AVENIO Tumor Tissue Analysis Kits V2

Reagent Instructions for Use

Version 1.6
Software Version 2.1.0

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1.1	2.0.0	May 2019	Clarified information and instructions, and added Bioanalyzer trace examples.
1.2	2.0.0	October 2020	Updated Illumina Sequencing kit and catalog number in consumables list purchased from other vendors.
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1.6	2.1.0	July 2023	The AVENIO Tumor Tissue Analysis kits were updated to V2 kits to align with both manufacturing/operational changes and in compliance with Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). Clarified information and instructions, and added additional instruments for sample analysis.

Edition notice

This publication is intended for AVENIO Tumor Tissue Analysis Kits V2.
Every effort has been made to ensure that all the information contained in this publication is correct at the time of publishing. However, the manufacturer of this product may need to update the publication information as output of product surveillance activities, leading to a new version of this publication.

Where to find information

This *AVENIO Tumor Tissue Analysis Kits V2 Reagent Instructions for Use* contains information about using the reagents, organized according to the normal operation workflow.

The *AVENIO Oncology Analysis Software User Guide* focuses on routine operation and maintenance, and contains important safety information. You must read the safety information before operating the instrument.

The *AVENIO Oncology Analysis Software Admin Guide* contains information about setting up and managing the analysis software for users.

General attention

To avoid incorrect results, ensure that you are familiar with the instructions before you use the product.

- Pay particular attention to all important notes.
- Always follow the instructions in this publication.
- Do not use the reagents in a way that is not described in this publication.
- Store all publications in a safe and easily retrievable place.

Screenshots

The screenshots in this publication have been added exclusively for illustration purposes. Configurable and variable data, such as tests, results, or path names visible therein must not be used for laboratory purposes.

Warranty for the reagent

Roche warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label.

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Feedback

Every effort has been made to ensure that this publication fulfills the intended use. All feedback on any aspect of this publication is welcome and is considered during updates. Contact your Roche representative, should you have any such feedback.

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Technical support

For product-related technical documents or to contact Roche Technical Support, go to *sequencing.roche.com/support.*

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Preface

Regulatory disclaimer

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

AVENIO Tumor Tissue Analysis Kits V2

The AVENIO Tumor Tissue Analysis Kits V2 include the reagents needed to isolate DNA from formalin-fixed paraffin-embedded (FFPE) tissue, prepare sequencing libraries, and enrich selected regions of interest using one of three panel options:

- AVENIO Tumor Targeted Panel V2 (17 genes)
- AVENIO Tumor Expanded Panel V2 (77 genes)
- AVENIO Tumor Surveillance Panel V2 (197 genes)

These ready-to-use kits use a hybrid-capture based workflow to profile the genomics of DNA derived from solid tumor FFPE tissue. The AVENIO Tumor Tissue Analysis Kits V2 help laboratories consistently purify and enrich targeted regions in solid tumor FFPE DNA for sequencing. The AVENIO Tumor Tissue Analysis Kits V2 provide laboratory professionals the ability to generate insights from all four mutation classes—SNVs, Indels, CNVs, and fusions—using a DNA-only workflow. Each kit includes reagents for processing up to 24 reactions in a single sequencing run on a NextSeq 500/550/550Dx.

Additional AVENIO products

Except where the context clearly indicated otherwise, the following product names and descriptors are used.

Product name	Descriptor
AVENIO Oncology Analysis Software	Analysis software

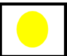
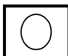
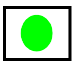
An on-premise Oncology Analysis Server (OAS) allows laboratory professionals to produce secondary analysis variant calls locally, review Quality Control (QC) metrics, and run analytical concordance reports. The OAS runs the AVENIO Oncology Analysis Software and is necessary to provide analysis setup and reporting functionality for the entire AVENIO family of next-generation oncology assays. The OAS is available for purchase from Roche.

Symbols, abbreviations, and acronyms

Symbols used in the publication

Symbol	Explanation
	Safety alert note: The safety alert symbol is used to alert you to potential physical injury hazards. To avoid possible injury or death, comply with all safety messages that follow this symbol.
	Important note: Highlights information that is critical for optimal performance of the system. May also indicate that loss of data or invalid data could occur if the precautions or instructions are not observed.
	Information note: Identifies items of general interest and additional information about the topic or procedure being described.

Symbols used on product

Symbol	Explanation
	A yellow top-dot sticker is used on vial caps, when possible, for reagents exclusive to the AVENIO Tumor Tissue Analysis Kits V2 workflow.
	A white top-dot sticker is used on vial caps, when possible, for reagents exclusive to the AVENIO ctDNA Analysis Kits V2 workflow.
	A Green top-dot sticker is used on the AVENIO Post-Hybridization Kit V2. The AVENIO Post-Hybridization Kit V2 is the only sub-kit that is jointly used by both the AVENIO Tumor Tissue and the AVENIO ctDNA workflows.
	Corrosion ▪ Skin corrosion/burns ▪ Eye damage ▪ Corrosive to metals
	Exclamation Mark ▪ Irritant (eye and skin) ▪ Skin sensitizer ▪ Acute toxicity ▪ Narcotic effects ▪ Respiratory tract irritant ▪ Hazardous to ozone layer (non-mandatory)
	Health Hazard ▪ Carcinogen ▪ Mutangenicity ▪ Reproductive toxicity ▪ Respiratory sensitizer ▪ Target organ toxicity ▪ Aspiration toxicity
	Skull and Crossbones ▪ Acute toxicity (fatal or toxic)

Abbreviations and acronyms

The following abbreviations and acronyms are used.

Abbreviation/acronym	Definition
bp	Base pair
CNV	Copy Number Variant
Cp	Crossing point
Cq	Quantification Cycle
CSV	Comma-Separated Values
Ct	Cycle threshold
FFPE	Formalin-Fixed Paraffin-Embedded
H&E	Hematoxylin and Eosin
Indel	Insertion and Deletion
min	Minutes
NTC	No Template Control
OAS	Oncology Analysis Server
QC	Quality Control

Abbreviation/acronym	Definition
qPCR	Quantitative PCR
RCF	Relative Centrifugal Force
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RPM	Revolutions Per Minute
sec	Seconds
SNV	Single Nucleotide Variant
∞	Hold

What is new in publication version 1.6

- The AVENIO Tumor Tissue Analysis Kits were updated to V2 Kits to satisfy Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) compliance requirements. The REACH compliant reagents are designed to provide optimal results with the new workflow conditions, therefore, using the previous workflow with V2 reagents will lead to suboptimal assay performance, delayed results and potential loss of sample, resulting in poor libraries and poor sequencing results, requiring the user to perform the workflow again.
- The AVENIO Tumor Tissue Analysis Kits V2 were updated to align with operational requirements and changes in manufacturing.
- The Hybridization workflow was optimized due to the newly launched REACH-compliant reagent. The volumes of the components changed for the Hybridization Master Mix to obtain optimal concentrations within 43 µL per reaction. The concentration of the panels changed due to manufacturing changes, and thus 4 µL is required per reaction. In addition, the incubation temperature for the Hybridization reaction, Hybridization Wash 1 and Stringent Wash Buffer were changed from 47°C to 60°C.
- Safety alert notes, important notes and information notes were added and updated throughout the document.
- The 4200 TapeStation and LabChip GX Touch HT Nucleic Acid Analyzer were added as alternative platforms for library quality assessment. In addition, NextSeq 500Dx was added as an alternative sequencing analyzer.
- Terminology corrected where Q-score was changed to Q-ratio, and Q-ratio changed to normalized Q-ratio.

Protocol information and safety

- Apply good laboratory practices, such as wearing appropriate personal protective equipment when handling biological material.
- Wear gloves and take precautions to avoid sample contamination.
- Change gloves when opening and closing strip tubes to minimize cross-contamination.
- Take precautions to avoid sample cross contamination, especially prior to the first PCR reaction, which is the step when unique sample barcodes are tagged to the DNA.
- It is recommended to perform DNA isolation and pre-PCR procedures in an amplicon-free area, preferably, a separate lab, to minimize contamination. Clean work area thoroughly before and after all lab procedures.
- Unless otherwise specified, all mixing steps are listed as “mix thoroughly” and indicate that the sample should be mixed by either vortexing for 5 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.
- Properly label tubes at all times to prevent sample mix-up.
- Check each reagent name and expiration date before use.
- Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.
- It is recommended to use a multi-channel pipettor to ensure sample consistency.
- Thaw components that contain enzymes on ice, and keep on ice during procedure. All other components, including primer pools, can be thawed at room temperature. Gently vortex and centrifuge before use.
- When provided, follow the recommended heated lid temperature for the thermocycler incubations. Otherwise, it is recommended to set the lid 10°C above the block temperature.
- Be careful when removing tubes from the thermocycler as the caps can pop open due to the high incubation temperature.
- Perform all centrifugations at room temperature (+15°C to +25°C), unless indicated otherwise.
- Laboratory temperature should be maintained at room temperature (+15°C to +25°C) to avoid freezing of Hybridization Buffer 2 V2.
- Safe stopping points of the assay are after the DNA extraction, after the PCR cleanup of the pre-capture library, and the final enriched library. Consult the procedures for the storage conditions of the stopping points.
- Ensure hazardous waste is properly disposed.
- Hybridization Buffer 1 and PCR Reaction Mix (2X) contain tetramethylammonium chloride. Practice safe laboratory practices when handling these reagents.

- The QC measurements by the Qubit dsDNA HS quantification assays can also be performed using the Qubit 1X dsDNA HS Assay Kit according to the manufacturer's instructions.
- The QC measurements performed by the Agilent 2100 Bioanalyzer High Sensitivity DNA trace analysis can be substituted with the Agilent D1000 / High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT Nucleic Acid Analyzer.
- Avoid using tissues from slides as much as possible, to minimize hazards associated with razor blade use. If razor blades must be used, a razor blade holder is recommended. A surgical scalpel may also be used with care.

Required equipment, labware, and consumables

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

Laboratory equipment

The catalog numbers referenced in the following table may be United States catalog numbers.

Equipment	Supplier	Catalog number
96-well thermocycler with programmable heated lid capable of 50°C, 85°C, and 105°C (Recommended: Veriti Dx 96-well Thermocycler, 0.2 mL, ThermoFisher Scientific, catalog number 4452300)	Multiple vendors	
0.2 mL PCR Strip Magnetic Separator	Permagen Labware	MSR812
DynaMag-2 Magnet	ThermoFisher Scientific	12321D
Serological pipettors (Capable of 2 to 10 mL volumes)	Multiple vendors	
Multi-channel pipettes (8-channels each, capable of 2 to 20 µL and 20 to 200 µL volumes)	Multiple vendors	
Single-channel pipettes (capable of 0.5 to 2 µL, 2 to 20 µL, 20 to 200 µL, and 200 to 1000 µL volumes).	Multiple vendors	
Qubit 3.0 or Qubit 4 Fluorometer	ThermoFisher Scientific	Q33216, Q33226
2100 Bioanalyzer or 4200 TapeStation System	Agilent Technologies	G2938C, G2940CA G2943CA, G2991BA
LabChip GX Touch HT Nucleic Acid Analyzer	PerkinElmer	CLS137031
Illumina NextSeq 500, NextSeq 550 or NextSeq 550Dx	Illumina	SY-415-1001, SY-415-1002, 20005715
Tabletop plate centrifuge (Capable of 1800 x g)	Multiple vendors	
Tabletop centrifuge (Capable of up to 20,000 x g for 1.5 mL and 2 mL microcentrifuge tubes)	Multiple vendors	
Tabletop micro/mini centrifuge (0.2 mL PCR strip tube compatible rotor)	Multiple vendors	
Vortex mixer	Multiple vendors	
384-well quantitative (real-time) PCR machine capable of SYBR Green I dye detection (Recommended: LightCycler® 480 Instrument II, 384-well, Roche, catalog number 05015243001). **Note**: These protocols are designed for use with the specified equipment, labware, and consumables.	Multiple vendors	
Thermomixer (capable of 75°C and 2000 RPM or heat block capable of 75°C)		

Reagents and consumables available from Roche Diagnostics

Three AVENIO Tumor Tissue Analysis Kits V2 are available that reflect three distinct panel options. Each AVENIO Tumor Tissue Analysis Kit V2 contains reagents for end-to-end processing of FFPE samples, from DNA extraction to an enriched library ready for sequencing.

Orderable kit name	Orderable kit number	Included sub-kit name
AVENIO Tumor Targeted Kits V2	09733710001 (with Sample Primer Plate A) 09733728001 (with Sample Primer Plate B)	AVENIO Tumor DNA Isolation and QC Kit
		AVENIO Tumor Cleanup and Capture Beads V2
		AVENIO Tumor Library Prep Kit V2
		AVENIO Tumor Sample Primers (Plate A or Plate B)
		AVENIO Tumor Enrichment Kit V2
		AVENIO Tumor Targeted Panel V2
		AVENIO Post-Hybridization Kit V2
AVENIO Tumor Expanded Kits V2	09733744001 (with Sample Primer Plate A) 09733752001 (with Sample Primer Plate B)	AVENIO Tumor DNA Isolation and QC Kit
		AVENIO Tumor Cleanup and Capture Beads V2
		AVENIO Tumor Library Prep Kit V2
		AVENIO Tumor Sample Primers (Plate A or Plate B)
		AVENIO Tumor Enrichment Kit V2
		AVENIO Tumor Expanded Panel V2
		AVENIO Post-Hybridization Kit V2
AVENIO Tumor Surveillance Kits V2	09733787001 (with Sample Primer Plate A) 09733809001 (with Sample Primer Plate B)	AVENIO Tumor DNA Isolation and QC Kit
		AVENIO Tumor Cleanup and Capture Beads V2
		AVENIO Tumor Library Prep Kit V2
		AVENIO Tumor Sample Primers (Plate A or Plate B)
		AVENIO Tumor Enrichment Kit V2
		AVENIO Tumor Surveillance Panel V2
		AVENIO Post-Hybridization Kit V2

Component	Catalog number
LightCycler® 480 Multiwell Plate 384, white with sealing foils (recommended)	04729749001

Reagents and consumables purchased from other vendors

The catalog numbers referenced in the following table may be United States catalog numbers.

Component	Supplier	Catalog number
0.2 mL 8-Strip PCR tubes (required tubes ordered from one of these two suppliers	Starlab International GmbH or USA Scientific (Western hemisphere)	I1402-3700 1402-4700
1.5 mL or 2 mL microcentrifuge tubes (low-bind tubes recommended)	Multiple vendors	
5.0 mL microcentrifuge tubes	Multiple vendors	
15 mL or 50 mL polypropylene conical tubes	Multiple vendors	
Sterile pipetting reservoirs	Multiple vendors	
Low-retention pipette tips	Multiple vendors	
5 mL or 10 mL volume serological pipettes	Multiple vendors	
Nuclease-free Water, PCR grade (not DEPC treated)	Multiple vendors	

Consumable	Supplier	Catalog number
Ethanol, 200 proof (absolute), molecular biology grade	Multiple vendors	
Qubit dsDNA HS Assay Kit or Qubit 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q32851 (100 assays) Q32854 (200 assays) Q33230 (100 assays) Q33231 (500 assays)
Qubit Assay Tubes	ThermoFisher Scientific	Q32856
Agilent High Sensitivity DNA Kit (for 2100 Bioanalyzer)	Agilent Technologies	5067-4626
Agilent High Sensitivity D1000 ScreenTape assay (for 4200 TapeStation)	Agilent Technologies	5067-5584 5067-5585 5067-5587
DNA NGS 3K Reagent Kit (for LabChip)	PerkinElmer	CLS960013
HT DNA X-Mark Chip (for LabChip)	PerkinElmer	CLS144006
96-well or 384-well skirted plate (for LabChip)	Multiple vendors	
1 M NaOH, molecular biology grade (for sequencing)	Multiple vendors	
200 mM Tris-HCl, pH 7.0, molecular biology grade (for sequencing)	Multiple vendors	
NextSeq 500/550 High Output v2.5 Kit (300 cycles)	Illumina	20024908
NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) IVD	Illumina	20028871

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Chapter 1. Getting started

This guide describes the use of the AVENIO Tumor Tissue Analysis Kits V2 to prepare sequencing-ready libraries from DNA isolated from FFPE (formalin-fixed paraffin-embedded) tissue to identify aberrations such as SNVs (single nucleotide variants), Indels (insertions and deletions), CNVs (copy number variants), as well as fusions from solid tumors.

About AVENIO Tumor Tissue Analysis Kits V2

The AVENIO Tumor Tissue Analysis Kits V2 include the reagents needed to isolate DNA from FFPE tissue, prepare sequencing libraries, and enrich selected regions of interest using one of three panel options:

- AVENIO Tumor Targeted Panel V2 (17 genes)
- AVENIO Tumor Expanded Panel V2 (77 genes)
- AVENIO Tumor Surveillance Panel V2 (197 genes)

These ready-to-use kits use a hybrid-capture based workflow to profile the genomics of DNA derived from solid tumor FFPE tissue. The AVENIO Tumor Tissue Analysis Kits V2 aim at purifying and enriching targeted regions in solid tumor FFPE DNA for sequencing. The AVENIO Tumor Tissue Analysis Kits V2 provide insights from classes of genomic alterations - SNVs, Indels, CNVs, and fusions—using a DNA-only workflow. Each kit includes reagents for processing up to 24 reactions in a single sequencing run on an Illumina NextSeq 500/550 platform and NextSeq 550Dx in RUO Mode.

Workflow

The AVENIO Tumor Tissue Analysis Kits V2 workflow includes the following high-level steps and associated sub-kits:

1. *Isolate DNA from FFPE tissue*
    - AVENIO Tumor DNA Isolation and QC Kit
    - AVENIO Tumor Cleanup and Capture Beads V2
2. *Prepare sequencing libraries*
    - AVENIO Tumor Library Prep Kit V2
    - AVENIO Tumor Sample Primers – Plate A or Plate B
    - AVENIO Tumor Cleanup and Capture Beads V2
3. *Perform the enrichment protocol*
    - AVENIO Tumor Enrichment Kit V2
    - AVENIO Tumor Panels V2 (assay-specific panel includes one of the following)
        - AVENIO Tumor Targeted Panel V2
        - AVENIO Tumor Expanded Panel V2
        - AVENIO Tumor Surveillance Panel V2
    - AVENIO Post-Hybridization Kit V2
    - AVENIO Tumor Cleanup and Capture Beads V2
4. *DNA Sequencing*

The following table details the approximate processing time of each step of the AVENIO Tumor Tissue V2 workflow:

Step	Processing time
Isolate DNA from FFPE tissue	2.5 hours
	Safe stopping point
Measure quality and concentration of the isolated DNA	2.5 hours
Prepare DNA for adapter ligation and initiate adapter ligation	3 hours
	Overnight ligation (16 to 18 hours)
Perform post-ligation cleanup	1 hour
PCR amplify ligated sample and cleanup	1.5 hours
	Safe stopping point
Hybridization of the sample	1 hour
	Overnight hybridization (16 to 20 hours)
Bind sample to Capture Beads and perform post-hybridization washes	1 hour
PCR amplify enriched sample and cleanup	1.5 hours
	Safe stopping point
Final library quantification and pooling for sequencing	4 hours

Chapter 2. Isolate DNA from FFPE tissue

This section of the protocol uses the AVENIO Tumor DNA Isolation and QC Kit and the AVENIO Tumor Cleanup and Capture Beads V2 to isolate DNA from FFPE tissue and to assess the quality of the extracted FFPE DNA. A normalized quality ratio (Q-ratio) is calculated and used to determine the quality of the DNA and optimal input into library preparation as described in *Chapter 3. Prepare sequencing libraries* on page 22.

Change gloves when opening and closing strip tubes to minimize cross-contamination.
Clearly label tubes at the required steps to minimize sample mix-up.
Always check the reagent for correct name and expiration date before use.

For all master mixes, prepare 1 extra reaction for every 8 samples, unless stated otherwise.

Preparing FFPE tissue

- The recommended input for FFPE DNA isolation is two 10 micron curls in one tube. The amount of DNA necessary is determined in *Determining DNA input for sequencing library generation* on page 21.
- If using slides, carefully scrape as much tissue as possible into 1.5 mL tubes with a razor blade.

Avoid using tissue from slides as much as possible to minimize hazards associated with razor blade use. Razor blade holder is recommended if razor blades must be used.

Curl(s) and slide(s) with different thickness can be used, but total sample thickness should be no more than 20 micron per extraction.

Isolating DNA from FFPE tissue

Before starting, thaw DNA Elution Buffer at room temperature for 3 hours until the solution is completely clear or at 4°C overnight. Crystals may be observed if the buffer is not thawed properly.

To isolate DNA from FFPE tissue

1. Place FFPE curls or scraped FFPE tissue in 1.5 mL tubes.
2. Prepare the Extraction Master Mix as follows:

Extraction Master Mix	
Reagent	**Volume per reaction**
Nuclease-free Water, PCR grade	88 µL
Extraction Buffer (10X)	10 µL
Extraction Enzyme	2 µL

3. Vortex and spin the master mix, and add 100 µL of the master mix to each tube containing FFPE tissue.
4. If the curl is not near the bottom of the tube, vortex or use a clean pipette tip to get the tissue as submersed in liquid as possible.
5. Incubate the tubes in a thermomixer at 75°C, and shake at 2000 RPM (revolutions per minute) with the lid on for 1 hour.

   If a thermomixer is not available, vortex the tubes as follows:

   a. Incubate at 75°C in a heat block for a total of 1 hour with intermittent vortexing as described in *step b* and *step c*.
   b. For the first 10 minutes, vortex the tubes for 5 seconds every 2 minutes.

   Repeat *step b* if wax does not dissolve.

   c. Vortex the tubes again at the 30 minute mark.

6. Following the 1 hour incubation, spin the tubes at 20,000 RCF (relative centrifugal force) for 5 minutes to pellet the remaining cellular debris. A visible and/or solid wax layer may form after the spin.
7. Pierce through the wax layer with a pipette tip, and transfer the liquid to 0.2 mL strip tubes.
   a. Carefully transfer each sample to a unique position in the 0.2 mL strip tube.
   b. Transfer as much liquid as possible while avoiding cellular debris/wax carryover.

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

Post-Extraction Cleanup

To clean up isolated FFPE DNA

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade the buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Thoroughly mix the Cleanup Beads V2 by vortexing.
3. Add 180 µL of Cleanup Beads V2 to each sample in the 0.2 mL strip tube, and carefully mix well by pipetting to avoid spill over.

The total volume will be 280 µL in 0.2 mL strip tubes. Take care to ensure no spillover occurs during mixing.

4. Incubate for 10 minutes at room temperature.
5. Pellet beads on a 0.2 mL magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
6. Discard the supernatant.
7. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
8. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
9. Repeat *step 7* and *step 8* for a second wash with the freshly prepared 80% ethanol.
10. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
11. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
12. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each pellet surface should have an uncracked matte appearance before resuspension.

13. Remove the tubes from the magnetic rack, and resuspend the beads in 40 µL of DNA Elution Buffer.
14. Incubate for 2 minutes at room temperature.
15. Quickly spin down the samples, pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
16. Transfer the eluate containing extracted DNA to new 0.2 mL strip tubes.

Safe stopping point: Freeze at –20°C for up to 1 month, or proceed to *Checking the quality of FFPE DNA*.

Checking the quality of FFPE DNA

The quality of each FFPE DNA sample is assessed by quantitative PCR (qPCR) using two PCR amplicons (66 bp and 191 bp). The normalized quality ratio (Q-ratio) is calculated using QC PCR DNA Standard of known quality.

To check the quality of FFPE DNA

1. Dilute the extracted DNA samples in nuclease-free water, PCR grade, to a 500-fold final dilution.

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

2. Dilute the QC PCR DNA Standard in nuclease-free water, PCR grade, to a 500-fold final dilution.

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

The diluted QC PCR DNA Standard must be included in each qPCR run.

3. Prepare the following master mix for each QC PCR Primer Mix separately (66 bp and 191 bp):

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

qPCR Master Mix	
Reagent	**Volume per reaction**
QC PCR Reaction Mix (2X)	5 µL
QC PCR Primer Mix (66 bp) or QC PCR Primer Mix (191 bp)	1 µL

4. Vortex and spin the master mix, and add 6 µL of the appropriate master mix to each well of a 384-well plate.
5. Add 4 µL of the diluted sample, the diluted QC PCR DNA Standard, and the nuclease-free water, PCR grade, (NTC) to the applicable wells.
6. Seal the plate well and centrifuge briefly.
7. Perform qPCR as follows using a qPCR machine that can detect SYBR Green I:

Thermocycler Profile			
Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	95°C	10 min	1
Denaturation	95°C	10 sec	40
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Cooling	40°C	30 sec	1

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

7. Use the 3 technical replicates of the Cp values per amplicon to calculate the Q-ratio for each sample, including the QC PCR DNA Standard, by using the following equation:

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

   For example, for a given sample, if its average (Cp66) = 17 and average (Cp191) = 16, then its Q-ratio = $2^{(17-16)}$ = 2.

In addition to Cp values, Cycle threshold (Ct) and Quantification Cycle (Cq) values can also be used for Q-ratio calculation.

8. Obtain a Normalized Q-ratio for each sample through the following equation:

   Normalized Q-ratio = sample Q-ratio/QC PCR DNA Standard Q-ratio

   For example, for a given sample, if its Q-ratio = 1 and QC PCR DNA Standard Q-ratio = 2, then the normalized Q-ratio for this sample = 1/2 = 0.5.

Avoid using the sample if its normalized Q-ratio is less than 0.04 as the sequencing results will not be optimal.

Determining DNA input for sequencing library generation

To determine DNA input for sequencing library generation

1. Utilize the normalized Q-ratio to determine the recommended input mass for each sample as follows:

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

   For example, for a given sample, if its normalized Q-ratio = 0.5, then its input mass in ng = 10/0.5 + 10 = 30.

Use Q-ratio of 1 in the formula if the Q-ratio is greater than 1.

2. Determine the concentration of each sample using the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
3. Use the Qubit concentration to determine the recommended input volume as follows:

   Input volume in µL = Input mass in ng/Qubit concentration in ng/µL

   For example, for a given sample, if its recommended input mass = 30 ng, and Qubit concentration = 5 ng/µL, then its recommended input volume in µL = 30/5 = 6.

The maximum sample input volume for this workflow is 30.5 µL. Avoid using the sample below the recommended input mass as the sequencing results will not be optimal.

Chapter 3. Prepare sequencing libraries

This section of the protocol uses the AVENIO Tumor Library Prep Kit V2, the AVENIO Tumor Sample Primers, and the AVENIO Tumor Cleanup and Capture Beads V2 to prepare sequencing libraries with unique Sample Primer IDs. Each sample will be amplified with a unique Sample Primer set, which enables the multiplexed sequencing of up to 24 samples per sequencing run.

For optimal assay performance, follow the recommended sample requirement:
Normalized Q-ratio ≥ 0.04
Input mass in ng = 10/normalized Q-ratio+10.

Each sample must receive a unique Sample Primer ID. Keep track of each sample to ensure that the proper Sample Primer ID is entered in the analysis software.

To ensure quality FFPE DNA is used to prepare sequencing libraries, it is recommended to use the AVENIO Tumor DNA Isolation and QC Kit following the instructions in *Chapter 2. Isolate DNA from FFPE tissue* on page 17. However, preparation of sequencing libraries can also be performed with FFPE DNA isolated using other methods. When alternative extraction methods are used, it is highly recommended to use the DNA Elution Buffer for the elution and the QC kit to determine the quality of the DNA and optimal input into library preparation. Performance is not guaranteed when using other sources of FFPE DNA or when using alternative QC methods.

Change gloves when opening and closing strip tubes to minimize cross-contamination.
Clearly label tubes at the required steps to minimize sample mix-up.
Always check the reagent for correct name and expiration date before use.

For all master mixes, prepare 1 extra reaction for every 8 samples, unless stated otherwise.

Performing DNA polishing

To perform DNA polishing

1. Add extracted DNA into 0.2 mL strip tube using the recommended amount, as described in *Determining DNA input for sequencing library generation* on page 21.
2. Adjust the volume in each tube to 30.5 µL using nuclease-free water, PCR grade.
3. Dilute 1 µL of the DNA Polishing Enzyme 50-fold using nuclease-free water, PCR grade, right before use. Discard unused diluted enzyme.

DNA Polishing Enzyme must be diluted fresh before each use.
Note, there is appropriate overfill volume of concentrated DNA Polishing Enzyme supplied to accommodate at least 4 usages. Briefly spin down the tube prior to use. If preparing less than 24 samples, retain the tube for future use.

4. Prepare the DNA Polishing Master Mix:

DNA Polishing Master Mix	
Reagent	**Volume per reaction**
Diluted DNA Polishing Enzyme	1 µL
Fragmentation Buffer (10X)	3.5 µL

5. Add 4.5 µL of DNA Polishing Master Mix to each sample.
6. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom.
7. Incubate on a thermocycler:

Temperature	Duration
37°C	30 min
4°C	∞

Set the thermocycler lid to 50°C and volume to 35 µL.

8. Proceed immediately to *Performing fragmentation*.

Performing fragmentation

To perform fragmentation

1. Place empty tubes on ice or cold block while setting up the fragmentation reaction.
2. Prepare the Fragmentation Master Mix:

Fragmentation Master Mix	
Reagent	**Volume per reaction**
Fragmentation Buffer (10X)	1.5 µL
Fragmentation Enzyme	10 µL
Nuclease-free Water, PCR grade	3.5 µL

The Fragmentation Master Mix is highly viscous. 1.5 extra reaction overage should be made for 8 samples, and 3 extra reaction overage should be made for 24 samples. Extra care is needed to mix the master mix well and to aliquot the right amount to each tube and mix well.

3. Add 15 µL of the Fragmentation Master Mix to each sample on ice or cold block.
4. Mix by vortexing, and briefly spin down the tubes to settle the liquid to the bottom.
5. Incubate on a thermocycler:

Temperature	Duration
37°C	30 min
4°C	∞

Set the thermocycler lid to 50°C and volume to 50 µL.

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

Do not let fragmentation continue for more than the 30 minute reaction time. Proceed immediately and ensure to keep the samples on ice or cold block between fragmentation and DNA preparation for ligation.

Preparing DNA for ligation

To prepare DNA for ligation

1. Remove the samples from the thermocycler and place on ice or cold block.
2. Prepare the DNA Preparation Master Mix:

DNA Preparation Master Mix	
Reagent	**Volume per reaction**
DNA Preparation Buffer	7 µL
DNA Preparation Enzyme V2	3 µL

3. Add 10 µL of the DNA Preparation Master Mix to each sample on ice.
4. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom.

5. Incubate on a thermocycler:

Temperature	Duration
65°C	30 min
4°C	∞

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

6. Proceed immediately to *Performing adapter ligation*.

Performing adapter ligation

To perform adapter ligation

1. Prepare the Ligation Master Mix:

Ligation Master Mix	
Reagent	**Volume per reaction**
Ligation Buffer	30 µL
DNA Ligase	10 µL

The Ligation Master Mix is highly viscous. Extra care is needed to mix the master mix well and to aliquot the right amount to each tube and mix well.

2. Add 10 µL of Universal Adapters to the 60 µL of prepared DNA product.
3. Mix by pipetting or vortexing and briefly spin down the tubes to settle the liquid to the bottom.
4. Add 40 µL of the Ligation Master Mix to each sample.

Add the Ligation Master Mix last to reduce adapter dimers formation.

5. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom.

6. Incubate on a thermocycler for 16 to 18 hours.

Temperature	Duration
16°C	∞

Set the thermocycler lid to 50°C and volume to 110 µL (100 µL volume is acceptable if limited by thermocycler parameter).

Preparing PCR primers

To prepare PCR primers

1. Take out Sample Primers (Plate A or Plate B) from cold storage, unwrap, and quickly spin down the plate for 30 seconds at 1000 RCF.

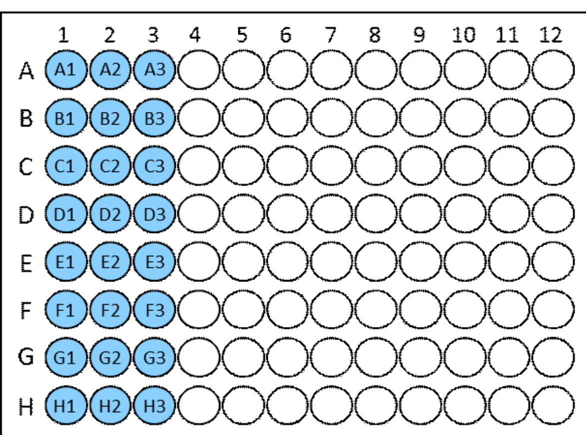
Primer plate usage switching is recommended to avoid cross-contamination so that subsequent sequencing runs do not use the same primers as the previous sequencing run. Recommend alternating between two Sample Primer plates (Plate A and Plate B) for subsequent 24-sample sequencing runs, if both plates are available.

2. Pierce the foil cover using a multichannel pipette with clean tips for the appropriate number of wells needed.

As shown in the plate layout below, Sample Primers are located in the first 3 columns of each plate, i.e., in well positions A1 to H1, A2 to H2, and A3 to H3. Take attentive care to ensure that well position A1 is on the upper-left side.

Note, there are no primers in Columns 4 to 12.

Record Sample Primer plate (Plate A or Plate B) and well position for Sample Primer ID tracking. For example, Sample Primer ID A-A1 for Sample Primer Plate A, well position A1.



3. Add 25 µL room temperature nuclease-free water, PCR grade, to the well, and pipette up and down 5 times.

Pipette carefully to avoid liquid splash-over to adjacent wells, and make sure the water reaches at the bottom of the well with no air bubbles. If a compatible centrifuge is available, spin down the plate at 1000 RCF.

4. Set the plate aside, and proceed to *Cleaning up post-ligation sample*. Resuspended primers will be used to elute the ligation product from the Cleanup Beads V2.

Cleaning up post-ligation sample

To clean up post-ligation sample

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Thoroughly mix the Cleanup Beads V2 by vortexing.
3. Add 110 µL of Cleanup Beads V2 to each sample.
4. Mix thoroughly by pipetting, and quickly spin down the liquid without pelleting the beads.

The total volume will be 220 µL in the 0.2 mL strip tubes. Take care to ensure no spillover during mixing.

5. Incubate for 10 minutes at room temperature.
6. Pellet beads on a 0.2 mL magnetic rack for 3 minutes. Visually inspect to ensure that all of the beads are collected to the side of the tube.
7. Discard the supernatant.
8. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
9. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
10. Repeat *step 8* and *step 9* for a second wash with the freshly prepared 80% ethanol.
11. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
12. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.

13. Leave the tubes open on the magnetic rack to air dry the beads for 5 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to a reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

14. Remove the tubes from the magnetic rack, and add 25 µL of prepared primer mix to each sample on the beads. Resuspend thoroughly by pipetting up and down.

Each sample must receive a unique Sample Primer ID. Sample Primers are named based on their plate names and well positions. Record the Sample Primer used for each sample to ensure that the proper Sample Primer ID is entered in the analysis software.

15. Incubate for 2 minutes at room temperature.
16. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
17. Transfer the eluate into a new 0.2 mL strip tube. The eluate contains the adapter-ligated DNA sample and Sample Primers.

Performing PCR amplification

During this step, the samples are molecularly tagged. While good lab practices are required all throughout the workflow, take special caution not to mix up or cross-contaminate samples prior to this step.

To perform PCR amplification

1. Pipette 25 µL of PCR Reaction Mix (2X) to the 25 µL of purified ligated sample and Sample Primers, bringing the total volume to 50 µL.

PCR Reaction Mix (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

2. Briefly vortex and spin down the tubes to settle the liquid to the bottom.
3. Perform PCR with the following cycling profile:

Thermocycler Profile			
Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	8
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 and volume to 50 µL.

4. Proceed to *Cleaning up post-PCR product*.

Cleaning up post-PCR product

To clean up post-PCR product

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Thoroughly mix the Cleanup Beads V2 by vortexing.
3. Add 50 µL of Cleanup Beads V2 to the PCR product.
4. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
5. Incubate for 10 minutes at room temperature.
6. Pellet beads on a 0.2 mL magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
7. Discard the supernatant.
8. Remove the tubes from the magnetic rack, and resuspend the beads in 50 µL of nuclease-free water, PCR grade.
9. Rebind the DNA by adding an additional 50 µL of Cleanup Beads V2 to the 50 µL of sample from *step 8* to enhance cleanup of the ligated DNA sample.
10. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
11. Incubate for 10 minutes at room temperature.
12. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
13. Discard the supernatant.

14. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
15. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
16. Repeat *step 14* and *step 15* for a second wash with the freshly prepared 80% ethanol.
17. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
18. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
19. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to a reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

20. Remove the tubes from the magnetic rack, and add 65 µL nuclease-free water, PCR grade, to each sample. Resuspend thoroughly by pipetting up and down.
21. Incubate for 2 minutes at room temperature.
22. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
23. Transfer the eluate into a new 0.2 mL strip tube. The eluate contains the pre-capture library.

Safe stopping point: Freeze at –20°C for up to 1 month, or proceed to *(Suggested) Assessing library quality before enrichment*.

(Suggested) Assessing library quality before enrichment

The library preparation and enrichment procedures have been optimized based on the recommended sample input. Library quantification and QC is not required to continue on to the enrichment protocol but may be useful for troubleshooting purposes. The volume unused for the enrichment procedure may be saved and used for library QC if troubleshooting is required.

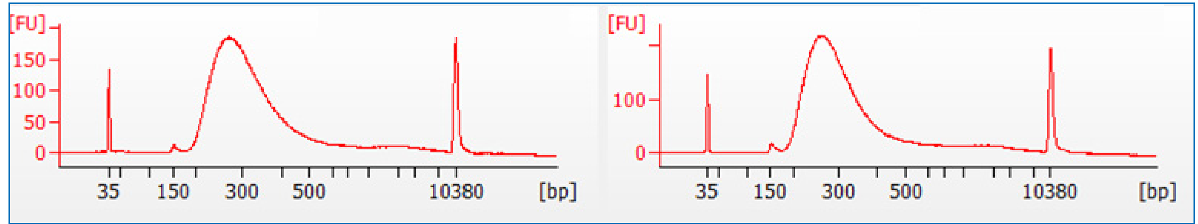
(Suggested) To assess library quality before enrichment

1. Quantify the library yield by measuring 1 µL with the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
2. Dilute 1 µL of the samples with nuclease-free water, PCR grade, to a recommended concentration for analysis using Agilent High Sensitivity DNA Kit. A 10-fold dilution is generally sufficient.
3. Assess the average library size using an Agilent High Sensitivity DNA Assay on a 2100 Bioanalyzer following the manufacturer's instructions.

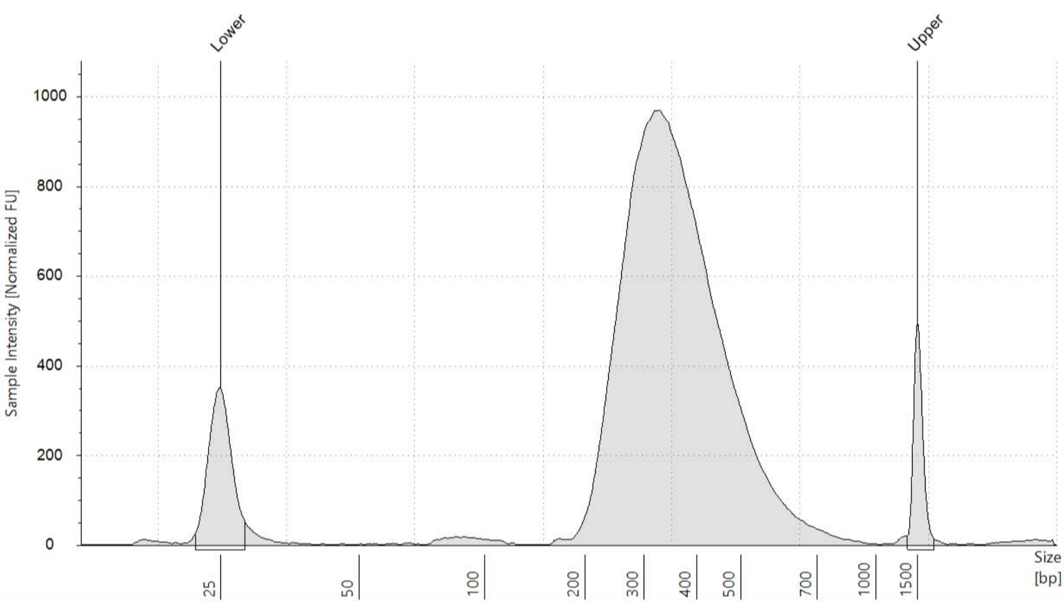
Alternatively, a Agilent High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

The enrichment protocol has been optimized to tolerate moderate levels of adapter dimers. Residual adapter dimers are excluded from the library during the enrichment procedure.

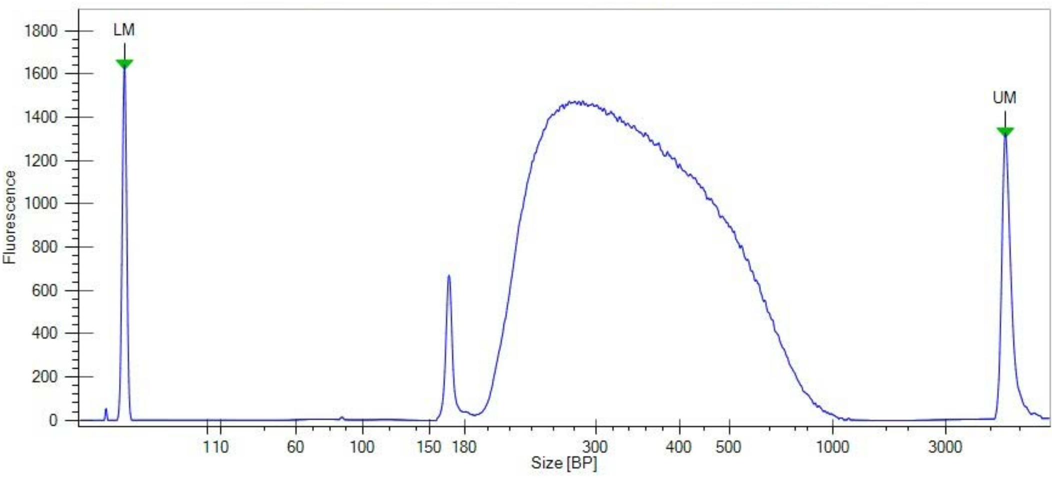
The following show examples of Bioanalyzer profiles for typical, good adapter-ligated library with minimal adapter dimers and a fragment peak near 300 bp, which indicates successful adapter ligation. The profiles provided are from internal data at Roche.



The following is an example of a TapeStation profile showing a typical, good adapter-ligated library.



The following is an example of a LabChip profile showing a typical, good adatper-ligated library.



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Chapter 4. Perform the enrichment protocol

This section of the protocol uses the AVENIO Tumor Enrichment Kit V2, the AVENIO Post-Hybridization Kit V2, the AVENIO Tumor Cleanup and Capture Beads V2 and one or more of the AVENIO Tumor Panels V2 (AVENIO Tumor Targeted, Expanded, or Surveillance Panel V2). The purpose of this section is to enrich genes of interest from sequencing libraries prepared from FFPE DNA samples produced in *Chapter 3. Prepare sequencing libraries* on page 22. Panels contain probes designed to target regions of interest for detection of cancer mutations present within FFPE DNA.

It is crucial to use the correct amount of V2 panel for hybridization. Not enough panel being added to the hybridization reactions can lead to low on-target and/or reduced coverage post sequencing, and hence suboptimal assay performance and delayed results. Adding too much panel leads to little to no significant impact on sequencing and results.

It is important to ensure the correct V2 panels are used for the new workflow conditions. The previous version of the panels are not designed to hybridize properly using the new workflow conditions, and will lead to poor libraries and poor sequencing results, suboptimal assay performance, delayed results and potential loss of sample.

It is essential to use the correct V2 reagents (check for appropriate kit name and material/lot number) because the new workflow is designed to provide optimal results with the REACH compliant reagents. The use of the previous version of reagents will lead to suboptimal assay performance, delayed results and potential loss of sample, resulting in poor libraries and poor sequencing results.

It is crucial to verify the shelf-life by checking the expiry date of reagents prior to use. Expired reagents/panel used in the workflow can lead to suboptimal assay performance, delayed results, potential loss of sample and creation of poor libraries and poor sequencing results.

It is essential to use the correct hybridization temperature which was optimized when using the new Hybridization Buffer 2 V2 and V2 panels. The use of an incorrect hybridization temperature will affect target enrichment, which may result in suboptimal assay performance, delayed results, and potential loss of sample, as well as poor sequencing results.

Change gloves when opening and closing strip tubes to minimize cross-contamination.
Clearly label tubes at the required steps to minimize sample mix-up.
Always check the reagent for correct name and expiration date before use.

For all master mixes, prepare 1 extra reaction for every 8 samples unless stated otherwise.

Preparing for hybridization

To prepare for hybridization

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer and lead to loss of sample, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

80% ethanol should be prepared fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Transfer 30 µL of pre-capture library from *step 23* of the procedure *To clean up post-PCR product* on page 30 to a new 0.2 mL strip tube to use for hybridization.

   Freeze the remainder of the pre-capture library at -20°C for up to 1 month, or proceed to *Chapter 4, Peforming the enrichment protocol.* This remainder can be used to repeat the enrichment if desired but will require additional hybridization reagents.

3. Add 20 µL of Hybridization Supplement to each sample. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom.
4. Thoroughly mix the Cleanup Beads V2 by vortexing.
5. Add 100 µL of Cleanup Beads V2.
6. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
7. Incubate for 10 minutes at room temperature.

8. While incubating, prepare the Hybridization Master Mix:

Hybridization Master Mix	
Reagent	**Volume per reaction**
Hybridization Buffer 1 (2X)	31.1 µL
Hybridization Buffer 2 V2	10.9 µL
Nuclease-free water, PCR grade	1 µL

Hybridization Buffer 1 (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

9. Pellet beads on a 0.2 mL magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
10. Discard the supernatant.
11. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
12. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
13. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
14. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
15. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

16. Remove the tubes from the magnetic rack. Add 13.4 µL of Universal Enhancing Oligos to each sample, and resuspend beads by pipetting.
17. After the beads are resuspended, add 43 µL of the Hybridization Master Mix.
18. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
19. Incubate for 2 minutes at room temperature.
20. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.

Dispersed pelleting of the beads is frequently observed. Gently aspirate the supernatant to promote collecting as many beads by the magnet.

21. Transfer the supernatant to a new 0.2 mL strip tube, and add 4 µL of the appropriate V2 Panel to each sample.

Check the label to ensure that the correct panel is used: AVENIO Tumor Targeted Panel V2 , AVENIO Tumor Expanded Panel V2, or AVENIO Tumor Surveillance Panel V2. The correct panel name must be noted and entered in the AVENIO analysis software to properly analyze the data after sequencing. Each sample may receive a different panel, but only one panel is to be used per sample.

22. Vortex to mix the sample, and quickly spin down the tubes to settle the liquid to the bottom.

23. Incubate on a thermocycler overnight for 16 to 20 hours using the following program:

Hybridization Program	
Temperature	**Duration**
95°C	10 min
60°C	∞

Set the thermocycler lid to 105°C and volume to 60 µL.

Preparing the Hybridization Wash Buffers

To prepare the Hybridization Wash Buffers

Hybridization Wash Buffers can be prepared immediately before performing the hybridization washes or up to 2 weeks prior to use. The 1X working solutions can be stored at room temperature for up to 2 weeks.

After preparing 1X working solutions of Stringent Wash Buffer, Hybridization Wash 1, Hybridization Wash 2, and Hybridization Wash 3, the buffers should be aliquoted into 0.2 mL strip tubes for ease of use.

1. Before the end of the hybridization, thaw the Hybridization Wash Buffers.

Ensure that stock wash buffers do not contain precipitates or are cloudy. If necessary, thoroughly vortex and gently warm precipitated or cloudy buffers at 37°C until buffers are completely clear. The Stringent Wash Buffer and Hybridization Wash 1 will have more precipitates.

2. Prepare the wash buffers according to the following table. The table indicates volumes required for 1 reaction. Include overage and scale accordingly for more reactions.

Buffer	Volume of stock buffer per reaction	Volume of PCR grade water per reaction	Total volume of 1X buffer prepared per sample	Temperature
Bead Wash Buffer (2.5X)	150 µL	225 µL	375 µL	Room temperature
Hybridization Wash 1 (10X)	10 µL	90 µL	100 µL	60°C
	20 µL	180 µL	200 µL	Room temperature
Stringent Wash Buffer (10X)	20 µL	180 µL	200 µL	60°C
	20 µL	180 µL	200 µL	60°C
Hybridization Wash 2 (10X)	20 µL	180 µL	200 µL	Room temperature
Hybridization Wash 3 (10X)	20 µL	180 µL	200 µL	Room temperature

3. Place the strips of the 1X working solutions of the Stringent Wash Buffers and Hybridization Wash 1 specified at 60°C in the thermocycler so that they may incubate for at least 15 minutes while the Capture Beads are being prepared.

Preparing the Capture Beads

To prepare the Capture Beads

1. Allow the Capture Beads to reach room temperature 15 minutes prior to use.
2. Vortex the Capture Beads thoroughly for 15 seconds before use to ensure they are resuspended well.
3. In a new 1.5 mL microcentrifuge tube, aliquot 75 µL of Capture Beads for each capture reaction (75 µL beads for 1 reaction; 300 µL beads for 4 reactions).

Prepare up to 4 reactions in each 1.5 mL microcentrifuge tube. 24 reactions will require 6 tubes.

4. Pellet the Capture Beads on a 1.5 mL magnetic rack until the solution is clear, and discard the supernatant.
5. Add 150 µL of 1X Bead Wash Buffer per capture (150 µL for 1 reaction; 600 µL for 4 reactions).
6. Thoroughly wash Capture Beads by vortexing 2 times for 10 seconds each, and quickly spin down the liquid without pelleting the beads.
7. Pellet the beads on the magnetic rack until the solution is clear, and discard the supernatant.
8. Repeat *step 5* through *step 7* for a total of two washes.
9. Remove the tubes from the magnetic rack, and resuspend the beads with 75 µL of 1X Bead Wash Buffer per capture (75 µL for 1 reaction; 300 µL for 4 reactions).
10. Mix thoroughly by vortexing 2 times for 5 seconds each, and aliquot 75 µL of the resuspended beads per sample into new 0.2 mL strip tubes.
11. Pellet the beads on a 0.2 mL magnetic rack until the solution is clear, and discard the supernatant.
12. Proceed immediately to *Performing the hybridization cleanup*.

Do not allow the beads to dry at this stage. Small amounts of residual 1X Bead Wash Buffer will not interfere with the subsequent steps.

Performing the hybridization cleanup

To perform the hybridization cleanup

Use prepared 1X buffers for all wash steps.

1. Ensure that the following buffers are aliquoted into 0.2 mL strip tubes at the indicated volumes, and incubate the buffers specified at 60°C in the thermocycler for at least 15 minutes prior to use.

Buffer	Volume	Temperature
Hybridization Wash 1	100 µL	60°C
Stringent Wash Buffer	200 µL	60°C
Stringent Wash Buffer	200 µL	60°C
Hybridization Wash 1	200 µL	Room temperature
Hybridization Wash 2	200 µL	Room temperature
Hybridization Wash 3	200 µL	Room temperature

2. Remove the hybridization reaction from the thermocycler. Working quickly, briefly spin down the liquid to the bottom of the tube, and transfer the 60.4 µL hybridization reaction onto the pelleted Capture Beads.

Work quickly to minimize cooling of the hybridization reaction, which may reduce assay performance.

3. Mix thoroughly by vortexing, and quickly spin down the liquid without pelleting the Capture Beads.
4. Place the samples back on the thermocycler, and incubate at 60°C for 15 minutes to bind the hybridized DNA to the Capture Beads.
5. Remove the strip of samples and the pre-heated Hybridization Wash 1 from the thermocycler.
6. Quickly transfer 100 µL of pre-heated Hybridization Wash 1 to the sample, and immediately mix thoroughly by pipetting up and down 10 times. **Do not vortex the samples.**

When pipetting up and down, avoid bubbles, which can reduce the washing efficiency. If necessary, adjust the pipetting volume to reduce bubbles.

7. Quickly spin down the liquid, and place the samples on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.

8. Quickly remove the pre-heated Stringent Wash Buffer from the thermocycler, and remove the strip of samples from the magnetic rack.
9. Immediately transfer 200 µL of pre-heated Stringent Wash Buffer to the sample, and mix thoroughly by pipetting up and down 10 times or by vortexing for 5 seconds.
10. Quickly spin down the liquid without pelleting the beads.
11. Incubate the samples at 60°C for 5 minutes on the thermocycler.
12. Remove the strip of samples from the thermocycler and quickly spin down the liquid. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.
13. Repeat *step 8* through *step 12* for a second Stringent Wash.
14. Remove the strip of samples from the magnetic rack, and add 200 µL of room temperature Hybridization Wash 1.
15. Mix the pellet thoroughly by pipetting up and down 10 times. **Do not vortex the samples.**
16. Quickly spin down the liquid without pelleting the beads and incubate for 1 minute.
17. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.
18. Remove the strip of samples from the magnetic rack.
19. Add 200 µL of room temperature Hybridization Wash 2 to each sample.
20. Mix the pellet thoroughly by pipetting up and down 10 times or by vortexing for 5 seconds, and quickly spin down the liquid without pelleting the beads.
21. Carefully transfer all of the contents to a new 0.2 mL strip tube. Incubate for 1 minute at room temperature.

Transferring to a fresh tube helps to ensure thorough washing. Ensure that all of the beads are transferred and no pellet is left behind.

22. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.
23. Remove the strip of samples from the magnetic rack and add 200 µL of room temperature Hybridization Wash 3 to each sample.
24. Mix the pellet thoroughly by pipetting up and down 10 times or by vortexing for 5 seconds.
25. Quickly spin down the liquid without pelleting the beads, and incubate for 1 minute at room temperature.
26. Place the tubes on the magnetic rack to pellet the beads for 1 minute. When the supernatant is clear, discard the supernatant.

Remove as much of the Hybridization Wash 3 as possible to avoid PCR inhibition.

27. Remove the tubes from the magnetic rack, and resuspend the Capture Beads by adding 20 µL of nuclease-free water, PCR grade, to each sample and incubate for 2 minutes.

Do not discard Capture Beads at this point. Enriched DNA remains bound to the Capture Beads.

28. Proceed to *Amplifying the enriched FFPE DNA sample*.

Amplifying the enriched FFPE DNA sample

To amplify the enriched FFPE DNA sample

1. Prepare the PCR Master Mix:

PCR Master Mix	
Reagent	**Volume per reaction**
PCR Reaction Mix (2X)	25 µL
PCR Primer Mix (10X)	5 µL

PCR Reaction Mix (2X) contains tetramethylammonium chloride. Follow safe laboratory practices when handling it.

2. Add 30 µL of the PCR Master Mix into the 20 µL of DNA-bound Capture Beads sample.
3. Briefly vortex and spin down the tubes to bring the liquid to the bottom.
4. Transfer the entire reaction to a new 0.2 mL strip tube.
5. Perform PCR on a thermocycler with the following cycling profile:

Thermocycler Profile			
Stage	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	
Annealing	60°C	30 sec	15
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
Hold	4°C	∞	1

Set the thermocycler lid to 105°C and volume to 50 µL.

6. After PCR, proceed to *Cleaning up post-capture PCR product*.

Cleaning up post-capture PCR product

To clean up post-capture PCR product

Cleanup Beads V2 must be stored at the correct storage temperature to prevent degradation. Bead degradation may lead to incomplete DNA-to-bead binding during purification steps of the workflow, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 must be equilibrated to room temperature and fully resuspended prior to use. If not, the probability for obtaining the correct bead-to-DNA ratio is low, which may result in capturing the incorrect size of DNA fragments, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade its buffer and lead to loss of sample, causing suboptimal assay performance, insufficient DNA yield, and loss of sample.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.

1. Remove the Cleanup Beads V2 from cold storage at least 15 minutes before use to bring them to room temperature.
2. Resuspend amplified samples containing the Capture Beads by vortexing, and quickly spin down the liquid without pelleting the beads.
3. Place the amplified samples containing the Capture Beads on the magnetic rack for 2 to 3 minutes until the supernatant is clear.
4. Carefully transfer the supernatant, which contains the PCR amplified, panel-enriched library to a new 0.2 mL strip tube. Discard the Capture Beads.

Pay attention to not discard the supernatant, which contains the PCR amplified, panel-enriched library.

A cloudy supernatant may be observed; however, this occurrence does not impact downstream yield or performance.

5. Thoroughly mix the Cleanup Beads V2 by vortexing.
6. Add 50 µL of Cleanup Beads V2 to the PCR product.
7. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
8. Incubate for 10 minutes at room temperature.
9. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
10. Discard the supernatant.
11. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
12. Incubate for at least 30 seconds at room temperature, and discard the ethanol.
13. Repeat *step 11* and *step 12* for a second wash.
14. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
15. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
16. Leave the tubes open on the magnetic rack to air dry the beads for 3 minutes.

Do not over dry the beads (visible by the formation of cracks on the pellet). Over drying the beads may lead to reduction in yield. Each bead pellet surface should have an uncracked matte appearance before resuspension.

17. Remove the tubes from the magnetic rack, and add 50 µL nuclease-free water, PCR grade, to each sample. Resuspend thoroughly by pipetting up and down.
18. Incubate for 2 minutes at room temperature.
19. Pellet beads on the magnetic rack for 2 to 3 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
20. Transfer the clear eluate into a new 0.2 mL strip tube. The eluate contains the final library.

Freeze at –20°C for up to 1 month, or proceed to *Assessing enriched library quality*.

Assessing enriched library quality

To assess enriched library quality

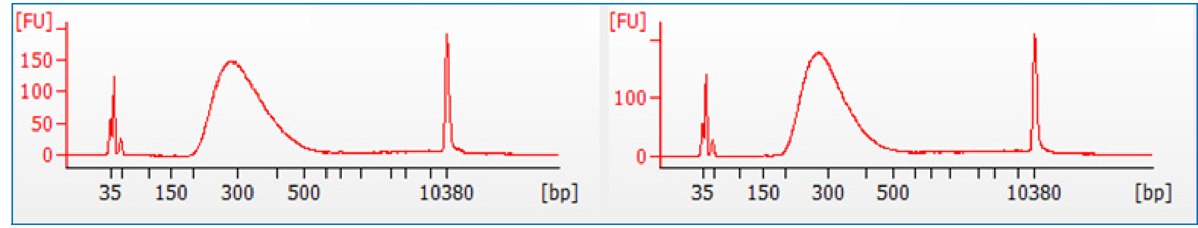
1. Quantify the concentration of individual samples with the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
2. If needed, dilute the samples with nuclease-free water, PCR grade, to a recommended concentration for analysis using Agilent High Sensitivity DNA Kit. A 5 to 10-fold dilution is generally sufficient.
3. Assess the average library size using an Agilent High Sensitivity DNA Kit on a 2100 Bioanalyzer following the manufacturer's instructions.
4. The library size and concentration will be used to calculate the molarity.

Alternatively, a D1000/High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

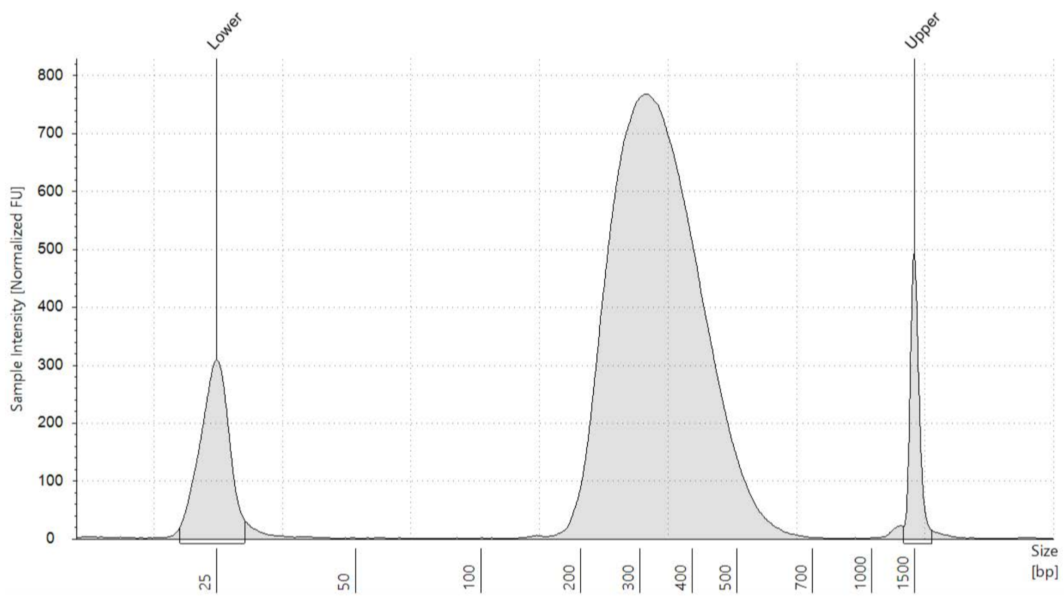
The typical concentration of the enriched library is between 0.5 ng/µL and 15 ng/µL based on recommended input.

There should be minimal to no adapter dimers present. Library size and major peak position of the library varies based on sample quality. A typical library size ranges from 200 to 1000 bp with a major peak around 300 bp.

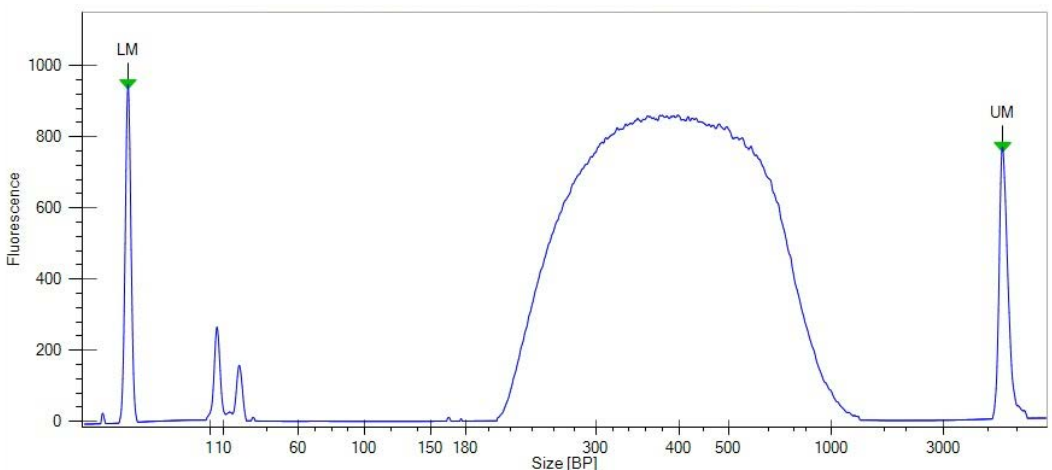
The following show examples of Bioanalyzer profiles for typical, good final enriched library with negligible adapter dimers and a fragment peak near 300 bp. The profiles provided are from internal data at Roche.



The following is an example of a TapeStation profile showing a typical, good final enriched library.



The following is an example of a LabChip profile showing a typical, good final enriched library.



Chapter 5. DNA sequencing

The AVENIO Tumor Tissue Analysis Kits V2 prepare sequencing-ready libraries from DNA isolated from FFPE tissue. After QC of the enriched libraries from *Chapter 4. Perform the enrichment protocol* on page 33, the samples are ready for pooling and sequencing with the Illumina NextSeq 500/550 instrument or NextSeq 550Dx instrument in RUO Mode.

The Illumina NextSeq 500/550/550Dx instrument is a third-party instrument. This guide makes no claims on the instrument, and it is not supported by Roche Customer Support.

Ensure that the following is available:

- The Illumina NextSeq 500/550/550Dx instrument is configured in Manual Mode
- Illumina Sequencing Kit listed under *Reagents and Consumables purchased from other vendors* on page 12
  - NextSeq 500/550 High Output Kit v2.5 (300 cycles)
  - NextSeq 550Dx High Output Kit v2.5 (300 cycles)
- PCR grade water
- 1 M NaOH, molecular biology grade
- 200 mM Tris-HCl, pH 7.0, molecular biology grade

Pooling enriched DNA samples for sequencing

Avoid pooling AVENIO Tumor Tissue libraries, AVENIO ctDNA libraries or non-AVENIO libraries in the same NextSeq run. Mixing libraries generated from different workflows will make the final sequencing data unanalyzable by the AVENIO Oncology Analysis Software.

Ensure that each sample was prepared with a unique Sample Primer ID. If samples from different batches of library preparation need to be pooled together, it is recommended that Qubit quantification be done on all samples to be pooled together using a single Qubit master mix to minimize the impact of Qubit measurement variability on pooling.

Up to 24 samples can be pooled and sequenced as 1 multiplexed pool per NextSeq 500/550/550Dx sequencing run.

To pool enriched DNA samples for sequencing

1. Use the concentration and the average library size determined in *Assessing enriched library quality* on page 42 to calculate the molarity of each sample in nM using the following equation:

   Library Molarity in nM = (library concentration in ng/µL) / ((average library size in bp*607.4)+157.9)*10^6

   For example, if library concentration = 10 ng/µL and average library size is 300 bp, then the Library Molarity in nM = 10/((300*607.4)+157.9)*10^6 = 54.83.

2. Pool an appropriate volume of each sample so that there are equal molar mass of each sample in the pool. Prepare enough total volume sufficient for Illumina's suggested denaturing protocol (for an example, refer to Illumina's *NextSeq System Denature and Dilute Libraries Guide*).

It is important to pool equal molar mass per sample, to ensure that each sample receives approximately an equal amount of sequencing reads for data analysis.

Ensure that each sample to be pooled for sequencing used a Sample Primer ID unique to the pool. Mixing more than one sample with the same Sample Primer ID will make final sequencing data for these samples unusable.

3. Vortex and spin down pooled library briefly.
4. Quantify the concentration of the pooled library using the Qubit dsDNA HS Assay Kit according to the manufacturer's instructions.
5. If needed, dilute the samples with nuclease-free water, PCR grade, to bring the concentration within the Bioanalyzer High Sensitivity specifications.

Alternatively, a High Sensitivity D1000 ScreenTape assay on a 4200 TapeStation or a LabChip NGS 3K reagent kit with HT DNA X-Mark Chip on a LabChip GX Touch HT can be used.

6. Assess the average fragment size of the pooled libraries by diluting to a recommended concentration for analysis using Agilent High Sensitivity DNA Kit on a 2100 Bioanalyzer following the manufacturer's instructions.
7. Calculate the molarity of the pooled library outlined in *step 1*.
8. Dilute the pooled library following Illumina's NextSeq guideline.
9. Proceed to *Denaturing enriched DNA pool and preparing for sequencing*.

Denaturing enriched DNA pool and preparing for sequencing

To denature enriched DNA pool and prepare for sequencing

1. Follow Illumina's suggested protocol to denature and dilute libraries for NextSeq sequencing. (For an example, refer to Illumina's *NextSeq System Denature and Dilute Libraries Guide*.)

Illumina recommends 1.8 pM for NextSeq High Output Kit loading. It is the user's responsibility to determine the optimal loading amount onto the NextSeq 500/550/550Dx instrument to attain Illumina's recommended cluster density.

No PhiX is necessary for these samples. While PhiX does not interfere with the sequencing and subsequent data analysis and can be used as a control, if desired, for Illumina customer support purposes, it takes up a significant amount of reads in the run.

Sequencing multiplexed libraries with the Illumina NextSeq 500/550/550Dx

To sequence multiplexed libraries with the Illumina NextSeq 500/550/550Dx

1. Use Illumina's NextSeq 500/550 System Guide or NextSeq 550Dx Instrument Guide for loading instructions for paired-end 150-cycle run.

The steps on the Run Setup screen differs based on the system configuration. The NextSeq 500/550 instrument must be configured to Manual Mode. If using the NextSeq 550Dx, use the instrument in the Research Mode. Use of Basespace mode is not supported.

2. Use the following run parameters:
   - **Run name**: User defined
   - **Library ID**: User defined
   - **Recipe**: NextSeq High
   - **Read 1**: 151 cycles
   - **Read 2**: 151 cycles
   - **Index 1**: 8 cycles
   - **Index 2**: 8 cycles

Performing Illumina NextSeq 500/550/550Dx maintenance

To perform Illumina NextSeq 500/550/550Dx maintenance

1. Follow Illumina's manufacturer recommendations for regular instrumentation maintenance.
2. Frequently perform the Manual Post-Run Wash according to Illumina's protocols.

Regular washing of the Illumina NextSeq 500/550/550Dx reduces the possibility of contamination of samples from past sequencing runs.

Performing post-sequencing data analysis

To perform post-sequencing data analysis

Refer to the *AVENIO Oncology Analysis Software User Guide*.

Appendix A. AVENIO Tumor Tissue Analysis Kits V2

The AVENIO Tumor Tissue Analysis Kits V2 include the following products:

- AVENIO Tumor Tissue Targeted Kit V2
- AVENIO Tumor Tissue Expanded Kit V2
- AVENIO Tumor Tissue Surveillance Kit V2

Each product contains a uniquely designed probe panel that target different regions in the genome. In addition, each product contains one of two primer plate options, Plate A or Plate B.

AVENIO Tumor Tissue Targeted Kit V2 (Plate A)

The AVENIO Tumor Tissue Targeted Kit V2 (Plate A option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Targeted Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate A)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Targeted Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Targeted Kit V2 (Plate B)

The AVENIO Tumor Tissue Targeted Kit V2 (Plate B option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Targeted Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate B)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Targeted Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Expanded Kit V2 (Plate A)

The AVENIO Tumor Tissue Expanded Kit V2 (Plate A option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Expanded Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate A)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Expanded Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Expanded Kit V2 (Plate B)

The AVENIO Tumor Tissue Expanded Kit V2 (Plate B option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Expanded Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate B)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Expanded Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Surveillance Kit V2 (Plate A)

The AVENIO Tumor Tissue Surveillance Kit V2 (Plate A option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Surveillance Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate A)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Surveillance Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Surveillance Kit V2 (Plate B)

The AVENIO Tumor Tissue Surveillance Kit V2 (Plate B option) includes:

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue Surveillance Kit V2	1	AVENIO Tumor DNA Isolation and QC Kit	–15°C to –25°C	–15°C to –25°C
	2	AVENIO Tumor Cleanup and Capture Beads V2	+2°C to +8°C	+2°C to +8°C
	3	AVENIO Tumor Library Prep Kit V2	–15°C to –25°C	–15°C to –25°C
	4	AVENIO Tumor Sample Primers (Plate B)	+2°C to +8°C	+2°C to +8°C
	5	AVENIO Tumor Enrichment Kit V2	–15°C to –25°C	–15°C to –25°C
	6	AVENIO Tumor Surveillance Panel V2	–15°C to –25°C	–15°C to –25°C
	7	AVENIO Post-Hybridization Kit V2	–15°C to –25°C	–15°C to –25°C

AVENIO Tumor Tissue Analysis Kits V2 – Sub-Kits

The AVENIO Tumor Tissue Targeted V2, Expanded V2, and Surveillance Kit V2 each contain the following sub-kits:

AVENIO Tumor DNA Isolation and QC Kit

The AVENIO Tumor DNA Isolation and QC Kit supports up to 48 samples, allowing two rounds of DNA isolation and QC for 24 samples, if needed.

Component	Volume	Quantity in Kit
Extraction Buffer (10X)	560 µL	1
Extraction Enzyme	110 µL	1
DNA Elution Buffer	40 mL	1
QC PCR Primer Mix (66 bp)	200 µL	1
QC PCR Primer Mix (191 bp)	200 µL	1
QC PCR Reaction Mix (2X)	4.8 mL	1
QC PCR DNA Standard	25 µL	1

AVENIO Tumor Cleanup and Capture Beads V2

The AVENIO Tumor Cleanup and Capture Beads V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
Capture Beads	2.7 mL	1
Cleanup Beads V2	15 mL	2

AVENIO Tumor Library Prep Kit V2

The AVENIO Tumor Library Prep Kit V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
DNA Polishing Enzyme	5 µL	1
Fragmentation Buffer (10X)	150 µL	1
Fragmentation Enzyme	300 µL	1
DNA Preparation Buffer	200 µL	1
DNA Preparation Enzyme V2	100 µL	1
Universal Adapters	280 µL	1
Ligation Buffer	900 µL	1
DNA Ligase	290 µL	1
PCR Reaction Mix (2X)	690 µL	1

AVENIO Tumor Sample Primers (Plate A or Plate B)

Each of the AVENIO Tumor Sample Primers (Plate A or Plate B) supports up to 24 samples.

Component	Volume	Quantity in Kit
Sample Primers – Plate A or Plate B	Dry-down	1

AVENIO Tumor Enrichment Kit V2

The AVENIO Tumor Enrichment Kit V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
Hybridization Supplement	1000 µL	2
Hybridization Buffer 1 (2X)	890 µL	1
Hybridization Buffer 2 V2	500 µL	1
Universal Enhancing Oligos	355 µL	1

AVENIO Tumor Targeted Panel V2

The AVENIO Tumor Targeted Panel V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
AVENIO Tumor Targeted Panel V2	134 µL	1

AVENIO Tumor Expanded Panel V2

The AVENIO Tumor Expanded Panel V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
AVENIO Tumor Expanded Panel V2	134 µL	1

AVENIO Tumor Surveillance Panel V2

The AVENIO Tumor Surveillance Panel V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
AVENIO Tumor Surveillance Panel V2	134 µL	1

AVENIO Post-Hybridization Kit V2

The AVENIO Post-Hybridization Kit V2 supports up to 24 samples.

Component	Quantity	Quantity in Kit
Bead Wash Buffer (2.5X)	12 mL	1
Hybridization Wash 1 (10X)	900 µL	1
Hybridization Wash 2 (10X)	580 µL	1
Hybridization Wash 3 (10X)	580 µL	1
Stringent Wash Buffer (10X)	1160 µL	1
PCR Reaction Mix (2X)	690 µL	1
PCR Primer Mix (10X)	138 µL	1

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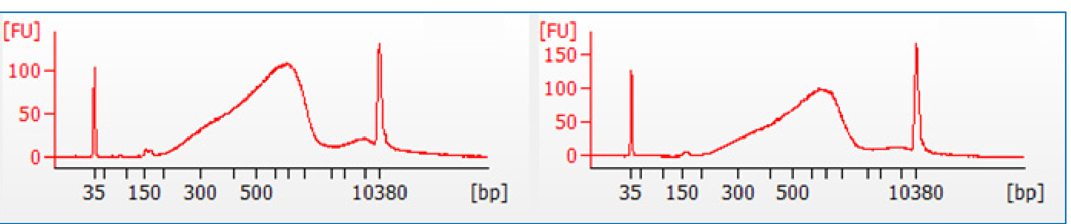
Appendix B. Troubleshooting

This appendix includes pre- and post-sequencing Quality Control (QC) troubleshooting information.

Pre-sequencing QC troubleshooting

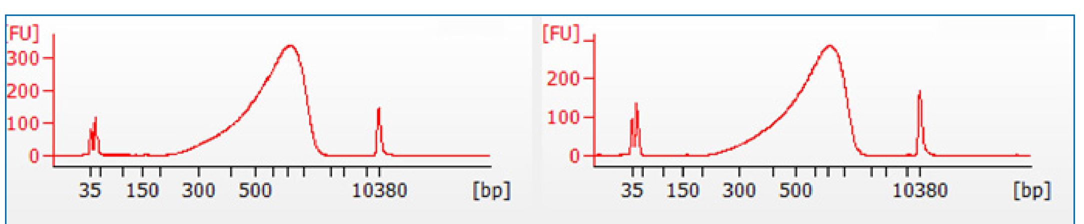
Issue	Cause	Possible resolution
No or low yield from DNA isolation	Poor tissue quality	▪ Ensure that a sufficient sized sample is used.
	▪ Forgot to add Extraction Enzyme to extraction master mix ▪ Incorrect incubation conditions (temperature, incubation times, etc.)	▪ Ensure proper volumes and incubation times are used during DNA isolation.
High adapter dimers in the pre-enrichment library after the first PCR	Insufficient DNA or poor quality DNA used in the assay	▪ Ensure proper quantification of the input DNA. ▪ Follow the recommended DNA amount according to the user guide.
	Poor cleanup	▪ Ensure proper volumes are used in the reaction. ▪ Clean the sample again, maintaining the sample-to-bead ratio. Note that this additional cleanup may result in some sample loss and lower Unique Depth.
Abnormal Bioanalyzer profile of the pre-enrichment library	Under-fragmentation	▪ Ensure that the Fragmentation Buffer is fully thawed and resuspended. ▪ Ensure proper mixing of viscous Fragmentation Enzyme in the master mix and in the fragmentation reaction with the samples. ▪ Ensure that the DNA Polishing Enzyme is diluted 50-fold using nuclease-free water, PCR grade before use.

The following examples show underfragmented adapter-ligated library Bioanalyzer profiles with the peak skewed above 500 bp. The profiles provided are from internal data at Roche. Similar profiles are observed on the TapeStation and Labchip (data not shown).



Issue	Cause	Possible resolution
Low yield of the pre-enrichment library (less than 10 ng/μL)	Poor ligation efficiency	▪ Ensure that the proper amount of input DNA and Universal Adapters is used. ▪ Ensure proper ligation incubation time and temperature are used.
	Poor PCR amplification	▪ High adapter dimers (visible near 150 bp) can inhibit the PCR reaction. Follow proper cleanup steps before PCR. ▪ Ensure that the Sample Primers are fully resuspended by carefully following the procedure *Preparing PCR primers* on page 26. ▪ Ensure that the first PCR reaction is set up properly.
	Sample loss	▪ Ensure that bead cleanup steps are performed properly.
Low yield of the final enriched library	Poor binding with the Capture Beads	▪ Ensure thorough washing by carefully following the procedure *Preparing the Capture Beads* on page 37.
	Sample loss	▪ Ensure that the Capture Beads containing bound libraries are not accidentally discarded during the enrichment procedure. ▪ Ensure that the final PCR step includes the Capture Beads, which have the enriched library bound to them. ▪ Ensure that bead cleanup steps are performed properly.
	Poor PCR amplification	▪ Ensure that the final PCR reaction is set up properly.
Abnormal Bioanalyzer profile of the final enriched library	Under-fragmentation	▪ See possible resolutions to the Under-fragmentation cause on page 53.

The following examples show underfragmented final enriched library Bioanalyzer profiles with peak skewed above 500 bp. The profiles provided are from internal data at Roche. Similar profiles are observed on the TapeStation and Labchip (data not shown).



Post-sequencing QC troubleshooting

Issue	Cause	Possible resolution
Poor On-target Rate on the Sample Metrics Report	Wrong Panel used	▪ Ensure that the intended panel was used and recorded in the analysis submission.
	Poor post-hybridization washes	▪ Ensure all wash buffers are fully thawed and resuspended before preparing 1x wash buffers. ▪ Ensure thorough washing by carefully following the procedure *Performing the hybridization cleanup* on page 38. ▪ Ensure that fresh tubes are used during post-hybridization washes when indicated in the user guide. ▪ Avoid long delays during the post-hybridization wash steps.
	Improper temperature for hybridization	▪ Ensure that hybridization occurred at the correct temperature.
	Improper volume for Universal Enhancing Oligos.	▪ Ensure proper volume of Universal Enhancing Oligos is used.
Low "Number of Read Pairs" on the Sample Metrics Report	Poor sequencing run	▪ Refer to an up-to-date Illumina manual. ▪ Ensure that samples are free of adapter dimers through Bioanalyzer analysis of the sample. ▪ Check cluster density and resequence if the total sequencing reads are impacted by poor clustering.
Low "Median Unique Depth" on the Sample Metrics Report	Poor on-target rate	▪ See possible resolutions to the *Poor On-target Rate on the Sample Metrics Report* cause.
	Poor ligation efficiency	▪ See possible resolutions to the *Poor ligation efficiency* cause on page 54.
	Under-fragmentation	▪ Check Bioanalyzer traces to confirm under-fragmentation showing up as larger average peak size. ▪ See possible resolutions to the *Under-fragmentation* cause on page 53.
Long "Fragment Length" on the Sample Metrics Report	Under-fragmentation	▪ See possible resolutions to the *Under-fragmentation* cause on page 53.
High "Error Rate" on the Sample Metrics Report	Poor DNA polishing efficiency	▪ Ensure that the DNA Polishing Enzyme is diluted fresh before each use and DNA polishing reaction is set up properly.

Issue	Cause	Possible resolution
Low “Percent Mapped Reads” on the Sample Metrics Report	Presence of non-human samples contaminating the sample	▪ Ensure good lab practices when processing the sample. ▪ Keep good Illumina NextSeq 500/550/550Dx maintenance. ▪ Perform a manual post-run wash according to Illumina’s manufacturer protocols before resequencing.