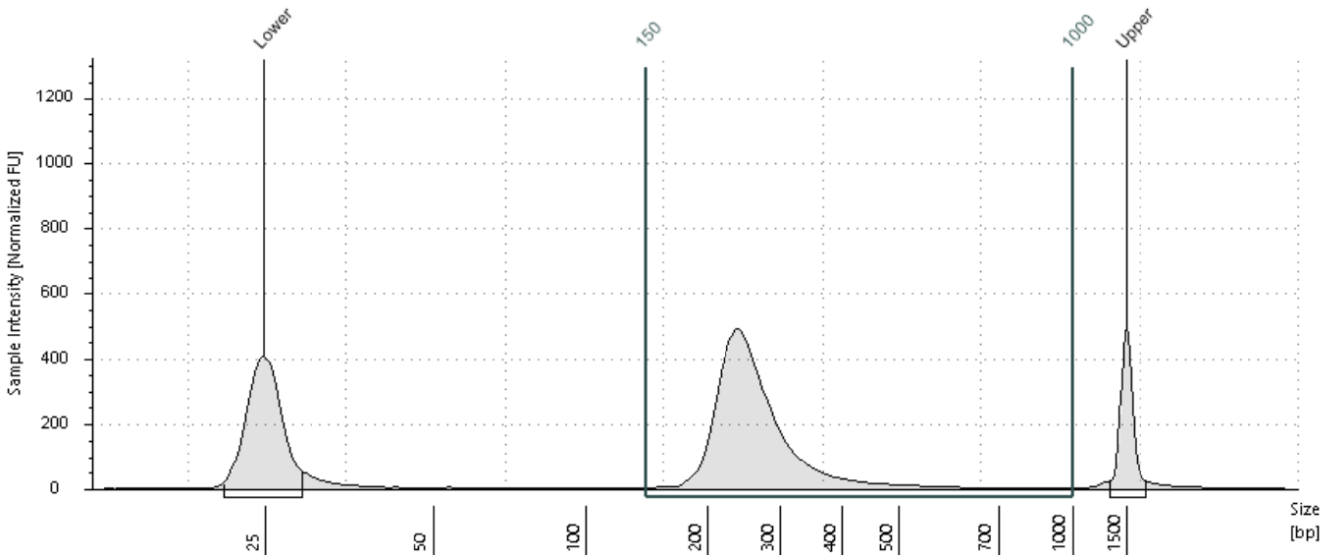
16. Transfer the eluate into a new strip tube. The eluate contains the pre-capture input library.

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 D1000 ScreenTape Assay to analyse the appropriate volume of the diluted amplified pre-capture input library (and any controls) as per manufacturer's instructions.

3. The undiluted amplified pre-capture input library should have a concentration of ≥ 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 libraries should have an average fragment size distribution between 250 and 350 bp, depending on input RNA quality. Figure 3 is an example library prepared from low quality FFPET RNA. 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	274	1190	7070	91.73		

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

Freeze at -20°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 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 RNA HyperPrep Kit for RNA 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 0.2 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.

1. 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 input library volume.

2. Add 35-40 µL of the Capture Extension Reaction Master Mix to the pre-capture input libraries for a final volume of 50 µL.
3. 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
4. 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.

5. During the primer annealing and extension incubation proceed to *Step 3. Prepare the Capture Beads.*
6. 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 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 *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 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 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 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. 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 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	∞

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
Primer Annealing	60°C	30 sec	

Extension	72°C	30 sec	below.
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
5-20kb	17
>20kb	16

PCR cycle numbers have been verified for the following panels

Panel	Panel Capture Target Size (kb)	Number of Cycles
Lung Cancer Fusion	18	17

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 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.

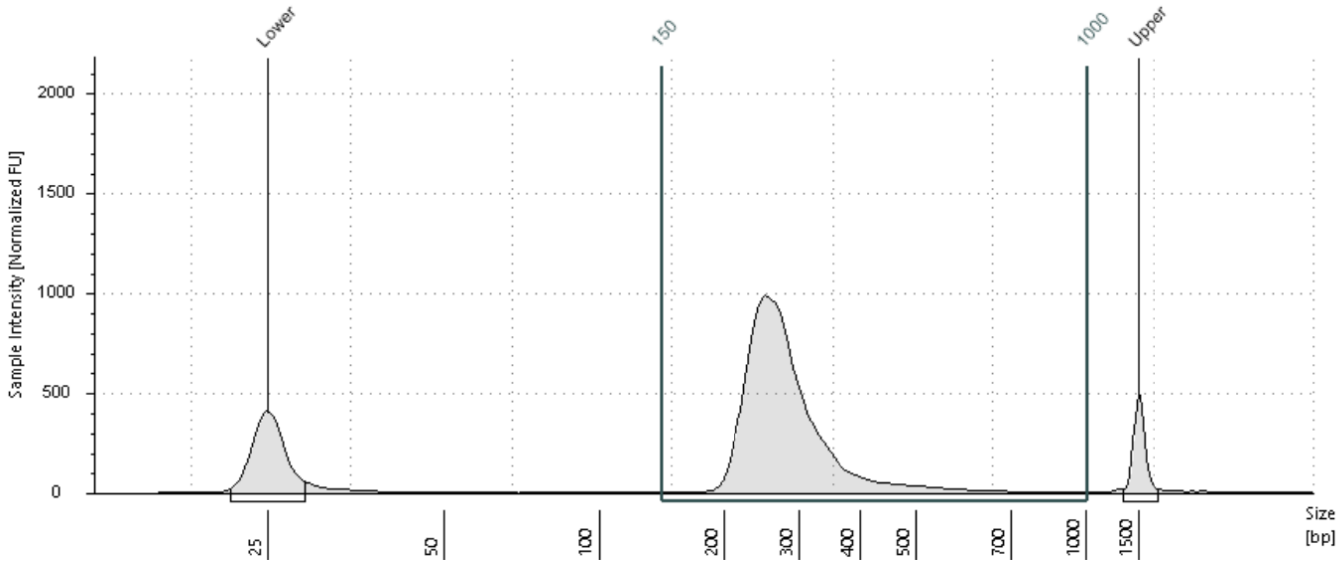
    Visually confirm that the beads are pelleted.

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 280 and 400 bp depending on RNA input quality. Figure 4 is an example Primer Extension Target Enrichment library prepared from a FFPET RNA 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	2660	14800	96.19		

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

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
Lung Cancer Fusion	18	Tissue RNA	1,800,000
Custom	Panel capture target size varies depending on the panel. Use the Estimated Coverage size given by HyperDesign and not the primary target size.	Tissue RNA	100 reads per capture target base (100 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

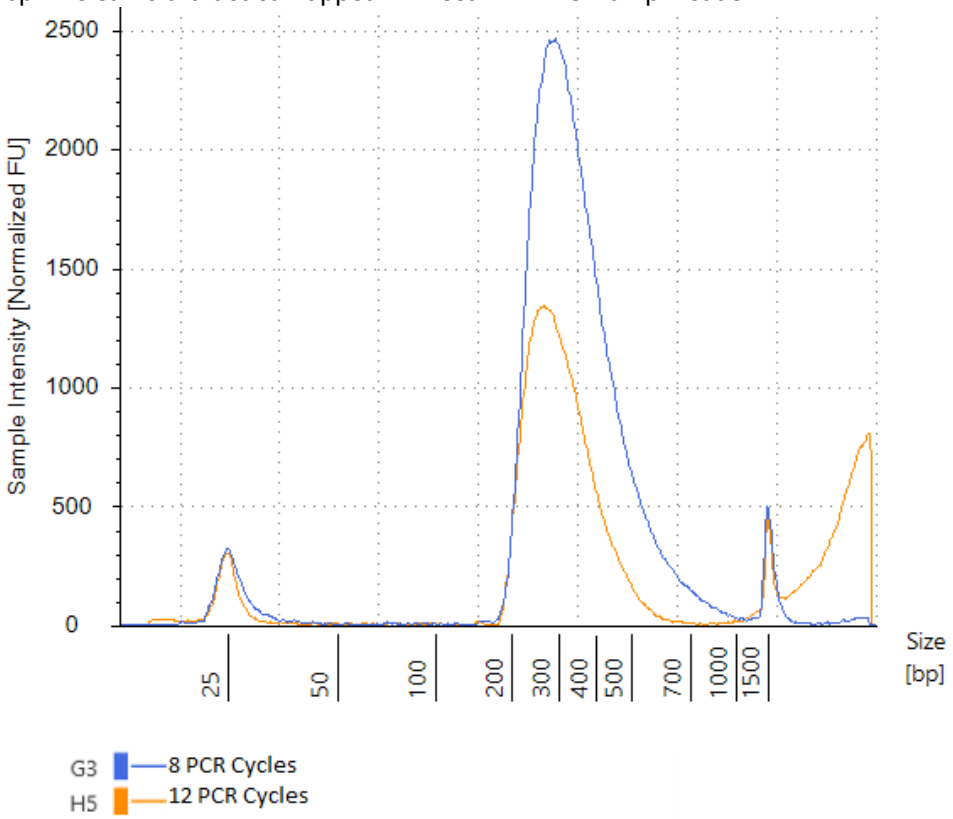
1. Store Primer Extension Target Enrichment libraries at -15°C to -25°C for up to 1 month.

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)
Library Preparation	
Using DV200 < 30% for FFPET RNA	Usage of RNA samples with a DV200 < 30% is not recommended and can result in over-fragmentation and low pre-capture input library yield.
Not using DV200	Failure to assess the quality of input RNA using DV200 is not recommended and can result in over-fragmentation and low pre-capture input library yield.
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 RNA 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 the recommended RNA input amount ● Ensure RNA samples have a DV200 > 30% ● 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 Pure Bead purification steps are performed properly ● Do not let KAPA Pure Beads overdry
Fragment distribution (analyzed using the Agilent Tapestation DNA High Sensitivity ScreenTape Assay D1000) shows that the average amplified fragment size is not within the size range of 150 to 1000 bp.	Poor fragmentation occurred. ● Repeat library preparation. Over-fragmentation: ● Use the recommended RNA input amount ● Ensure RNA samples have a DV200 > 30%
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	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

set of fragments (Figure A)	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-enrichment library after the first PCR	Insufficient RNA or poor quality RNA used in the assay: ● Ensure proper quantification of the input RNA. ● Use the recommended RNA input amount ● Ensure RNA samples have a DV200 > 30% Poor ligation efficiency: ● Ensure that the proper amount of input RNA 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 Pure 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 Pure 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 RNA 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 Pure Bead purification steps are performed properly. ● Do not let KAPA Pure Beads overdry.
Sequencing Performance Metrics	
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 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
Lower or higher number of sequencing reads than expected	Insufficient RNA or poor quality RNA used ● Ensure proper quantification and/or qualification of the input RNA. ● Follow the recommended RNA 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 2uL and 20uL
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

Appendix B.

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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