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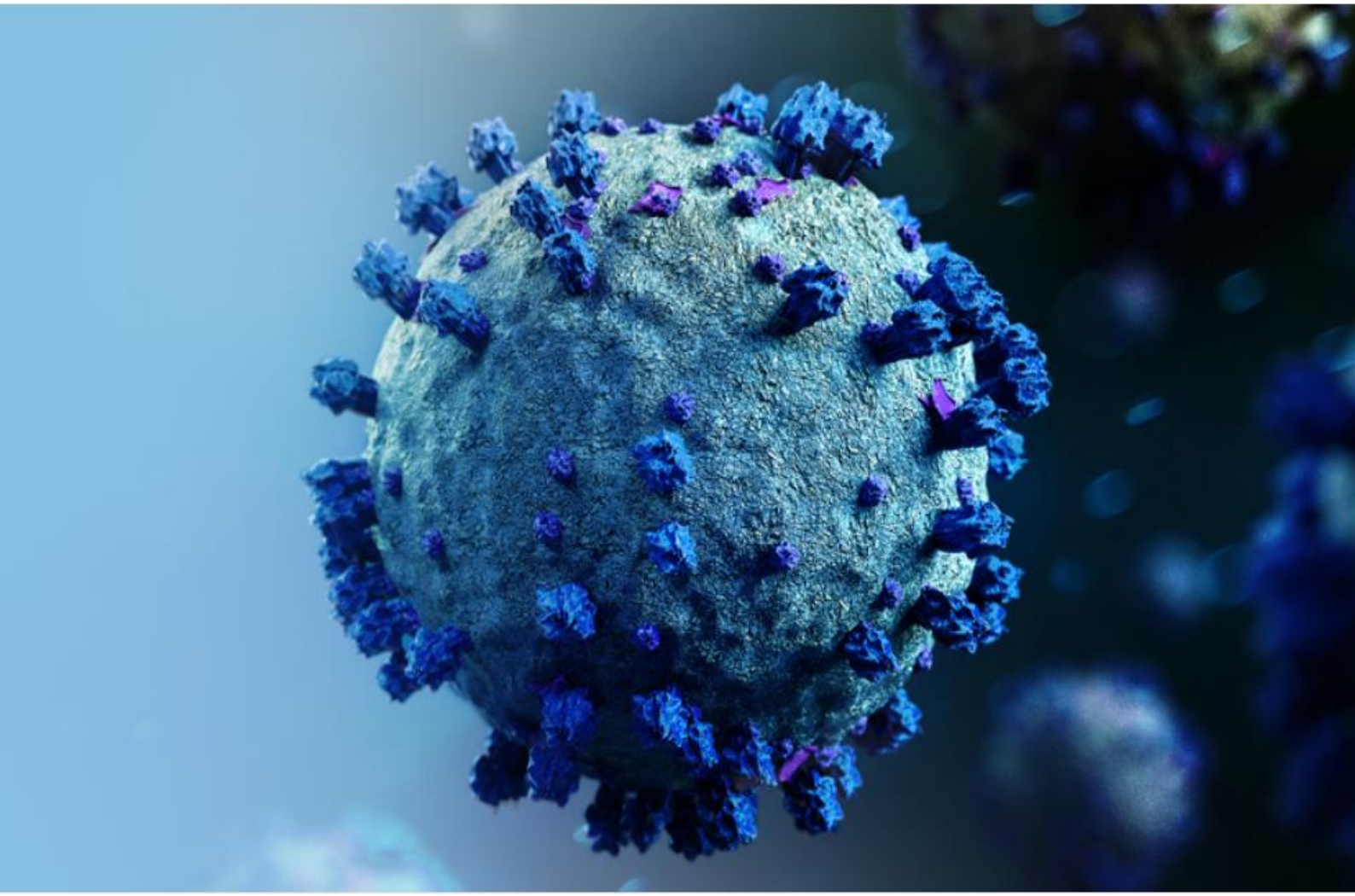
KAPA RNA HyperCap Workflow v1.1

Instructions For Use with the

KAPA HyperCap SARS-CoV-2 Panel



Contents

The KAPA HyperCap SARS-CoV-2 Panel contains:

Component	Description
KAPA HyperCap SARS-CoV-2 probes	Biotinylated capture oligos designed against the SARS-CoV-2 viral genome.

Storage and Stability

- KAPA HyperCap SARS-CoV-2 Probes are provided lyophilized and will need to be resuspended prior to first use, with the KAPA Probes Resuspension Buffer (sold separately). It is recommended to aliquot the probes into single-use volumes and freeze at –15°C to –25°C.
- KAPA HyperCap SARS-CoV-2 Probes are stable at –15°C to –25°C until the expiration date printed on the label, lyophilized or resuspended.

Application

KAPA HyperCap SARS-CoV-2 probes enable targeted enrichment of the SARS-CoV-2 viral genome and deliver high-performance enrichment in terms of coverage and uniformity. The KAPA HyperCap SARS-CoV-2 Panel covers:

› 184 sequences for SARS-CoV-2 from NCBI (1 RefSeq sequence and 183 GenBank sequences contributed by the scientific community).

› 100% of the reference genome (MN908947.3) by design

› >99.7% of the other 183 genomic sequences that are publicly available by design

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 laboratory area. Do not pipette by mouth.
- Wear the appropriate personal protective equipment, such as gloves, lab coat and safety glasses, to avoid direct contact while handling the reagents.
- Wash hands thoroughly after handling samples and reagents.
- In the event of a spill, clean up the solution with absorbent pads, allow to dry, and dispose of pads.

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

Formatting changes.

Ordering Information

For complete overview of Roche Sequencing products, go to *sequencing.roche.com/products*.

Trademarks

Contact and Support

This instruction set is not fully validated and not fully supported. If you have questions, contact your local Roche Technical Support. Go to *sequencing.roche.com/support* for contact information.

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Editions

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Use Restrictions

For patent license limitations for individual products, refer to: *www.technical-support.roche.com*.

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Preface

Regulatory Disclaimer

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KAPA HyperCap SARS-CoV-2 Panel

KAPA HyperCap SARS-CoV-2 Panel is a solution-based capture reagent that enables enrichment of the SARS-CoV-2 genome. Throughout this document, 'KAPA HyperCap Target Enrichment Probes' refers to the KAPA HyperCap SARS-CoV-2 Panel.

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	Highlights a resource in a different area of this manual or on a web site.
Italic type	Identifies the external general resources or names.
Bold type	Identifies names of paragraphs, sections or emphasized words.

Chapter 1. Before You Begin

This Instructions for Use document describes the process for enrichment of individual or multiplexed cDNA sample libraries using the KAPA HyperCap SARS-CoV-2 Panel and the amplification of these sample libraries by ligation-mediated PCR. Specifically, these Instructions for Use provide a protocol for the workflow outlined in *Figure 1* using the KAPA RNA HyperPrep Kit. Modification of certain workflow steps may be appropriate for individual experimental needs (contact your local support). The output of this protocol are enriched viral libraries that can be directly sequenced using an Illumina sequencing instrument.

The KAPA HyperCap SARS-CoV-2 Panel supported by the KAPA RNA HyperCap Workflow v1 provides:

- High performance based on proprietary probe design algorithms and optimized kits and reagents:
  - Enhanced capture uniformity
  - Fewer PCR duplicates
  - Deeper target coverage
  - Higher sensitivity in SNP detection
- An easy to use, streamlined, and automation friendly workflow with minimal resource requirements:
  - Moderate hybridization and wash temperatures
  - Independent of a vacuum concentrator
  - Streamlined washes
- Single vendor service and support for NGS sample preparation:
  - KAPA RNA HyperPrep Kits
  - KAPA HyperPure Beads
  - KAPA Universal adapter and UDI Primer Mixes 1-384
  - KAPA HyperCapture Reagent and Bead Kits

Overview of the KAPA RNA HyperCap Workflow v1.1

The KAPA RNA HyperCap Workflow v1.1 involves:

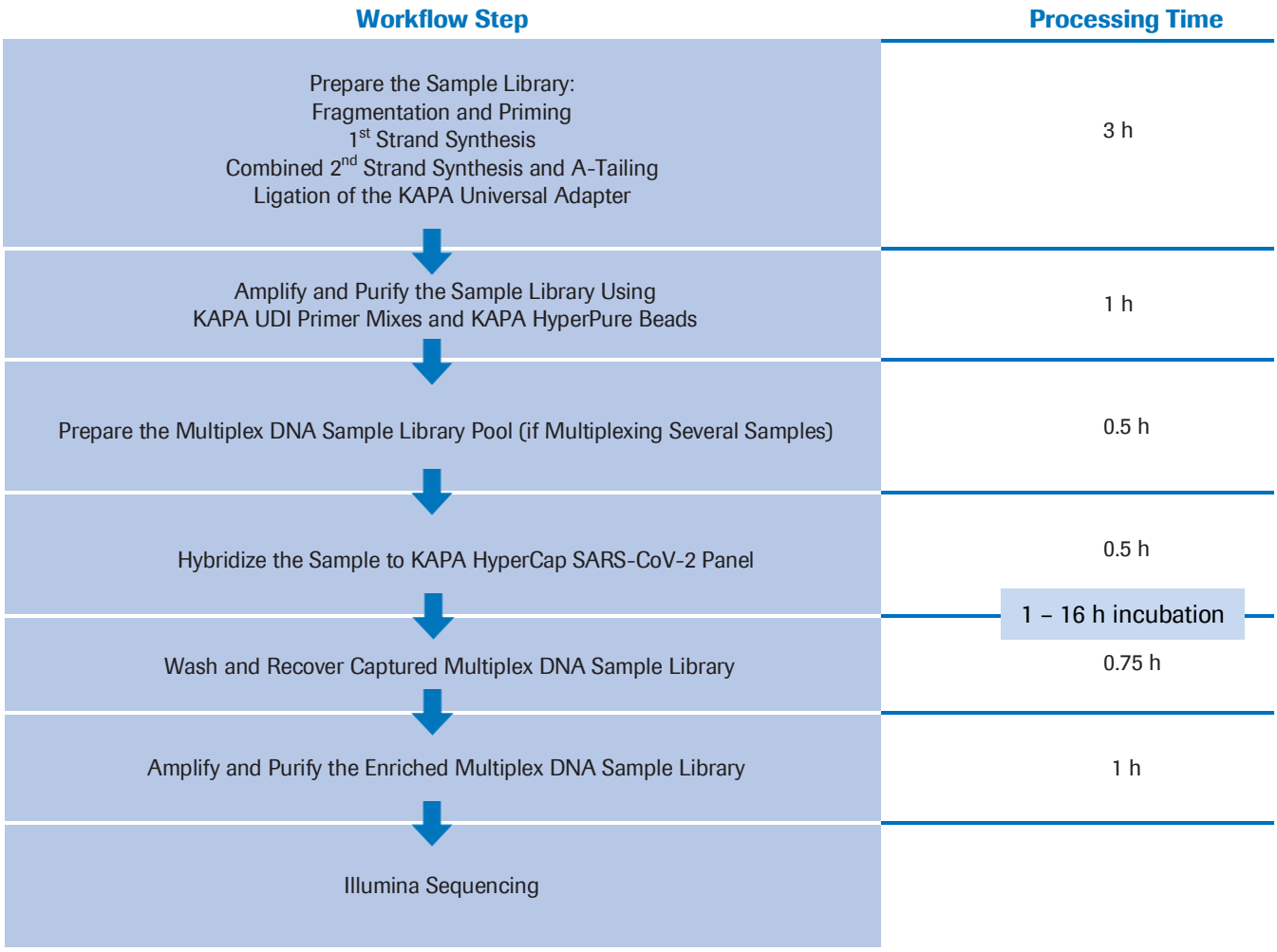


Figure 1: KAPA RNA HyperCap Workflow. Where applicable, incubation times are indicated between steps. Processing time may vary based on number of samples processed and multiplexing levels.

Protocol Information & Safety

- Wear gloves, lab coat, safety glasses and take precautions to avoid sample contamination.
- Perform all centrifugations at room temperature (+15 to +25°C) unless indicated otherwise.
- 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.

Terminology

Target Enrichment (or Capture): The process of enriching targeted regions from genomic DNA or RNA. In the context of this document, the hybridization of the Amplified Sample Library to the KAPA HyperCap Target Enrichment Probes, and subsequent washing steps.

KAPA HyperCap Target Enrichment Probes: The complete set of biotinylated 120 bp oligonucleotide probes provided by Roche (KAPA HyperCap SARS-CoV-2 Probes) to perform target enrichment.

Sample Library: The initial library generated from RNA by 1st Strand Synthesis, combined 2nd Strand Synthesis and A-tailing, and ligation. In the context of this document, this is the Sample Library before amplification by PCR and prior to capture.

Amplified Sample Library: The sample library after amplification by PCR but before capture.

Universal Adapter: Truncated Y-shaped adapter with T overhang

UDI Primer Mixes: Unique Dual-Indexed Primer pairs used for sample barcoding

RNA Handling

RNases are ubiquitous and special care should be taken throughout the procedure to avoid RNase contamination. To avoid airborne RNase contamination, keep all reagents and RNA samples closed when not in use.

Use a laminar flow hood if available, or prepare a sterile and RNase-free area. Clean the workspace, pipettes and other equipment with an RNase removal product (e.g., RNaseZAP, Ambion Inc.) according to manufacturer's recommendations.

To avoid RNase contamination, always wear gloves when handling reagents, and use certified RNase-free plastic consumables. Change gloves after making contact with equipment or surfaces outside of the RNase-free working area.

To mix samples containing RNA, gently pipette the reaction mixture several times. Vortexing may fragment the RNA, resulting in lower quantity and a reduced library insert size.

To avoid degradation, minimize the number of freeze thaw cycles and always store RNA in RNase-free water.

Prepare the Following Equipment and Reagents

- Thermocyclers should be programmed with the following:
- KAPA RNA HyperPrep Kit
  - Fragmentation and Priming program (*Chapter 3*, Prepare the Sample Library, Step 1.3)
  - 1st Strand Synthesis Program (*Chapter 3*, Prepare the Sample Library, Step 1.5.d)
  - 2nd Strand Synthesis and A-Tailing Program (*Chapter 3*, Prepare the Sample Library, Step 1.6.d)
  - Pre-Capture PCR program (*Chapter 4*, Amplify the Sample Library Using the KAPA UDI Primer Mixes, Step 2.1)
  - Hybridization incubation program (*Chapter 5*, Hybridize the Sample and KAPA HyperCap Target Enrichment Probes, Step 3.19)
  - Post-Capture LM-PCR program (*Chapter 7*, Amplify Enriched Multiplex DNA Sample, Step 3.1)

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 Instruction for Use. If further guidance is needed, please contact *Roche Technical Support*.

- The following steps should be taken before beginning the workflow:
  - Resuspend and aliquot the KAPA HyperCap Target Enrichment Probes (*Chapter 2*, Store the KAPA HyperCap Reagents, Step 2)
  - Preparation of the KAPA UDI Primer Mixes (*Chapter 2*. Prepare and store the KAPA HyperCap Reagents, Step 3)
  - Resuspend the Post-Capture PCR Oligos (*Chapter 7*, Amplify Enriched Multiplex DNA Sample, Step 1)

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

Required Equipment, Labware & Consumables

You assume full responsibility when using the equipment, labware, and consumables described below. This protocol is designed for use with the specified equipment, labware, and consumables.

Laboratory Equipment

Equipment	Supplier	Catalog No.
DynaMag-2 Magnet (16 x 2.0 mL tube holder) (optional)	Thermo Fisher	12321D
DynaMag-96 Side Magnet, or 0.2 mL PCR Strip Magnetic Separator Rack	Thermo Fisher, or Permagen	12331D, or MSR812
Microcentrifuge (16,000 x *g* capability)	*Multiple Vendors*	
Qubit Fluorometer	ThermoFisher	Multiple models
Bioanalyzer 2100	Agilent	
Thermocycler (capable of maintaining +55°C for 16 - 20 hours), with programmable heated lid	*Multiple Vendors*	
Vortex mixer	*Multiple Vendors*	
Plate Centrifuge which can reach at least 280 x *g*	*Multiple Vendors*	

Consumables Available from Roche

Component	Package Size/Contents	Catalog No.
KAPA Universal Adapter	96 reactions 384 reactions*	09 063 781 001 09 063 790 001
KAPA UDI Primer Mixes, 1-96 KAPA UDI Primer Mixes, 97-192 KAPA UDI Primer Mixes, 193-288 KAPA UDI Primer Mixes, 289-384	96 reactions 96 reactions 96 reactions 96 reactions	09 134 336 001 09 329 838 001 09 329 846 001 09 329 854 001
KAPA HyperCapture Bead Kit	24 reactions 96 reactions 384 reactions*	09 075 780 001 09 075 798 001 09 075 909 001
KAPA HyperCapture Reagent Kit	24 reactions 96 reactions 384 reactions*	09 075 810 001 09 075 828 001 09 075 917 001
KAPA RNA HyperPrep Kit	24 reactions 96 reactions	08 098 093 702 08 098 107 702
KAPA Hybrid Enhancer Reagent	1 mL	09 075 763 001
KAPA Probes Resuspension Buffer	1 mL 5 mL	09 075 879 001 09 075 887 001
KAPA HyperCap SARS-CoV-2 Panel†	12 reactions 24 reactions 48 reactions 96 reactions	09 436 499 001 09 436 502 001 09 436 529 001 09 436 537 001

*** Virtual kits, consist of 4 x 96 reaction kits.**

† Larger reaction packs available up to 4,000 reactions

Consumables Purchased from Other Vendors

Component	Supplier	Package Size	Catalog No.
Agilent DNA 1000 Kit	Agilent	1 kit	5067-1504
Agilent High Sensitivity DNA Kit (recommended)	Agilent	1 kit	5067-4626
10 mM Tris-HCl, pH 8.0	*Multiple Vendors*		
Ethanol, 200 proof (absolute), for molecular biology	Sigma-Aldrich	500 mL	E7023-500ML
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 or strip-tubes, (preferably low-bind) ■ 1.5 mL microcentrifuge tubes (optional)	*Multiple Vendors*		
Water, PCR Grade	Sigma-Aldrich	1 x 25 mL 25 x 1 mL 4 x 25 mL	03 315 959 001 03 315 932 001 03 315 843 001

Use nuclease-free, PCR Grade water for all described protocol steps. Working with a liquid handler system may require excess volumes from various reagents.

Chapter 2. Prepare and Store the KAPA HyperCap Reagents

This chapter describes the storage conditions for the following kits:

- KAPA HyperCapture Bead Kit
- KAPA HyperPure Beads
- KAPA HyperCapture Reagent Kit
- KAPA Universal Adapter
- KAPA UDI Primer Mixes
- KAPA Probes Resuspension Buffer
- KAPA RNA HyperPrep Kit

Step 1. Store the Reagent Kits

Reagent Kit	Storage Temperature
KAPA HyperCapture Bead Kit	+2 to +8°C
KAPA HyperPure Beads	+2 to +8°C
KAPA HyperCapture Reagent Kit	-15 to -25°C
KAPA Universal Adapter	-15 to -25°C
KAPA UDI Primer Mixes or KAPA UDI Primer Mixes (resuspended)	+2 to +8°C or -15 to -25°C
KAPA Probes Resuspension Buffer	-15 to -25°C
KAPA Hybrid Enhancer Reagent (if using non-human samples)	-15 to -25°C
KAPA RNA HyperPrep Kit	-15 to -25°C

The KAPA HyperCapture Bead kit must not be frozen.

Step 2. Resuspend and Aliquot the KAPA HyperCap Target Enrichment Probes

Store the KAPA HyperCap Target Enrichment Probes in their lyophilized form at -15°C to -25°C until ready to use. Before first use of the KAPA HyperCap Target Enrichment Probes, undertake the following steps to ensure the highest performance and to avoid multiple freeze/thaw cycles or potential accidental contamination:

1. Review the KAPA HyperCap Target Enrichment Probes tube label to verify the probe reaction number and resuspension volume.
2. Spin the KAPA HyperCap Target Enrichment Probes tube at 10,000 x *g* for 30 seconds to ensure the contents are at the bottom of the tube.
3. Add the recommended volume of KAPA Probes Resuspension Buffer provided on the tube label to the KAPA HyperCap Target Enrichment Probes tube.
4. Vortex the tube for 1 minute to resuspend the probe pool.
5. Centrifuge the tube at 10,000 x *g* for 30 seconds to ensure that the liquid is at the bottom of the tube before removing the cap.
6. Aliquot the KAPA HyperCap Target Enrichment Probes into single-use aliquots (4 µL/capture) into 0.2 mL PCR tubes and store at -15 to -25 °C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.
7. When ready to perform the hybridization portion of the experiment, thaw the required number of single-use probe pool aliquots on ice.

The KAPA HyperCap Target Enrichment Probes should not undergo freeze/thaw cycles after aliquoting. Ensure that you properly label and record the expiration date of the aliquoted probes.

Step 3. Preparation of the KAPA UDI Primer Mixes

Before use of the KAPA UDI Primer Mixes, undertake the following steps to resuspend the primers:

1. Take out the KAPA UDI Primer Mixes plate from storage (+2 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 PCR Grade water directly to the bottom of each well and discard tips after dispensing.

   A new pipette tip must 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. Ensure every well contains 10 µL of PCR Grade water and cover the plate with one of the adhesive foil seals provided in the kit.

   Make sure the foil seal is properly aligned and 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 ensuring all wells are mixed well.

   Ensure wells at the corners of the plate are mixed well by vortexing the corners of the plate. Keep the plate upright.

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 to -25°C. To avoid repeated freeze/thaw cycles you may transfer the resuspended primers to separate tubes or tube strips for storage.

    Ensure aliquoted KAPA UDI Primer Mixes are correctly labeled.

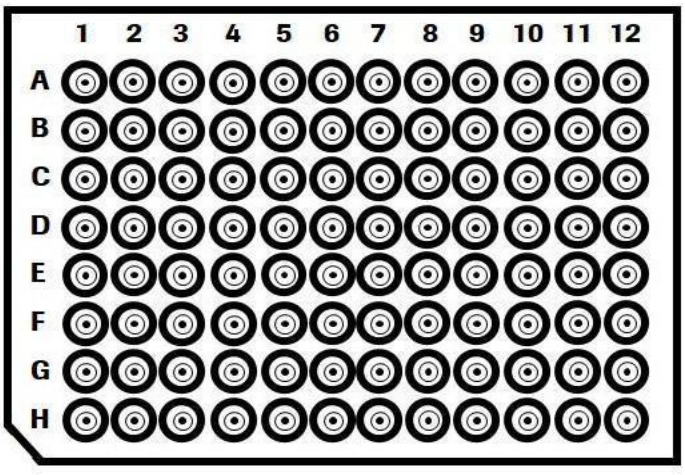


Figure 2: KAPA UDI Primer Mixes plate layout.

Chapter 3. Prepare the Sample Library

This chapter describes the RNA sample library preparation method, and requires use of components from the following kits:

- KAPA RNA HyperPrep Kit
- KAPA Universal Adapter
- KAPA UDI Primer Mixes

Ensure that the following are available:

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

Sample Requirements

This workflow was tested with 50 ng and 100 ng of total RNA for sample library preparation. The RNA should be quantified by using the Agilent Bioanalyzer RNA Assay. Lower input amounts and sample quality may not yield equivalent results. For guidance on lower input amounts or sample quality, contact *Roche technical support*.

Step 1. Prepare the Sample Library

- KAPA RNA HyperPrep Kit

KAPA RNA HyperPrep Kit

Make sure the KAPA HyperPure/Pure Beads are removed from storage to allow time for proper equilibration to room temperature. For best performance, store the beads protected from light when not in use.

RNA in volumes >10 µL should be concentrated to 10 µL prior to use by either ethanol precipitation, bead purification (e.g., KAPA Pure Beads), or column-based methods. Note that some loss of material is inevitable when using any of the above methods to concentrate RNA.

When concentrating RNA, elute in 12 µL of RNase-free water to ensure that 10 µL is available for use with this protocol.

1. Prepare the required volume of **Fragment, Prime and Elute Buffer** (1X) at room temperature as follows:

Component	Volume
50 - 100 ng Purified total RNA	10 µL
Fragment, Prime and Elute Buffer (2X)	10 µL
Total	**20 µL**

RNA is fragmented by incubating at a high temperature in the presence of magnesium before carrying out 1st Strand cDNA Synthesis.

Fragmentation conditions given in the Library Construction Protocol should be used as a guideline and may require adjustment based upon the quality and size distribution of the input RNA. It is recommended that a non-precious, representative sample of RNA be evaluated in order to identify the optimal fragmentation conditions.

2. Mix thoroughly by gently pipetting up and down several times.

Do not vortex to avoid RNA degradation.

3. Place the plate/tube(s) in a thermocycler and carry out the fragmentation and priming program for desired size and sample quality. For high quality intact RNA, fragmentation and priming for 6 min at +94°C resulted in library insert sizes of about 200 bp. For varying quality input RNA refer to the table below:

Set the thermocycler lid to 105°C and volume to 20 µL.

Input RNA Type	Desired mean library insert size (bp)	Fragmentation
Intact	100-200	8 min @ +94°C
Partially degraded	100-300	1-6 min @ +85°C
Degraded	100-200	1 min @ +65°C

4. Place the plate/tube(s) on ice and proceed immediately to next step
5. Perform **1st Strand Synthesis Reaction** as follows:
   a. Prepare the 1st Strand Synthesis Master Mix of the following reagents:

1st Strand Synthesis Master Mix	Per Individual Sample (includes 20% excess)
1st Strand Synthesis Buffer	11 µL
KAPA Script	1 µL
Total	**12 µL**

b. Add only 10 µL of 1st Strand Synthesis Master Mix to the fragmented, primed RNA sample for a total volume of 30 µL.

Final Reaction Composition	Per Reaction
1st Strand Synthesis Master Mix	10 µL
Fragmented, Primed RNA	20 µL
Total Reaction Volume	**30 µL**

c. Keeping the plate/tube(s) on ice, mix thoroughly by gently pipetting the reaction up and down several times.

d. Incubate the plate/tube(s) using the following protocol:

Set the thermocycler lid to 85°C and volume to 30 µL.

1. Step 1: 10 minutes at +25°C
2. Step 2: 15 minutes at +42°C
3. Step 3: 15 minutes at +70°C
4. Step 4: Hold at +4°C

e. Place the plate/tube(s) on ice and proceed immediately to 2nd Strand Synthesis and A-Tailing.

6. On ice, perform **2nd Strand Synthesis and A-Tailing Reaction** as follows:

a. Prepare the 2nd Strand Synthesis and A-Tailing Master Mix of the following reagents:

2nd Strand Synthesis and A-Tailing Master Mix	Per Individual Sample (includes 10% excess)
2nd Strand Marking Buffer	31 µL
2nd Strand Synthesis & A-Tailing Enzyme Mix	2 µL
Total	**33 µL**

b. Add only 30 µL of 2nd Strand Synthesis Master and A-Tailing Master Mix to the 1st Strand Synthesis Product for a total volume of 60 µL.

Final Reaction Composition	Per Reaction
1st Strand Synthesis Product	30 µL
2nd Strand Synthesis and A-tailing Master Mix	30 µL
Total Reaction Volume	**60 µL**

c. Keeping the plate/tube(s) on ice, mix thoroughly by gently pipetting the reaction up and down several times.

d. Incubate the plate/tube(s) using the following protocol:

Set the thermocycler lid to +85°C and volume to 60 µL.

1. Step 1: 30 minutes at +16°C
2. Step 2: 10 minutes at +62°C
3. Step 3: Hold at +4°C

e. Place the plate/tube(s) on ice and proceed immediately to Adapter Ligation.

7. Perform **Adapter Ligation** according to the instructions

   a. On ice, set up the Adapter Ligation Master Mix as follows:

Adapter Ligation Master Mix	Per Individual Sample (includes 10% excess)
KAPA HyperPrep Ligation Buffer	40 µL
KAPA HyperPrep DNA Ligase Enzyme	10 µL
Total	**50 µL**

The KAPA HyperPrep Ligation Buffer is very viscous and requires special attention during pipetting.

   b. On ice, prepare a fresh working solution of 1.5 µM from the KAPA Universal Adapter by diluting the stock at 1:10 with 10 mM Tris-HCl pH 8.0:

KAPA Universal Adapter Working Solution (1.5 µM)	For 2 reactions
KAPA Universal Adapter (15 µM)	1 µL
10 mM Tris-HCl pH 8.0	9 µL
Total Volume	**10 µL**

   c. Add 5 µL of KAPA Universal Adapter Working Solution (1.5 µM) to the 2nd Strand Synthesis Product.

   d. Add 45 µL of Adapter Ligation Master Mix to obtain a total volume of 110 µL.

Final Reaction Composition	Per Reaction
2nd Strand Synthesis Product	60 µL
KAPA Universal Adapter (1.5 µM)	5 µL
Adapter Ligation Master Mix	45 µL
Total Reaction Volume	**110 µL**

The KAPA Universal Adapter Working Solution (1.5 µM) must be added to each well individually prior to addition of the Adapter Ligation Master Mix to prevent the formation of adapter dimers.

   e. Keeping the plate/tube(s) on ice, mix thoroughly by pipetting the reaction up and down several times.

   f. Incubate the plate/tube(s) at 20°C for 15 min.

   g. Proceed immediately to the Post-Ligation Cleanup.

8. Perform a single **Post-Ligation Cleanup** as follows:

   a. Perform a 0.7X bead-based cleanup by combining the following:

Post-Ligation Cleanup	Per Individual Sample
Ligation Reaction	110 µL
KAPA Pure Beads from the KAPA RNA HyperPrep Kit	77 µL
Total	**187 µL**

   b. Mix the Ligation Reaction and the KAPA Pure Beads mixture thoroughly and perform a quick spin.

Make sure that you use KAPA Pure Beads and not HyperPure Beads at this step.
Ensure that the solution is thoroughly mixed and appears homogeneous.
Insufficient mixing may compromise recovery and size selection.

c. Incubate the sample at room temperature for 5 minutes to allow the DNA to bind to the beads.
d. Place the plate/tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
e. Carefully remove and discard the supernatant.
f. Keeping the plate/tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
g. Incubate the plate/tube(s) at room temperature for ≥30 seconds.
h. Carefully remove and discard the ethanol.
i. Keeping the plate/tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
j. Incubate the plate/tube(s) at room temperature for ≥30 seconds.
k. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
l. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

Caution: Over-drying the beads may result in dramatic yield loss.

m. Remove the plate/tube(s) from the magnet.
n. Thoroughly resuspend the beads in 22 µL of PCR Grade water.
o. Incubate the plate/tube(s) at room temperature for 2 minutes to allow the DNA to elute off the beads.
p. Place the plate/tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
q. Transfer 20 µL of the eluate to a fresh tube/well. The eluate contains the Adapter-Ligated cDNA Sample Library.
r. Proceed immediately to *Chapter 4*. Amplify The Sample Library Using the KAPA UDI Primer Mixes.

Sample indexes are incorporated in the Pre-Capture Amplification step.
Precautions should be taken to avoid sample cross contamination.

Chapter 4. Amplify the Sample Library Using the KAPA UDI Primer Mixes

This chapter describes how to amplify the sample library using the KAPA UDI Primer Mixes in preparation for hybridization to the KAPA HyperCap Target Enrichment Probes. This chapter requires the use of the components from the following kits:

- KAPA HiFi HotStart ReadyMix (KAPA RNA HyperPrep Kit)
- KAPA UDI Primer Mixes
- KAPA Pure Beads

Ensure that the following is available:

- Freshly-prepared 80% ethanol
- PCR Grade water

References

- *Thermocycler Manual*
- *Qubit Fluorometer Manual*
- *Qubit dsDNA HS Assay Kit Guide*
- *Agilent 2100 Bioanalyzer Instrument Manual*
- *Agilent Bioanalyzer DNA Kits Guide*

Step 1. Prepare the Pre-Capture PCR Reaction

We recommend the inclusion of negative (water) and positive (previously amplified library) controls in the Pre-Capture PCR step.

For guidance on Pre-Capture and Post-Capture sample multiplexing, please refer to the KAPA UDI Primer Mixes Instructions for Use.

Make sure the KAPA Pure Beads are removed from storage to allow time for proper equilibration to room temperature. For best performance, store the beads protected from light when not in use.

Record the well position of the KAPA UDI Primer Mix used for each sample.

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

   If piercing th8e 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 each individual sample library.
5. Add 25 µL of KAPA HiFi HotStart ReadyMix to each combined sample library and KAPA UDI Primer Mixes.
6. Mix thoroughly and perform a quick spin. Immediately proceed to amplification.

   If only using a subset of the KAPA UDI Primer Mixes, remove and discard residual primers from the well and 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' utilization at a later date.

Step 2. Perform the Pre-Capture PCR Amplification

1. Place the sample in the thermocycler and amplify the sample library using the following Pre-Capture PCR program with the lid temperature set to +105°C and volume to 50 µL:
   - Step 1: 45 seconds at +98°C
   - Step 2: 15 seconds at +98°C
   - Step 3: 30 seconds at +60°C
   - Step 4: 30 seconds at +72°C
   - Step 5: Go to Step 2, Variable (see table below for recommendation)
   - Step 6: 1 minute at +72°C
   - Step 7: Hold at +4°C

Starting material	Go to Step 2:
Total RNA (100 ng)	12 times (13 total cycles)
Total RNA (50 ng)	14 times (15 total cycles)

Optimal cycling will need to be determined by the end user.

2. Proceed immediately to the next step.

Step 3. Purify the Amplified Sample Library using KAPA Pure Beads

1. Add 50 µL of room temperature, thoroughly resuspended, KAPA Pure Beads to each amplified sample library.
2. Mix the amplified sample library and KAPA Pure Beads thoroughly and perform a quick spin.

Ensure that the solution is thoroughly mixed and that it appears homogeneous. Insufficient mixing may compromise recovery and size selection.

3. Incubate the sample at room temperature for 5 minutes to allow the DNA to bind to the beads.
4. Place the plate/tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant.
6. Keeping the plate/tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
7. Incubate the plate/tube(s) at room temperature for ≥30 seconds.
8. Carefully remove and discard the ethanol.
9. Keeping the plate/tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
10. Incubate the plate/tube(s) at room temperature for ≥30 seconds.
11. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
12. Allow the beads to dry at room temperature, sufficiently for all of the ethanol to evaporate.

Over-drying the beads may result in dramatic yield loss.

13. Remove the sample from the magnet.
14. Thoroughly resuspend the beads in 32 µL of PCR Grade water.
15. Incubate the plate/tube(s) at room temperature for 2 minutes to allow the DNA to elute off the beads.
16. Place the plate/tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
17. Transfer 30 µL of the eluate to a new tube/well.
18. Purified, Amplified Sample Libraries can be stored at +2°C to +8°C for 1-2 weeks or at -15°C to -25°C for up to one month.

Step 4. Qualify the Amplified Sample Library

1. Make a 10-fold dilution of the Amplified Sample Library by combining 2 µL of library with 18 µL of PCR Grade water.
2. Utilize 5 µL of the diluted library with the Qubit dsDNA HS Assay Kit to obtain the concentration of the diluted library. Multiply by 10 to obtain results for the undiluted Amplified Sample Library.

Accurate quantification is **essential** when working with samples that will be pooled for hybridization (*i.e.* Pre-Capture multiplexing). Slight differences in the mass of each sample combined to form the 'Multiplex DNA Sample Library Pool' will result in variations in the sequencing reads obtained for each sample in the library pool.

   - The undiluted Amplified Sample Library should contain ≥ 1000 ng of total DNA. If the sample library contains <1000 ng of total DNA, please refer to the *troubleshooting section* for guidance.
   - The negative control yield should be <1 ng/µL.
3. Use 1 µL of the diluted Amplified Sample Library (and any controls) with an Agilent Bioanalyzer DNA High Sensitivity Kit. If using the Agilent Bioanalyzer DNA 1000 Kit, refer to the user manual for guidance.
   - Pre-Capture libraries should have an average fragment size distribution at ~300 bp (*Figure 3*) with a range setting at 150 to 1000 bp on the Bioanalyzer. 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.
   - The negative control should not show any signal above baseline within the 150 to 500 bp size range, which could indicate contamination between amplified sample libraries, but it may exhibit sharp peaks visible below 150 bp. If the negative control reaction shows a positive signal by the Qubit, but the Bioanalyzer trace indicates only the presence of a sharp peak below 150 bp in size, then the negative control should not be considered contaminated.
4. If the Amplified Sample Library meets requirements, proceed to *Chapter 5*. If the Amplified Sample Library does not meet these requirements, repeat the library preparation.

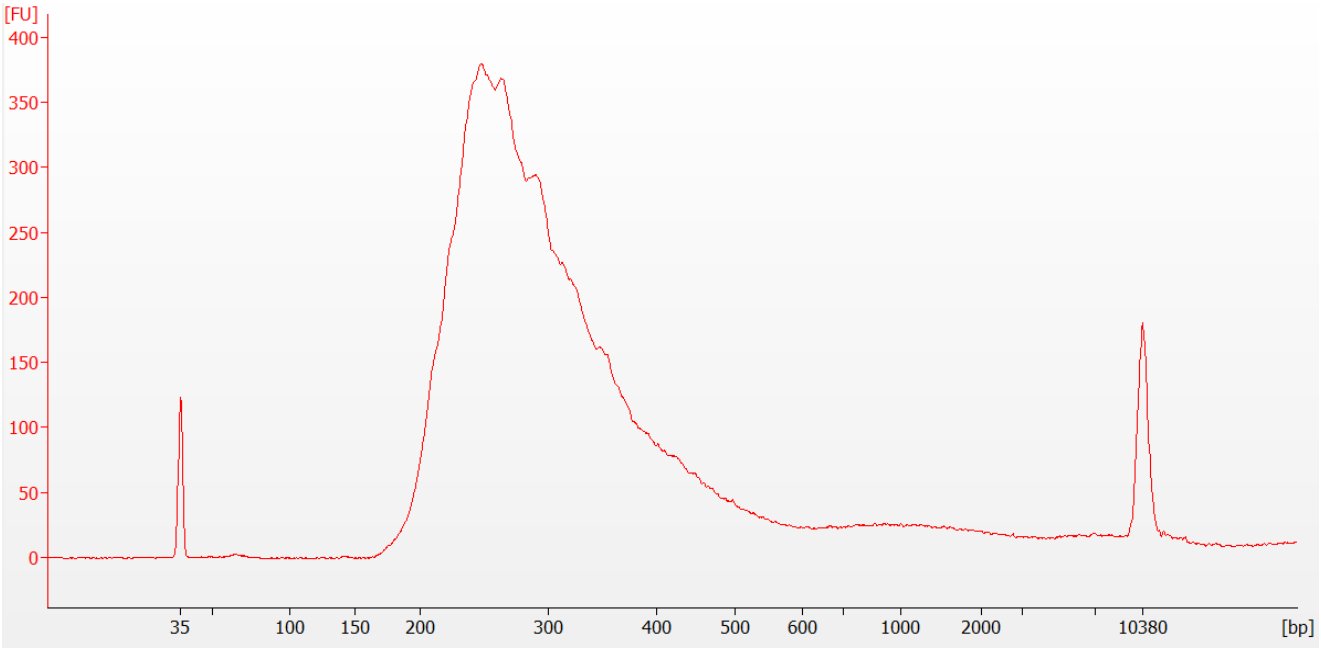


Figure 3: Example of an Amplified RNA HyperPrep Sample Library analyzed using the Agilent Bioanalyzer High Sensitivity DNA assay.

Chapter 5. Hybridize the Sample to KAPA HyperCap Target Enrichment Probes

This chapter describes the Roche protocol for the hybridization of the amplified sample libraries to the KAPA HyperCap Target Enrichment Probes. The following protocol provides instructions based on the capture target size of the KAPA HyperCap SARS-CoV-2 Panel (found in coverage_summary.txt design deliverable file) which is ~29.9 Kbp. This chapter requires the use of the components from the following kits:

- KAPA HyperCap SARS-CoV-2 Panel
- KAPA HyperCapture Reagent Kit
- KAPA HyperCapture Bead Kit

Ensure that the following is available:

- Freshly-prepared 80% ethanol

The hybridization protocol requires a thermocycler capable of maintaining +55°C for 1 to 20 hours. A programmable heated lid is required.

Note: In this chapter we use the term 'Multiplex DNA Sample Library Pool', however a single DNA sample library may be enriched using similar instructions. It is not required to capture more than one library at a time.

Step 1. Prepare for Hybridization

1. Remove the appropriate number of 4 µL KAPA HyperCap SARS-CoV-2 Panel aliquots (one per hybridization) from the -15°C to -25°C storage and allow them to thaw on ice.

Step 2. Prepare the Multiplex DNA Sample Library Pool

1. Thaw on ice the amplified DNA sample libraries that will be included in the capture experiment (generated in *Chapter 4*).
2. Prepare the Single / Multiplex DNA Sample Library by doing the following:
   a. For multiplex samples, mix together equal amounts (by mass) of each uniquely indexed DNA sample libraries to obtain a combined DNA mass of 1.5 µg (*i.e.* for two-plex DNA Sample Library, mix together 750 ng of each uniquely indexed amplified DNA sample library for a total mass of 1.5 µg). This mixture will subsequently be referred to as the 'Multiplex DNA Sample Library Pool'.

To obtain equal numbers of sequencing reads from libraries in the Multiplex DNA Sample Library Pool, it is very important to combine identical mass of each independently amplified DNA sample library at this step. Accurate quantification and pipetting are critical.

Differences in viral copy number (C_t values) input, in the library preparation, may create significant imbalance in the sequencing reads distribution. Optimal pooling based on input viral copy number (C_t values) will need to be determined by the end user.

   b. If capturing a single sample, utilize 1000 ng of a uniquely indexed amplified DNA sample library.

3. Add PCR Grade water to achieve a final volume 45 µL.

If the DNA Sample Library Pool exceeds 45 µL, please refer to the *troubleshooting section* for further guidance.

Step 3. Prepare the Hybridization Sample

This step outlines how to prepare the sample for hybridization by using KAPA HyperPure Beads.

Make sure the KAPA HyperPure Beads are removed from storage to allow time for equilibration to room temperature. For best performance, store the beads protected from light when not in use.

Note: When working with non-human gDNA background, consider using the KAPA Hybrid Enhancer Reagent (catalog number 09 075 763 001) in place of COT Human DNA. Optimization is needed when using the KAPA Hybrid Enhancer Reagent, but a good starting point is to add 20 µL of this reagent to each hybridization instead of COT Human DNA.

1. Add 20 µL of COT Human DNA to the Amplified Sample Library Pool, for a total volume of 65 µL.
2. Add 130 µL of KAPA HyperPure Beads to each tube/well containing the Amplified Sample Library pool and COT Human DNA mixture.
3. Mix thoroughly by vortexing for 10 seconds and perform a quick spin.
4. Incubate at room temperature for 10 minutes to ensure the Amplified Sample Library and COT Human DNA bind to the beads.
5. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
6. Carefully remove and discard the supernatant.
7. Keeping the tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
8. Incubate the tube(s) at room temperature for ≥30 seconds.
9. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads.
10. Allow the beads to dry at room temperature, sufficiently for all the ethanol to evaporate.

    Caution: Over-drying the beads may result in dramatic yield loss.
11. Add 13.4 µL of the Universal Enhancing Oligos to the bead-bound DNA sample.
12. Remove the tube(s) from the magnet and mix thoroughly by vortexing. It is important that sufficient mixing is performed to result in a homogenous mixture.

13. Prepare the Hybridization Master Mix following the table below.
    a. For the KAPA HyperCap SARS-CoV-2 Panel, prepare a master mix of the following reagents:

Hybridization Master Mix	Per Individual Capture
Hybridization Buffer	28 µL
Hybridization Component H	12 µL
PCR Grade water	3 µL
Total	**43 µL**

14. Add 43 µL of the Hybridization Master Mix to the tube(s) from step 12 above.
15. Mix thoroughly and perform a quick spin. Incubate at room temperature for 2 minutes.
16. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
17. Transfer 56.4 µL of the eluate (entire volume) into a new tube/well containing 4 µL of the KAPA HyperCap SARS-CoV-2 Panel.

    Slight bead carryover may be observed when transferring the supernatant. This is unlikely to impact results.

18. Mix thoroughly by vortexing for 10 seconds and perform a quick spin.
19. Perform the hybridization incubation in a thermocycler with the following program and the lid temperature set to +105°C:
    - +95°C for 5 minutes
    - +55°C for 1 to 16 hours

    The hybridization must remain at +55°C until it is transferred to the Capture Beads in *Chapter 6*. Wash and Recover Captured DNA Sample, Step 3.

20. Continue to *Chapter 6*. Wash and Recover Captured Multiplex DNA Sample For Washing and Recovery.

Chapter 6. Wash and Recover Captured DNA Sample

This chapter describes the process of the washing and recovery of the captured DNA Sample after hybridization to the KAPA HyperCap Target Enrichment Probes.

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

- KAPA HyperCapture Reagent Kit
- KAPA HyperCapture Bead Kit

Ensure that the following is available:

- Additional PCR Grade water for buffer preparation and elution

Step 1. Prepare Wash Buffers

Volumes for an individual capture are shown here. When preparing 1X buffers for processing multiple reactions, prepare an excess volume of ~5% (automated liquid handling systems may require an excess of ~20%).

1. Before completion of the hybridization incubation, thaw the Hybridization Wash Buffers.

   Ensure that stock wash buffers do not contain precipitates or are cloudy. Allow sufficient time for the buffers to thaw. Thoroughly vortex and warm cloudy buffers at 37°C until buffers are completely clear.

2. Dilute 10X Wash Buffers (I, II, III and Stringent) and 2.5X Bead Wash Buffer, contained in the KAPA HyperCapture Reagent Kit, to create 1X working solutions. Volumes listed below are sufficient for one capture.

Concentrated Buffer	Volume of Concentrated Buffer	Volume of PCR Grade Water	Total Volume of 1X Buffer*	Temperature
10X Stringent Wash Buffer	40 µL	360 µL	400 µL	+55°C
10X Wash Buffer I	10 µL	90 µL	100 µL	+55°C
	20 µL	180 µL	200 µL	Room temp.
10X Wash Buffer II	20 µL	180 µL	200 µL	Room temp.
10X Wash Buffer III	20 µL	180 µL	200 µL	Room temp.
2.5X Bead Wash Buffer	120 µL	180 µL	300 µL	Room temp.

***Store working solutions at room temperature (+15°C to +25°C) for up to 2 weeks. The volumes in this table are calculated for a single experiment; scale up accordingly if multiple samples are processed.**

It is expected that excess volume of 1X Bead Wash Buffer will remain after the following Capture Bead preparation protocol.

3. To pre-warm the 1X Stringent Wash Buffer, make two aliquots of 200 µL in 0.2 mL tubes and place the tubes into a thermocycler set to +55°C.
4. To pre-warm the 1X Wash Buffer I, make one aliquot of 100 µL into a 0.2 mL tube and place the tube into a thermocycler set to +55°C.
5. Pre-warm the buffers for a minimum of 15 minutes.

Pre-warming buffers can be performed in the same thermocycler used in the probe hybridization incubation step.

Step 2. Prepare the Capture Beads

1. Allow the Capture Beads to equilibrate to room temperature prior to use.
2. Vortex the Capture Beads for 15 seconds before immediate use to ensure a homogeneous mixture.

3. Aliquot 50 µL of beads per capture reaction into a 0.2 mL or 1.5 mL tube (i.e. for one capture use 50 µL beads and for two captures use 100 µL beads, etc.). Beads for up to two captures can be prepared in a single 0.2 mL tube or up to twelve captures can be prepared in a single 1.5 mL tube.
4. Place the tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
5. Remove and discard the supernatant being careful not to disturb the beads.
6. Keeping the tube(s) on the magnet, add 2X the initial beads' volume of 1X Bead Wash Buffer (e.g. for one capture use 100 µL of buffer and for two captures use 200 µL buffer, etc.).
7. Remove tube(s) from the magnet and mix thoroughly by vortexing. Perform 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, perform a second wash by adding 2X the initial volume of beads of 1X Bead Wash Buffer (*e.g.* for one capture use 100 µL buffer and for four captures use 400 µL buffer, *etc.*).
11. Remove tube(s) from the magnet and mix thoroughly by vortexing. Perform 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 1X the initial volume of beads of 1X Bead Wash Buffer (*i.e.* 50 µL buffer per capture).
15. Remove the tube(s) from the magnet and mix thoroughly by vortexing for 10 seconds. Perform a quick spin.
16. If preparing Capture Beads for more than one capture in a single tube, aliquot 50 µL of resuspended beads into a new tube/well for each capture.
17. Place the tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
18. Carefully remove and discard the supernatant.
19. The Capture Beads are now ready to bind the hybridized DNA. Proceed immediately to the next step.

Do not allow the Capture Beads to dry out. Small amounts of residual 1X Bead Wash Buffer will not interfere with binding of DNA to the Capture Beads.

Step 3. Bind Hybridized DNA to the Capture Beads

1. Transfer each hybridization sample into a single tube/well with prepared Capture Beads from the previous step.
2. Mix thoroughly by vortexing for 10 seconds and perform a quick spin, avoiding pellet formation.
3. Incubate the hybridization reaction by placing the sample in a thermocycler set to +55°C for 15 minutes, with the thermocycler lid temperature set to +105°C.

This step can be performed in the same thermocycler used in the probe hybridization incubation step.

4. Following the 15 minutes incubation, proceed immediately to the next step.

Step 4. Wash the Capture Beads Plus Bead-Bound DNA

Thermocycler should remain at +55°C with the heated lid set to +105°C for the following steps.

1. Add 100 µL of pre-warmed 1X Wash Buffer I to the Capture reaction.
2. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin.
3. Place the tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
4. Remove and discard the supernatant without disturbing the beads.

5. Add 200 µL of pre-warmed 1X Stringent Wash Buffer to each sample.
6. Remove the tube(s) from the magnet.
7. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin.
8. Place in the thermocycler pre-heated to +55°C, close the lid (set to +105°C) and incubate for 5 minutes.
9. After the 5 minutes incubation, remove the tube(s) from the thermocycler and place on the magnet to collect the beads. Incubate until the liquid is clear.
10. Remove and discard the supernatant being careful not to disturb the beads.
11. Add 200 µL of pre-warmed 1X Stringent Wash Buffer to each tube.
12. Remove the tube(s) from the magnet.
13. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin.
14. Place in the thermocycler pre-heated to +55°C, close the lid (set to +105°C) and incubate for 5 minutes.
15. After the 5 minutes incubation, remove the tube(s) from the thermocycler and place on the magnet to collect the beads. Incubate until the liquid is clear.
16. Remove and discard the supernatant being careful not to disturb the beads.
17. Add 200 µL of room temperature 1X Wash Buffer I.
18. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin.
19. Incubate at room temperature for 1 minute.
20. Place the tube(s) on the magnet to capture the beads. Incubate until the liquid is clear.
21. Remove and discard the supernatant being careful not to disturb the beads.
22. Add 200 µL of room temperature 1X Wash Buffer II.
23. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin and transfer the contents to a new tube.

Transferring each sample to a new tube is essential.

24. Incubate at room temperature for 1 minute.
25. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
26. Remove and discard the supernatant being careful not to disturb the beads.
27. Add 200 µL of room temperature 1X Wash Buffer III.
28. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous. Perform a quick spin.
29. Incubate at room temperature for 1 minute.
30. Place the tube(s) on the magnet to collect the beads. Incubate until the liquid is clear.
31. Remove and discard the supernatant being careful not to disturb the beads.
32. Remove the tube(s) from the magnet.
33. Add 20 µL PCR Grade water to each tube/plate well and mix thoroughly, Perform a quick spin.
34. Proceed to *Chapter 7*. Amplify Enriched DNA Sample.

No elution is performed at this step. The enriched DNA sample library remains bound to the Capture Beads and will be used as the template in the PCR as described in *Chapter 7*.

Chapter 7. Amplify Enriched DNA Sample

This chapter describes the amplification of the enriched DNA sample and requires the use of components from the following kits:

- KAPA HyperCapture Reagent Kit
- KAPA HyperCapture Bead Kit

In addition, ensure that the following are available:

- Freshly-prepared 80% ethanol
- PCR Grade water

References

- *Thermocycler Manual*
- *Agilent 2100 Bioanalyzer Instrument Manual*
- *Qubit Fluorometer Manual*
- *Agilent Bioanalyzer DNA Analysis Kit Guide*
- *Qubit dsDNA HS Assay Kit Guide*

Step 1. Resuspend the Post-Capture PCR Oligos

1. Briefly spin the lyophilized Post-Capture PCR Oligos, contained in the KAPA HyperCapture Reagent Kit, to allow the contents to pellet at the bottom of the tube. Note that both oligos are contained within a single tube.
2. Add 480 µL PCR Grade water to the tube of centrifuged oligos.
3. Briefly vortex the resuspended oligos and spin down the tube to collect the contents.
4. Store resuspended oligos at -15°C to -25°C.

Step 2. Prepare the Post-Capture PCR Master Mix

Instructions for preparing individual PCR reactions are shown here. When assembling a master mix for processing multiple samples, prepare an excess volume of ~5% to allow for complete pipetting (automated liquid handling systems may require an excess of ~20%).

Make sure KAPA HyperPure Beads are removed from storage to allow time for proper equilibration to room temperature. For best performance, store the beads protected from light when not in use.

1. Prepare a master mix of the following reagents.

Post-Capture PCR Master Mix	Per Individual PCR Reaction
KAPA HiFi HotStart ReadyMix (2X)*	25 µL
Post-Capture PCR Oligos*	5 µL
Total	**30 µL**

* **Note: The Post-Capture PCR Oligos and the KAPA HiFi HotStart ReadyMix (2X) are contained in the KAPA HyperCapture Reagent Kit.**

2. Add 30 µL of Post-Capture PCR Master Mix to a 0.2 mL tube or well of a PCR plate.
3. Take the bead-bound DNA from *Chapter 6* and mix thoroughly by vortexing for 10 seconds. Perform a quick spin.
4. Transfer 20 µL of the bead-bound DNA as template into the tube/well with the 30 µL Post-Capture PCR Master Mix. (If performing a negative control, add 20 µL PCR Grade water to this tube/well).
5. Mix thoroughly by pipetting up and down several times.

Step 3. Perform the Post-Capture PCR Amplification

1. Place the sample in the thermocycler.
   - Step 1: 45 seconds at +98°C
   - Step 2: 15 seconds at +98°C
   - Step 3: 30 seconds at +60°C
   - Step 4: 30 seconds at +72°C
   - Step 5: Go to Step 2, 17 times (18 cycles total)
   - Step 6: 1 minute at +72°C
   - Step 7: Hold at +4°C

   The Post-Capture PCR cycling conditions are recommendations and can be adjusted to individual experimental needs.
2. Proceed immediately to the next step.

Step 4. Purify the Amplified Enriched DNA Sample using KAPA HyperPure Beads

1. Ensure the KAPA HyperPure Beads are equilibrated at room temperature.
2. Mix the Amplified Enriched DNA Sample from Step 3 by vortexing. Perform a quick spin. Avoid pellet formation.
3. Place the tube(s) on a magnet to collect the beads. Incubate until the liquid is clear.
4. Carefully transfer the supernatant to a new tube.

   Do not discard the supernatant. It contains the Amplified Enriched DNA Sample Library.
5. Vortex the KAPA HyperPure Beads for 10 seconds before use to ensure a homogenous mixture.
6. Add 70 µL of KAPA HyperPure Beads to the 50 µL Amplified Enriched DNA Sample library.
7. Mix thoroughly by vortexing for 10 seconds, ensure that the mixture is homogeneous and perform a quick spin. Incubate at room temperature for 5 minutes to allow the sample to bind to the beads.
8. Place the tube(s) on a 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 200 µL of freshly-prepared 80% ethanol sample.
11. Incubate at room temperature for ≥30 seconds.
12. Remove and discard the ethanol.
13. Keeping the tube(s) on the magnet, add 200 µL of freshly-prepared 80% ethanol.
14. Incubate the tube(s) at room temperature for ≥30 seconds.
15. Carefully remove and discard the ethanol. Remove residual ethanol without disturbing the beads and allow the beads to dry at room temperature with the tube lid open.

    Over-dryin9g of the beads can result in yield loss.
16. Remove the tube(s) from the magnet. Resuspend the bead pellet using 22 µL of PCR Grade water.
17. Vortex for at least 10 seconds to ensure that all of the beads are resuspended. Perform a quick spin.
18. Incubate at room temperature for 2 minutes.
19. Place the tube(s) back on the magnet and allow the liquid to clear.
20. Transfer 20 µL of the eluate to a new tube/well. This tube contains the Amplified Enriched and purified DNA Sample Library.

Step 5. Qualify the Amplified Enriched DNA Sample Library

1. Measure the concentration of the Amplified Enriched DNA Sample Library using a Qubit Fluorometer:
   a. Make a 10-fold dilution combining 2 µL of the sample with 18 µL of PCR Grade water.
   b. Utilize 5 µL of the diluted sample using the Qubit dsDNA HS Assay Kit to obtain the concentration of the diluted sample. Multiply by 10 to obtain results for the undiluted sample. Reserve at least 1 µL of the diluted sample to be analyzed using the Agilent Bioanalyzer DNA High Sensitivity Kit.
   - The Amplified Enriched DNA Sample Library yield should be ≥ 100 ng.
   - If the negative control yields show significant amplification, this would indicate contamination.
2. Analyze 1 µL of each diluted sample (and any controls) using an Agilent Bioanalyzer DNA High Sensitivity assay according to manufacturer's instructions. If using the Agilent Bioanalyzer DNA 1000 Kit, diluting the Amplified Enriched Sample Library is not necessary.
   - The average fragment size distribution should be ~300 bp (*Figure 4*) with a range setting of 150 to 1000 bp on the Bioanalyzer.
3. The Amplified Enriched DNA Sample Library is ready for sequencing.

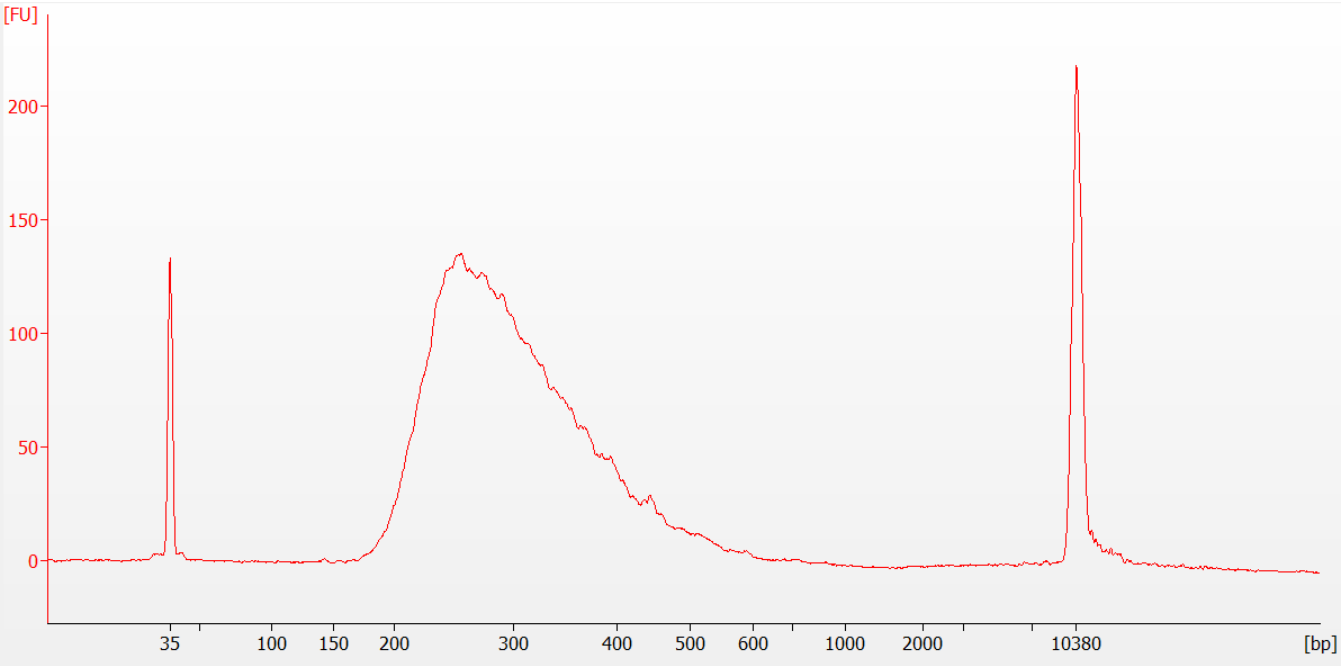


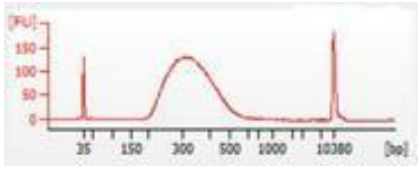
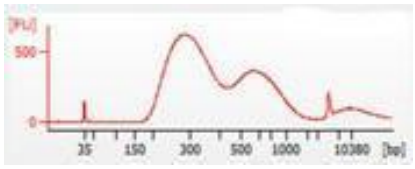
Figure 4: Example of a successfully Amplified Enriched DNA Sample Library using the KAPA RNA HyperPrep Kit and analyzed using an Agilent Bioanalyzer High Sensitivity DNA Chip.

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)
Sample Library Preparation	
Less than 50 ng of input total RNA is available for library preparation.	Libraries generated using <50 ng of input total RNA can produce high quality capture results; however, several adjustments summarized below, will increase the probability of success. ■ Adjust the adapter concentration to preserve the adapter: insert molar ratio in order to maintain high ligation efficiency. For more information, contact Roche Technical Support. ■ Increase the number of PCR cycles during Pre-Capture PCR by 1 – 3 cycles, depending on starting RNA amount. Performance of these cycle number recommendations may vary for your particular sample. **Note:** There is a possibility that these steps will not lead to success with lower input amounts. For the most current guidance on working with lower input amounts, contact *Roche Technical Support*.
Amplified Sample Library (Pre-Capture PCR Product)	
Yield is <1 µg for multiplex or <1.5 µg for singleplex hybridizations (yield should be ≥1 µg or ≥1.5 µg, respectively).	Poor quality input sample. 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.
Fragment distribution (analyzed using an Agilent HS DNA chip) shows that the average amplified fragment size is not within the size range of 150 to 500 bp.	Poor fragmentation occurred. Repeat library preparation.
Fragment distribution (analyzed using an Agilent DNA 1000 chip) is bimodal, with a larger set of fragments observed in addition to, or instead of, the expected set of fragments.	See the section entitled “Amplified Enriched DNA Sample (Post-Capture PCR Product)” in this appendix.

Observation	Cause(s) / Recommendation(s)
The negative control yield measured by the Qubit is >1 ng/µL.	The measurement may be high due to the presence of adapter dimers carried over from previous steps/PCR. This carryover will be apparent as one or more sharp peaks visible less than 150 bp in size when examining the data from the Agilent Bioanalyzer HS DNA chip. This carryover is not a sign of contamination.
The Agilent Bioanalyzer HS DNA chip 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 but could lead to overestimation of the amplified library yield when interpreting the data from Qubit. Repeat the PCR cleanup.
The Agilent Bioanalyzer HS DNA chip indicates that the average amplified material is 150 to 500 bp in size in the negative control for sample library amplification.	This material could represent cross contamination between amplified sample libraries. Test reagents for contamination and replace if necessary. Repeat library construction using fresh genomic DNA.
If only the Agilent Bioanalyzer DNA 1000 chip is available for qualifying sample following Pre-Capture PCR amplification and purification.	The Agilent Bioanalyzer DNA 1000 chip can be used to analyze the Pre-Capture Sample Library. Please refer to the manufacturer's user manual for further guidance.
Prepare the Multiplexing the Sample Library Pool and KAPA HyperCap Target Enrichment Probes	
The Multiplex DNA Sample Library volume is greater than 45 µL.	For KAPA HyperPure bead based library concentration, transfer the Multiplex DNA Sample Library to a 1.5 mL tube and add 20 µL of COT DNA. Add 2X KAPA HyperPure Beads to the mixture and proceed with step 3, point 3, on page 23, section "Step: Prepare the Sample for Hybridization." For vacuum concentration (if the required bead volume is very large) of the Multiplex DNA Sample Library, use the following recommendations: 1) Mix the Multiplex DNA Sample Library with 20 µL of COT DNA. 2) Concentrate the sample with a vacuum concentrator to a volume of 65 µL. 3) Once concentrated proceed with step 3, point 2, on page 23, section Step 3. Prepare the Sample for Hybridization."
Amplified enriched Multiplex DNA Sample (Post-Capture PCR Product)	
Yield is <100 ng (yield should be ≥100 ng).	Library construction or Pre-Capture PCR failed. Pre-Capture PCR yield should be ≥ 1 µg. Repeat with a DNA sample that was previously processed with success. Incorrect hybridization or wash temperatures were used. Make sure the correct hybridization and wash temperatures were used. If temperatures were not correct, repeat the experiment from hybridization. PCR reagents are compromised. Verify that the positive control worked. If the positive control did not work, repeat hybridization and re-amplify using fresh PCR reagents. **Note:** Experiments designed to capture less genomic DNA (*i.e.* a smaller cumulative target size) may be successful even though they can generate lower PCR yields than experiments designed to capture larger targets. Target size should be taken into consideration when evaluating low Post-Capture PCR yield.
Fragment distribution (analyzed using an Agilent HS DNA chip) shows that the average amplified fragment size is not within the size range of 150 to 500 bp.	Poor fragmentation occurred. Repeat library preparation. Consider implementing the gel-cut size selection option.
Fragment distribution (analyzed using an Agilent HS DNA chip) is bimodal, with a larger set of fragments observed in addition to (Fig B), the expected set of fragments (Fig A): A.  B. 	Primer depletion due to over-amplification of sample library relative to the amount of primers available in the reaction results in single stranded amplification products. These products can anneal to each other via adapter homology on both ends of the fragments to form heteroduplexes, and migrate as larger products on an Agilent HS DNA chip than their actual length in base pairs. The artifact can be resolved by increasing primer concentration or reducing cycle number in the PCR reaction, however the products themselves are perfectly acceptable for use in sequence capture and sequencing, and this artifact will not affect capture performance. Care should be taken to quantify the area under both peaks if quantification will be performed using the Bioanalyzer image. The Agilent HS DNA chip traces shown in Fig. A and Fig. B, show the result of amplification of the same enriched DNA sample library following Post-Capture PCR amplification for 14 or 20-cycles, respectively. The same artifact can appear in Pre-Capture PCR amplification.

Observation	Cause(s) / Recommendation(s)
If only the Agilent Bioanalyzer DNA 1000 chip is available for qualifying sample following Post-Capture PCR amplification and purification.	The Agilent Bioanalyzer DNA 1000 chip can be used to analyze the Pre-Capture Sample Library. Please refer to the manufacturer's user manual for further guidance.
Sequencing Performance Metrics	
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. ■ Enough material is present to accurately quantify after PCR clean-up. ■ For the Pre-Capture PCR, enough amplified library is produced for at least 1 µg or 1.5 µg respectively for multiplex or singleplex hybridization. Increasing input into hybridization may improve duplicate rates. Take the following points into consideration when adjusting sample input into hybridization. ■ Enough material is produced from the Pre-Capture PCR reaction(s) to increase sample input into hybridization. **Note:** There is a possibility that these steps will not lead to success in reducing duplicate rates. For the most current guidance, contact *Roche Technical Support*.

Appendix B. Limited Warranty

1. Limited Warranty

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

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

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D. Any action by Customer against Roche for Roche's breach of this warranty must be commenced within 12 months following the date of such breach. Notwithstanding such 12-month period, within twenty (20) days of the delivery of Data and/or Products to Customer, Customer must notify Roche in writing of any nonconformity of the Services and Products, describing the nonconformity in detail; otherwise all Services and Products shall be conclusively deemed accepted without qualification.

2. FURTHER LIABILITY LIMITATION

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

If you have any questions concerning service of this product, contact your local Roche Technical Support. Go to sequencing.roche.com/support.html for contact information.

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

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