



CleanPlex® Comprehensive Hereditary Cancer Panel

User Guide

This user guide is for the following product for sequencing on Illumina® and Ion Torrent™ platforms:

- CleanPlex® Comprehensive Hereditary Cancer Panel

For other Ready to use Panels not listed, see product document page for appropriate user guides

Get the latest user guide at: www.paragongenomics.com/product_documents/

UG1005-04

January 2024

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

This document and its contents are proprietary to Paragon Genomics, Inc., and are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Paragon Genomics. Paragon Genomics does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The contents of this document are provided "AS IS." This document may be updated or changed without notice at any time. This information could contain technical inaccuracies, typographical errors and out-of-date information. Use of the information is therefore at your own risk. In no event shall PARAGON GENOMICS, INC. be liable for any special, indirect, incidental or consequential damages resulting from or related to the use of this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s). FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY, AND WILL VOID ANY WARRANTY APPLICABLE TO THE PRODUCT(S).

PARAGON GENOMICS DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN.

CleanPlex® NGS Panels are intended for **Research Use Only**. It may not be used for any other purposes including, but not limited to, use in diagnostics, therapeutics, or in humans. CleanPlex NGS Panels may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products without prior written approval of Paragon Genomics, Inc.

All trademarks are the property of Paragon Genomics, Inc. or their respective owners.

©2024 Paragon Genomics, Inc. All Rights Reserved.

Revision History

Document	Date	Description of Change
UG1005-04	January 2024	<ul style="list-style-type: none">• Initial version• Adapted from MGI version.

Table of Content

Overview	4
Product Information	4
Applications	5
Compatible Sequencing Instruments	5
Kit Contents	6
Required Materials and Equipment Not Included	6
Storage, Handling, and Usage	9
Workflow	10
Protocol	11
Best Practices	11
Input DNA Requirements	13
1A. Multiplex PCR (mPCR) Reaction	14
1B. Post-mPCR Purification	16
2A. Digestion Reaction	19
2B. Post-Digestion Purification	21
3A. Second PCR Reaction	23
3B. Post-Second PCR Purification	26
Quality Control Prior to Sequencing	28
Recommended Sequencing Length and Depth	28
Supporting Information	30
Dual-Indexed PCR Primers for Illumina	30
Single-Indexed PCR Primers for Ion Torrent	33
Troubleshooting Guide	34
Data Analysis Recommendations for Illumina	36
Data Analysis Recommendations for Ion Torrent	38
Technical Support	40

Overview

Product Information

CleanPlex® NGS Panels are a fast, robust, and versatile solution for target enrichment and library preparation for next-generation sequencing (NGS) on Illumina® and Ion Torrent™ sequencing platforms. The CleanPlex Comprehensive Hereditary Cancer Panel is a targeted resequencing assay designed for analyzing genes associated with an increased risk of developing hereditary cancers. The panel is expertly curated using the latest research findings to target entire genes and both single nucleotide variants (SNVs) and insertion-deletion mutations (indels). This panel is specifically designed to detect inherited mutations and is not appropriate for the detection of other types of mutations in acquired cancers. Starting with 20 ng of high-quality genomic DNA (10 ng per primer pool), sequencing-ready libraries can be prepared using a streamlined workflow in just 3 hours.

The CleanPlex Comprehensive Hereditary Cancer Panel provides enhanced performance and further streamlined workflow. The panel is capable of detecting Boland inversion, and the number of multiplex PCR primer pools has been reduced from 4 pools to just 2 pools.

The CleanPlex Comprehensive Hereditary Cancer Panel is powered by Paragon Genomics' proprietary CleanPlex Technology, which combines an advanced primer design algorithm and an innovative background cleaning chemistry to generate best-in-class target enrichment performance and efficient use of sequencing reads. The patented CleanPlex background cleaning chemistry effectively removes non-specific PCR products to enable ultra-high multiplexing of amplicons. More than 20,000 amplicons can be multiplexed per primer pool to build large NGS panels that can interrogate megabase-size genomic regions to unlock new applications using a streamlined workflow.

The CleanPlex Comprehensive Hereditary Cancer Panel features a simple and streamlined workflow that can be completed in less than 3 hours from input DNA to indexed and amplified NGS libraries. The workflow involves just 3 steps, each consisting of a thermal-cycling or incubation reaction followed by a library purification using magnetic beads. Tube-to-tube transfer is minimized throughout the protocol to preserve DNA material and prevent handling errors and sample mix-up.

The first step of the CleanPlex workflow is a multiplex PCR reaction that uses target-specific primers to amplify targets of interest. The second step is a digestion reaction that performs background cleaning by removing non-specific PCR products. The last step is a PCR reaction that uses CleanPlex Indexed PCR Primers to amplify and add sample-level indexes to the NGS libraries. CleanMag® Magnetic Beads are recommended for library purification. See the Workflow section for a detailed depiction of the CleanPlex workflow.

CleanPlex Comprehensive Hereditary Cancer Panel Target Enrichment and Library Preparation Workflow

3 hours of total assay time, 75 minutes of hands-on time



Applications

The CleanPlex Comprehensive Hereditary Cancer Panel can be used to detect germline mutations for analyzing genes associated with an increased risk of developing hereditary cancers.

The table below shows some of the sample types and applications of the CleanPlex Comprehensive Hereditary Cancer Panel. This is not meant to be an exhaustive or restrictive list.

Organism	Sample Types	Applications
<ul style="list-style-type: none"> Human 	<ul style="list-style-type: none"> Degraded DNA from FFPE tissues. Note: highly degrade FFPE DNA will likely produce low-quality libraries. Genomic DNA from whole blood, tissues, biofluids, buccal swab, and dried blood spots (DBSs). 	<ul style="list-style-type: none"> Disease predisposition

Compatible Sequencing Instruments

CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex Hereditary Panel v2 libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, and MiSeq® System. Choose a sequencing instrument that will meet the sequencing and sample multiplexing requirements of the experiment.

CleanPlex Indexed PCR Primers for Ion Torrent are used to generate CleanPlex target-enriched NGS libraries that are compatible all Ion Torrent sequencing platforms, including Ion PGM System, Ion Proton System, and Ion GeneStudio S5 Series.

Kit Contents

The protocol outlined in the CleanPlex Comprehensive Hereditary Cancer Panel User Guide requires the following components, which need to be ordered separately:

- CleanPlex Comprehensive Hereditary Cancer Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)

Panel Specifications					
Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Amplicon Length	Average Library Length
CleanPlex Comprehensive Hereditary Cancer Panel	2	5X	2454	105 to 275 bp (223 bp on average)	330 bp

CleanPlex Comprehensive Hereditary Cancer Panel — Kit Contents, Store at –20°C					
Panel	SKU	Size (Reactions)	Components		
			Primer Pool 1	Primer Pool 2	CleanPlex Targeted Library Kit
CleanPlex Comprehensive Hereditary Cancer Panel	930015	96	192 µl	192 µl	2-pool, 96rxns
	930013	384	768 µl	768 µl	2-pool, 384rxns
	930011	1152	2304 µl	2304 µl	2-pool, 1152rxns

CleanPlex Targeted Library Kit — Kit Contents, Store at –20°C (not sold separately)				
		Configuration 2-Pool		
	Size	96 Rxns	384 Rxns	1152 Rxns
Component	Cap Color	SKU 930015	930013	930011
5X Ultra mPCR Mix	Green	384 µl	1536 µl	4604 µl
CP Reagent Buffer	White	192 µl	768 µl	2304 µl
CP Digestion Reagent	Yellow	192 µl	768 µl	2304 µl

Stop Buffer	Red	768 µl	3072 µl	9216 µl
5X 2nd PCR Mix	Blue	768 µl	3072 µl	9216 µl
TE Buffer	Clear	5 mL	20 mL	50 mL

Required Materials and Equipment Not Included

- CleanPlex Indexed PCR Primers (visit www.paragongenomics.com/store for more indexing options)
- CleanMag Magnetic Beads, or equivalent — eg. Agencourt™ AMPure™ XP Kit (Beckman Coulter, A63880, A63881, or A63882)

CleanMag Magnetic Beads — Store at 2–8°C			
	SKU	Size (Volume)	2 Pool Panel Reactions
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~8
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~44
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~520
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,950

- For PCR tubes or strips, CleanMag Magnetic Rack, for 0.2 ml 8/12-tube PCR Strip (SKU 719001) or equivalent magnetic racks designed for PCR strip workflows
- For 96-well PCR plates, CleanMag Magnetic Plate, for 96-well PCR plates (SKU 719002) or equivalent magnetic plates designed for PCR plate workflows

CleanMag Magnetic Rack & Plate		
	SKU	Compatibility
CleanMag Magnetic Rack	719001	2 rows of 12 tubes each for PCR strip tubes
CleanMag Magnetic Plate	719002	96 well PCR plates, full or semi- skirted compatible

- 70% ethanol (freshly prepared)
- Nuclease-free water
- Nuclease-free, low bind, thin-wall PCR strip tubes with attached caps, or 96-well PCR plate with adhesive film

- Pipettors and low-retention filtered pipette tips
- Thermal cycler
- Mini-centrifuge, 96-well plate centrifuge
- Qubit® Fluorometer and dsDNA HS (high sensitivity) Assay Kit, or equivalent
- Agilent® 2100 BioAnalyzer® Instrument and Agilent High Sensitivity DNA Kit, or equivalent

Storage, Handling, and Usage

CleanPlex NGS Panels and CleanPlex Indexed PCR Primers are shipped on blue ice (ice packs). Upon receipt, immediately store CleanPlex NGS Panels and CleanPlex Indexed PCR Primers at -20°C in a constant-temperature freezer. Do not store in a freezer with auto-defrost or frost-free features. Do not store at -80°C.

CleanMag Magnetic Beads is shipped at room temperature or on blue ice (ice packs). Upon receipt, immediately store CleanMag Magnetic Beads at 2°C to 8°C in a constant-temperature refrigerator. Do not freeze. Do not store at -20°C.

Always ensure that all frozen components are fully thawed and have been vortexed and spun down to bring all liquids to the bottom of the tubes prior to use.

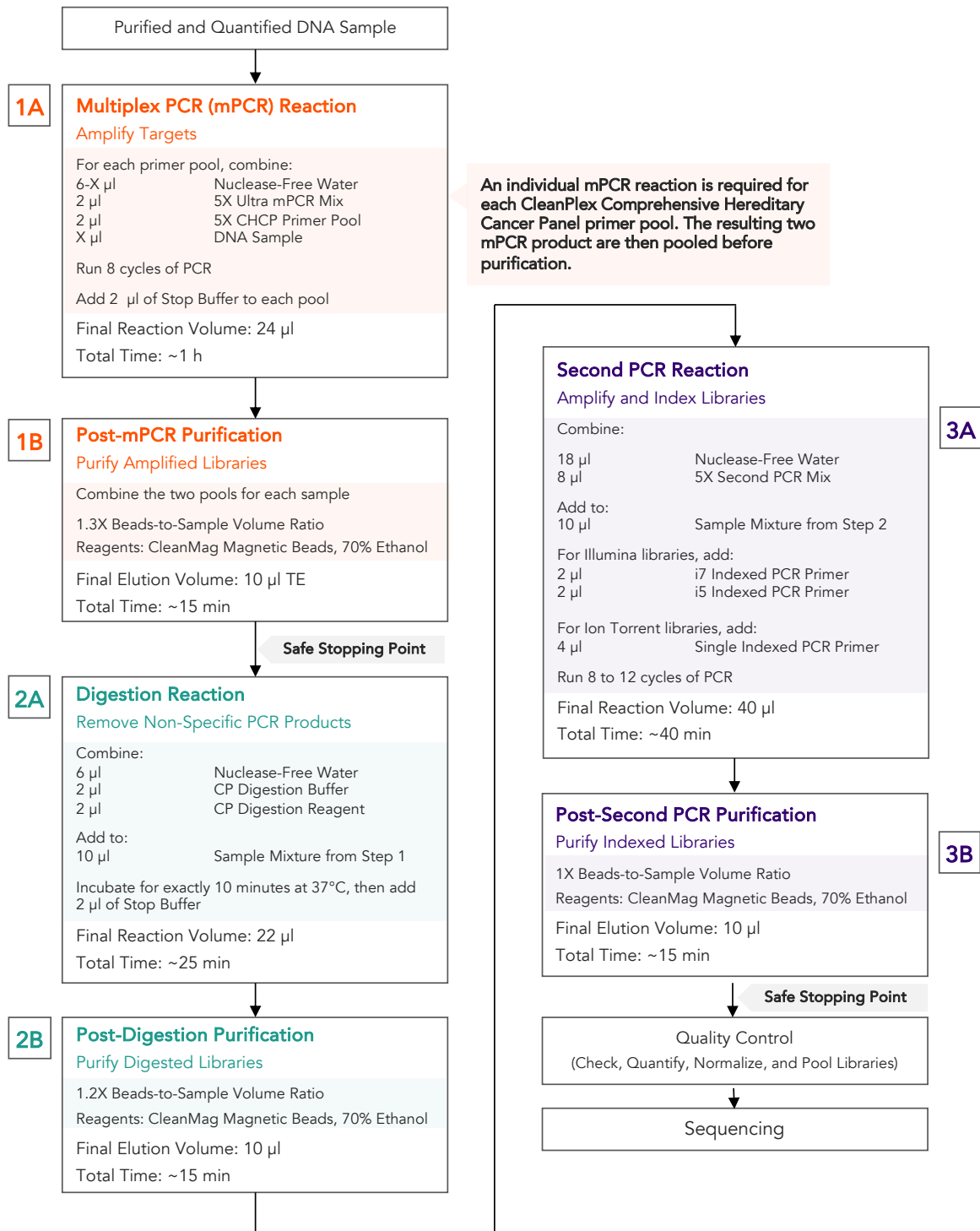
The components containing enzymes (5X Ultra mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

CleanPlex NGS Panels are developed, designed and sold exclusively for research use only. None of the products or their individual components have been tested for use in diagnostic procedures.

For hazard information, please refer to the Safety Data Sheet (SDS), which is available upon request.

Workflow

The following diagram illustrates CleanPlex Comprehensive Hereditary Cancer Panel targeted NGS library preparation workflow.



Protocol

Best Practices

- When using the kit for the first time, briefly vortex and spin the tubes in the kit to bring the liquid to the bottom of the tubes. Store the tubes containing enzymes (5X Ultra mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) on ice during their respective procedures. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spun-down before use.
- Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination between samples, multiplex PCR (mPCR) primers, or indexed PCR primers. Always change pipette tips between samples and change gloves frequently. Clean all workstations and tools with 10% bleach followed by water, then alcohol at the end of each work day.
- Use a calibrated PCR thermal cycler as specified by the manufacturer's user guide. Validated thermal cyclers include Bio-Rad C1000, Eppendorf Mastercycler series, and Applied Biosystems GeneAmp PCR System 9700. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second and higher, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed, or use the default setting (no ramp adjustment).
- To ensure accurate assembly of reactions, withdraw viscous solution (such as 5X Ultra mPCR Mix, CP Digestion Reagent, and 5X 2nd PCR Mix) slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly mix each assembly to ensure solutions are homogeneous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.
- Always keep working solutions, PCR tubes and plates, and PCR products on ice until needed. Combine PCR mixes just immediately prior to use and do not prolong storage of combined PCR mixes and PCR products.
- The protocol is designed to minimize the number of tube-to-tube transfer in order to avoid or reduce sample loss. For a single-pool CleanPlex NGS Panel, the entire protocol is performed following a single-tube workflow, with no tube-to-tube transfers. For a multi-pool CleanPlex NGS Panel, the individual mPCR products from the primer pool-specific reactions are combined into one tube, and the remaining protocol is carried out using a single-tube workflow.
- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross contamination between samples.

- Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for a successful bead purification
- Always pre-warm thermal cycles, pre-warm a water bath or heat block to 37°C.
- Assign sample indexes to specific samples before starting the protocol.
- Always prepare a master mix of reagents when working with multiplex reactions. Prepare ~5% excess of each master mix to allow for pipetting losses. A master mix calculation sheet can be downloaded from the Product Documents page on the Paragon Genomics website at www.paragongenomics.com/product_documents/.

Input DNA Requirements

Refer to the following chart for the amounts of input DNA.

Panel	DNA Input Range (per pool)	Recommended gDNA Input (per pool)	Recommended FFPE DNA Input (per pool)
CleanPlex Comprehensive Hereditary Cancer Panel	5–40 ng	10 ng	20 ng

- The maximum volume of DNA input per Multiplex PCR Reaction is 6 µl. For the CleanPlex Comprehensive Hereditary Cancer Panel, which has 2 primer pools, an individual Multiplex PCR Reaction is required for each primer pool.
- Qubit dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) or an equivalent fluorometric method is recommended for measuring DNA concentration. UV spectrophotometry methods (e.g. NanoDrop™ spectrophotometer) are not recommended because it can significantly overestimate the DNA concentration.
- 5–40 ng of human genomic DNA from normal or FFPE tissue is recommended for each Multiplex PCR Reaction depending on the panel and application.
- When DNA quality is low or unknown (such as DNA from FFPE tissues), higher DNA input can be used to produce better library quality and reach lower limits of detection.
- Avoid diluting DNA samples to < 10 ng/µl for prolonged storage as lower concentrations of DNA are less stable in solution. Avoid freeze-thawing dilute DNA samples when possible, and measure sample concentrations immediately prior to use to avoid inaccurate sample input.

1A. Multiplex PCR (mPCR) Reaction

- 1A.1. Using thin-wall PCR strip tubes (or a 96-well PCR plate), prepare the mPCR Reaction Mixture by adding components in the following order on ice or a cold block. Prepare an individual reaction for each of the two primer pools.

Note: When working with multiple samples, normalize all DNA samples to the same concentration, and prepare a master mix of mPCR Reaction Mixture for each primer pool. Mix well, aliquot into individual tubes, then add DNA samples to each reaction.

Note: When working with multi-pool panels, the recommended amount of DNA is to be added to each pool, and not divided between the pools.

mPCR Reaction Mixture		
Reagent	Cap Color	Volume per reaction (gDNA)
Nuclease-Free Water	—	6 – X μ l
5X Ultra mPCR Mix	Green	2 μ l
5X CleanPlex Comprehensive Hereditary Cancer Panel Primer Pool	Varies	2 μ l
DNA Sample	—	X μ l
Total Volume per reaction		10 μl



Important! 5X Ultra mPCR Mix and 5X CleanPlex Comprehensive Hereditary Cancer Panel Primer Pools are viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution when handling these reagents.

- 1A.2. Close the caps of the PCR tubes or seal the PCR plate with adhesive film, spin briefly to bring down the liquid, mix thoroughly by pipetting up and down at least 5 times, or by vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can decrease yield and increase nonspecific product formation.

Note: If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during thermal cycling.

- 1A.3. Load the tubes/plate in the thermal cycler and run the following thermal cycling protocol to amplify target DNA regions. Refer to the table below for the mPCR thermal cycling conditions

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	98 °C	30 sec	-	-
Denaturation	98 °C	15 sec	3 °C/s	8
Annealing/Extension	60 °C	5 min	2 °C/s	
Hold	10 °C	∞		
* For thermal cyclers without adjustable ramp speed, use the default settings.				

- 1A.4. Immediately add 2 µl of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is approximately 12 µl.

Note: Do not allow samples to hold at 10 °C for longer than 30 minutes before adding Stop Buffer.

- 1A.5. Proceed to Step 1B. Post-mPCR Purification.



Important. Do not stop and store PCR products after multiplex PCR reaction. Add Stop Buffer then proceed to Step 1B, Post-mPCR Purification immediately.

1B. Post-mPCR Purification



Important! Bring magnetic beads to room temperature and vortex thoroughly to disperse the beads before each use. Dispense slowly and carefully. Keep the outside of the pipette free from droplets.



Important! Use freshly prepared 70% ethanol. Prepare the 70% ethanol solution by mixing 100% ethanol with nuclease-free water at volume ratios of 7 parts 100% ethanol 3 parts nuclease-free water. Do not top off one liquid with the other in a volumetric container because the volumetric ratio will not be accurate. When water and ethanol are mixed, the final volume will be less than the sum of individual volumes.

- 1B.1. Open the tubes or carefully remove the adhesive film from the PCR plate. Combine the 12 µl multiplex PCR products for each sample for a total of 24 µl volume.
- 1B.2. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1.3X** bead-based purification by adding magnetic beads to the combined sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Note: Accurate dispensing of magnetic beads is critical for each purification step. Aspirate and dispense slowly, taking care to completely transfer the required volume of beads and do not allow droplets of beads on the outside of the tip to be added to the sample.

Note: Ensure the mixture is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Incomplete mixing can result in lowered yields and/or increased background in the final library.

Post-mPCR Purification — 1.3X Beads-to-Sample Volume Ratio	
	Pool 2
Volume of mPCR Product Mixture per reaction	24 µl (12 µl of mPCR Product per pool)
Volume of Magnetic Beads per reaction	31 µl

- 1B.3. Incubate the mixture for 5 minutes at room temperature.
- 1B.4. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/well. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.

Note: *If the magnetic beads are aspirated into the pipette tip at any point during the purification process, dispense the solution back into tube, remove the tip, allow the beads to migrate toward the magnet again, then repeat.*

Note: *When working with 96-well PCR plates, avoid touching the side of the well with the pipette tip when removing supernatant to minimize wicking up of liquid to the side of the well.*

Note: *If it takes significantly longer than 2 minutes for the beads to completely clear, your magnetic rack is low performing and you should consider getting a different rack or plate.*

- 1B.5. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

Note: *Removing all residual supernatant from the Multiplex PCR Reaction prior to washing is critical to obtaining a clean, high-quality library.*

Note: *When working with 96-well PCR plates, a strong compatible magnetic plate is essential. If your magnetic rack cannot collect the beads effectively, please look into a replacement.*

- 1B.6. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Remove the tubes/plate from the magnetic rack and place the clear side of the tubes/wells (the side without beads) against the magnet to move the beads from one side of each tube/well to the other. **Do not vortex**. Carefully remove and discard the supernatant without disturbing the beads.

- 1B.7. Repeat step 1B.6.

- 1B.8. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnet rack to gather the beads. Carefully remove the residual ethanol in each tube/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry**.

Note: *Over-dried beads can dislodge from the side of the tube and lead to cross contamination, and they are also more difficult to resuspend during elution. Residual ethanol inhibits PCR and will result in reduced yield. Especially when working with 96-well PCR plates, make sure all residual ethanol is dried (may take longer than 5 minutes) before going to the next step.*

- 1B.9. Add 10 μ l TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads. The DNA will be immediately released from the beads (there is no need to remove the beads). Spin briefly to collect the liquid and the beads.
- 1B.10. Proceed to Step 2A. Digestion Reaction.



Safe Stopping Point. Purified products may be stored at 2-8°C for up to 1 hour or at -20°C for up to 24 hours.

2A. Digestion Reaction

Note: After starting the Digestion Reaction, the samples cannot be stored and must continue to Post-Digestion Purification and Second PCR Reaction. Plan accordingly.

Note: When thawing CP Reagent Buffer, ensure no precipitate is visible. Bring to room temperature and vortex to dissolve the precipitate.

Note: Pre-warm a thermal cycler to 37°C before beginning the Digestion Reaction.

- 2A.1. Prepare Digestion Reaction Master Mix and add to each purified sample from Step 1B. The magnetic beads in the sample mixture do not affect the Digestion Reaction.

Note: When working with multiple reactions, prepare a master mix. Mix well, then add 10 µl of master mix to each sample.

Digestion Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	6 µl
CP Reagent Buffer	White	2 µl
CP Digestion Reagent	Yellow	2 µl
Total Volume per reaction		10 µl

Digestion Reaction Mixture	
Reagent	Volume per reaction
Digestion Reaction Master Mix	10 µl
Purified Sample from Step 1B	10 µl
Total Volume per reaction	20 µl

- 2A.2. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: It is crucial that the reaction mixture is homogeneous prior to incubation. Incomplete mixing can cause digestion of the library or under-digestion of nonspecific products.

Note: If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during incubation.

- 2A.3. Incubate at 37°C for exactly 10 minutes. **Do not** incubate shorter or longer than 10 minutes.
- 2A.4. Immediately add 2 µl of Stop Buffer (red cap) to each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is approximately 22 µl.
- 2A.5. Proceed to Step 2B. Post-Digestion Purification immediately.



Important! Do not stop and store samples after the Digestion Reaction. Proceed to Step 2B. Post-Digestion Purification immediately.

2B. Post-Digestion Purification

- 2B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1.2X** bead-based purification by adding **26.4 µl** of magnetic beads to each sample. Mix by pipetting up and down least 5 times or vortex vigorously at least 5 seconds until homogeneous.

Note: Accurate dispensing of magnetic beads is critical for each purification step. Aspirate and dispense slowly, taking care to completely transfer the required volume of beads and do not allow droplets of beads on the outside of the tip to be added to the sample.

Note: Ensure the mixture is thoroughly mixed before proceeding, especially when working with 96-well PCR plates.

Post-Digestion Purification — 1.2X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Digestion Reaction Product	22 µl
Magnetic Beads	26.4 µl

- 2B.2. Incubate the mixture for 5 minutes at room temperature.
- 2B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/wall. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.
- 2B.4. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

Note: Removing all supernatant after the Digestion Reaction is critical to obtaining a clean, high-quality library.

- 2B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Remove the tubes/plate from the magnetic rack and place the clear side of the tubes/wells (the side without beads) against the magnet to move the beads from one side of each tube/well to the other. **Do not vortex**. Carefully remove and discard the supernatant without disturbing the beads.
- 2B.6. Repeat step 2B.5.

- 2B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tube/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: *Over-dried beads can dislodge from the side of the tube and lead to cross contamination, and they are also more difficult to resuspend during elution. Residual ethanol inhibits PCR and will result in reduced yield. Especially when working with 96-well PCR plates, make sure all residual ethanol is dried (may take longer than 5 minutes) before going to the next step.*

- 2B.8. Add 10 µl TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads and elute DNA from the beads (there is no need to remove the beads). Spin briefly to collect the liquid.
- 2B.9. Proceed to Step 3A. Second PCR Reaction immediately.



Important! Do not stop and store samples after Post-Digestion Purification. Proceed to Step 3A. Second PCR Reaction immediately.

3A. Second PCR Reaction

Note: Remember to assign a specific index to each sample before starting this step.

- 3A.1. Thaw Indexed PCR Primers, vortex thoroughly, then briefly spin to collect the liquid. Prepare Second PCR Reaction Master Mix and add to each purified sample from Step 2B. Then add a unique (combination of) Indexed PCR Primer(s) to each sample.

Note: When working with multiple samples, prepare a master mix. Mix well, then add 26 μ l of master mix to each sample.

Second PCR Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	18 μ l
5X 2nd PCR Mix	Blue	8 μ l
Total Volume per reaction		26 μl

Second PCR Reaction Mixture	
Reagent	Volume per reaction
Second PCR Reaction Master Mix	26 μ l
Purified Sample from Step 2B	10 μ l
CleanPlex Indexed PCR Primer	4 μ l
Total Volume per reaction	40 μl



Important! When handling Indexed PCR Primers, take extra care to prevent cross contamination by opening one tube at a time and changing pipette tips and gloves as necessary. Avoid touching the opening and inside of the tubes with your hands, pipette channel, or anything non-disposable.

- 3A.2. Close the caps of the PCR tubes or seal the PCR plate with adhesive film. Spin briefly to collect the liquid, then mix thoroughly by pipetting up and down at least 5 times, or by vortexing vigorously for at least 5 seconds or until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can cause decreased yield and increase non-specific product formation.

Note: If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during thermal cycling.

- 3A.3. Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. The Second PCR thermal cycling protocol depends on the starting DNA input amount and DNA quality. Generally, lower quality DNA or lower DNA input amount requires more PCR cycles. See tables below for thermal cycling protocol and cycle number suggestions for CleanPlex Comprehensive Hereditary Cancer Panel.

Note: Use the amount of DNA input per primer pool to determine the correct cycles, not the combined DNA input from all pools. For example, if 10 ng of DNA was added to each of the two primer pools of the CleanPlex Comprehensive Hereditary Cancer Panel, use 10 ng to determine the Second PCR cycle number.

Second PCR Reaction — Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	10**
Annealing/Extension	Illumina: 60 °C Ion Torrent: 68°C	75 sec	2 °C/s	
Hold	10 °C	∞		
* For thermal cyclers without adjustable ramp speed, use the default settings.				
** When using 10 ng of high quality gDNA.				

Second PCR Reaction — Input Specific Cycle Number	
Input DNA per pool	Change in Cycle Number
5 ng	+ 1
10 ng	-
20 ng	- 1
40 ng	- 2
Low Quality DNA (eg. FFPE DNA)	+ 1 to + 2

- 3A.4. Proceed to Step 3B. Post-Second PCR Purification immediately after cycle completion. Do NOT allow PCR product to hold at 10 °C for more than 30 minutes.



Important. Do not stop and store PCR product after Second PCR. Proceed to 3B, Post-Second PCR purification immediately.

3B. Post-Second PCR Purification

- 3B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1X** bead-based purification by adding **40 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogeneous.

Note: Accurate dispensing of magnetic beads is critical for each purification step. Aspirate and dispense slowly, taking care to completely transfer the required volume of beads and do not allow droplets of beads on the outside of the tip to be added to the sample.

Note: Ensure the solution is thoroughly mixed before proceeding.

Post-Second PCR Purification — 1X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Magnetic Beads	40 µl
Second PCR Reaction Product	40 µl

- 3B.2. Incubate the mixture for 5 minutes at room temperature.
- 3B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/wall. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.
- 3B.4. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.
- Note:** Removing all supernatant is critical in obtaining a clean, high-quality library.
- 3B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Remove the tubes/plate from the magnetic rack and place the clear side of the tubes/wells (the side without beads) against the magnet to move the beads from one side of the tube/well to the other. **Do not vortex**. Carefully remove and discard the supernatant without disturbing the beads.
- 3B.6. Repeat step 3B.5.

- 3B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tubes/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: Over-drying and under-drying the beads can lead to reduced yield.

- 3B.8. Add 10 μ l TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads and elute DNA from the beads. Spin briefly to collect the liquid. At this point the library is complete and can be stored with beads at -20°C .

Note: To perform QC and sequencing, use a magnetic rack to separate the beads. Avoid transferring the beads when pipetting the clear supernatant for QC or sequencing.



Safe Stopping Point. Purified products may be stored with beads at -20°C until ready to sequence. When taking the library out for QC and sequencing, vortex briefly and place the tubes or plate on a magnetic rack to pull the beads to one side. Avoid pipetting the beads.

Quality Control Prior to Sequencing

Quantify each amplified library using one of the following methods:

1. Fluorometric methods, such as Qubit™ Fluorometer with dsDNA HS Assay Kit
2. Fragment Analysis, such as Agilent™ 2100 Bioanalyzer with High Sensitivity DNA Kit
3. qPCR quantification methods

The typically library yield is 5,000pM to 30,000pM, but can vary depending on your sample input, sample quality, and PCR cycles used. Yield does not indicate library quality. Refer to the troubleshooting guide towards the end of the user guide for more information.

After confirmation of library concentration, libraries of different indexes can be normalized and pooled for sequencing based on the sequencing provider's instructions.

Fluorometric and qPCR methods will yield absolute concentration and will not be able to differentiate potential background from the actual library. It is recommended that a high sensitivity fragment analyzer is used to visualize the peak shape, size, and potential background concentration to ensure library preparation was performed correctly prior to sequencing, and aid in troubleshooting. The presence of a peak at ~330bp indicates successful amplification of the targeted regions. If other major peaks exist, refer to the troubleshooting guide towards the end of this user guide.

If qualitative confirmation is not required prior to sequencing, a fragment analysis is not necessary for high-throughput workflows after ensuring that high quality libraries with little nonspecific product can be consistently produced.

Recommended Sequencing Length and Depth

The CleanPlex Comprehensive Hereditary Panel was designed to be compatible with PE 150 bp reads (2x150 bp).

For detecting germline mutations, the recommended average sequencing read depth is 500X. For detecting somatic mutations down to 1% minor allele frequency, the recommended average sequencing

Recommended Sequencing Depth			
Panel	Application	Average Read Depth	Total Read/Sample
CleanPlex Comprehensive Hereditary Cancer Panel	Somatic Mutations	2,000X	5M
	Germline Mutations	500X	1.25 M

read depth is 2,000X.

Terminology

Coverage	Also known as depth of coverage, is achieved with one set of paired end reads that cover the entire length of the amplicon.
Read	One continuous act of sequencing. For example, 1 million clusters on a flow cell would produce 1 million single reads and 2 million paired-end reads. Paired end reads count as two individual reads, and required for 1X coverage.

When calculating how many samples can be sequenced in one chip, it is Important to refer to the correct total read capacity of the chip.

For example:

For **1000X** coverage of a panel with **100** amplicons, it requires a total of 100,000 cluster reads or 200,000 paired end reads per sample. With a **Miseq V2 kit** (~15 million single reads or 30 million paired end reads) you can sequence about **150** samples per run.

For a sample multiplexing calculator and recommended sample multiplexing on various Illumina NGS instruments, please refer to tools section and panel-specific product sheets at www.paragongenomics.com/product_documents/.

Supporting Information

Dual-Indexed PCR Primers for Illumina

Best Practices

- Employ best laboratory practices to avoid cross contamination of indexed primers. Upon removing the primers from -20C storage, centrifuge the plate at room temperature (e.g. for 1min at 280 x g), ensure ALL the liquid is collected in the bottom of the wells, then use accordingly. We do not recommend vortexing the adapter plates as it could result in cross-contamination of the indexed primers. Pipette mix to resuspend individual adapters prior to use.
- The foil cover is pierceable and peelable. If peeling the cover off, take care to slowly remove the foil cover to avoid cross-contamination and to remove the foil evenly.
- Upon first use, carefully remove the foil cover to avoid cross-contamination. Discard the original foil cover and do not reuse. If only using a subset of the adapters, partial use of the foil is not recommended. Please replace the seal entirely once it has been opened.

Primer Sequences

Each sample is indexed by a pair of Indexed PCR Primers for sequencing on Illumina platforms. XXXXXXXX denotes the index region of the primer. Index sequences are listed below.

i5 Indexed Primer

5' -AATGATACGGCGACCAACGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

i7 Indexed Primer

5' -CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

Set A and Set B have mutually exclusive sequences and allow use together to form 384 combinatorial index pairs for sequencing up to 384 samples on one Illumina flow cell.

CleanPlex Targeted Library Kits and Panels are not compatible with most other commercially available index primer kits. Please use CleanPlex PCR Primers for Illumina for best results.

Index Sequences



Additional sequence and Sample sheet information can be downloaded from [Product Document Page](#) under Indexes and Useful Tools.

CleanPlex Dual-Indexed PCR Primers for Illumina Set T 3 x 3 indexes, 9 reactions (SKU 716021)
A selection of three i5 Indexes and three i7 Indexes from Illumina Set A to create 9 unique index combinations. Index selection may vary from kit to kit.

CleanPlex Dual-Indexed PCR Primers for Illumina Set A 12 x 8 indexes, 96 reactions (SKU 716006) 12 x 8 indexes, 384 reactions (SKU 716017)						
i7 Index	Index Sequence	i7 Bases for Sample Sheet All Illumina Systems	i5 Index	Index Sequence	i5 Bases for Sample Sheet	
					MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
A701	GTCGTGAT	ATCACGAC	A501	TGAACCTT	TGAACCTT	AAGGTTCA
A702	ACCACTGT	ACAGTGGT	A502	TGCTAAGT	TGCTAAGT	ACTTAGCA
A703	TGGATCTG	CAGATCCA	A503	TGTTCTCT	TGTTCTCT	AGAGAACA
A704	CCGTTTGT	ACAAACGG	A504	TAAGACAC	TAAGACAC	GTGTCTTA
A705	TGCTGGGT	ACCCAGCA	A505	CTAATCGA	CTAATCGA	TCGATTAG
A706	GAGGGGTT	AACCCCTC	A506	CTAGAACA	CTAGAACA	TGTTCTAG
A707	AGGTTGGG	CCCAACCT	A507	TAAGTTCC	TAAGTTCC	GGAACCTA
A708	GTGTGGTG	CACCACAC	A508	TAGACCTA	TAGACCTA	TAGGTCTA
A709	TGGGTTTC	GAAACCCA				
A710	TGGTCACA	TGTGACCA				
A711	TTGACCCT	AGGGTCAA				
A712	CCACTCCT	AGGAGTGG				

CleanPlex Dual-Indexed PCR Primers for Illumina Set B 12 x 8 indexes, 96 reactions (SKU 716018) 12 x 8 indexes, 384 reactions (SKU 716019)						
i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems		i5 Index	Index Sequence	i5 Bases for Sample Sheet MiSeq, NovaSeq, HiSeq 2000/2500 MiniSeq, NextSeq, HiSeq 3000/4000
Q7005	ATATTCAC	GTGAATAT		Q5001	AGCGCTAG	AGCGCTAG CTAGCGCT
Q7006	GCGCCTGT	ACAGGCGC		Q5002	GATATCGA	GATATCGA TCGATATC
Q7007	ACTCTATG	CATAGAGT		Q5007	ACATAGCG	ACATAGCG CGCTATGT
Q7008	GTCTCGCA	TGCGAGAC		Q5008	GTGCGATA	GTGCGATA TATCGCAC
Q7015	AGTAGAGA	TCTCTACT		Q5009	CCAACAGA	CCAACAGA TCTGTTGG
Q7016	GACGAGAG	CTCTCGTC		Q5010	TTGGTGAG	TTGGTGAG CTCACCAA
Q7017	AGACTTGG	CCAAGTCT		Q5013	AACCGCGG	AACCGCGG CCGCGGTT
Q7018	GAGTCCAA	TTGGAATC		Q5014	GGTTATAA	GGTTATAA TTATAACC
Q7023	AATTCTGC	GCAGAATT				
Q7024	GGCCTCAT	ATGAGGCC				
Q7025	ATCTTAGT	ACTAAGAT				
Q7026	GCTCCGAC	GTCGGAGC				

Single-Indexed PCR Primers for Ion Torrent

Primer Sequences

Each sample is indexed by uniquely designed Indexed PCR Primers for sequencing on Ion Torrent platforms. Only CleanPlex Single-Indexed PCR Primers for Ion Torrent are compatible with CleanPlex workflow for generating libraries intended for Ion Torrent sequencing.

Index Sequences

CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent Sets C, D, E, and F contain index sequences identical to the sequences of the equivalent IonCode™ barcodes. These indexed PCR primers are provided in sealed 96-well PCR plates and arrange in ascending order in columns.



For plated indexes v2 sets C, D, E, and F, plate map and sequences information can be downloaded from [Product Document Page](#) under Indexes.

Troubleshooting Guide

Potential Causes for Extra Peaks and Suggested Solutions

Peaks around 70 bp are index primer dimers from the Second PCR Reaction and result from incomplete removal of low molecular weight DNA fragments during the final magnetic bead purification (Post-Second PCR Purification). When these peaks are high, it usually indicates one or more of the following:

- Inaccurate pipetting of magnetic beads when making a large number of libraries in a short period of time.
- Insufficient removal of supernatant and/or ethanol washes during the last purification.
- Adding more than specified amount of indexed primers to Second PCR Reaction.

Peaks around 100–140 bp are residues of digested non-specific amplification products and adapter dimers. They come from incomplete removal of low molecular weight DNA fragments during the Post-Digestion Purification or nonspecific products formed during or after the Multiplex PCR (mPCR) Reaction due to deviation from protocol or high concentration of panel. The digestion reagent degrades non-specific amplification products into small pieces, which are then removed during magnetic bead purification. These peaks are usually caused by one or more of the following:

- Inaccurate pipetting of magnetic bead volume.
- Insufficient mixing of reaction solutions or bead and sample mixture.
- Insufficient removal of supernatant and ethanol washes during purification.
- Allowing mPCR products to sit for too long (and form nonspecific products) before purification.
- CleanPlex Custom NGS Panels may require panel titration for optimal performance. Often dimers will decrease by reducing the panel concentration in the mPCR Reaction Mixture.
- Incorrect annealing time, specifically unnecessarily longer annealing time.

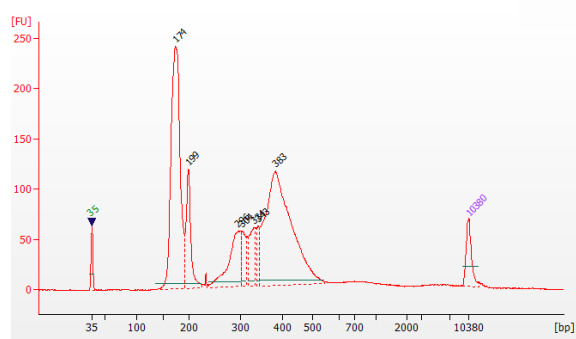
Broad peaks spread across 500 – 10,000 bp range are nonspecific products due to overamplification. Double check that the correct mPCR and Second PCR cycle numbers were used. PCR cycles are determined based on the amplicon count per pool of your panel, DNA input amount, and DNA quality. Try reducing the Second PCR cycles by 2-3 cycles if issue persists. If you're working with a custom panel for organisms with a much smaller genome than human's, consider reducing the DNA input and Second PCR cycles accordingly.

Removing nonspecific products from final libraries

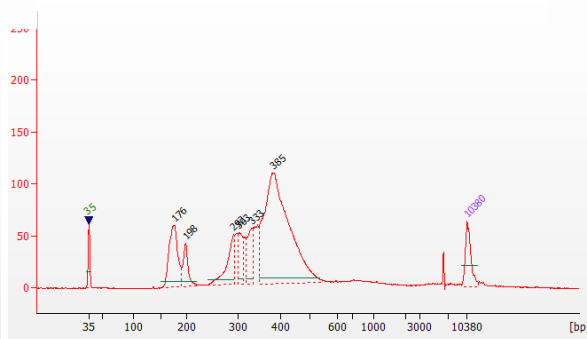
If short nonspecific products described above are present in significant amounts, they can be reduced by pooling indexed libraries (that will be sequenced in the same lane) and performing one additional round of 1X magnetic bead-based purification if the pooled library **concentrations is >3,000 pM** and the **total volume is >5 µL**. Low volume and low concentration libraries should not be bead purified again as it will lead to significant loss of the library of interest. In the figures below, the Agilent Bioanalyzer trace on the left shows a CleanPlex

Hereditary Cancer NGS library that contains significant adapter dimers due to poor library preparation. The Bioanalyzer trace on the right shows the same library after an additional 1X magnetic bead-based purification. The bead purification can be repeated once more if volume and concentration still meet the criteria above. We recommend keeping short adapter dimer peaks to less than 5% for best results. When possible it's always better to repeat the library preparation with the corrected steps so nonspecific products do not form in the first place.

Before purification



After additional 1X bead-based purification



Potential Reasons for No Peaks

- 30% ethanol instead of 70% ethanol was used in DNA purification with magnetic beads.
- Magnetic beads were not added for one or more of the purification steps.
- Stop Buffer was not added or was added too late after Digestion Reaction, resulting in over-digestion of the samples. This may happen when handling a large number of samples.
- DNA quantification was inaccurate, especially if using spectrophotometric methods, such as the NanoDrop instrument. Try using more input DNA.
- DNA quality is extremely degraded. Try using more input DNA.
- Incompatible indexed PCR primers were using in the Second PCR Reaction. Only use CleanPlex Indexed PCR Primers with CleanPlex NGS Panels.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do **not** use magnetic racks designed for 1.5 ml tubes.

Additional Resources

Please visit www.paragongenomics.com/product/faq/ for additional troubleshooting resources.

Data Analysis Recommendations for Illumina

We recommend on-instrument analysis using Illumina's DNA Amplicon Module for the simplest and most streamlined experience for downstream QC and variant calling. Please follow Illumina's latest user guide for step-by-step instructions, which is available in their support center. Manifest files are automatically provided for hg19 designs (in DesignDetails.zip) and upon request, Paragon Genomics can provide the manifest file for other genomes.

As an alternative, the Broad Institute's GATK Best Practice (<https://software.broadinstitute.org/gatk/best-practices/>) offers general guiding principles for sequencing data analysis.

For off-instrument analysis, please refer to the following recommended steps for analyzing CleanPlex NGS libraries sequenced on Illumina platforms.

1. **BED Files.** Visit <https://www.paragongenomics.com/my-account/downloads/> to download panel-specific amplicon insert BED file. For CleanPlex Custom Panels, the BED file is available for download via the associated order account through our design portal: <https://www.paragongenomics.com/panel-design-my-design-requests/>

2. Adapter Trimming.

For panel with amplicon inserts that fall less than 150bp lengths, it is recommended to trim leftover adapter sequences for 2X150 sequencing before read mapping.

Following is an example command with open-source software cutadapt (<https://cutadapt.readthedocs.io/en/stable/>).

```
cutadapt -g CCTACACGACGCTCTTCCGATCT \
-a AGATCGGAAGAGCACACGTCTGAA \
-A AGATCGGAAGAGCGTCGTGTAGG \
-G TTCAGACGTGTGCTCTTCCGATCT \
-e 0.1 -O 9 -m 20 -n 2 \
-o R1_out.fq.gz -p R2_out.fq.gz R1_in.fq.gz R2_in.fq.gz \
> cutadapt_report.output.txt
```

3. Map reads to reference genome.

Ready to use panels are designed against GRCh37 (hg19) reference genome. For custom panels, please refer to your specified reference genome. Bwa mem is recommended for read mapping and de-duplication procedure shall be skipped.

4. Trim primer sequences.

Before construction of a consensus genome sequence, it is recommended to remove primer sequences. The trimming should be based primarily on reads' mapping starts/ends positions and primers 3' ends' coordinates in ampInsert.bed files. We recommend using the DNA amplicon workflow on Illumina instruments for trimming primers and calling variants. An alternative approach for primer trimming is using the software package fgbio. It requires primer genomic coordinates in a tab delimited file which can be provided by Paragon Genomics to customers.

Following is an example command:

```
java -jar fgbio-1.2.0-e7ac607-SNAPSHOT.jar TrimPrimers -i input.bam -o  
output.primerTrim.bam -p primer_info.tab -H true
```

5. Calculate QC metrics.

In order to assess the quality of the sequencing results, it is recommended to assign mapped reads to amplicons based on mapping position. Subsequently, the following metrics can be used to measure general performance of the panel.

- **Mapping Rate:** Percentage of reads mapped to reference genome. It assesses primer-dimers and other PCR artifacts.
- **On-Target Rate:** Percentage of mapped reads that aligned to the targeted regions. It assesses binding/amplification specificity of designed primers.
- **Coverage Uniformity:** Percentage of amplicons with read depth equal to or greater than 20% of mean read depth of all amplicons in the panel. It measures performance uniformity of amplicons in the panel.

Data Analysis Recommendations for Ion Torrent

We recommend customers to use analysis software provided by Ion Torrent. A few suggestions specific to Paragon Genomics panels are given below.

1. **BED Files.** Visit <https://www.paragongenomics.com/my-account/downloads/> to download panel-specific amplicon insert BED file. For CleanPlex Custom Panels, the BED file is available for download via the associated order account through our design portal: <https://www.paragongenomics.com/panel-design-my-design-requests/>
2. **Extra Trimming settings.** When working in the Ion environment, such as the Torrent Suite, Ion Reporter, or VariantCaller Plugin, discard any custom argument and revert to default arguments for your chip type and application combination, then try adding to both Pre-BaseCaller Args and BaseCaller Args : `--extra-trim-left 24 --extra-trim-right 24` , to avoid misassignment of read quality.

Setting	Description
Pre Basecaller args for calibration	<code>--extra-trim-left 24 --extra-trim-right 24</code>
Basecaller args	<code>--extra-trim-left 24 --extra-trim-right 24</code>

3. **Trim tag sequences.** If working with FASTQ directly without BaseCaller trimming setting above, tag sequences can be trimmed with the following instructions. If you're working with already trimmed BAM files, additional trimming with cutadapt is not necessary.

As shown in the diagram of library structure below, a short tag sequence (TAG-1 and TAG-2 respectively) is attached to both ends of an amplicon. The tag sequences are added during primer synthesis and they serve as priming site in second PCR reaction where Ion Torrent adapters are added. It is the best to trim those sequences from sequencing read before read mapping.



It is recommended to trim the tag sequences with open-source software cutadapt using the following options:

```
cutadapt -g CCTACACGACGCTCTTCCGATCT \
-g TTCAGACGTGTGCTCTTCCGATCT \
-a AGATCGGAAGAGCGTCGTGTAGG \
-a AGATCGGAAGAGCACACGTCTGAA \
-e 0.1 -O 9 -m 20 -n 2 \
-o out.fq.gz in.fq.gz \
> cutadapt_report.output.txt
```

The above command would trim left-over adapter sequences as well.

4. **Trim primer sequences.** For a single-pool panel, we don't recommend to trim primer sequences from sequencing reads. For a two-pool panel, however, trimming primer sequences with a software is recommended to avoid false positive calls from primer binding sites. The trimming should be based primarily on reads' mapping starts/ends positions and primers 3' ends' coordinates in amplInsert bed files. We recommend using the Torrent Variant Caller plugin for trimming primers and calling variants. For other applications that require actual primer sequences, please contact Paragon Genomics to acquire them.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

Phone: 650-822-7545

Email: support@paragongenomics.com

Website: www.paragongenomics.com

Paragon Genomics

5020 Brandin Court, 2nd Floor Fremont, CA

94538-3140, USA 1-510-363-9918

contact@paragongenomics.com

www.paragongenomics.com

© 2024 Paragon Genomics, Inc. All rights reserved.