AVENIO Tumor Tissue CGP Kit V2

Reagent Instructions for Use

Version 1.0

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

Publication information

Publication version	Software version	Revision date	Change description
1.0	1.0	July 2024	First version

Edition notice

This publication is intended for the AVENIO Tumor Tissue CGP Kit 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 CGP Kit V2 Instructions for Use v1.0 contains information about using the AVENIO Tumor Tissue CGP Kit V2 reagents, and instructions for the operation workflow for the AVENIO Tumor Tissue CGP Kit V2.

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

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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

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AVENIO Tumor Tissue CGP Kit V2

The AVENIO Tumor Tissue CGP Kit V2 includes the reagents needed to isolate DNA from FFPE tissue, prepare sequencing libraries, and enrich selected regions of interest using the AVENIO Tumor Tissue CGP Panel V2. It also includes a cloud-based secondary data analysis software called the FoundationOne® Analysis Platform as well as a data management software called AVENIO Connect.

This ready-to-use kit uses a hybrid-capture based workflow for molecular profiling of DNA derived from FFPE solid tumor tissue. The AVENIO Tumor Tissue CGP Kit V2 helps labs consistently purify and enrich targeted regions in DNA from FFPE solid tumor tissue for sequencing. It provides lab professionals the ability to generate meaningful genomics insights from all classes of genomic alterations - Single-Nucleotide Variants, Insertions and Deletions, Copy Number Alterations, and Rearrangements - as well as relevant genomic signatures such as Tumor Mutational Burden, Microsatellite Instability, genomic Loss of Heterozygosity and Homologous Recombination Deficiency signature, using a DNA workflow. Each kit includes reagents for processing up to 24 reactions pooled into two to three sequencing runs of up to 8-12 libraries on an Illumina® NextSeq 500/550 or Illumina NextSeq 550 Dx in RUO Mode.

Summary of product components

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

Product name	Descriptor
AVENIO Tumor Tissue CGP Kit V2	Collection of reagents needed for DNA isolation, library preparation and target enrichment.
FoundationOne® Analysis Platform	Cloud-based secondary analysis software
AVENIO Connect	Cloud-based data management software

Symbols, abbreviations, and acronyms

Symbols used in the publication

Symbol	Explanation
	**Safety alert symbol**: 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.

Abbreviations and acronyms

The following abbreviations and acronyms are used.

Abbreviation/acronym	Definition
CGP	Comprehensive Genomic Profiling
CN	Copy Number
CNA	Copy Number Alteration
Cp	Crossing point
CpG	Cytosine-phosphate-Guanine
Cq	Quantification Cycle
Ct	Cycle threshold
FFPE	Formalin-Fixed Paraffin-Embedded
gLOH	genomic Loss of Heterozygosity
H&E	Hematoxylin and Eosin
HRD	Homologous Recombination Deficiency
HRDsig	Homologous Recombination Deficiency signature
Indel	Insertion and Deletion
MSI	Microsatellite Instability
ncRNA	non-coding RNA
NTC	No Template Control
QC	Quality Control
qPCR	quantitative Polymerase Chain Reaction
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
SNV	Single Nucleotide Variant
TMB	Tumor Mutational Burden

Protocol information and safety

- Check the reagents, panel, and kit names before use. Ensure AVENIO CGP Kit V2 reagents are used, and not the original AVENIO CGP Kit (version 1). The original (version 1) and V2 reagents are not interchangeable and may lead to suboptimal assay performance.
- Ensure to follow this Instructions for Use when using AVENIO Tumor Tissue CGP Kit V2 reagents. The original (V1) and V2 protocol conditions are not interchangeable and may lead to suboptimal assay performance.
- Wear gloves and take precautions to avoid sample contamination.
- Change gloves when opening and closing strip tubes to minimize cross-contamination.
- Perform all centrifugations at room temperature (+15°C to +25°C) unless indicated otherwise.
- 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.
- 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.
- Apply good laboratory practices, such as wearing appropriate personal protective equipment when handling biological material.
- 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.
- Properly label tubes at all times to prevent sample mix-up.

- Check expiration date before use. Do not use reagents if they are expired. Expired reagents will affect sample preparation and may lead to suboptimal assay performance.
- Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade.
- Ensure hazardous waste is properly disposed.
- It is recommended to use a multi-channel pipettor to ensure sample consistency.
- Hybridization Buffer 1 and PCR Reaction Mix (2x) contain tetramethylammonium chloride. Practice safe laboratory practices when handling them.
- Hybridization Buffer 2 contains dimethyl sulfoxide. Practice safe laboratory practices when handling it.
- While both slides and curls can be processed, avoid using tissues from slides if curls are available, 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.
- 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.
- The QC measurements by the Qubit dsDNA HS Assay Kit can also be performed by the Qubit 1X dsDNA HS Assay Kit according to manufacturer's instructions. The Qubit Fluorometer or Qubit Flex Fluorometer can be used.
- The QC measurements performed by the Agilent Bioanalyzer can be substituted by the Agilent TapeStation DNA ScreenTape or TapeStation High Sensitivity DNA ScreenTape.
- Safe stopping points of the assay are after the DNA extraction, after the first PCR of Chapter 3, and the final enriched library. Consult the procedures for the storage conditions of the stopping points.
- 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.
- While not required, it is good laboratory practice to run a known positive DNA process control when performing the library preparation procedures, to ensure execution of the laboratory procedures. A cell line DNA, such as HD753 from Horizon Diagnostics can be used as a positive process control. When using a high quality DNA cell line DNA, 50 ng can be used as input into the library preparation procedure. Note, while variant calling may not be reliable when using an artificial cell line DNA, a positive control can be used to assess library QC measurements and general sequencing metrics.
- Follow the AVENIO Connect Software User Assistance and select the correct analysis pipeline for the AVENIO Tumor Tissue Analysis CGP Kit V2.

Required equipment, labware, and consumables

You assume full responsibility when using the equipment, labware, and consumables described below. These protocols are designed for use with the specified equipment, labware, and consumables.

Laboratory equipment

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 Thermal Cycler, 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 or Qubit Flex Fluorometer	ThermoFisher Scientific	Q33216 Q33238 Q33327
2100 Bioanalyzer or 4200 TapeStation System	Agilent Technologies	Q33216 G2940CA G2943CA G2991BA

2100 Bioanalyzer or 4200 TapeStation System	Agilent Technologies	Q33216 G2940CA G2943CA G2991BA
Illumina NextSeq 500 or NextSeq 550 or NextSeq 550Dx	Illumina	SY-415-1001 SY-415-1002, 20005715
Tabletop plate centrifuge (Capable of 1,000 x g or 1,000 RCF)	Multiple vendors	
Tabletop centrifuge (Capable of up to 1,000 x g or 1,000 RCF for 1.5 mL microcentrifuge tubes)	Multiple vendors	
Tabletop micro/mini centrifuge (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)	Multiple vendors	

Reagents and Consumables available from Roche Diagnostics

The AVENIO Tumor Tissue CGP Kit 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 #	Included Sub-kit Name
AVENIO Tumor Tissue CGP Kit V2	10240814001 (with Sample Primer Plate A) 10240822001 (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 Tissue CGP Panel Kit V2
		AVENIO Post-Hybridization Kit V2

Component	Catalog number
LightCycler® 480 Multiwell Plate 384, white with sealing foils (recommended)	04729749001
FoundationOne® Analysis Platform - 1.x (for support purposes only, not orderable)	09537546001
AVENIO Connect CGP Kit - RUO	09345442001
AVENIO Connect RUO - 1.x (for support purposes only, not orderable)	09326596001
AVENIO Connect Gateway	09428925001

Consumables purchased from other vendors

Consumables	Supplier	Catalog number
0.2 mL PCR 8-Strips (required tubes ordered from one of these two suppliers)	Starlab International GmbH or USA Scientific (Western hemisphere)	I1402-3700 1402-4700
1.5 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	

Additional reagents and consumables	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	Q32854 or Q33230
Qubit Assay Tubes	ThermoFisher Scientific	Q32856 or Q33252
Agilent High Sensitivity DNA Kit or Agilent D1000 or High Sensitivity D1000 ScreenTape, Reagents, Ladder, Sample Buffer	Agilent Technologies	5067-4626 5067-5582 5067-5583 5067-5602 5067-5584 5067-5585 5067-5587 5067-5603
1 N 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)	Illumina	20028871

Chapter 1. Getting started

This Instructions for Use describes the recommended steps for use of the AVENIO Tumor Tissue CGP Kit V2 to prepare sequencing-ready libraries from DNA isolated from FFPE (formalin-fixed paraffin-embedded) tissue to identify genomic aberrations such as SNVs (single nucleotide variants), Indels (insertions and deletions), CNAs (copy number alterations), rearrangements, TMB (tumor mutational burden), MSI (microsatellite instability), and gLOH (genomic Loss of Heterozygosity), and HRDsig (Homologous Recombination Deficiency signature) from solid tumors.

About AVENIO Tumor Tissue CGP Kit V2

The AVENIO Tumor Tissue CGP Kit V2 is a next generation sequencing based Research Use Only assay for detection of Single Nucleotide Variants, Insertion and Deletions, Copy Number Alterations and Rearrangements, as well as genomic signatures such as Tumor Mutational Burden, Microsatellite Instability, genomic Loss of Heterozygosity, and Homologous Recombination Deficiency signature in 335 genes using DNA extracted from formalin-fixed paraffin embedded tumor tissue specimens.

AVENIO Tumor Tissue CGP Kit V2 assay workflow includes manual lysis of tissue sections (curls or slides) derived FFPE tissue samples, FFPE tissue DNA purification, hybridization-based capture of regions of interest for 335 reportable targeted genes, preparation of sequencing-ready sample libraries, and library pooling prior to sequencing preparation. Each kit includes reagents for processing up to 24 reactions pooled into two to three sequencing runs of up to 8-12 libraries on an Illumina® NextSeq 500/550 or Illumina NextSeq 550 Dx in RUO Mode.

Sequencing data is processed using the Foundation Medicine Inc. (FMI) developed cloud-based FoundationOne® Analysis Platform, designed to detect various genomic alterations and four genomic signatures. The kit solution also includes the AVENIO Connect software that is a standalone software that manages sequencing data analysis from raw data uploading to result retrieval.

Workflow

The AVENIO Tumor Tissue CGP Kit V2 workflow includes the following 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 Tissue CGP Panel Kit 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 CGP Kit V2 reagent workflow:

Step	Processing time
Isolate DNA from FFPE tissue	Recommended: 4.5 hours - including 3 hour incubation (option for 2.5 hours with 1 hour incubation)
	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
	1 hour ligation (option for overnight ligation)
Perform post-ligation cleanup	1 hour
PCR amplify ligated sample	1 hour
	Safe stopping point
Post-PCR cleanup and hybridization of the sample	2 hours
	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

The recommended extraction incubation time is 3 hours for maximal DNA yield. The recommended library preparation protocol is performing ligation for 1 hour, for fastest 2-day library preparation. Optional incubation times and stopping points are available, as indicated in this Instructions for Use, noted at the appropriate step, for greater schedule flexibility.

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 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 21.

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

- Acceptable samples are Formalin-Fixed Paraffin-Embedded (FFPE) tissue, cut as curls or mounted on glass slides. The acceptable fixation method should be formalin based, such as a 10% neutral-buffered formalin for 6-72 hours.
    - Do not use slides that were prepared with non-formalin fixatives.
    - Do not use use samples that were baked or decalcified. Do not decalcify the tissue mounted on glass slides.
    - Do not stain the slides that will be used for DNA extraction.
- If available, it is recommended to use curls rather than tissue sections mounted on slides to minimize the hazards associated with razor blades used during tissue scraping techniques.
- The tissue specifications for the extraction procedure are:
    - >20% tumor content is minimally required for the assay. >30% tumor content is optimal. Tumor content should be assessed by cells containing nuclei. Hematoxylin and Eosin (H&E) staining of an adjacent slide can be used to determine tumor content. Do not use an H&E stained slide for DNA extraction or for library preparation.
    - >25 mm^2 tissue surface area (length x height) is the minimum size for the extraction procedure, and >50 mm^2 or larger is recommended. It is ideal to use a large surface area to maximize the extracted DNA yield for the assay - the larger the surface area, the greater the extraction yield. It is recommended to extract the DNA with the recommended 3 hour incubation time, especially for smaller samples. Ultimately, the DNA input requirement for the assay follows the instructions in Determining DNA input for sequencing library generation.
    - For curls, use two curls with 10 micron thickness.
    - For slides, use five slides with 4-5 micron thickness tissues mounted on positively charged glass slides. The slides should be unbaked.
- Tissue samples should be collected into a 1.5 mL tube prior to use of this extraction procedure.
- Suggestions when using tissues on slides:
    - When collecting tissue from slides, it is very important to consider proper scraping technique. Scraping tissues can be challenging for users unfamiliar with proper technique. It is recommended to practice the procedure before scraping critical samples mounted on slides. For users unaccustomed to scraping tissue, it is recommended to start using the AVENIO Tumor Tissue CGP Kit V2 with tissue curls rather than tissues from slides.
    - A razor blade or surgical scalpel can be used for scraping. Practice safe handling procedures and adhere to local safety guidelines and requirements.

- When scraping tissue from slides, ensure that the majority of the tissue is collected into a 1.5 ml tube.
- Several methods can be used for scraping tissues for slides for collection. There is no universal method, and individual users should determine their preferred method. Below are some possible methods:
  - Recommended method: Tissues can be scrapped, dry, directly from the slide using a flat razor blade. The blade can be glided across the slide in one swift motion, collecting the tissue. All five slides can be scrapped in succession. Take attention to avoid static disturbance of the tissues. Forceps can collect the five tissue sections off the blade, and placed into a clean 1.5 mL tube.

  - Pre-wetting the blade or tissue can make it easier to collect the tissue, however, using the wrong buffer can inhibit the extraction reaction. The Extraction Master Mix used in the section Isolating DNA from FFPE tissue can be prepared immediately prior to scraping slides. Prior to scraping, the surgical blade can be dipped into 100 µl of the room temperature Extraction Master Mix. The tissue can then be placed into the same tube of 100 µl Extraction Master Mix. When all the samples have been collected, follow the heated incubation steps in the workflow instructions.

  - Tissue can also be scrapped into 700 µl of 100% ethanol. Pre-wet the surgical blade with the ethanol. Slides can be scraped and transfered one at a time into the tube of ethanol. After collection, add 300 µl of additional 100% ethanol. Centrifuge the sample for 20,000 x g for 20 minutes. Remove and discard the excess ethanol, saving the spun down tissue. Residual ethanol will inhibit the extraction reaction, so allow the samples to dry overnight in the opened tube. Ensure that all the ethanol is evaporated before proceeding to the DNA isolation step.

When using a razor blade or surgical scalpel, practice safe handling procedures and adhere to local safety guidelines and requirements. If permissible by local guidelines, use a razor blade holder if a razor blade must be used.

Do not exceed a combined 20 microns (µm) of tissue thickness in the extraction procedure.

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 tissue 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 at the bottom of the tube, vortex to dislodge the tissue, 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 with the lid on for 3 hours. If a thermomixer is not available, vortex the tubes as follows:
   a. Incubate at 75°C in a heat block for a total of 3 hours 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 paraffin wax does not dissolve.

   c. Vortex the tubes again every 30 minutes.

Alternatively, Step 5a can be performed for 1 hour at 75°C, instead of 3 hours. Incubation should occur while shaking at 2000 RPM or vortexing according to Steps 5b and 5c. This provides flexibility for the operator's schedule.

A 3 hour extraction is the recommended duration to ensure maximum DNA yield, especially for challenging tissue samples, such as older samples or fibrous breast tissues. Note, the 3 hour extraction can be utilized with the 2 day library preparation (1 hour ligation) workflow for efficient overall schedule.

Note, only 1 hour or 3 hour incubations are permissible for step 5a, which were optimized with the analysis algorithms. It is not recommended to alter the incubation duration.

6. Following the incubation duration, spin the tubes at 20,000 RCF for 5 minutes to pellet the remaining cellular debris. A visible and/or solid paraffin wax layer may form after the spin.
7. Pierce through the paraffin wax layer with a pipette tip, and transfer the liquid to strip tubes.
   a. Carefully transfer each sample to a unique position in the strip tubes, taking caution to avoid cross sample contamination, and keeping track of the sample identification.
   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 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade buffer and lead to loss of sample. Ensure proper storage of Cleanup Beads V2 as improper storage conditions may lead to suboptimal assay performance.

Prepare 80% ethanol fresh on the day of usage using 100% ethanol and nuclease-free water, PCR grade. For time efficiency, the 80% ethanol can be prepared during the 1 hour or 3 hours extraction incubation.

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 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 5 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.
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. Visually inspect the drying beads, and an overly dry lab may require less drying time to prevent over-drying.

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 5 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 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 2 PCR amplicons (66 bp and 191 bp). The normalized quality ratio (Q-ratio) is calculated using a QC PCR DNA standard of known quality.

To check the quality of FFPE DNA

1. Dilute the extracted DNA samples and 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 QC PCR DNA Standard must be included in each qPCR run.

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

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

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

3. Vortex and spin the master mix, and add 6 µL of the appropriate master mix to each well of a 384-well plate.

   A 96-well plate can also be used for less samples.

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

Thermocycler Profile			
Stage	**Temp (°C)**	**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 score for each sample including the QC PCR DNA standard by using the following equation:

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

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

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

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

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

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

Avoid using the samples with Q-ratio of less than 0.04 as the sequencing results will not be optimal.

A template file is available to assist in calculating the Q-ratio. Consult your local Roche representative.

Determining DNA input for sequencing library generation

To determine DNA input for sequencing library generation

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

   Input mass in ng = 20/(Q-ratio) + 30

   For example, for a given sample, if its Q-ratio = 0.5, then its input mass in ng = 20/0.5 + 30 = 70.

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

   For maximal performance, take attention to use the proper amount of extracted DNA for the library generation, indicated in this section.

   In order to save time, it is possible to perform the Qubit procedure during the qPCR cycling reaction.

2. Determine concentration of each sample using the Qubit dsDNA HS Assay Kit according to manufacturer's instructions. If the sample concentration is higher than the quantitative range, dilute the sample with nuclease-free water, PCR grade, and repeat the quantitation.

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 = 70 ng, and Qubit concentration = 10 ng/µL, then its recommended input volume in µL = 70/10 = 7.

   The maximum sample input volume for library preparation is 30.5 µL; therefore, some samples may be too dilute to meet the required input mass. When available, input the recommended input mass into the workflow for maximum variant detection results. It may be possible to still create sequencing libraries when less than the recommended input amount of DNA is used. However, using less than the recommended input mass may result in lower median sequencing coverage and lower variant detection performance.

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 Beads V2 to prepare sequencing libraries with unique Sample Primer IDs. Each sample will be amplified with a unique Sample Primer set, and enables the multiplexed sequencing of up to 12 samples per sequencing run.

For optimal assay performance, follow the recommended sample requirement: Q-ratio>=0.04 Input mass in ng=20/Q-ratio+30.

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.

Although 24 samples can be prepared at the same time, when sequencing, multiplex a maximum of 12 samples per sequencing run.

To ensure optimally qualified FFPE tissue derived DNA is used to prepare sequencing libraries, it is recommended to only use the AVENIO Tumor DNA Isolation and QC Kit following the instructions in "Chapter 2. Isolate DNA from FFPE tissue". Alternative extraction methods are not recommended, however, if alternative extraction methods are used, ensure the DNA is in the DNA Elution Buffer from the AVENIO Tumor DNA Isolation and QC Kit. Use the AVENIO Tumor DNA Isolation and QC kit to determine the quality of the DNA and optimal input into library preparation. Performance is not guaranteed when using other FFPE tissue DNA isolation 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. Transfer the appropriate amount of extracted DNA into 0.2 mL strip tube, using the recommended amount, as described in "Determining DNA input for sequencing library generation" on page 20.
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, the vial label of the concentrated DNA Polishing Enzyme is 5 µL, sufficient for more than 24 samples. However, the actual filled volume is approximately 20 µL. Always vortex and briefly spin down the tube prior to usage. If preparing less than 24 samples, retain the tube for future usage.

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

Note, the Fragmentation Buffer (10X) is used twice: during the DNA Polishing and also the Fragmentation steps.

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, leaving it on ice or cold block.

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

Note, 10 µL of the Fragmentation Enzyme should be used, not 1.5 µL.

The Fragmentation Master Mix is highly viscous. 1.5 extra reaction overage should be made for 8 samples, and 4 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. Be cautious to not create too many bubbles when handling the mixture.

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

Temperature	Duration
37°C	20 min
4°C	∞

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

Note that the thermocycler is properly set to 20 minutes at 37°C.

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 20 minutes. Make sure 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.
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 1 hour.

Temperature	Duration
20°C	1 hour
4°C	∞

1 hour ligation incubation is the recommended protocol for a quicker 2 day library preparation workflow. However, it is possible to incubate for 16-18 hours at 16 °C , to allow for flexibility in the user's schedule. Set the thermocycler for 16 °C, duration ∞. There is no performance difference between 1 hour and 16-18 hours.

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.

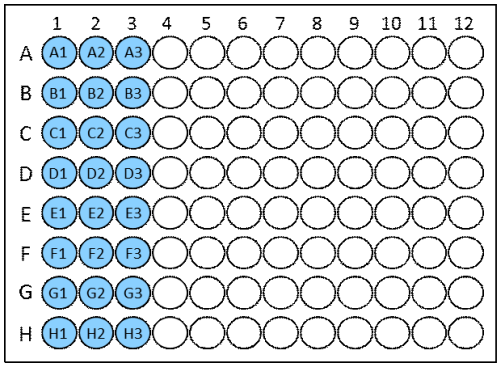
Primer plate usage switching is recommended to avoid cross-contamination so that subsequent sequencing runs do not use the same primers as the previous sequencing run. Recommend alternating between two Sample Primer plates (Plate A and Plate B) for subsequent 24-sample library preparations, if both plates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

2. Pierce the foil cover using a multichannel pipette with clean tips for the appropriate number of wells needed. Take careful attention to not disturb the pelleted lyophilized primers at the bottom of the plate wells.

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 proper primer plate orientation, to ensure that the plate is not upside down. The well labels are visible from behind the primer plate, through the transparent plastic. The corner of the foil can be written on with a marker to ensure proper orientation. Note, the notch may not always be in the same location on the plate.

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

Record the Sample Primers 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 5-10 times.

Pipette carefully to avoid liquid splash-over to adjacent wells, and make sure the water reaches the bottom of the well with no air bubbles. If a compatible centrifuge is available, spin down the plate at 1000 RCF. It is recommended to apply a plate cover when centrifuging to avoid contamination. Take care when removing the plate cover.

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.

Cleaning up post-ligation sample

To clean up post-ligation sample

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade buffer and lead to loss of sample. Ensure proper storage of Cleanup Beads V2 as improper storage conditions may lead to suboptimal assay performance.

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 or vortexing, and quickly spin down the liquid without pelleting the beads.

The total volume will be 220 µL in 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 5 minutes, and 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.
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 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. Visually inspect the drying beads, and an overly dry lab may require less drying time to prevent over-drying.

14. Remove the tubes from the magnetic rack, and add 25 µL of prepared primer mix to each bead-bound sample. Resuspend thoroughly by pipetting up and down, or vortexing and briefly spinning down the sample.

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. It is critical to use the correct Sample Primer to avoid sample swap errors.

15. Incubate for 2 minutes at room temperature.
16. Pellet beads on the magnetic rack for 5 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 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	**Temp (°C)**	**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. Save this 50 µL post-PCR product. Proceed to: (Optional) Accessing post-PCR library quality or Chapter 4 Perform the enrichment protocol.

Safe stopping point: Freeze PCR product (non-cleaned up) at –20°C for up to 2 weeks.

(Optional) Assessing post-PCR library quality

The library preparation and enrichment procedures have been optimized based on the recommended sample input. Library quantification and QC of the pre-enrichment PCR product is not required to proceed forward with the enrichment protocol. Optionally, a 1 µL aliquot of the non-cleaned PCR product can be retained for future QC purposes. The retained QC aliquot can optionally be assessed at the same time as the final QC of the enriched library, or retained for QC in case of troubleshooting purposes.

(Optional) To assess library quality before enrichment:

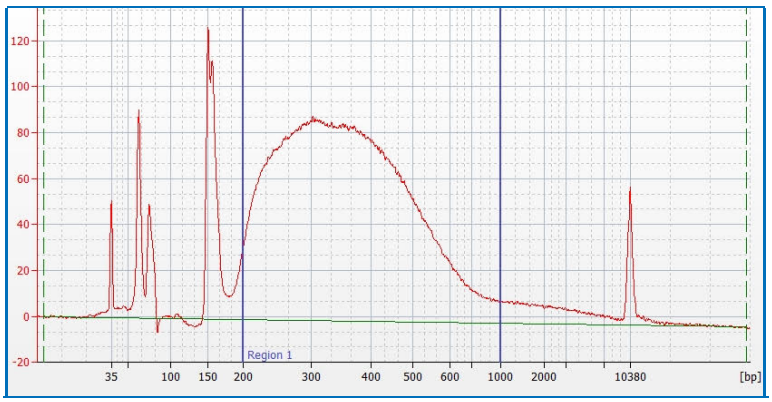
1. Prior to performing the Cleaning up post-PCR product and preparing for hybridization of Chapter 4, create a QC aliquot by pipetting 1 µL of the PCR product and transferring into a new tube containing 9µl of nuclease-free water.
2. Vortex and spin down the diluted QC aliquot.
3. To proceed to QC, continue to step 4. Or to perform QC at a later stage, such as the end of the procedure or for troubleshooting purposes, store the diluted QC aliquot for future QC (4 °C overnight or -20 °C for longer than overnight). To skip this optional QC procedure entirely, proceed directly to Cleaning up post-PCR product and preparing for hybridization of Chapter 4.
4. Quantify the library yield with the Qubit dsDNA HS Assay Kit or Qubit 1x dsDNA HS Assay Kit according to the manufacturer's instructions.

The concentration of the post-PCR product (the diluted 1:10 aliquot) can range between 1 ng/µL and 35 ng/µL, based on the input DNA quality and amount.

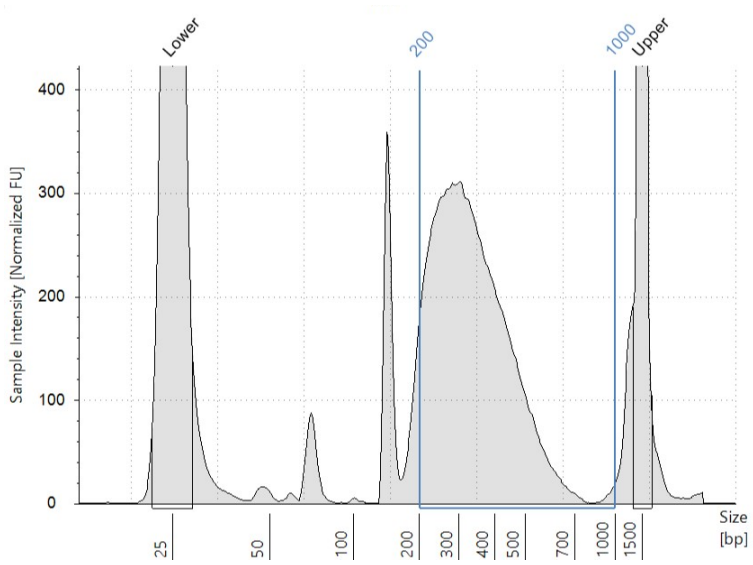
5. Dilute the samples to 1-5ng/µL range, and assess the average library size using Agilent High Sensitivity DNA Kit on the Bioanalyzer instrument following the manufacturer's instructions. Alternatively, assess the average library size using Agilent D1000 ScreenTape Assay on the TapeStation instrument following the manufacturer's instructions, diluting the samples within the range of 1 – 50 ng/µL with nuclease free water if necessary.

The enrichment protocol has been optimized to tolerate moderate levels of adapter dimers. Residual adapter dimers can proceed forward and will be cleaned up during the enrichment procedure. The peak of the library can be between 200 to 500 bp, depending on the quality of the extracted input DNA.

The following example shows the QC of the non-cleaned-up post PCR product. It shows a typical, good adapter-ligated library Bioanalyzer profile with a broad fragment peak near 300 to 500 bp, which indicates successful adapter ligation. Primer dimers may be visible adjacent to the fragment peak, which is still a successful library.



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



Chapter 4. Perform the enrichment

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 the AVENIO Tumor Tissue CGP Panel Kit V2. The purpose of this section is to clean up the post-PCR product and enrich genes of interest from the DNA prepared in "Chapter 3. Prepare sequencing libraries" on page 21. The panel contains probes designed to target regions of interest for detection of cancer mutations present within FFPE DNA.

This section of the protocol uses the post-PCR product created in Chapter 3.

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.

Cleaning up post-PCR product and preparing for hybridization

To clean up post-PCR product and prepare for hybridization:

Note, this section Cleaning up post-PCR product and preparing for hybridization uses an optimized procedure and updated reagent volumes. Take attentive care to follow the procedures in this Instructions for Use.

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade buffer and lead to loss of sample. Ensure proper storage of Cleanup Beads V2 as improper storage conditions may lead to suboptimal assay performance.

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

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. Obtain the 50 µL post-PCR product from Chapter 3 (the volume is 49 µL if 1 µL was optionally retained for the QC aliquot). Ensure that the sample is thawed if it is frozen.
4. Thoroughly mix the Cleanup Beads V2 by vortexing. Add 50 µL of Cleanup Beads V2 to each post-PCR sample.
5. Mix thoroughly by pipetting or vortexing, and quickly spin down the liquid without pelleting the beads.
6. Incubate for 10 minutes at room temperature.
7. Pellet beads on a 0.2 mL magnetic rack for 5 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
8. Discard the supernatant, and retain the beads.
9. To the beads, add 60 µL of Hybridization Supplement.
10. Mix by pipetting or vortexing, and briefly spin down the tubes to settle the liquid to the bottom without pelleting the beads.
11. Add 60 µL of Cleanup Beads V2 to each sample.
12. Mix thoroughly by pipetting. Avoid vortexing.

The total volume will be 120 µL in strip tubes. Take care to ensure no spillover during mixing.

13. Incubate for 10 minutes at room temperature..
14. While incubating, prepare the Hybridization Master Mix:

Hybridization Master Mix	
Reagent	**Volume per reaction**
Hybridization Buffer 1 (2X)	31 µL
Hybridization Buffer 2 V2	10.9 µL
Nuclease-free water	1.1 µL

Hybridization Buffer 1 (2X) contains tetramethylammonium chloride.

15. Pellet beads on a 0.2 mL magnetic rack for 5 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
16. Discard the supernatant.
17. With beads still on the magnetic rack, wash the beads with 200 µL of freshly prepared 80% ethanol.
18. Incubate for at least 30 seconds at room temperature and discard the ethanol.
19. Spin down the tubes quickly to bring residual ethanol to the bottom without pelleting the beads.
20. Place the tubes on the magnetic rack to collect the beads. Remove residual ethanol using a P20 pipette.
21. 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. Visually inspect the drying beads, and an overly dry lab may require less drying time to prevent over-drying.

22. Remove the tubes from the magnetic rack. Add 13.4 µL of Universal Enhancing Oligos to each sample, and resuspend beads by pipetting, or vortexing briefly and spinning down quickly to collect the liquid to the bottom without pelleting the beads.
23. After the beads are resuspended, add 43 µL of the Hybridization Master Mix.
24. Mix thoroughly by vortexing, and quickly spin down the liquid without pelleting the beads.
25. Incubate for 2 minutes at room temperature.
26. Pellet beads on the magnetic rack for 5 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
27. Transfer 56.4 µL of the supernatant to a new strip tube, and add 4 µL of the AVENIO Tumor Tissue CGP Panel V2 to each sample.

Check the label to ensure that the correct panel is used: AVENIO Tumor Tissue CGP Panel V2.

28. Vortex to mix the sample, and briefly spin down the tubes to settle the liquid to the bottom.

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

Hybridization Program	
Temperature	**Duration**
95°C	10 min
61°C	∞

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

Preparing the Hybridization Wash Buffers

Review this section carefully to use the appropriate temperatures.

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. 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 strip tubes for ease of use.

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

Ensure that stock wash buffers are not precipitated or cloudy. If necessary, thoroughly vortex and gently warm cloudy buffers at 37°C until buffers are completely clear.

2. Prepare the wash buffers according to the following table. The table indicates volumes required for 1 reaction.

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	61°C
	20 µL	180 µL	200 µL	Room temperature
Stringent Wash Buffer (10X)	20 µL	180 µL	200 µL	61°C
	20 µL	180 µL	200 µL	61°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 61°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; up to 600 µL beads for 8 reactions).

Prepare up to 8 reactions in each 1.5 mL microcentrifuge tube. 24 reactions will require 3 tubes.

4. Pellet the Capture Beads on a 1.5 mL tube magnetic rack for 2 minutes until the solution is clear and discard the supernatant.
5. To each tube of Capture Beads, add 150 µL of 1X Bead Wash Buffer per capture reaction (150 µL for 1 reaction; 1200 µL for 8 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 1.5 mL tube magnetic rack for 2 minutes until the solution is clear, and discard the supernatant.
8. Repeat step 5 through step 7 for a total of two washes with 150 µL of 1X Bead Wash Buffer per capture reaction (150 µL for 1 reaction; 1200 µL for 8 reactions).
9. Remove the tubes from the magnetic rack, and resuspend the beads with 75 µL of 1X Bead Wash Buffer per capture reaction (75 µL for 1 reaction; 600 µL for 8 reactions).
10. Mix thoroughly by vortexing 2 times for 5 seconds each. Into new strip tubes, carefully transfer 75 µL of the resuspended beads per strip tube.
11. Pellet the beads on a 0.2 mL tube magnetic rack for 1 minute 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

Review this section carefully to use the appropriate temperatures.

To perform the hybridization cleanup

Use prepared 1X Buffers for all wash steps.

1. Ensure that the following buffers are aliquoted into strip tubes at the indicated volumes, and incubate the buffers specified at 61°C in the thermocycler for at least 15 minutes prior to use.

Buffer	Volume	Temperature
Hybridization Wash 1	100 µL	61°C
Stringent Wash Buffer	200 µL	61°C
Stringent Wash Buffer	200 µL	61°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 61°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. Avoid creating bubbles.

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

Strip tube caps may be loose. Avoid vortexing the tubes.

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. Quickly transfer 200 µL of pre-heated Stringent Wash Buffer to the sample, and immediately mix thoroughly by pipetting up and down 10 times. Avoid creating bubbles.
10. Quickly spin down the liquid without pelleting the beads.
11. Incubate the samples at 61°C for 5 minutes on the thermocycler.
12. 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.
13. Repeat step 8 through step 12 for a second Stringent Wash.
14. Remove the 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, avoid creating bubbles.
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 samples from the magnetic rack, and add 200 µL of room temperature Hybridization Wash 2.
19. Mix the pellet thoroughly by pipetting up and down 10 times. Avoid creating bubbles. Quickly spin down the liquid without pelleting the beads.
20. 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.

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

26. Remove the tubes from the magnetic rack, and resuspend the Capture Beads with 20 µL of nuclease-free water, PCR grade.
27. Incubate for 2 minutes at room temperature.


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. Pipette 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 strip tube.
5. Perform PCR on a thermocycler with the following cycling profile:

Thermocycler Profile			
Stage	**Temp (°C)**	**Duration**	**Cycles**
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	15
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.

Cleaning up post-capture PCR product

To clean up post-capture PCR product

Cleanup Beads V2 are light-sensitive and should be protected from light when not in use. Excess light exposure may degrade buffer and lead to loss of sample. Ensure proper storage of Cleanup Beads V2 as improper storage conditions may lead to suboptimal assay performance.

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 0.2 mL magnetic rack for 3 minutes until the supernatant is clear.

In the event that not all the beads are bound to the magnet, while the strip tube is on the magnetic rack, pipet the remaining unbound beads 20 times to help collect the remaining beads. Incubate for an additional 5 minutes until the elution is clear. If there are still residual beads unbound , it is okay to move on to the next step even with residual beads carried over, which will not interfere with the procedures.

4. Carefully transfer the supernatant, which contains the PCR amplified, panel-enriched library to a new strip tube.

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

5. Thoroughly mix the Cleanup Beads V2 by vortexing.
6. Add 50 µL of Cleanup Beads V2 to each sample.
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 5 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. Visually inspect the drying beads, and an overly dry lab may require less drying time to prevent over-drying.

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, or vortexing and quickly spinning to collect the liquid without pelleting the beads.
18. Incubate for 2 minutes at room temperature.
19. Pellet beads on the magnetic rack for 5 minutes, and visually inspect to ensure that all of the beads are collected to the side of the tube.
20. Transfer the eluate into a new strip tube. The eluate contains the final library.

Safe stopping point: 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 to 1-5 ng/µL range, and assess the average library size using Agilent High Sensitivity DNA Kit on the Bioanalyzer instrument following the manufacturer's instructions.
   Alternatively, the Agilent 4200 TapeStation can be used. Dilute the samples to 0.1-50 ng/µL if using the D1000 ScreenTape or 10-1000 pg/µL if using the High Sensitivity D1000 ScreenTape and follow the manufacturer's instructions.

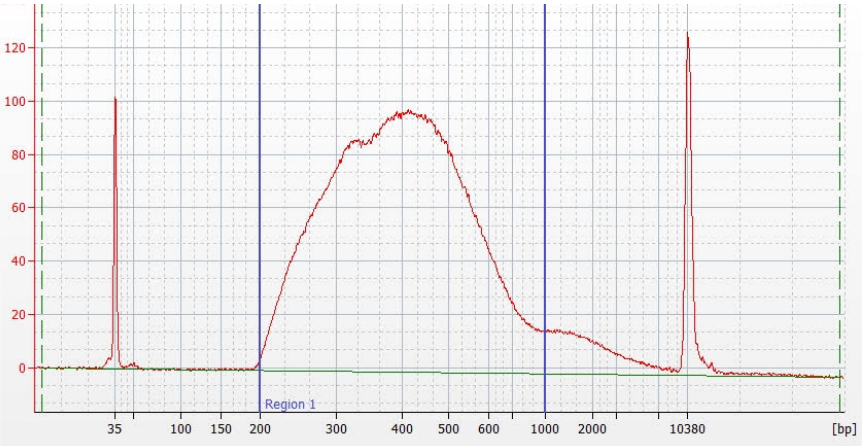
> (Optional) Assessing post-PCR library quality from Chapter 3: If a QC aliquot of the post-PCR library from Chapter 3 was retained, and the user optionally wishes to review it, the user can also assess the QC aliquot along with the quality assessment of the enriched library from Chapter 4. By combining the assessment of the optional post-PCR QC aliquot with the final library QC, the user can save time performing the QC assessments.

3. The size and concentration of the enriched library will be used to calculate the molarity.

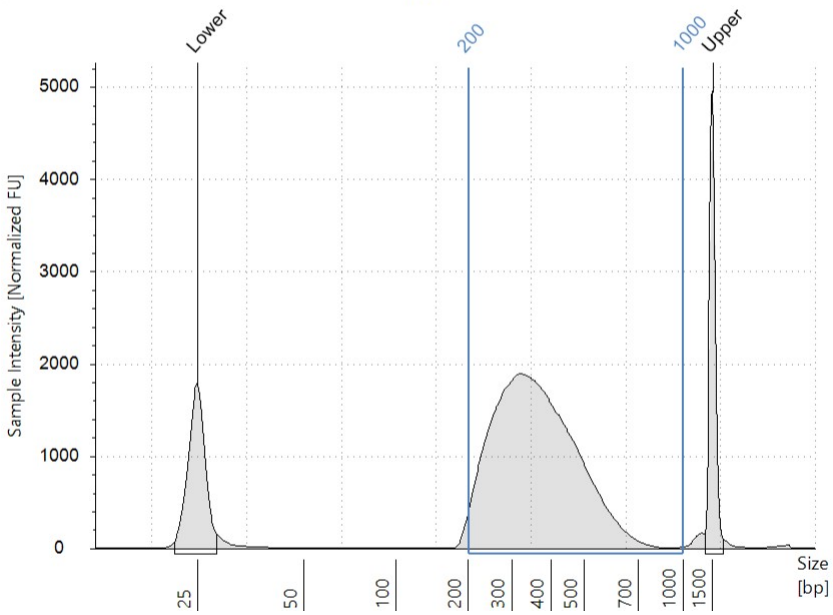
> **The concentration of the enriched library can range approximately between 15 ng/µL and 70 ng/µL based on recommended input. Avoid sequencing samples with <1 ng/µL because they are too dilute to include in the pool and will yield insufficient sequencing coverage for that sample.**

> 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 to 500 bp, depending on the quality of the extracted input DNA.

The following example shows a typical, good final enriched library Bioanalyzer profiles with negligible adapter dimers and fragment peak near 300 to 500 bp.



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



Chapter 5. DNA sequencing

The AVENIO Tumor Tissue CGP Kit V2 procedure produces sequencing-ready libraries from DNA isolated from FFPE tissue. After Quality Control (QC) of the enriched libraries from "Chapter 4. Perform the enrichment protocol" on page 34, 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 "Consumables purchased from other vendors" on page 11
  - NextSeq 500/550 High Output Kit v2.5 (300 cycles)
  - NextSeq 550Dx High Output Kit v2.5 (300 cycles)
- Nuclease-free, PCR grade water
- 1 N NaOH, molecular biology grade
- 200 mM Tris-HCl, pH 7.0, molecular biology grade

Pooling enriched DNA samples for sequencing

It is not recommended to pool libraries generated by the AVENIO Tumor Tissue CGP Panel V2 with libraries generated by other panels and assays.

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.

8 - 12 samples can be pooled and sequenced per NextSeq 500/550/550Dx sequencing run. Do not load more than 12 samples, as it will reduce the sequencing performance for the samples. For a 24 sample library preparation, multiplexing with pools of 12 samples will require two Nextseq High Output flowcells, and pools of 8 samples will require three Nextseq High Output flowcells. A 12 pool multiplex will require greater accuracy of quantitation, pooling consistency, sequencing loading, and sequencer performance. Optimization of sequencer loading procedures may be required. While a multplex of 8 or 12 will yield similar variant detection performance, the 8 pool multiplex may receive greater Total reads and Median coverage, and have greater tolerance for pooling unevenness.

To pool enriched DNA samples for sequencing

1. Use the concentration and the average library size determined in "Assessing enriched library quality" on page 38 to calculate the molarity of each sample in nM using the following equation:

$$\text{Library Molarity in nM} = \left[\frac{library\ concentration\ in\ ng/\mu L}{((average\ library\ size\ in\ bp * 607.4) + 157.9))}\right] * 10^6$$

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 is an equal moles 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 moles 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. Assess the size of the pool by diluting the pool to 1-5 ng/µL range and analyzing it on the Bioanalyzer instrument with an Agilent High Sensitivity DNA Kit following the manufacturer's instructions.
   Alternatively, the Agilent 4200 TapeStation can be used. Dilute the pool to 0.1–50 ng/µL if using the D1000 ScreenTape or 10–1000 pg/µL if using the High Sensitivity D1000 ScreenTape and follow the manufacturer's instructions.
6. Calculate the molarity of the pooled library outlined in step 1.
7. Dilute the pooled library following Illumina's NextSeq guideline.
8. 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. However, it is the user's responsibility to determine 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 a paired-end 150-cycle run.

The steps on the Run Setup screen differs based on the system configuration. The NextSeq 500/550/550Dx instrument must be configured to Manual Mode. If using the NextSeq 550Dx, use the instrument in the Research Mode. The 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

Do not select any custom primers on the Run Set up interface.

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.

Chapter 6. Secondary Analysis Outputs and Content

Introduction

This chapter includes information and instructions for assessing analysis results provided via the analysis output files and metadata. This guide also includes general troubleshooting information and methodology.

About the FoundationOne® Analysis Platform

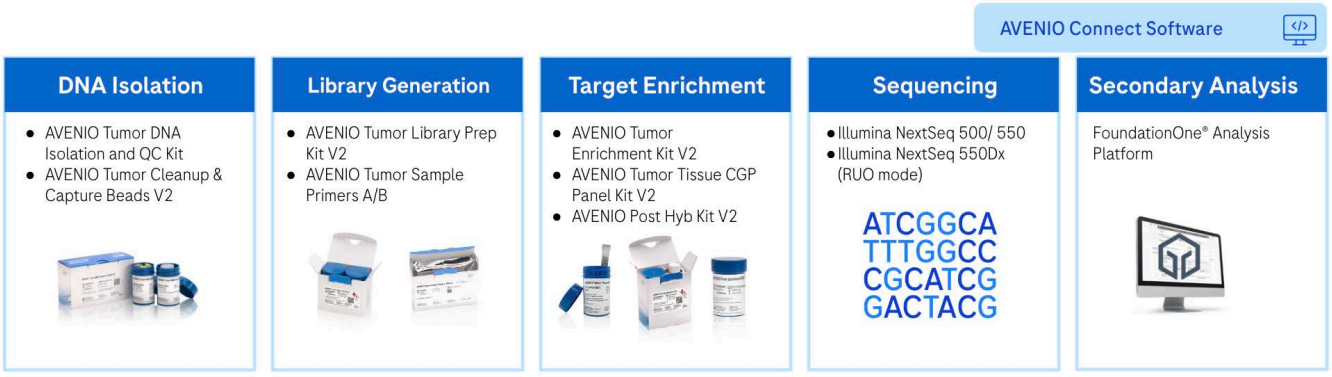
The FoundationOne® Analysis Platform enables the end-to-end workflow from sample extraction to data analysis. The software is for research use only. The FoundationOne® Analysis Platform allows labs to quickly and easily transform data from enriched DNA processed on an Illumina NextSeq into meaningful insights for comprehensive genomic profiling.

The FoundationOne® Analysis Platform is a cloud-based software that processes the sequencing data from the NextSeq instruments, and produces files with filtered variant calls and QC metrics which are made available through the AVENIO Connect Software.

The AVENIO Connect Software is a cloud solution and the user interface for the FoundationOne® Analysis Platform. The AVENIO Connect Software is used to manage analyses, view sample results, and download results output files. The AVENIO Connect Software is configured for on-premise shared network storage to enable upload of NextSeq BCL files and download results output files.

The AVENIO Connect Software and FoundationOne® Analysis Platform are required and available from Roche.

The AVENIO Connect Software User Assistance is available to provide guidance on the use of the software. Please consult your local Roche representative.



Sample Metrics Results Interpretation

QC metrics are aggregated and compared to specified thresholds through an automated process. The QC status of the metrics are included in the QC Metrics and Variant CSV output file.

Field	Description	Passing Criteria
Median Coverage	The median value of the average unique coverage in each exon.	>=500X
Coverage >250X	The percentage of exons with >250X unique coverage following the calculation of the average coverage of each exon.	>=95%
Computational Purity	The percentage of cells in the tumor sample that are derived from tumor based on the copy number model. Note, "Computational Purity" is not the same as "Tumor content %", which is the user-defined input based on a pathological observation of the tissue prior to the execution of this protocol.	>25%
Gender	Biological sex of the specimen as determined by the pipeline. Sequenced gender must match the gender input in sample metadata. If the result is N/A, this is due to the gender not being provided prior to analysis or not being able to be calculated.	Matched with the sample's known gender, self-reported by the user during analysis setup
Contamination	Presence of DNA from multiple distinct human sources, detected by allelic fractions of common germline SNPs that cannot occur if the sample contains only DNA from a single person.	<1%
Nonhuman Contamination	Percentage of DNA from a foreign source to the sample being analyzed, derived from a non-human source, excluding certain viruses. (e.g. HPV).	<0.5%
SNP-filtered sub err	Percentage of bases that are not identical to the reference sequence post mapping exclusive of biological variants.	<=0.1%
Plate gender mismatch	A quality control metric to assess sample processing issues. Occurs when 2 or more samples in the sequencing run shows a gender QC mismatch. This could suggest there was a sample swap during processing.	<2 gender mismatches
Total reads	The total number of reads used in analysis. Count may be inflated slightly as it includes secondary alignments and might be limited by down sampling to approximately 90M reads.	>=20M (10M read pairs) Recommended Total reads per sample is >=60M (30M Read pairs)
Percent selected bases	Defined as the percentage of aligned bases that are either on or near the target region.	>=30%
Mean insert size	The average length of the DNA insert between the ligated adapters. The insert size can be influenced by the quality of the DNA as measured by the Q-ratio. Also, large sizes can be indicative of poor fragmentation.	>100 bp and <300 bp
CpG Transition Error Rate	The error rate of C>T and G>A substitutions in CpG context. This error rate is only shown when the level of CpG transition error is elevated. If the CpG transition error level is low, the value will show as 0.	= 0
Normalized Coverage Noise	Measure of noisy coverage normalization for CNA modeling. This is showing the average distances of normalized target coverage ratio to their segment coverage ratio.	<=0.25
Normalized Coverage Noise Ratio	Measure of noisy coverage normalization for CNA modeling. This is showing the average weighted standard deviation of the normalized target coverage ratio is divided by the weighted standard deviation of the SNP allele frequencies in the same segment.	<=2.0
Sample Quality Set Status	An overall assessment of the sample's QC Metrics, considering all of the above individual metrics, except: Total Reads, Mean insert Size, CpG Transition Error Rate, and Plate gender mismatch.	This metric will show "Fail" when any of the above metrics show a "Fail", except for: Total Reads, Mean insert Size, CpG Transition Error Rate, and Plate gender mismatch.

Variant Calling

Variant calling consists of generating a genomic alteration profile from the processed paired-read data. Genomic alterations and signatures are identified using proprietary Foundation Medicine methods.

Gene List

The following genes are designed to be assessed by the AVENIO Tumor Tissue CGP Kit V2. The reference genome hg19 is used for the alignment and annotation.

Table 1: Genes with full coding exonic regions for the detection of substitutions, insertion deletions (InDels), and copy number alteration (CNAs)

ABL1	BRD4	CIC	ETV6	GNAQ	KLHL6	MYC	PDK1	RB1	SYK
ACVR1B	BRIP1	CREBBP	EZH2	GNAS	KMT2A (MLL)	MYCL (MYCL1)	PIK3C2B	RBM10	TBX3
AKT1	BTG1	CRKL	FANCA	GRM3	KMT2D (MLL2)	MYCN	PIK3C2G	REL	TEK
AKT2	BTG2	CSF1R	FANCC	GSK3B	KRAS	MYD88	PIK3CA	RET	TENT5C (FAM46C)[1]
AKT3	BTK	CSF3R	FANCG	H3-3A (H3F3A)	LTK	NBN	PIK3CB	RICTOR	TET2
ALK	CALR	CTCF	FANCL	HDAC1	LYN	NF1	PIK3R1	RNF43	TGFBR2
ALOX12B	CARD11	CTNNA1	FAS	HGF	MAF	NF2	PIM1	ROS1	TIPARP
AMER1 (FAM123B or WTX)[1]	CASP8	CTNNB1	FBXW7	HNF1A	MAP2K1 (MEK1)	NFE2L2	PLCG2	RPTOR	TNFAIP3
APC	CBFB	CUL3	FGF10	HRAS	MAP2K2 (MEK2)	NFKBIA	PMS2	RUNX1	TNFRSF14
AR	CBL	CUL4A	FGF12	HSD3B1	MAP2K4	NKX2-1	POLD1	SDHA	TP53
ARAF	CCND1	CXCR4	FGF14	ID3	MAP3K1	NOTCH1	POLE	SDHB	TP53BP1
ARFRP1	CCND2	CYP17A1	FGF19	IDH1	MAP3K13	NOTCH2	PPARG	SDHC	TSC1
ARID1A	CCND3	DAXX	FGF23	IDH2	MAPK1	NOTCH3	PPP2R1A	SDHD	TSC2
ASXL1	CCNE1	DDR1	FGF3	IGF1R	MCL1	NOTCH4	PPP2R2A	SETD2	TYRO3
ATM	CD22	DDR2	FGF4	IKBKE	MDM2	NPM1	PRDM1	SF3B1	U2AF1
ATR	CD274 (PD-L1)	DICER1	FGF6	IKZF1	MDM4	NRAS	PRKAR1A	SGK1	VEGFA
ATRX	CD70	DIS3	FGFR1	IKZF2	MED12	NSD2 (WHSC1 or MMSET)	PRKCI	SMAD2	VHL
AURKA	CD79A	DNMT3A	FGFR2	IKZF3	MEF2B	NSD3 (WHSC1L1)	PRKN (PARK2)	SMAD4	WT1
AURKB	CD79B	DOT1L	FGFR3	INPP4B	MEN1	NT5C2	PTCH1	SMARC A4	XPO1
AXIN1	CDC73	EED	FGFR4	IRF2	MERTK	NTRK1	PTEN	SMARC B1	XRCC2
AXL	CDH1	EGFR	FH	IRF4	MET	NTRK2	PTPN11	SMO	ZEB2
B2M	CDK12	EMSY (C11orf30)	FLCN	IRS2	MITF	NTRK3	PTPRO	SNCAIP	ZNF217
BAP1	CDK4	EP300	FLT1	JAK1	MKNK1	P2RY8[1]	QKI	SOCS1[1]	ZNF703

BARD1	CDK6	EPHA3	FLT3	JAK2	MLH1	PALB2	RAC1	SOX2	ZRSR2
BCL2	CDK8	EPHB1	FOXL2[1]	JAK3	MPL	PARP1	RAD21	SOX9	
BCL2L1	CDKN1A	EPHB4	FUBP1	JUN	MRE11 (MRE11A)	PARP2	RAD51	SPEN	
BCL2L2	CDKN1B	ERBB2	GABRA6	KDM5A	MSH2	PARP3	RAD51B	SPOP	
BCL6	CDKN2A	ERBB3	GATA3	KDM5C	MSH3	PAX5	RAD51C	SRC	
BCOR	CDKN2B	ERBB4	GATA4	KDM6A	MSH6	PBRM1	RAD51D	SRSF2	
BCORL1	CDKN2C	ERCC4	GATA6	KDR	MST1R	PDCD1 (PD-1)	RAD52	STAG2	
BRAF	CEBPA	ERG	GID4 (C17orf39)	KEAP1	MTAP	PDCD1LG2 (PD-L2)	RAD54L	STAT3	
BRCA1	CHEK1	ERRFI1	GNA11	KEL	MTOR	PDGFRA	RAF1	STK11	
BRCA2	CHEK2	ESR1	GNA13	KIT	MUTYH	PDGFRB	RARA	SUFU	

[1] AMER1 (FAM123B or WTX), FOXL2, P2RY8, SOCS1, and TENT5C (FAM46C) do not report CNA.

Table 2: Genes with additional select intronic regions for the enhanced detection of gene rearrangements, a promoter region and a ncRNA (non-coding RNA) gene.

ALK introns 18, 19	BRCA1 introns 2, 7, 8, 12, 16, 19, 20	ETV4 introns 5, 6	EZR introns 9-11	KIT intron 16	MYC intron 1	NUTM1 intron 1	RET introns 7-11	SLC34A2 intron 4
BCL2 3'UTR	BRCA2 intron 2	ETV5 introns 6, 7	FGFR1 intron 1, 5, 17	KMT2A (MLL) introns 6-11	NOTCH2 intron 26	PDGFRA introns 7, 9, 11	ROS1 introns 31-35	TERC ncRNA
BCR introns 8, 13, 14	CD74 introns 6-8	ETV6 introns 5, 6	FGFR2 intron 1, 17	MSH2 intron 5	NTRK1 introns 8-10	RAF1 introns 4-8	RSPO2 intron 1	TERT Promoter
BRAF introns 7-10	EGFR introns 7, 15, 24-27	EWSR1 introns 7-13	FGFR3 intron 17	MYB intron 14	NTRK2 intron 12	RARA intron 2	SDC4 intron 2	TMPRSS2 introns 1-3

Genomic Alterations

- **Short Variants (SNVs and indels)**
  SNVs and indels are detected using a custom local assembly of each targeted region. Any sequence variant found entirely within a targeted region will be reported as a short variant, while larger events, including large deletions, will be reported as rearrangements. Any positions or genes not reported are considered to be wildtype.
- **Genomic Rearrangements**
  Rearrangements are detected using the same methodology as used for short variants. Any candidate breakpoint sequences detected within or near a targeted region are mapped to the reference genome to determine a likely partner sequence. Quality filters are applied to ensure that only rearrangements with well-defined partners and with sufficient read support are reported.

The AVENIO Tumor Tissue CGP Kit will use the term rearrangements instead of fusions previously used by the current on-market kits. This type of variants consists not only of gene fusions but also of other relevant structural variants such as larger truncating deletion/inversion/translocation events in tumor suppressor genes.

Description of Genomic Rearrangements

Description	Structural Change
Truncation	Missing the 3' or 5' end of the gene
Fusion	Predicted fusion gene
Deletion	Deletion of several internal exons
Duplication	Duplication of several exons
Rearrangement	Any other large structural variant

- **Copy Number Alterations (CNAs)**

  CNAs are called based on a sample-wide copy number (CN) model, which consists of a purity level, a segmentation, and an assignment of copy number states to each segment.

The term "equivocal" is used when the FoundationOne® Analysis Platform determines that a copy number alteration is very near the threshold for calling, and is expected to have reduced reproducibility on repeat testing. However, the presence of the amplification is true. When this situation occurs, the results will indicate "TRUE" under the "equivocal" column of the .csv results file.

The AVENIO Tumor Tissue CGP Kit V2 uses the term CNA (Copy Number Alteration) instead of CNV (Copy Number Variation).

Genomic Signatures

- **Microsatellite Instability (MSI)**

  Microsatellite Instability (MSI) genomic signature score is calculated as the fraction of unstable loci out of the total number of evaluable loci.

  Categorical Status

  For each sample a qualitative/categorical status is assigned:

MSI Score Range	Categorical Status
≥ 0.0124	MSI-H (MSI-High)
=>0.0041 and <0.0124	MS-Equivocal
< 0.0041	MSS (MS Stable)
Fail	Fail due to insufficient number of loci having adequate coverage

MSI status is determined through internal testing of a research data set to analyze and set the MSI-H/MS-Equivocal/MSS threshold. This data is for research use only. In addition to the MSI status, the user is provided the MSI score for each sample and can set the MSI threshold per their own research requirements.

Samples with Microsatellite status of MS-Equivocal should be retested with an orthogonal (alternative) method.

- **Tumor Mutational Burden (TMB)**

  Tumor Mutational Burden (TMB) is measured by counting all synonymous and non-synonymous somatic variants present at 5% allele frequency or greater. This measurement is conducted after filtering out potential germline variants according to dbSNP, ExAC, and FMI's somatic/germline algorithm. Additionally, known and likely driver mutations are filtered out to exclude bias of the data set. The resulting mutation number is then divided by the assessed coding region and is communicated as mutations per megabase (mut/Mb).

  If Tumor Mutation Burden Status of "Fail" is returned, this is due to sample quality issues including low coverage, low tumor purity or high contamination.

- **Genomic Loss of Heterozygosity (gLOH)**

  Genomic Loss of Heterozygosity (gLOH) score is measured by the percent of gLOH in the tumor genome. For each tumor, the fraction of the genome with gLOH is computed and reported as the total length of non-excluded gLOH regions divided by the total length of non-excluded regions of the genome.

  If genomic Loss of Heterozygosity status of "Fail" is returned, this is due to sample quality issues including high-level contamination, low tumor purity, and samples with unusual copy number profiles where the biomarker cannot be reliably determined.

- **Homologous Recombination Deficiency (HRD):**

  The Homologous Recombination Deficiency signature (HRDsig) employs a DNA scar-based approach to calculate a score based on copy number (CN) features. The method is intended to provide predictions independent of genetic alterations (such as deleterious BRCA short variants) thus enabling detection of both genomic and non-genomic mechanisms of HRD. HRDsig provides a model score which ranges from 0 to 1.

  If HRDsig status is "fail", this is due to sample quality issues including high-level contamination, low tumor purity, and samples with unusual copy number profiles where the biomarker cannot be reliably determined.

CSV results file column descriptions

Column Header	Example	Description
reference-genome	hg19	The reference sequence used by the pipeline – The current reference is Human Genome version 19 (hg19)
quality-control_sample-quality-set_status	Pass/Fail	The overall sample's QC status, considering various individual metrics. This metric will show "Fail" when any of the Quality Set Metrics fail, except for Total Reads, Mean insert Size, CpG Transition Error Rate, and Plate gender mismatch.
quality-control_sample-quality-set_metrics_Computational Purity	69,30%	The percentage of cells in the tumor sample that are derived from tumor based on the copy number model.
quality-control_sample-quality-set_metrics_Computational Purity status	Pass/Fail	Metric QC status, showing Pass when Computational purity is >25%.
quality-control_sample-quality-set_metrics_Contamination	10.20% or NA	Presence of DNA from multiple distinct human sources, detected by allelic fractions of common germline SNPs that cannot occur if the sample contains only DNA from a single person.
quality-control_sample-quality-set_metrics_Contamination status	Pass/Fail	Metric QC status, showing Pass when Contamination is <1%.
quality-control_sample-quality-set_metrics_Coverage >250X	99,93%	The percentage of exons with >250X unique coverage following the calculation of the average coverage of each exon.
quality-control_sample-quality-set_metrics_Coverage >250X status	Pass	Metric QC status, showing Pass when Coverage >250X is >=95%.
quality-control_sample-quality-set_metrics_Gender	M, F, unknown	Biological sex of the specimen as determined by the pipeline. Sequenced gender must match the gender input in sample metadata to have a pass status
quality-control_sample-quality-set_metrics_Gender status	Pass/Fail/NA	Metric QC status, showing Pass when the sequenced matches the input gender, or NA when no gender was input by the user
quality-control_sample-quality-set_metrics_Mean insert size	136,99	The average length of the DNA insert between the ligated adapters.
quality-control_sample-quality-set_metrics_Mean insert size status	Pass/Fail	Metric QC status, showing Pass when Mean insert size is >100 bp and <300 bp.
quality-control_sample-quality-set_metrics_Median coverage	2028	The median value of the average unique coverage in each exon.
quality-control_sample-quality-set_metrics_Median coverage status	Pass/Fail	Metric QC status, showing Pass when Median coverage is >=500X.
quality-control_sample-quality-set_metrics_Nonhuman Contamination	NA	Percentage of DNA from a foreign source to the sample being analyzed, derived from a non-human source, excluding certain viruses. (e.g. HPV).
quality-control_sample-quality-set_metrics_Nonhuman Contamination status	Pass/Fail	Metric QC status, showing Pass when Nonhuman contamination is <0.5%

quality-control_sample-quality-set_metrics_Normalized coverage noise	0,25	Measure for effectiveness of normalization, for CNA calling. Average distances of normalized target coverage ratio to their segment coverage ratio. The higher the value, the poorer the normalization.
quality-control_sample-quality-set_metrics_Normalized coverage noise status	Pass/Fail	Metric QC status, showing Pass when Normalized coverage noise status is <=0.25.
quality-control_sample-quality-set_metrics_Normalized coverage noise ratio	0,892	Measure for effectiveness of normalization, for CNA calling. The average weighted standard deviation of the normalized target coverage ratio is divided by the weighted standard deviation of the SNP allele frequencies in the same segment. The higher the value, the poorer the normalization.
quality-control_sample-quality-set_metrics_Normalized coverage noise ratio status	Pass/Fail	Metric QC status, showing Pass when Normalized coverage noise ratio status is <=2.0
quality-control_sample-quality-set_metrics_Percent selected bases	93,47%	Defined as the percentage of aligned bases that are either on or near the target region.
quality-control_sample-quality-set_metrics_Percent selected bases status	Pass/Fail	Metric QC status, showing Pass when Percent selected bases >=30%
quality-control_sample-quality-set_metrics_Plate gender mismatch	0	A quality control metric to assess sample processing issues. Occurs when 2 or more samples in the sequencing run shows a gender QC mismatch. This could suggest there was a sample swap during processing.
quality-control_sample-quality-set_metrics_Plate gender mismatch status	Pass/Fail	Metric QC status, showing Pass when <2 gender mismatches within the batch of samples.
quality-control_sample-quality-set_metrics_SNP-filtered sub err	0,02%	Percentage of bases that are not identical to the reference sequence post mapping exclusive of biological variants.
quality-control_sample-quality-set_metrics_SNP-filtered sub err status	Pass/Fail	Metric QC status, showing Pass when SNP-filtered sub err <=0.1%
quality-control_sample-quality-set_metrics_Total reads	91051974	The total number of reads used in analysis. Count may be inflated slightly as it includes secondary alignments and might be limited by down sampling to approximately 90M reads.
quality-control_sample-quality-set_metrics_Total reads status	Pass/Fail	Metric QC status, showing Pass when Total reads <20M reads.
quality-control_sample-quality-set_metrics_CpG transition error rate	1,34%	Error rate of CpG transitions which are known to be related to UNG treatment
quality-control_sample-quality-set_metrics_CpG transition error rate status	Pass/Fail	Pass/Fail status of CpG transition error rate
Flags	"various flags"	Further description when any of the sample QC metrics fails
short-variants_allele-fraction	0,4883	The relative frequency of the variant allele. FMI calculates allele fraction based on re-assembled, filtered reads, which may be different from what is displayed in IGV. Fraction of reads where the alteration is present over the total number of reads at that position

short-variants_alternate-sequence	“C”	The non-reference bases that have been changed or inserted. For simple insertions and deletions, we include a leading reference/anchor base style).
short-variants_chromosome	“chr8”	The chromosome involved in this short variant (displays which chromosome is affected by the alteration).
short-variants_depth	938	The local sequencing depth at the genomic position of this short variant.
short-variants_functional-effect	frameshift	The dominant effect type of the primary annotation. Describes the functional consequence of the observed mutation, types include frameshift, missense, nonframeshift, nonsense, promoter, splice, unknown
short-variants_genomic-end	41565529	1-based coordinate of the last base of the reference sequence.
short-variants_genomic-start	41565529	1-based Coordinate of the first base of the reference sequence.
short-variants_position	chr22:41565529	The genomic position of the alteration (Chromosome name and coordinate or coordinate interval, that is the first base position of the variation on the given sequence).
short-variants_reference-sequence	G	Shows the original base or bases on the reference sequence, that have been changed by this short variant. For simple insertions and deletions, we include a leading reference/anchor (VCF base style).
short-variants_status	known	The inferred relevance of this variant in cancer, based on literature or prevalence in tumor specimens. See below.
short-variants_strand	"+"	The strand/orientation (positive-sense (+) or negative-sense (-)) of the gene associated with this variant.
short-variants_subclonal	WAHR	Indicates when the variant allele fraction =< 10% of Computational Purity, indicative of emerging subclones.
short-variants_variant-type	"single-nucleotide-substitution"	String describing the type of genomic alteration.
short-variants_short-variant-intergenic-annotation_gene_symbol	“NSD3”	The NCBI authoritative symbol for the targeted gene, if it exists, otherwise the NCBI internal symbol
short-variants_short-variant-intergenic-annotation_gene_ncbi-id	GeneId:54904	The NCBI id associated with the gene
short-variants_short-variant-intergenic-annotation_gene_hgnc-id	HGNC:12767	The HGNC id associated with the gene if it exists
short-variants_short-variant-intergenic-annotation_hgvs-transcript-effect	c.757A>G	The transcript-level effect (nucleotide change notation), based on HGVS recommendations, of this short variant in the transcript used for annotation. Same as the value of the hgvs-protein-effect-abbreviated attribute without the abbreviation.
short-variants_short-variant-intergenic-annotation_hgvs-transcript-effect-abbreviated	c.757A>G	Contextualized full description of the variant at the transcript level (nucleotide sequence) based on HGVS recommendations. This is a concatenation of a transcript-refseq-id and a hgvs-transcript-effect string and may also include the refseq-id for the corresponding genomic reference sequence if, for example, intronic reference nucleotides are affected. hgvs-transcript-effect, except for some long insertions/deletion

short-variants_short-variant-intergenic-annotation_primary	WAHR	Indicates if this annotation is considered primary. The annotation on the primary transcript is used to populate the summary fields in the parent element (where still available). Evaluates to true or false. To remove duplicated calls that are only different in annotation, the TRUE primary call can be used as the main call.
short-variants_short-variant-intergenic-annotation_coding	WAHR	Indicates if this short variant overlaps with an exon between the start and stop codons. Evaluates to true or false
short-variants_short-variant-intergenic-annotation_transcript-functional-tags	["exonic", "missense"]	Combination of functional effect + affected-component-type (intonic, exonic)
short-variants_short-variant-intergenic-annotation_transcript_accession	"NM_023034"	The unversioned NCBI accession/identifier of the reference transcript. A transcript can be canonical or non-canonical. 'Canonical' typically refers to the sequence of DNA, RNA or amino acids that reflects the most common choice of base or amino acid at each position. 'Non-canonical' refers to the transcript produced from alternative splicing and is less commonly utilized. Both canonical and non-canonical transcripts are routinely used for sequencing and variant calling. Most often, the canonical transcript is utilized. However, the non-canonical transcript may be used when an identified alteration in a tumor suppressor gene has a more deleterious effect in the non-canonical transcript than the canonical.
short-variants_short-variant-intergenic-annotation_transcript_coding	WAHR	Evaluates to true or false: Indicates if this transcript translates into a protein
short-variants_short-variant-intergenic-annotation_transcript_transcript-refseq-id	"NM_023034.1"	The versioned NCBI accession/identifier of the reference transcript
short-variants_short-variant-intergenic-annotation_transcript_protein-refseq-id	"NP_075447.1"	The versioned NCBI accession/identifier of the translated protein
short-variants_short-variant-intragenic-annotation_gene_symbol	“NSD3”	The NCBI authoritative symbol for the targeted gene, if it exists, otherwise the NCBI internal symbol
short-variants_short-variant-intragenic-annotation_gene _ncbi-id	GeneId:54904	The NCBI id associated with the gene
short-variants_short-variant-intragenic-annotation_gene _hgnc-id	HGNC:12767	The HGNC id associated with the gene if it exists
short-variants_short-variant-intragenic-annotation_hgvs-transcript-effect	"c.757A>G"	The transcript-level effect (nucleotide change notation), based on HGVS recommendations, of this short variant in the transcript used for annotation. Same as the value of the hgvs-protein-effect-abbreviated attribute without the abbreviation.

short-variants_short-variant-intragenic-annotation_hgvs-transcript-effect-abbreviated	"c.757A>G"	Contextualized full description of the variant at the transcript level (nucleotide sequence) based on HGVS recommendations. This is a concatenation of a transcript-refseq-id and a hgvs-transcript-effect string and may also include the refseq-id for the corresponding genomic reference sequence if, for example, intronic reference nucleotides are affected. hgvs-transcript-effect, except for some long insertions/deletions
short-variants_short-variant-intragenic-annotation_hgvs-protein-effect	"p.I253V"	The protein-level effect (amino acid change notation), based on HGVS recommendations, of this short variant in protein used for annotation. Same as the value of the hgvs-protein-effect-abbreviated attribute without abbreviation. The value is not specified for variants affecting non-coding transcripts.
short-variants_short-variant-intragenic-annotation_hgvs-protein-effect-abbreviated	"p.I253V"	Contextualized full description of the variant at the protein level based on HGVS recommendations. This is a concatenation of a protein-refseq-id and a hgvs-protein-effect string. The value is not specified for variants affecting non-coding transcripts. Same as hgvs-protein-effect, except for some long insertions/deletions
short-variants_short-variant-intragenic-annotation_primary	true	Indicates if this annotation is considered primary. The annotation on the primary transcript is used to populate the summary fields in the parent element (where still available). Evaluates to true or false. To remove duplicated calls that are only different in annotation, the TRUE primary call can be used as the main call.
short-variants_short-variant-intragenic-annotation_coding	true	Indicates if this short variant overlaps with an exon between the start and stop codons. Evaluates to true or false
short-variants_short-variant-intragenic-annotation_transcript-functional-tags	["exonic", "missense"]	Combination of functional effect + affected-component-type (intonic, exonic)
short-variants_short-variant-intragenic-annotation_transcript_accession	"NM_023034"	The unversioned NCBI accession/identifier of the reference transcript. A transcript can be canonical or non-canonical. 'Canonical' typically refers to the sequence of DNA, RNA or amino acids that reflects the most common choice of base or amino acid at each position. 'Non-canonical' refers to the transcript produced from alternative splicing and is less commonly utilized. Both canonical and non-canonical transcripts are routinely used for sequencing and variant calling. Most often, the canonical transcript is utilized. However, the non-canonical transcript may be used when an identified alteration in a tumor suppressor gene has a more deleterious effect in the non-canonical transcript than the canonical.
short-variants_short-variant-intragenic-annotation_transcript_coding	WAHR	Evaluates to true or false: Indicates if this transcript translates into a protein

short-variants_short-variant-intragenic-annotation_transcript_transcript-refseq-id	"NM_023034.1"	The versioned NCBI accession/identifier of the reference transcript
short-variants_short-variant-intragenic-annotation_transcript_protein-refseq-id	"NP_075447.1"	The versioned NCBI accession/identifier of the translated protein
copy-number-alterations_copy-number	46	The model-based estimated absolute copy number level for the copy number alteration (CNA) in the tumor fraction of the specimen.
copy-number-alterations_equivocal	FALSCH	A boolean flag indicating that the data supporting this variant is borderline, meaning there is some, but not unambiguous, evidence that the copy number of a gene exceeds the threshold for identifying copy number amplification.
copy-number-alterations_gene_symbol	DDR2	The NCBI authoritative symbol for the targeted gene, if it exists, otherwise the NCBI internal symbol
copy-number-alterations_gene_ncbi-id	GeneId:4921	The NCBI id associated with the gene
copy-number-alterations_gene_hgnc-id	HGNC:2731	The HGNC id associated with the gene if it exists
copy-number-alterations_number-of-exons	5 of 5	The number of exons (hence, number of targets) of the gene that are contained in the copy number alteration.
copy-number-alterations_position	chr2:16081000-16087219	The genomic position (chromosome name and coordinate interval) of this copy number alteration variant.
copy-number-alterations_ratio	5,53	The log (base 2) of the normalized tumor/reference coverage ratio (ratio between the measured coverage for a given target in the test sample vs the corresponding coverage in a control sample) for the copy number alteration.
copy-number-alterations_status	known	The inferred relevance of this variant in cancer, based on literature or prevalence in tumor specimens (one of: known, likely, unknown, ambiguous)
copy-number-alterations_type	amplification	The type of copy number alteration (amplification or deletion)
copy-number-alterations_non-focal	true or false	True identifies large portions (greater than 20MB and/or 50% of one chromosomal arm) of the genome which have been contiguously altered. False indicates that the segment is smaller than 20MB and 50% of its chromosomal arm.
copy-number-alterations_cn-ploidy-ratio	3	The ratio of the two modeled values, absolute copy-number and ploidy. In the context of this product, most tumors are assumed to be diploid. However, since polyploidy is a common characteristic of hepatocytes, twice as many tumor cells may be needed to obtain enough tumor DNA for analysis – higher tumor content may be required because hepatocyte nuclei have twice the DNA content of other somatic nuclei.

rearrangements_description	ROS1(NM_002944)-SLC34A2(NM_006424) fusion (R31; S5)	This column provide a description of the rearrangement. The numbers indicate the intron/exon or intergenic region where the breakpoint(s) for the rearrangement is occurring.
rearrangements_in-frame	Yes	Refers to if the reading frame of codons is maintained from the 5'-gene to the 3'-gene of a fusion.
rearrangements_other-gene	ROS1	The non-targeted or secondary targeted gene, if any, involved in this rearrangement. Could be set to "N/A" if the partner breakpoint is intergenic. In the example case ROS1 is listed as the target gene.
rearrangements_pos1	chr4:25666629	The genomic annotation position (chromosome name and 1-based coordinate interval) corresponding to the target-gene breakpoint.
rearrangements_pos2	chr6:117658326	The genomic annotation position (chromosome name and 1-based coordinate interval) corresponding to the other-gene breakpoint.
rearrangements_status	known	The inferred relevance of this variant in cancer, based on literature or prevalence in tumor specimens (one of: known, likely, unknown, ambiguous)
rearrangements_supporting-read-pairs	46	The number of semi-mapped read pairs supporting this rearrangement.
rearrangements_targeted-gene	SLC34A2	The targeted gene (or primary targeted gene) involved in this rearrangement. In the example case SLC34A2 is listed as the target gene.
rearrangements_type	fusion, deletion, duplication, truncation, or rearrangement	An internal FMI definition of various rearrangements that take into account genomic type, targeted gene, and effect on the selected targeted gene. Fusion: In-strand and in-frame event involving two genes; Deletion: Intragenic event where exons are lost/deleted; Duplication: Intragenic event where exons are duplicated; Truncation: Out-of-strand non-duplication events that disrupt either the 3-prime or 5-prime end of the targeted gene or a deletion with one breakpoint outside of the gene; Rearrangement: Any event not falling into one of the above categories
rearrangements_allele-fraction	0,0333	The relative frequency of the variant.
rearrangements_comment	5'-ROS1(ex1-31 NM_002944) -SLC34A2 (ex5-13 NM_ 006424) Breakpoints ROS1 intron 31, SLC34A2 intron 4; Reciprocal: unknown	A more in-depth description of the rearrangement, including additional information, such as the direction of the mapping reads (5′ or 3′ direction), the NCBI transcript ID, the introns or exons that are involved, or if it is in an untranslated region (UTR) and if it is a reciprocal event or not. Human readable, not intended for parsing out information.
rearrangements_chimeric-junctions_genomic-type	Deletion, duplication, inversion or translocation	Deletion: Deletion/loss of sequence on a single chromosome Duplication: Duplication of sequence on a single chromosome Inversion: Inversion of sequence on a single chromosome Translocation: Rearrangement between multiple chromosomes.
rearrangements_chimeric-junctions_intragenic	True or False	Set to true if the entire rearrangement occurs within a single gene. From first base of 5'_utr to last base of 3'_utr of primary transcript.

rearrangements_chimeric-junctions_chimeric-junction_in-strand	Yes/No or unknown	At the chimeric-junction, the 3'_end of one partner connects to the 5'_end of the other partner.
biomarkers_microsatellite-instability_score	0,1131	The score on which the microsatellite-instability status is based. Calculated as the fraction of unstable loci out of the total number of evaluable loci
biomarkers_microsatellite-instability_status	MSI-H/MS-Equivocal/MSS	The microsatellite-instability classification for the specimen. Samples with Microsatellite status of MS-Equivocal should be retested with an orthogonal (alternative) method.
biomarkers_tumor-mutation-burden_score	2,41	The score on which the tumor mutation burden status is based. Calculated as mutation number divided by the assessed coding region.
biomarkers_tumor-mutation-burden_status	Pass/Fail	pass/fail (failure can be due to high contamination, low tumor purity, or the presence of certain unusual copy number profiles.)
biomarkers_genomic-loss-of-heterozygosity_score	0,0839	The score on which genomic Loss of Heterozygosity (gLOH) status is based. The gLOH score is summarized as the fraction of the tumor genome displaying LOH (scored from 0 – 1).
biomarkers_genomic-loss-of-heterozygosity_status	Pass/Fail	Quality control status of LOH. Failure can be due to high contamination, low tumor purity, or the presence of certain unusual copy number profiles.)
biomarkers_homologous-recombination-deficiency_score	0,064	A scar-based measure of HRD using a machine learning-based algorithm incorporating copy number and indel features.
biomarkers_homologous-recombination-deficiency_status	Pass/Fail	Quality Control status of HRDsig. Failure can be due to high contamination, low tumor purity, or the presence of certain unusual copy number profiles.)
non-human-content_organism	NA	The name of the non-human organism detected.
non-human-content_reads-per-million	NA	The normalized abundance of sequence read data supporting the non-human content call.
non-human-content_status	NA	The inferred relevance of the non-human content observation based on read-per-million level

Appendix A. AVENIO Tumor Tissue CGP Kit V2 Contents

The AVENIO Tumor Tissue CGP Kit V2 includes the following products:

This product contains one of two primer plate options, Plate A or Plate B.

AVENIO Tumor Tissue CGP Kit V2 A

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

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue CGP 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 Tissue CGP Panel Kit 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 CGP Kit V2 B

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

Orderable kit name	Box #	Sub-kit name	Shipping	Storage
AVENIO Tumor Tissue CGP 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 Tissue CGP Panel Kit 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 CGP Kit V2 – Sub-Kits

The AVENIO Tumor Tissue CGP Kit V2 contains 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 (66bp)	200 µL	1
QC PCR Primer Mix (191bp)	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 supports up to 24 samples.

Component	Volume	Quantity in Kit
Capture Beads	2.7 mL	1
Cleanup Beads V2	12 mL	2

AVENIO Tumor Library Prep Kit V2

The AVENIO Tumor Library Prep Kit 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	90 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	Dried-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)	810 µL	1
Hybridization Buffer 2 V2	325 µL	1
Universal Enhancing Oligos	355 µL	1

AVENIO Tumor Tissue CGP Panel Kit V2

The AVENIO Tumor Tissue CGP Panel Kit V2 supports up to 24 samples.

Component	Volume	Quantity in Kit
AVENIO Tumor Tissue CGP Panel V2	115 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

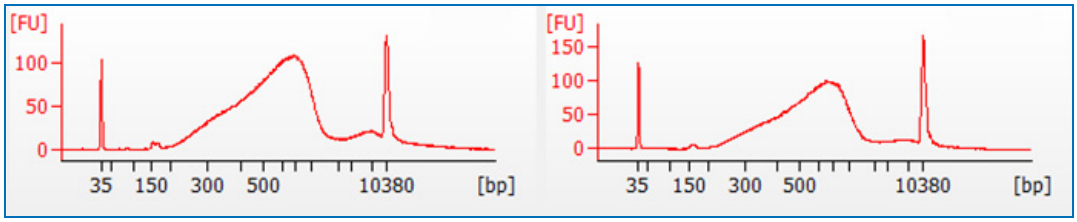
Appendix B. Troubleshooting

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

Pre-sequencing QC troubleshooting

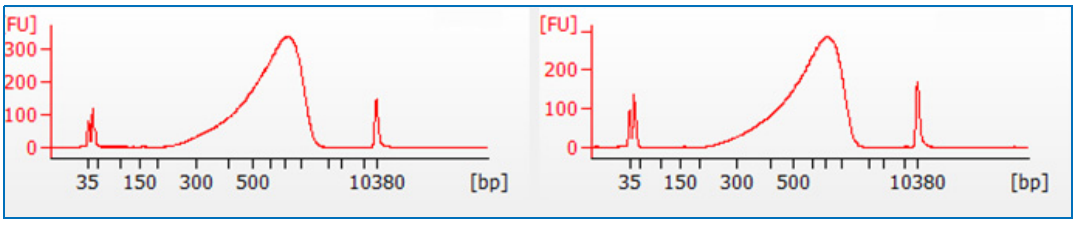
Issue	Cause	Possible resolution
No or low yield from DNA isolation	Poor tissue quality	▪ Ensure that a sufficient sized sample with adequate tumor content is used.
	▪ Forgot to add Extraction Enzyme to Extraction Master Mix ▪ Incorrect incubation conditions (temperature, incubation times, etc.)	▪ Ensure proper volumes and incubation times are used during DNA isolation.
Extremely 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	▪ Moderate amount of adapter dimers are suitable for the assay. ▪ Ensure proper volumes are used in the reaction. ▪ Clean the sample again, maintaining the sample to beads 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 peak skewed above 500 bp.



Issue	Cause	Possible resolution
Low yield of the pre-enriched, post-PCR retained QC aliquot, (less than 1 ng/ul)	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	▪ Extremely high adapter dimers (visible near 150 bp) can inhibit the PCR reaction. Follow proper cleanup steps before PCR. Moderate levels of adapter dimers are suitable for the assay. ▪ Ensure that the Sample Primers are fully resuspended by carefully following the procedure "Preparing PCR primers" on page 25. ▪ 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 33.
	Sample loss	▪ Ensure that the DNA is 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 61.

The following examples show underfragmented final enriched library Bioanalyzer profiles with peak skewed above 500 bp.



Post-sequencing QC troubleshooting

Issue	Cause	Possible resolution
Poor Percent Selected Bases in the CSV output	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 34. ▪ 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.
	Improper volume of Hybridization Supplement	▪ Ensure 60 µL of Hybridization Supplement is used.
	Improper volume of panel and supporting reagents used	▪ Ensure that 4 µL of panel is used and the correct volume of the supporting reagents.
Low "Total Reads" in the CSV output	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 Coverage" in the CSV output	Poor ligation efficiency	▪ Ensure overnight ligation at the proper temperature. ▪ Ensure that the Q-ratio is properly calculated and enough DNA is input into the assay, according to the input mass formula.
	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 61.

Issue	Cause	Possible resolution
Long “Fragment Length” on the Sample Metrics Report	Under-fragmentation	▪ See possible resolutions to the "Under-fragmentation" cause on page 61.
High “SNP-filtered sub err” (substitution error) in the CSV output	Poor DNA polishing efficiency	▪ Ensure that the DNA Polishing Enzyme is diluted fresh before each use and DNA polishing reaction is set up properly.
Failed "Gender Status" or "Plate Gender Mismatch" in the CSV output	Potentially an unexpected sample mix-up due to an expected gender of the sample. When Plate Gender Mismatch is failed, at least 2 samples in the Run had the wrong gender, potentially indicating a sample swap.	▪ Ensure proper tissue and sample handling during processing.
High "Contamination" in the CSV output	Possible sample contamination from neighboring samples during FFPE tissue handling or spillover during the library preparation workflow.	▪ Ensure proper tissue and sample handling during processing.
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.
Failed CpG Transition Error Rate	Presence of high levels of CpG transition errors, due to poor quality tissues or DNA.	▪ Ensure the input tissue/DNA sample is of sufficient quality.