



CleanPlex® NGS Panel

User Guide

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

- CleanPlex® OncoZoom® Panel
- CleanPlex® BRCA1 & BRCA2 Panel
- CleanPlex® TP53 Panel
- CleanPlex® Mitochondrial Disease Panel
- CleanPlex® CFTR Panel
- CleanPlex® ACE2 & TMPRSS2 Panel
- CleanPlex® Custom NGS 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/

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Revision History

Document	Date	Description of Change
UG1001-01		<ul style="list-style-type: none"> Initial version
UG1001-02	September 2018	<ul style="list-style-type: none"> Changed document template Reorganized information and revised wording to enhance clarity Added instructions for CleanPlex Mitochondrial Disease Panel and CleanPlex Hereditary Cancer Panel Added instructions for CleanPlex Indexed PCR Primers for Ion Torrent Added index sequences for CleanPlex Indexed PCR Primers
UG1001-03	December 2018	<ul style="list-style-type: none"> Fixed typos Updated Required Materials and Equipment Not Provided section Added instructions for working with 96-well PCR plates
UG1001-04	February 2019	<ul style="list-style-type: none"> Updated Workflow diagram to remove two safe stopping points for more robust results Included additional tips under Best Practices
UG1001-05	July 2019	<ul style="list-style-type: none"> Added index information for new CleanPlex Single-Indexed PCR Primers for Ion Torrent Updated protocol to include addition of Stop Buffer at the end of the Multiplex PCR Reaction Revised wording to improve clarity Added Data Analysis Recommendations for Ion Torrent
UG1001-06	October 2019	<ul style="list-style-type: none"> Removed Hereditary panel because updated Hereditary panel v2 will have specific user guide. Revised wording and reformatted section to improve clarity Relocated some notes to a new section "set up and preparations" to allow users to plan ahead accordingly. Added CFTR ready to use panel information.
UG1001-07	September 2020	<ul style="list-style-type: none"> Revised wording and added additional notes to improve clarity Updated location of BED files in the Analysis guidelines. Updated user guide to include ACE2/TMPRSS2 Panel
UG1001-08	January 2021	<ul style="list-style-type: none"> Revised wording and added additional notes to improve clarity throughout protocols. Updated Index information to reference Illumina and Ion Torrent Index sheets from product download page. Added Trademark notice.
UG1001-09	December 2021	<ul style="list-style-type: none"> Updated Ion Torrent Data Analysis Recommendations

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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. CleanPlex NGS Panels generate highly accurate data from as little as 1 ng of DNA using a fast and simple workflow. CleanPlex Ready-to-Use NGS Panels are expertly optimized with predesigned primers to generate valuable insights in key cancer and disease research areas. CleanPlex Custom NGS Panels are made-to-order to target user-defined genomic regions of interest.

CleanPlex NGS Panels are 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.

CleanPlex NGS Panels feature a fast and simple workflow that can be completed in about 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. A single-pool CleanPlex NGS Panel, such as the CleanPlex OncoZoom Cancer HotSpot Panel, has no tube-to-tube transfer and thus offers the many benefits of a single-tube workflow.

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 Target Enrichment and Library Preparation Workflow

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

Applications

The CleanPlex NGS Panels can be used to detect germline and somatic mutations in a wide variety of applications ranging from tumor profiling and early cancer detection to high-throughput genotyping and gene expression analysis. CleanPlex Custom NGS Panels can be built from 7 to 20,000 amplicons per primer pool to target either or both hotspot position and full genes. Amplicons can be designed with size ranging from 80 to 500 bp to accommodate different sample types and the needs of specific applications.

The table below shows some of the organisms, sample types, and applications that users have studied using CleanPlex NGS Panels. This is not meant to be an exhaustive or restrictive list.

Organisms	Sample Types	Applications
<ul style="list-style-type: none"> • Human • Mammals • Fish • Insects • Plants • Microorganisms 	<ul style="list-style-type: none"> • Genomic DNA from whole blood, tissues, biofluids, buccal swab, dried blood spots (DBSs), feces, circulating cells, and cultured cells • Degraded DNA from FFPE tissues • Cell-free DNA (cfDNA) from plasma and other biofluids • cDNA from bulk or single cell RNA samples 	<ul style="list-style-type: none"> • Tumor profiling • Variant detection and discovery • Disease predisposition • Species identification • High-throughput genotyping • Gene expression profiling

Other Applications

For other targeted sequencing applications, Paragon Genomics also offer a while variety of both ready to use panel and custom panels for infectious disease, oncology, drug discovery, and more.

Visit our applications page for additional information: <https://www.paragongenomics.com/applications/>

Other Ready to Use Panels			
Panel	SKU	Application	Description
CleanPlex SARS-CoV-2 Research and Surveillance Panel	918011	Infectious Disease	Original SARS-CoV-2 full viral genome sequencing panel
CleanPlex SARS-CoV-2 FLEX Research and Surveillance Panel	918014	Infectious Disease	FLEX panel for frontline SARS-CoV-2 mutation research
CleanPlex Respiratory Research Panel	918301	Infectious Disease	Respiratory Panel contains additional viral content for concurrent Influenza and SARS-CoV-2 detection and flu subtyping

CleanPlex ACE2&TMPRSS2 Panel	916121	Infectious Disease	Host (human) germline panel for mutation analysis of <i>ACE2</i> and <i>TMPRSS2</i> and associated eQTL for expression level related research, and susceptibility investigations.
CleanPlex OncoZoom Cancer HotSpot Panel	916002	Oncology	Target 2,900+ hotspots in 65 genes with known cancer associations.
CleanPlex BRCA1 & BRCA2 Panel v3	916006	Oncology	Target the entire coding region including 20 bases of padding around all targeted coding exons in <i>BRCA1&2</i>
CleanPlex TP53 Panel	916009	Oncology	Target the entire coding region and flanking intronic sequences of <i>TP53</i> for somatic mutations detection low as 1%
CleanPlex UMI Lung Cancer Panel	916066	Oncology	Interrogate 23 genes containing variants that are frequently mutated in lung cancer using a hotspot panel designed to work with cell-free DNA (cfDNA). Detect low-frequency variants below 0.5% MAF.
CleanPlex TMB 500 Panel	916074	Oncology	Interrogate 516 genes associated with the major solid tumor types to assess tumor mutational burden and characterize mutation profile.
OmniFusion™ RNA Lung Cancer Panel	917101	Oncology	Interrogate >530 known RNA fusion and additional novel RNA fusion using a single-primer method.
AccuFusion™ RNA Lung Cancer Panel	917104	Oncology	Interrogate ~280 known RNA fusions using a target specific dual-primer multiplex PCR method.
CleanPlex Mitochondrial Disease Panel	916063	Genetic Disease	Detect and investigate important variants with complete coverage of the entire human mitochondrial genome.
CleanPlex Hereditary Cancer Panel v2	916115	Genetic Disease	Interrogate 37 genes associated with cancers of the breast, ovary, uterus, skin, prostate, and gastrointestinal system, including rs12516 and rs8176318 in <i>BRCA1</i> and Boland inversion in <i>MSH2*</i> .
CleanPlex CFTR Panel	916117	Genetic Disease	Target the entire exon and include 20 bases of padding around all targeted coding exons for <i>CFTR</i> .
CleanPlex Custom NGS Panels	Varies	Variable	One-on-one service to design a custom panel specific to your applications and needs.

Compatible Sequencing Instruments

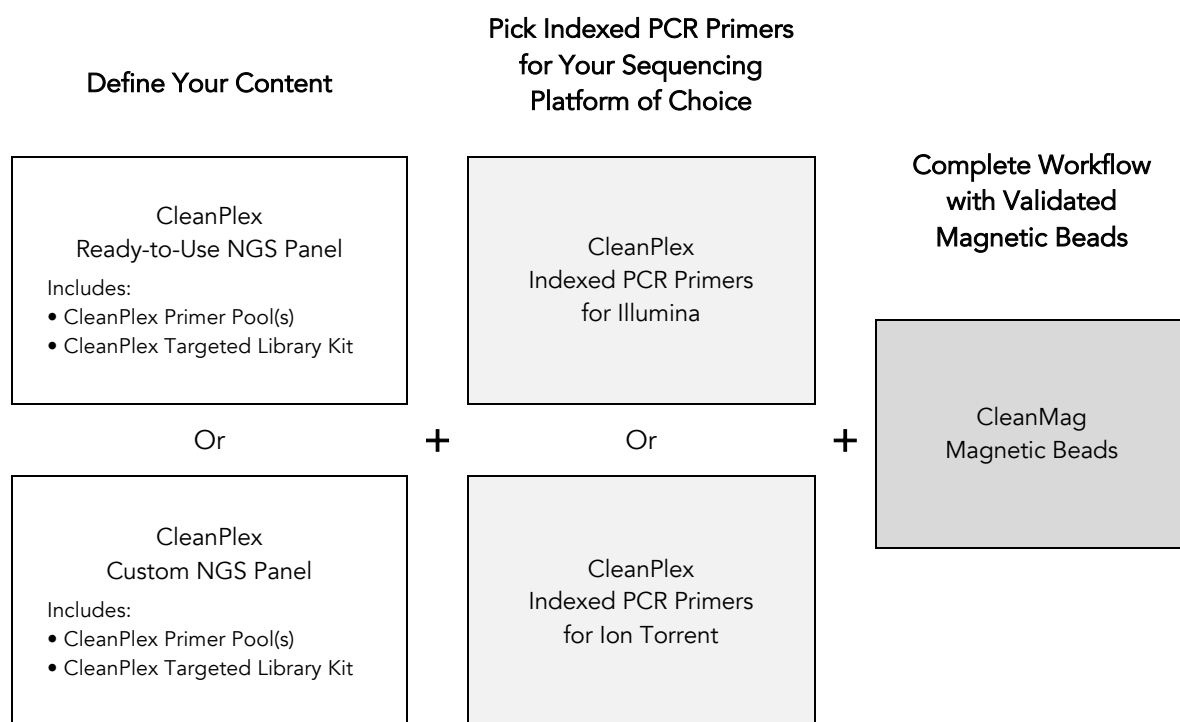
CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, MiSeq® System, MiniSeq® System, and iSeq® System.

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 NGS Panel User Guide requires the following components, which need to be ordered separately:

- CleanPlex Ready-to-Use NGS Panel or CleanPlex Custom NGS Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)



Panel Specifications					
Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Average Amplicon Length	Average Library Length
CleanPlex OncoZoom Cancer HotSpot Panel	1	5X	601	146 bp	282 bp
CleanPlex BRCA1 & BRCA2 Panel	2	5X	218	158 bp	294 bp
CleanPlex TP53 Panel	2	5X	29	133 bp	269 bp
CleanPlex Mitochondrial Disease Panel	2	5X	102	254 bp	390 bp
CleanPlex CFTR Panel	2	5X	65	222 bp	358 bp
CleanPlex ACE2 & TMPRSS2 Panel	2	5X	210	225 bp	361 bp
CleanPlex Custom NGS Panels	Varies	5X	Varies	Varies	Varies

CleanPlex NGS Panel — Kit Contents, Store at –20°C					
Panel	SKU	Size (Reactions)	Components		
			Primer Pool 1	Primer Pool 2	CleanPlex Targeted Library Kit
CleanPlex OncoZoom Cancer HotSpot Panel	916001	8	16 µl	---	1-pool, 8 rxns
	916002	96	192 µl	---	1-pool, 96 rxns
CleanPlex BRCA1 & BRCA2 Panel	916005	8	16 µl	16 µl	2-pool, 8 rxns
	916006	96	192 µl	192 µl	2-pool, 96 rxns
CleanPlex TP53 Panel	916008	8	16 µl	16 µl	2-pool, 8 rxns
	916009	96	192 µl	192 µl	2-pool, 96 rxns
CleanPlex Mitochondrial Disease Panel	916107	8	16 µl	16 µl	2-pool, 8 rxns
	916063	96	192 µl	192 µl	2-pool, 96 rxns
CleanPlex CFTR Panel	916116	8	16 µl	16 µl	2-pool, 8 rxns
	916117	96	192 µl	192 µl	2-pool, 96 rxns
CleanPlex ACE2 & TMPRSS2 Panel	916120	8	16 µl	16 µl	2-pool, 8 rxns
	916121	96	192 µl	192 µl	2-pool, 96 rxns
CleanPlex Custom NGS Panels	Varies	96+	Varies		Varies

A CleanPlex Targeted Library Kit is included in every CleanPlex Ready-to-Use NGS Panel and CleanPlex Custom NGS Panel. Please note that the CleanPlex Targeted Library Kit is **not** sold separately.

CleanPlex Targeted Library Kit — Kit Contents, Store at –20°C (not sold separately)								
Configuration			1-Pool		2-Pool		4-Pool	
		Size	8 Rxns	96 Rxns	8 Rxns	96 Rxns	8 Rxns	96 Rxns
Component	Cap Color	SKU	816001	816002	816006	816007	816023	816024
5X mPCR Mix	Green		16 µl	192 µl	32 µl	384 µl	64 µl	768 µl
CP Reagent Buffer	White		16 µl	192 µl	16 µl	192 µl	16 µl	192 µl
CP Digestion Reagent	Yellow		16 µl	192 µl	16 µl	192 µl	16 µl	192 µl
Stop Buffer	Red		32 µl	384 µl	48 µl	576 µl	80 µl	960 µl
5X 2nd PCR Mix	Blue		64 µl	768 µl	64 µl	768 µl	64 µl	768 µl
TE Buffer	Clear		500 µl	4 ml	500 µl	4 ml	500 µl	4 ml

Required Materials and Equipment Not Included

- CleanPlex Indexed PCR Primers (visit www.paragongenomics.com/store for more indexing options)

CleanPlex Dual-Indexed PCR Primers for Illumina — Store at –20°C

	SKU	Size (Reactions)	Format
CleanPlex Dual-Indexed PCR Primers for Illumina Set T	716021	9	Random 3 i7 indexes + 3 i5 indexes (9 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set A	716006 716017	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set B	716018 716019	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)

CleanPlex Dual-Indexed PCR Primers for Illumina — Store at –20°C

	SKU	Size (Reactions)	Format
CleanPlex Dual-Indexed PCR Primers for Illumina Set T	716021	9	Random 3 i7 indexes + 3 i5 indexes (9 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set A	716006 716017	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set B	716018 716019	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set A	716033 716045	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set B	716034 716046	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set C	716035 716047	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set D	716036 716048	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set C	716037 716049	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set D	716038 716050	96 384	96 i7 indexes + i5 indexes (96-well plate)

CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set E	716039 716051	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set F	716040 716052	96 384	96 i7 indexes + i5 indexes (96-well plate)

CleanPlex Single-Indexed PCR Primers for Ion Torrent — Store at –20°C

	SKU	Size (Reactions)	Format
CleanPlex® Single-Indexed PCR Primers v2 for Ion Torrent Set T	716016	8	8 tubes
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set C	716053	192	96 indexes, (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set D	716054	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set E	716055	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set F	716056	192	96 indexes (96-well plate)

- 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)	Reactions 1-pool panels	Reactions 2-pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~9	~8
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~45	~44
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~180	~173
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~540	~520
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~4,050	~3,950

- For PCR tubes or strips, CleanMag Magnetic Rack (SKU 719001) or equivalent magnetic racks designed for PCR strip workflows
- For 96-well PCR plates, CleanMag Magnetic Plate (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 Bead solution is shipped at room temperature or on blue ice (ice packs). **Magnetic beads may be frozen during transit.** This single freeze thaw will not affect the performance of the beads. 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 with other reagents. 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.

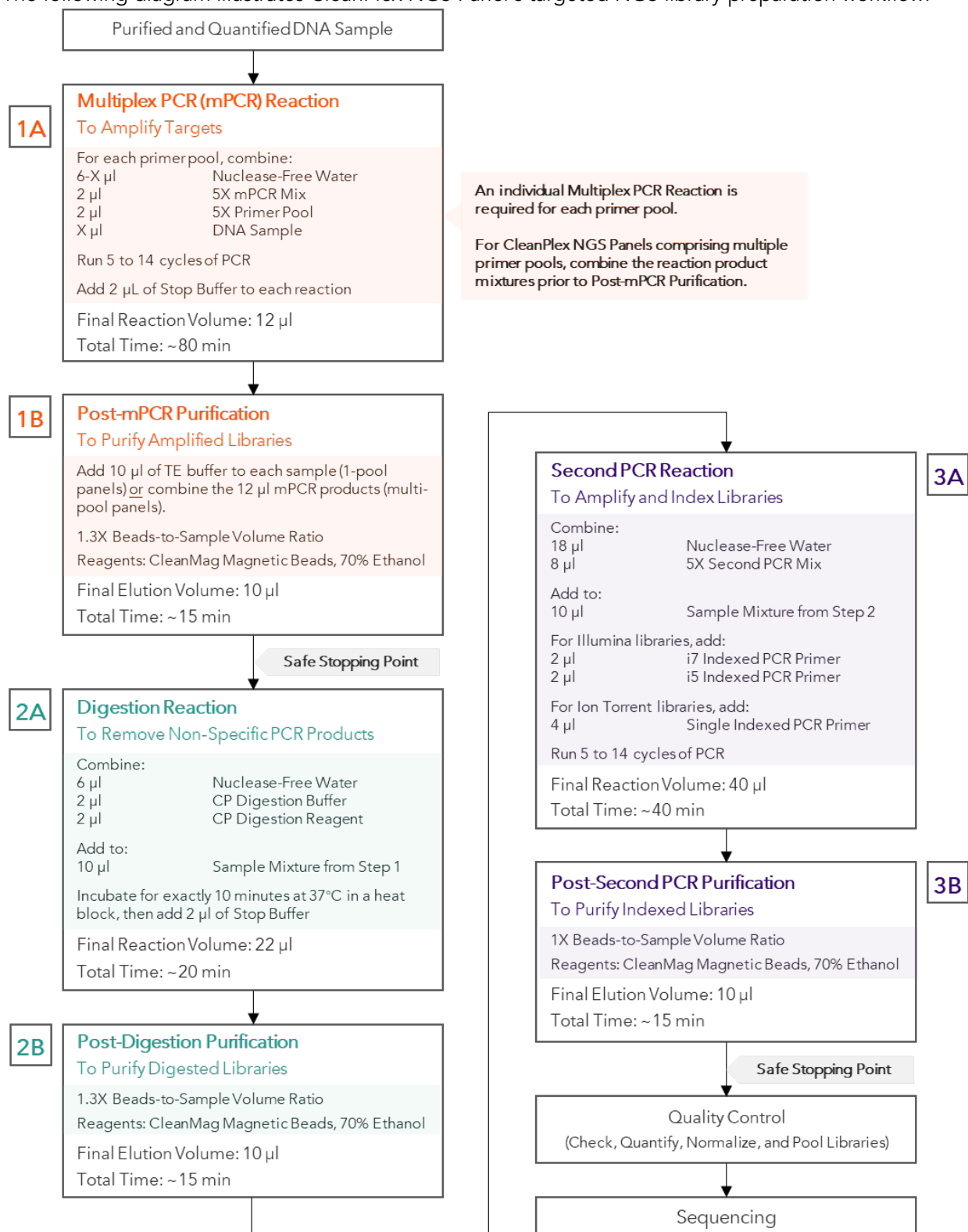
The components containing enzymes (5X mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

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 NGS Panel's 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 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 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 pipette or vortex 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 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 cyclers, 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 OncoZoom Cancer HotSpot Panel	10–40 ng	10 ng	20 ng
CleanPlex BRCA1 & BRCA2 Panel	10–40 ng	10 ng	20 ng
CleanPlex TP53 Panel	10–40 ng	10 ng	20 ng
CleanPlex Mitochondrial Disease Panel	1–10 ng	10 ng	10 ng
CleanPlex CFTR Panel	5–40 ng	10 ng	20 ng
CleanPlex ACE2 & TMPRSS2 Panel	10–40 ng	10 ng	20 ng
CleanPlex Custom NGS Panels (Human genotyping)	1–40 ng	10 ng	20 ng
CleanPlex Custom NGS Panels (Human somatic mutation detection)	10–40 ng	10 ng	20 ng

- The maximum volume of DNA input per Multiplex PCR Reaction is 6 µl. For CleanPlex NGS Panels with multiple 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.
- After DNA extraction, avoid diluting DNA samples to < 10 ng/µl when possible for prolonged storage since DNA is less stable in solution at lower concentrations.
- Avoid freeze-thawing dilute DNA samples when possible, and measure sample concentrations immediately prior to use to avoid inaccurate sample input.
- 1–40 ng of human genomic DNA from normal or FFPE tissue is recommended for each Multiplex PCR Reaction depending on the panel and application.
- A minimum of 10 ng of human genomic DNA is recommended for detecting somatic variants with 1% frequency.
- 1–10 ng of DNA is recommended for genotyping applications that do not require low allele frequency detection. In rare cases where DNA is extremely limited and the application allows, 0.1 ng of DNA can be used.
- 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.

- For panels specific to organisms other than humans, DNA input need will vary based on the genome size of the organism. For example, significantly less DNA input is needed for bacteria panels as each nanogram of DNA contains many more copies of the target DNA. An input titration can be done to determine minimum DNA input.
- Compatible DNA buffering systems are Tris HCl or TE.

Set up and Preparations

- When working with multiple samples, it is recommended to 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 the recommended DNA input to each reaction.
- Bring Magnetic bead solution to room temperature for at least 30 minutes before use. Replace in 2-8 °C storage at the end of the day.
- Freshly prepare 10mL (per8 reactions) of 70% Ethanol by combining 7mL of 100% ethanol and 3mL nuclease-free water at volume ratios of 7 to 3 respectively. 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. Lower concentration of ethanol will affect final library yield.
- For all samples to be sequenced together, assign a specific index (CleanPlex Single-Indexed PCR Primers for Ion Torrent) or index combination (CleanPlex Dual-Indexed PCR Primers for Illumina) to each sample.
- Note the safe stopping points after mPCR purification and 2nd PCR purification. After starting Digestion Step, samples must be carried through to 2nd PCR purification without stopping. Plan accordingly.
- Upon initial receipt, Stop Buffer can be thawed and stored continuously at room temperature.

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. For CleanPlex NGS Panels with multiple primer pools, prepare individual reaction for each primer pool.

Note: When working with multi-pool panels, such as CleanPlex BRCA1 & BRCA2 Panel, the recommended amount of DNA is to be added to **each** pool, and not divided between the pools.

Note: Thaw and keep Stop Buffer and Magnetic Beads at room temperature for use at the end of step 1A and beginning of 1B.

mPCR Reaction Mixture		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	6 – X μ l
5X mPCR Mix	Green	2 μ l
5X mPCR Primer Pool	Varies	2 μ l
DNA Sample	—	X μ l
Total Volume per reaction		10 μ l



Important! 5X mPCR Mix and 5X mPCR Primer Pool(s) 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 cause decreased 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 with a heated lid at 105 °C and run the following thermal cycling protocol to amplify target DNA regions. Use the table below to determine the mPCR thermal cycling conditions for specific CleanPlex NGS Panels.

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	Refer to table below for cycle numbers
Annealing/Extension	60 °C	See table below	2 °C/s	
Hold	10 °C	∞		

* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/S.

Note: For CleanPlex Custom NGS panels, use per pool amplicon number to determine the appropriate annealing/extension times using the table below. *Typically, a custom multi-pool gene panel from a single design will evenly split the total amplicons into each primer pool. Please refer to the "ampinsert" file of your custom design to determine the amplicon count per pool, or contact Technical Support if you have any questions.*

Panel Specific mPCR Thermal Cycling Conditions		
Panel	Annealing/Extension Time	mPCR Cycle Number
CleanPlex OncoZoom Cancer HotSpot Panel	5 min	10
CleanPlex BRCA1 & BRCA2 Panel	5 min	10
CleanPlex TP53 Panel	5 min	14
CleanPlex Mitochondrial Disease Panel	5 min	5
CleanPlex CFTR Panel	5 min	13
CleanPlex ACE2 & TMPRSS2 Panel	5 min	10
CleanPlex Custom NGS Panels	5 min for 7–2,000 amplicons per pool 8 min for 2,000–5,000 amplicons per pool 16 min for 5,000–20,000 amplicons per pool	10

- 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 now 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! Ensure the Magnetic Bead Solution has come to room temperature before use.



Important! Use freshly prepared 70% ethanol. Poorly stored mixture can cause have reduced ethanol composition and may result in lower yields.

- 1B.1. Open the tubes or carefully remove the adhesive film from the PCR plate.
- For CleanPlex NGS Panels comprising of one primer pool, add 10 µl of TE buffer to each sample.
 - For CleanPlex NGS Panels with multiple primer pools, combine the 12 µl multiplex PCR products for each pool for each sample into one tube and perform purification on the combined product. See table below for examples.
- 1B.2. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a **1.3X** bead-based purification by adding magnetic beads to the 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.

Post-mPCR Purification — 1.3X Beads-to-Sample Volume Ratio				
	1-Pool	2-Pool	3-Pool	4-Pool
Volume of Combined Sample per reaction	22 µl (12 µl of sample + 10 µl of TE Buffer)	24 µl	36 µl	48 µl
Volume of Magnetic Beads per reaction	29 µl	31 µl	47 µl	62 µl



Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets, **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

- 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.*

- 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.



Important! Removing all residual supernatant from the Multiplex PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin step ensures complete removal of supernatant.

***Note:** A strong compatible magnetic rack is essential. If your magnetic rack or plate cannot collect the magnetic beads effectively, please look into a replacement.*

- 1B.6. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **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 open 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. 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. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove 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, then Second PCR Reaction. Plan accordingly.

Note: When thawing CP Reagent Buffer, bring the tube to room temperature. Ensure all visible precipitate is dissolved by vortexing as needed.

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

Note: If not already, keep magnetic beads and freshly prepared 70% ethanol ready at RT

- 2A.1. Prepare Digestion Reaction Master Mix and add directly into the same wells of each purified sample from Step 1B. No tube-to-tube transfer is needed and the magnetic beads from the previous reaction are carried onto the next steps and do not affect future reactions.

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 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 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



Important! Ensure the Magnetic Bead Solution has come to room temperature before use.



Important! Use freshly prepared 70% ethanol. Poorly stored mixture can have reduced ethanol composition and may result in lower yields.

- 2B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1.3X** bead-based purification by adding **29 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-Digestion Purification — 1.3X Beads-to-Sample Volume Ratio

Reagent	Volume per reaction
Digestion Reaction Product	22 µl
Magnetic Beads	29 µl



Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets, **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

- 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.



Important! Removing all residual supernatant from the Digestion Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin step ensures complete removal of supernatant.

- 2B.5. Add 180 μ l of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **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 open 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. Remove tubes/wells from the magnetic rack and add 10 μ l TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.
- 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 or unique combination of dual index to each sample before starting this step. Either combinatorial or Unique Dual-Index primers can be used for any CleanPlex libraries.

- 3A.1. Thaw Indexed PCR Primers, vortex thoroughly, then spin briefly 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.

Note: For Illumina indexes in plates, the i5 and i7 indexes are already combined. Use 4 μ l of the pooled index per sample. For separate indexes in tubes, use 2 μ l each as show below.

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 μ l

Second PCR Reaction Mixture		
Reagent	Illumina Volume per reaction	Ion Torrent Volume per reaction
Second PCR Reaction Master Mix	26 μ l	26 μ l
Purified Sample from Step 2B	10 μ l	10 μ l
i5 Indexed PCR Primer for Illumina	2 μ l	—
i7 Indexed PCR Primer for Illumina	2 μ l	—
Single-Indexed PCR Primer for Ion Torrent	—	4 μ l
Total Volume per reaction	40 μ l	40 μ l

Note: For Illumina indexes in plates, the i5 and i7 indexes are already combined. Use 4 μ l of the pooled index per sample. For separate indexes in tubes, use 2 μ l each as shown above.



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 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 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. Use the three tables below for thermal cycling protocol and cycle number suggestions for CleanPlex Ready-to-Use NGS Panels and CleanPlex Custom NGS Panels.

Note: The Second PCR thermal cycling protocol depends on the starting DNA input amount and DNA quality. Generally, lower quality DNA, lower DNA input amount, or fewer amplicons in a panel requires more PCR cycles.

Note: Use the per primer pool DNA input to determine the correct cycles in the tables below. BRCA1 & BRCA2 Panel for example recommends 10 ng of DNA per primer pool. Refer to 10 ng to determine the Second PCR cycle number, not 20 ng of DNA.

Note: Nanogram input suggestions below are based on Human Genome DNA samples. For custom panels amplifying target regions of other organisms, use the genome size vs human to calculate nanogram equivalent as needed.

Note: For CleanPlex Custom NGS Panels, use the total number of amplicons to determine the appropriate Second PCR cycles.

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	Refer to table below for cycle numbers
Annealing/Extension	Illumina 60 °C	Ion Torrent 68 °C	75 sec	2 °C/s	
Hold	10 °C		∞		

* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/Ss..

CleanPlex Ready-to-Use NGS Panels — Second PCR Cycle Number		
Panel	10 ng of High Quality gDNA per Pool	10 ng of Low Quality DNA per Pool (eg. FFPE DNA)
CleanPlex OncoZoom Cancer HotSpot Panel	9	10
CleanPlex BRCA1 & BRCA2 Panel	13	14
CleanPlex TP53 Panel	10	11
CleanPlex Mitochondrial Disease Panel	8	9
CleanPlex CFTR Panel	9	10
CleanPlex ACE2 & TMPRSS2 Panel	10	11

CleanPlex Custom NGS Panels — Second PCR Cycle Number		
Total Number of Amplicons* in Panel	10 ng of High Quality gDNA per Pool	10 ng of Low Quality DNA per Pool (eg. FFPE DNA)
7 - 100	13	14
101 - 200	11	12
201 - 500	10	11
501 - 1,000	9	10
1,001 - 2,000	8	9
2,001 - 5,000	7	8
5,001 - 12,000	6	7
12,001 - 20,000	5	6

Note: For optimal yield, CleanPlex Custom NGS panels may require adjustment to Second PCR cycle numbers based on the sample quality, panel design, amplicon number, and application.

Second PCR Reaction — Input Specific Cycle Number	
Input DNA per Pool	Change in Cycle Number
0.1 ng	+ 7
1 ng	+ 3 to + 4
5 ng	+ 1
10 ng	-
20 ng	- 1
40 ng	- 2
Low Quality 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.

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



Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets, **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

- 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.



Important! Removing all residual supernatant from the Second PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin step ensures complete removal of supernatant.

- 3B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **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. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads. 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, which will affect QC and sequencing.

Quality Control Prior to Sequencing

Check library quality and concentration using a high sensitivity fragment analyzer such as Agilent 2100 Bioanalyzer Instrument and Agilent High Sensitivity dsDNA Kit, or a qPCR-based method. The final library can also be quantified using a Qubit Fluorometer or equivalent. However, this method will only give you the absolute yield and not differentiate potential background from the actual library. To confirm the quality of the DNA, it is highly recommended that a high sensitivity fragment analyzer is used to visualize the peak shape, size, and potential background concentration.

CleanPlex Ready to Use typically have a yield of 6 nM to 30nM, depending on DNA sample quantity/quality and cycle numbers. Custom panel yield might be more variable. Library quantity is not indicative of library quality. Additional input or 2nd PCR cycles can be adjusted accordingly for optimal yield for your downstream processing or sequencing needs.

Expected library peak size and shapes for Ready-to-use kits can be found in the troubleshooting guide at the end of this user guide. The expected amplicon size distribution for Custom Panels can be found in the design files folder, available for download via your account through our website:

Custom panels → **ParagonDesigner Portal** → [View Designs](#).

Please see troubleshooting guide towards the end of this document if you observe nonspecific peaks or unexpected yield.

After confirmation of library quality, the libraries can be normalized using library peak(s) concentrations only, not including the nonspecific product(s) concentration(s). Then samples with unique index combinations can be pooled for sequencing.

Recommended Sequencing Length and Depth

All CleanPlex Ready-to-Use NGS Panels are designed to be compatible with Illumina PE 150 bp reads (2x150 bp) and 200bp on Ion Torrent. CleanPlex Custom NGS Panels also have standard 2x150 bp designs unless otherwise decided and communicated between the customer and the Paragon Genomics panel design team. It is recommended to follow Illumina's suggestions for PhiX concentration according to sequencer used and incorporate that calculate in the final sample multiplexing calculation.

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 read depth is 5,000X. Based on results, depth can be further reduced to maintain sufficient coverage but also increase sample multiplexing efficiency.

Recommended Sequencing Depth		
Panel	Application	Suggested Average Read Depth (Paired End Reads)
CleanPlex OncoZoom Cancer HotSpot Panel	Somatic Mutations	2,000-5,000X
CleanPlex BRCA1 & BRCA2 Panel	Somatic Mutations	2,000-5,000X
	Germline Mutations	100x-500X
CleanPlex TP53 Panel	Somatic Mutations	2,000-5,000X
CleanPlex Mitochondrial Disease Panel	Germline Mutations	100x-500X
CleanPlex CFTR Panel	Germline Mutations	100x-500X
CleanPlex ACE2 & TMPRSS2 Panel	Germline Mutations	100x-500x

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

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' -CAAGCAGAAGACGGCATAACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-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	TTGGACTC	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.



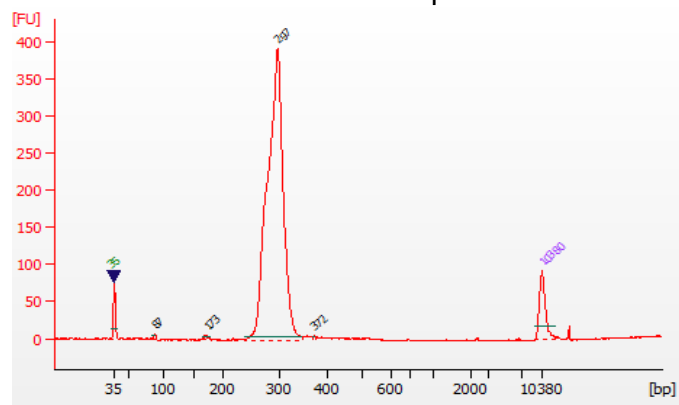
For plated indexes v2 sets C, D, E, and F, plate map and sequences information can be downloaded from [Product Document Page](#) under Indexes.

Troubleshooting Guide

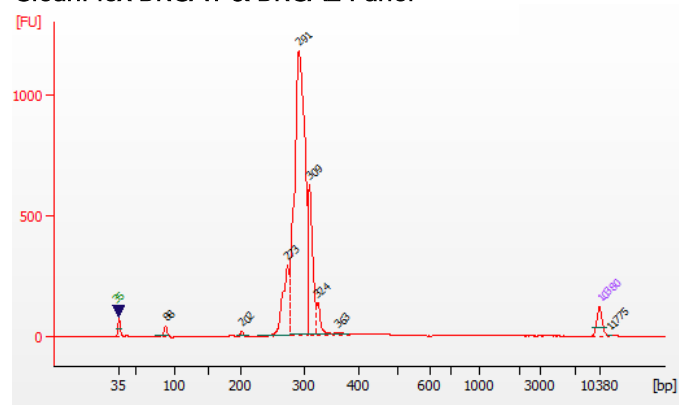
Examples of Libraries Prepared with CleanPlex NGS Panels

Depending on the CleanPlex NGS Panel used, library peak(s) should be between 200 and 400 bp. Below are representative Agilent Bioanalyzer traces generated for various CleanPlex Ready-to-Use Panels using 10 ng of gDNA as input.

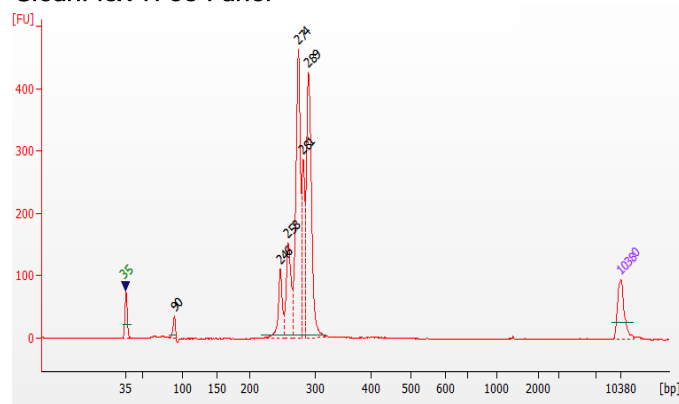
CleanPlex OncoZoom Cancer HotSpot Panel



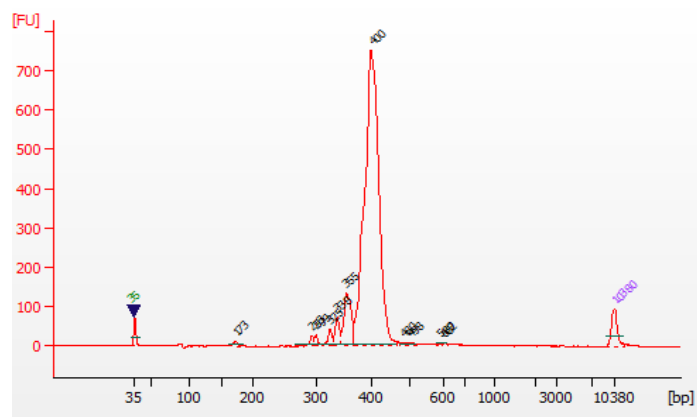
CleanPlex BRCA1 & BRCA2 Panel



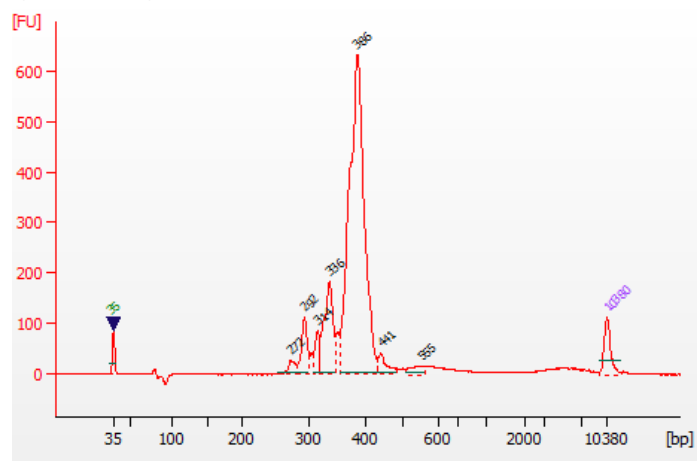
CleanPlex TP53 Panel



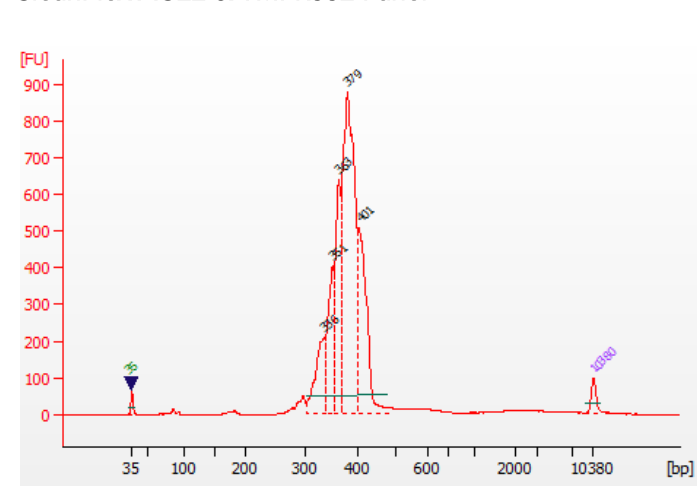
CleanPlex Mitochondrial Disease Panel



CleanPlex CFTR Panel



CleanPlex ACE2 & TMPRSS2 Panel



Potential Causes for Extra Peaks and Suggested Solutions

Peaks around 70–90 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 the specified amount of indexed primers to Second PCR Reaction.

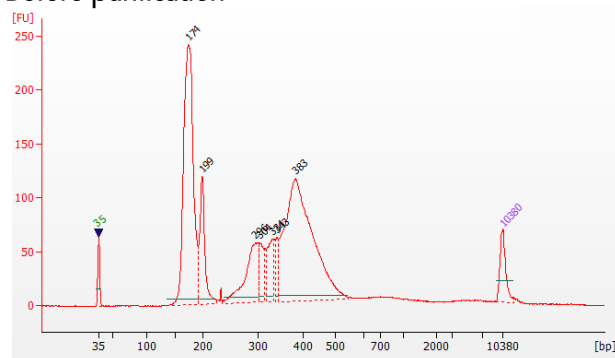
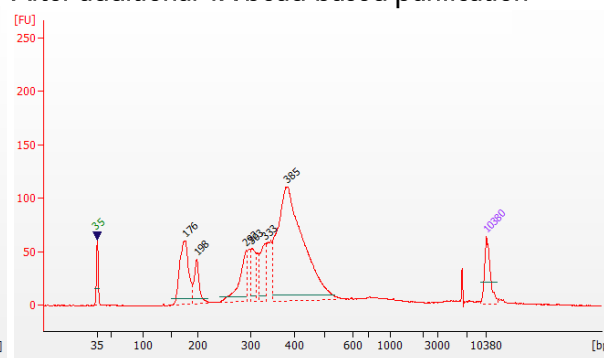
Peaks around 150–190 bp are residues of digested non-specific amplification products and adapter dimers. They come from incomplete removal of low molecular weight DNA fragments during the Post-Digestion Purification or nonspecific products formed during or after the Multiplex PCR (mPCR) Reaction due to deviation from protocol or high concentration of the 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 this 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 >20 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 used in the Second PCR Reaction. Only use CleanPlex Index primers with CleanPlex library prep. Use the appropriate index primers for the sequencer of choice.
- 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 the Broad Institute's GATK Best Practice (<https://software.broadinstitute.org/gatk/best-practices/>) as general guiding principles for sequencing data analysis. Analysis can also be performed using Illumina's DNA Amplicon Module, following Illumina's user guide.

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 customer panels, please refer to your specified reference genome. Bwa mem is recommended for read mapping and de-duplication procedure shall be skipped. For c

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 ampInsert 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.

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