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® 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/

UG1001-06
October 2019

Revision History

Document	Date	Description of Change
UG1001-01		• Initial version
UG1001-02	September 2018	• 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	• Fixed typos • Updated Required Materials and Equipment Not Provided section • Added instructions for working with 96-well PCR plates
UG1001-04	February 2019	• Updated Workflow diagram to remove two safe stopping points for more robust results • Included additional tips under Best Practices
UG1001-05	July 2019	• 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	• 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.

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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
• Human • Mammals • Fish • Insects • Plants • Microorganisms	• 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	• Tumor profiling • Variant detection and discovery • Disease predisposition • Species identification • High-throughput genotyping • Gene expression profiling

Compatible Sequencing Instruments

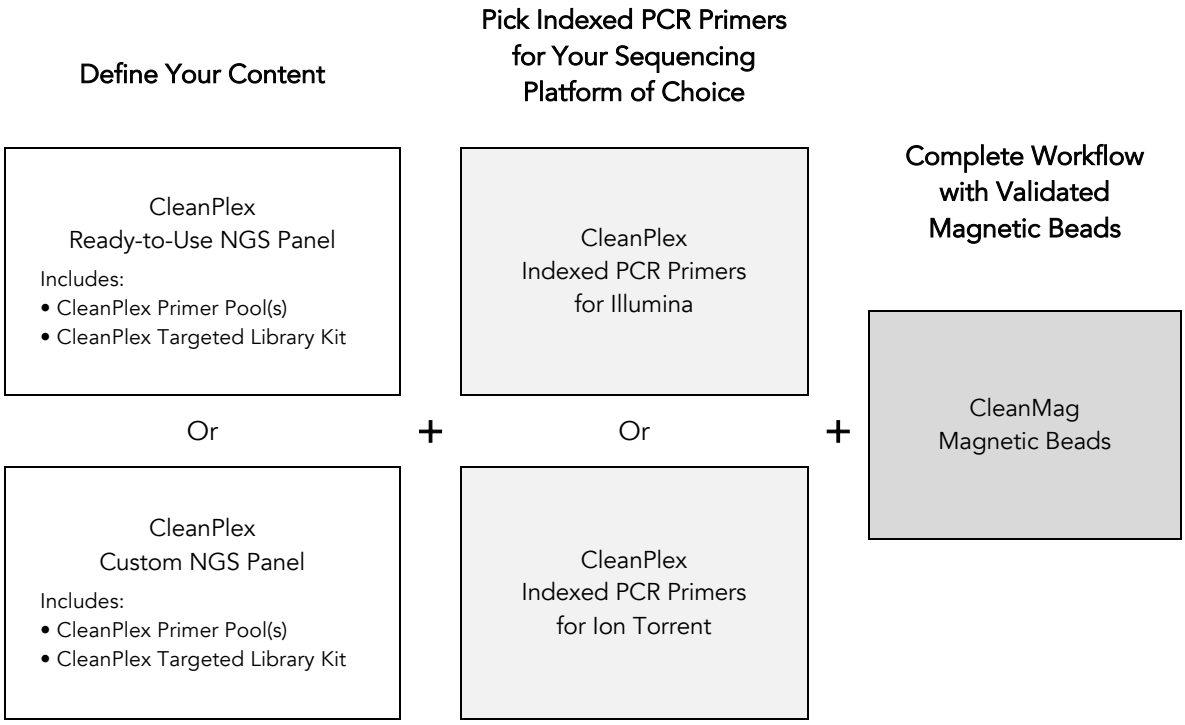
CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, MiSeq® System, MiniSeq® System, and iSeq® 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 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	Primer Pool 3	Primer Pool 4	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 Custom NGS Panels	---	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 A1	716005	16	4 i7 indexes + 4 i5 indexes (8 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 Single-Indexed PCR Primers for Ion Torrent — Store at –20°C

	SKU	Size (Reactions)	Format
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set A	716007 716008	32 96	16 indexes (16 tubes)
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set B	716009 716010	32 96	16 indexes (16 tubes)
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set C	716025 716029	192 960	96 indexes (96-well plate)
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set D	716026 716030	192 960	96 indexes (96-well plate)
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set E	716027 716031	192 960	96 indexes (96-well plate)
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set F	716028 716032	192 960	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	Reactions 4-pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~9	~8	~7
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~45	~44	~32
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~540	~520	~445
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~4,050	~3,950	~3,400

- 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). 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.

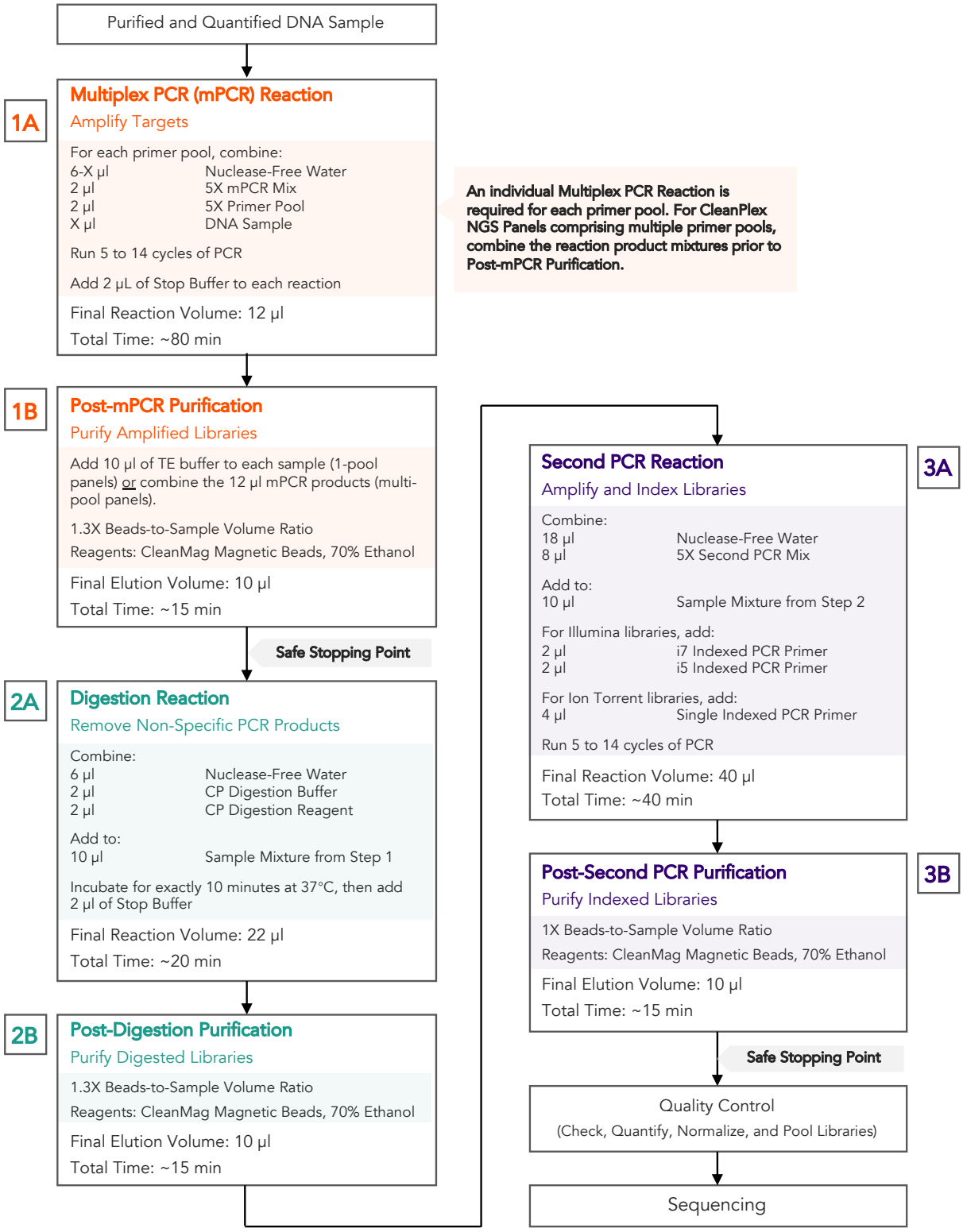
The components containing enzymes (5X mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

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 mix each assembly to ensure solutions are homogenous 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 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 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	20 ng
CleanPlex CFTR Panel	5–40 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 70% Ethanol by combining 100% ethanol and 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.

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.*

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

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 homogenous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: *It is crucial that the reaction mixture is homogenous 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 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, use the default settings.

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 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. Lower concentration ethanol 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 sample. See table below for examples.

1B.2. Vortex the magnetic beads suspension vigorously until homogenous. 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 homogenous.

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 and remove 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 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. 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.*

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 vortexing vigorously for at least 5 seconds until homogenous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: *It is crucial that the reaction mixture is homogenous 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 **1.3X** 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 homogenous.

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 Multiplex PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin and remove step ensures complete removal of supernatant.

2B.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.

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.*

1B.11. 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.

2B.8. 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.*

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 µl of master mix to each sample.*

Second PCR Reaction Master Mix

Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	18 µl
5X 2nd PCR Mix	Blue	8 µl
Total Volume per reaction		**26 µl**

Second PCR Reaction Mixture

Reagent	Illumina Volume per reaction	Ion Torrent Volume per reaction
Second PCR Reaction Master Mix	26 µl	26 µl
Purified Sample from Step 2B	10 µl	10 µl
i5 Indexed PCR Primer for Illumina	2 µl	—
i7 Indexed PCR Primer for Illumina	2 µl	—
Single-Indexed PCR Primer for Ion Torrent	—	4 µl
Total Volume per reaction	**40 µl**	**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 vortexing vigorously for at least 5 seconds until homogenous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: *It is crucial that the reaction mixture is homogenous 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 ablow. 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*: 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, use the default settings.

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 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 homogenous.

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 for 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 and remove 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. 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.

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 PE 150 bp reads (2x150 bp). 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.

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.

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

For recommended sample multiplexing on various Illumina NGS instruments, refer to 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'-AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATC*T**-3'**

i7 Indexed Primer
5'-CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T**-3'**

Index Sequences

CleanPlex Dual-Indexed PCR Primers for Illumina Set A1
4 x 4 indexes, 16 reactions (SKU 716005)

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	i5 Bases for Sample Sheet 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

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	i5 Bases for Sample Sheet 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	GGAACTTA
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
Q7005	ATATTCAC	GTGAATAT
Q7006	GCGCCTGT	ACAGGCGC
Q7007	ACTCTATG	CATAGAGT
Q7008	GTCTCGCA	TGCGAGAC
Q7015	AGTAGAGA	TCTCTACT
Q7016	GACGAGAG	CTCTCGTC
Q7017	AGACTTGG	CCAAGTCT
Q7018	GAGTCCAA	TTGGACTC
Q7023	AATTCTGC	GCAGAATT
Q7024	GGCCTCAT	ATGAGGCC
Q7025	ATCTTAGT	ACTAAGAT
Q7026	GCTCCGAC	GTCGGAGC

i5 Index	Index Sequence	i5 Bases for Sample Sheet	
		MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q5002	GATATCGA	GATATCGA	TCGATATC
Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q5009	CCAACAGA	CCAACAGA	TCTGTTGG
Q5010	TTGGTGAG	TTGGTGAG	CTCACCAA
Q5013	AACCGCGG	AACCGCGG	CCGCGGTT
Q5014	GGTTATAA	GGTTATAA	TTATAACC

Single-Indexed PCR Primers for Ion Torrent

Primer Sequences

Each sample is indexed by two pairs of Indexed PCR Primers for sequencing on Ion Torrent platforms. XXXXXXXXX denotes the index region of the primer. Index sequences are listed below.

Indexed PCR Primers

5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG**XXXXXXXXX**CGATTTCAGACGTGTGCTCTTCCGATC*T**-3′**
5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG**XXXXXXXXX**CGATCCTACACGACGCTCTTCCGATC*T**-3′**

Universal PCR Primers

5′-CCGCTTTCCTCTCTATGGGCAGTCGGTGATTTCAGACGTGTGCTCTTCCGATC*T**-3′**
5′-CCGCTTTCCTCTCTATGGGCAGTCGGTGATCCTACACGACGCTCTTCCGATC*T**-3′**

Index Sequences

CleanPlex Single-Indexed PCR Primers for Ion Torrent Sets A and B contain index sequences identical to the sequences of the equivalent Ion Xpress™ barcodes. These indexed PCR primers are provided in individual tubes.

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set A 16 indexes, 32 reactions (SKU 716007) 16 indexes, 96 reactions (SKU 716008)	
Index	**Index Sequence**
1	CTAAGGTAAC
2	TAAGGAGAAC
3	AAGAGGATTC
4	TACCAAGATC
5	CAGAAGGAAC
6	CTGCAAGTTC
7	TTCGTGATTC
8	TTCCGATAAC
9	TGAGCGGAAC
10	CTGACCGAAC
11	TCCTCGAATC
12	TAGGTGGTTC
13	TCTAACGGAC
14	TTGGAGTGTC
15	TCTAGAGGTC
16	TCTGGATGAC

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set B 16 indexes, 32 reactions (SKU 716009) 16 indexes, 96 reactions (SKU 716010)	
Index	**Index Sequence**
17	TCTATTCGTC
18	AGGCAATTGC
19	TTAGTCGGAC
20	CAGATCCATC
21	TCGCAATTAC
22	TTCGAGACGC
23	TGCCACGAAC
24	AACCTCATTC
25	CCTGAGATAC
26	TTACAACCTC
27	AACCATCCGC
28	ATCCGGAATC
29	TCGACCACTC
30	CGAGGTTATC
31	TCCAAGCTGC
32	TCTTACACAC

CleanPlex Single-Indexed PCR Primers 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.

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set C – Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set C
96 Indexes, 192 reactions (SKU 716025)
96 Indexes, 960 reactions (SKU 716029)

Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
1	CTAAGGTAAC	GGTGAT	25	CCTGAGATAC	GGTGAT
2	TAAGGAGAAC	GGTGAT	26	TTACAACCTC	GGTGAT
3	AAGAGGATTC	GGTGAT	27	AACCATCCGC	GGTGAT
4	TACCAAGATC	GGTGAT	28	ATCCGGAATC	GGTGAT
5	CAGAAGGAAC	GGTGAT	29	CGAGGTTATC	GGTGAT
6	CTGCAAGTTC	GGTGAT	30	TCCAAGCTGC	GGTGAT
7	TTCGTGATTC	GGTGAT	31	TCTTACACAC	GGTGAT
8	TTCCGATAAC	GGTGAT	32	TTCTCATTGAAC	GGTGAT
9	TGAGCGGAAC	GGTGAT	33	TCGCATCGTTC	GGTGAT
10	CTGACCGAAC	GGTGAT	34	TAAGCCATTGTC	GGTGAT
11	TCCTCGAATC	GGTGAT	35	CTTGAGAATGTC	GGTGAT
12	TAGGTGGTTC	GGTGAT	36	TGGAGGACGGAC	GGTGAT
13	TCTAACGGAC	GGTGAT	37	TAACAATCGGC	GGTGAT
14	TTGGAGTGTC	GGTGAT	38	CTGACATAATC	GGTGAT
15	TCTAGAGGTC	GGTGAT	39	TTCCACTTCGC	GGTGAT
16	TCTGGATGAC	GGTGAT	40	AGCACGAATC	GGTGAT
17	TCTATTCGTC	GGTGAT	41	TTGGAGGCCAGC	GGTGAT
18	AGGCAATTGC	GGTGAT	42	TGGAGCTTCCTC	GGTGAT
19	TTAGTCGGAC	GGTGAT	43	TCAGTCCGAAC	GGTGAT
20	CAGATCCATC	GGTGAT	44	TAAGGCAACCAC	GGTGAT
21	TCGCAATTAC	GGTGAT	45	TTCTAAGAGAC	GGTGAT
22	TTCGAGACGC	GGTGAT	46	TCCTAACATAAC	GGTGAT
23	TGCCACGAAC	GGTGAT	47	CGGACAATGGC	GGTGAT
24	AACCTCATTC	GGTGAT	48	TTGAGCCTATTC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set C (Continued)					
Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
49	CCGCATGGAAC	GGTGAT	73	CTAGGACATTC	GGTGAT
50	CTGGCAATCCTC	GGTGAT	74	CTTCCATAAC	GGTGAT
51	TCCACCTCCTC	GGTGAT	75	CCAGCCTCAAC	GGTGAT
52	CAGCATTAATTC	GGTGAT	76	CTTGGTTATTC	GGTGAT
53	TCCTTGATGTTC	GGTGAT	77	TTGGCTGGAC	GGTGAT
54	TCTAGCTCTTC	GGTGAT	78	TCCTGAATCTC	GGTGAT
55	TCACTCGGATC	GGTGAT	79	CTAACCACGGC	GGTGAT
56	TTCCTGCTTCAC	GGTGAT	80	CGGAAGGATGC	GGTGAT
57	CCTTAGAGTTC	GGTGAT	81	CTTGTCCAATC	GGTGAT
58	CTGAGTTCCGAC	GGTGAT	82	TCCGACAAGC	GGTGAT
59	TCCTGGCACATC	GGTGAT	83	CGGACAGATC	GGTGAT
60	CCGCAATCATC	GGTGAT	84	CCTTGAGGCGGC	GGTGAT
61	TTCAATTGGC	GGTGAT	85	TTCTTCCTCTTC	GGTGAT
62	CCTACTGGTC	GGTGAT	86	TTCTTCAAGATC	GGTGAT
63	TGAGGCTCCGAC	GGTGAT	87	CTTGGAACTGTC	GGTGAT
64	CGAAGGCCACAC	GGTGAT	88	TCGGCCGGAATC	GGTGAT
65	TCTGCCTGTC	GGTGAT	89	TGGAGATAATTC	GGTGAT
66	CGATCGGTTC	GGTGAT	90	TGAATTCCGGAC	GGTGAT
67	TCAGGAATAC	GGTGAT	91	CTTGCCACCGTC	GGTGAT
68	CGGAAGAACCTC	GGTGAT	92	CTAACAATTCAC	GGTGAT
69	CGAAGCGATTC	GGTGAT	93	TTCGCAATGAAC	GGTGAT
70	CAGCCAATTCTC	GGTGAT	94	TTCCGCACGGC	GGTGAT
71	TCGAAGGCAGGC	GGTGAT	95	TTGGCCAATTGC	GGTGAT
72	CCTGCCATTCGC	GGTGAT	96	TCTAGTTCAAC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set D – Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set D
96 Indexes, 192 reactions (SKU 716026)
96 Indexes, 960 reactions (SKU 716030)

Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
97	TGAGAAGAATTC	GGTGAT	121	CCAAGGCGAATC	GGTGAT
98	CCTCAACCATC	GGTGAT	122	CTTAGATCGGTC	GGTGAT
99	CCTGCTGGATTC	GGTGAT	123	CCGGTCCGATTC	GGTGAT
100	TGGCAGGAATTC	GGTGAT	124	TTGGAGCGAC	GGTGAT
101	CGCTTCGATTC	GGTGAT	125	CTTGTTCCGGC	GGTGAT
102	TTCCAGATTGC	GGTGAT	126	TCCGGCAAGATC	GGTGAT
103	TCCGGAGTCTTC	GGTGAT	127	TTCCTATCCGAC	GGTGAT
104	GCAACACGAC	GGTGAT	128	CTAATTGAATC	GGTGAT
105	TAAGCAATTCTC	GGTGAT	129	TTCGACACCAC	GGTGAT
106	CTGATCCATTC	GGTGAT	130	CCAGTTCCTC	GGTGAT
107	TAGGAACAATC	GGTGAT	131	TAACAATAATTC	GGTGAT
108	AACCGGAATTC	GGTGAT	132	CTGAAGTCGGAC	GGTGAT
109	CCGGAGGTAATC	GGTGAT	133	AAGGAATGGAAC	GGTGAT
110	TTCAGGACCTTC	GGTGAT	134	TTCCGAACCGAC	GGTGAT
111	TCTAACCAATGC	GGTGAT	135	TTCACCAGGATC	GGTGAT
112	TCCGAGCTGATC	GGTGAT	136	CTACAACTTC	GGTGAT
113	TTACCATGTTC	GGTGAT	137	CTGAGGCATCAC	GGTGAT
114	CTCATTCCGGTC	GGTGAT	138	CCAGCATCATTC	GGTGAT
115	TCGAGGCCTGGC	GGTGAT	139	CCGGCTTGAAC	GGTGAT
116	TGGAAGGTTGC	GGTGAT	140	TCAGGCAGATTC	GGTGAT
117	TAGGATTCCGAC	GGTGAT	141	TTCTGCACGATC	GGTGAT
118	TTGAAGCTCCGC	GGTGAT	142	TCCGAAGATAAC	GGTGAT
119	TTCAACTTCTTC	GGTGAT	143	CCTCATCGTTC	GGTGAT
120	TTAGGCTCAAC	GGTGAT	144	TGCAACCAAC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set D (Continued)					
Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
145	CGGAATCCGGTC	GGTGAT	169	CTTAAGGCTGAC	GGTGAT
146	TCTTGAGGAAGC	GGTGAT	170	CTGGAGAACCAC	GGTGAT
147	CCGCCACCAATC	GGTGAT	171	TACTTGGAATC	GGTGAT
148	AAGGTTATTC	GGTGAT	172	CTAGGCCTCCTC	GGTGAT
149	TCTCCATCAATC	GGTGAT	173	CCGAGAACAAC	GGTGAT
150	TGGAGCCAACAC	GGTGAT	174	TTAAGACGTC	GGTGAT
151	TCTAATCGATTC	GGTGAT	175	TGGCTTCATC	GGTGAT
152	CCACCAATAC	GGTGAT	176	CGAACAATTGTC	GGTGAT
153	CTTGGATTCGAC	GGTGAT	177	TTCAAGGTGTTC	GGTGAT
154	TTCTGGATTATC	GGTGAT	178	CTTAACCACCAC	GGTGAT
155	TTCTTCTGGC	GGTGAT	179	TCCGGACCGTTC	GGTGAT
156	TCCTGAGACTC	GGTGAT	180	CCTTGAGCATGC	GGTGAT
157	CTGGAACAAGAC	GGTGAT	181	TCTTAGATATTC	GGTGAT
158	TCTTGCTTAATC	GGTGAT	182	CCTGAATTAC	GGTGAT
159	CTCCAATTGGAC	GGTGAT	183	AAGCCAACCAAC	GGTGAT
160	CTAAGGAAGGTC	GGTGAT	184	TCTGGCAACGGC	GGTGAT
161	TGAAGGCACCTC	GGTGAT	185	CTAGGAACCGC	GGTGAT
162	ACAATCCGGTTC	GGTGAT	186	TTAAGCGGTC	GGTGAT
163	TCCTTACAGAAC	GGTGAT	187	TTGGCATCTC	GGTGAT
164	TGAATCGAAC	GGTGAT	188	TTGGTTCCAAC	GGTGAT
165	CTTGAAGCCGTC	GGTGAT	189	TTAGGCTGATTC	GGTGAT
166	TTGAGATCAATC	GGTGAT	190	TGGAACCACGTC	GGTGAT
167	CAGCAATTCGAC	GGTGAT	191	AGGCAACGGAAC	GGTGAT
168	CGAAGCTAATC	GGTGAT	192	TCCTCCTCCAC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set E – Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set E
96 Indexes, 192 reactions (SKU 716027)
96 Indexes, 960 reactions (SKU 716031)

Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
193	TCTCATTCATTC	GGTGAT	217	TCTTAGGTAATC	GGTGAT
194	TTCGGAACGTTC	GGTGAT	218	TCCAGGCTTATC	GGTGAT
195	TTAAGATTATC	GGTGAT	219	TGAATTCTTC	GGTGAT
196	CCTTATGGATTC	GGTGAT	220	AAGGCCTCGAAC	GGTGAT
197	CTTGAACAGGTC	GGTGAT	221	TTCCTTCAACAC	GGTGAT
198	CCGAACCTATC	GGTGAT	222	TGGTTGGATTC	GGTGAT
199	CCAGGACGTC	GGTGAT	223	CGGCAACATTC	GGTGAT
200	CCTTGTCGTC	GGTGAT	224	TGCATTCCGGTC	GGTGAT
201	CCTGGCTCAATC	GGTGAT	225	CGGAAGCATCAC	GGTGAT
202	CTGAGGCTTGTC	GGTGAT	226	CTTGGAGTCCTC	GGTGAT
203	CCGAACCAACGC	GGTGAT	227	CGGACCACGGAC	GGTGAT
204	CCTAGATTAATC	GGTGAT	228	TTGCCAACCGGC	GGTGAT
205	TCAACCACAAC	GGTGAT	229	CTGTTCGAAC	GGTGAT
206	AGGCCATTGATC	GGTGAT	230	CCGAGTGGTC	GGTGAT
207	TCGAGAATCGGC	GGTGAT	231	CAAGGCTTCCAC	GGTGAT
208	TTCTGCCACTTC	GGTGAT	232	TCTTCATGAATC	GGTGAT
209	CTAAGCCATCTC	GGTGAT	233	TTGACATTAATC	GGTGAT
210	CCTTAGCTCGGC	GGTGAT	234	TCAGGCCGAAC	GGTGAT
211	TCTTAGGACGGC	GGTGAT	235	TTCCGCATTGAC	GGTGAT
212	CTTGCAATGGAC	GGTGAT	236	TGGAAGGTCCAC	GGTGAT
213	TCTAATGGTC	GGTGAT	237	TTAGCAACATTC	GGTGAT
214	CCTCCACGATC	GGTGAT	238	TCTTAGCGATC	GGTGAT
215	CCTAAGGCAGGC	GGTGAT	239	AAGCAATCCATC	GGTGAT
216	TCTGGAAGTCGC	GGTGAT	240	CCAAGTTGTTC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set E (Continued)					
Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
241	TGGACTCAATTC	GGTGAT	265	TCCTGGAGTAAC	GGTGAT
242	TCTGTAATTC	GGTGAT	266	TGACCAATCCAC	GGTGAT
243	TTGAAGGATCGC	GGTGAT	267	TGGAGGCCGGTC	GGTGAT
244	TTCTACCGGC	GGTGAT	268	TAGCATCCGGC	GGTGAT
245	TGGAAGAAGGAC	GGTGAT	269	TAAGTCGATC	GGTGAT
246	CCTGCCGGAATC	GGTGAT	270	TCGGCCATAC	GGTGAT
247	CGGCCTTCGGTC	GGTGAT	271	TTCCAATCCGTC	GGTGAT
248	CCTTGGCCTGGC	GGTGAT	272	TAGAATTCCGTC	GGTGAT
249	CTAGTCGAATTC	GGTGAT	273	CGAGATGAAC	GGTGAT
250	TAGACGGAATTC	GGTGAT	274	CCTGGTTGTC	GGTGAT
251	TCCTCCAAGTTC	GGTGAT	275	TTCTGAGCGTTC	GGTGAT
252	CCTAAGCTAC	GGTGAT	276	TCCAAGGACAC	GGTGAT
253	CTAACCGATTC	GGTGAT	277	CCAAGCAACGGC	GGTGAT
254	CCACATCGAAC	GGTGAT	278	TTCTTAAGTATC	GGTGAT
255	TCTTCCTTCCGC	GGTGAT	279	TTCGACTCGTC	GGTGAT
256	TGGACAATTGAC	GGTGAT	280	TCCGGAACTAC	GGTGAT
257	TTAGCCTTAAC	GGTGAT	281	CGGCCTCAATTC	GGTGAT
258	TCACCTCGTTC	GGTGAT	282	CAGCCTCCGGAC	GGTGAT
259	TCTGACATTCGC	GGTGAT	283	TCTGACGGAATC	GGTGAT
260	TCCGCTCGGAC	GGTGAT	284	TCCTTATTGAC	GGTGAT
261	TTCTTGATCATC	GGTGAT	285	TTCTCCATTATC	GGTGAT
262	CTGACTCCGGC	GGTGAT	286	AACTTCCGGATC	GGTGAT
263	GAAGATCTTC	GGTGAT	287	TCCACAATTAAC	GGTGAT
264	TTCCGAAGTCAC	GGTGAT	288	AAGGCTCGGTTC	GGTGAT

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set F – Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

CleanPlex Single-Indexed PCR Primers for Ion Torrent Set F
96 Indexes, 192 reactions (SKU 716028)
96 Indexes, 960 reactions (SKU 716032)

Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
289	CCGAGTCGATTC	GGTGAT	313	TCTTCAGGATTC	GGTGAT
290	TTCTTCACCAAC	GGTGAT	314	TTCCTTGGTCAC	GGTGAT
291	CTAGGCCAGGAC	GGTGAT	315	TTCCTTGCCGTC	GGTGAT
292	CCTGAAGGCTGC	GGTGAT	316	CTTAGGCAAGTC	GGTGAT
293	CTTGAAGGTCTC	GGTGAT	317	CGAGGATCCGTC	GGTGAT
294	CTCTCCGATTC	GGTGAT	318	TCCTCTTCCTC	GGTGAT
295	CTTAACATCCTC	GGTGAT	319	TCGAAGCTTCGC	GGTGAT
296	TTGAATGGTC	GGTGAT	320	CTAAGGTTCGAC	GGTGAT
297	TGAGGAATTCAC	GGTGAT	321	TTAGGAATCCGC	GGTGAT
298	TGGAAGCAAGTC	GGTGAT	322	TGGCCAATCGAC	GGTGAT
299	CTGAGCAATCTC	GGTGAT	323	CTTAAGCATTAC	GGTGAT
300	TCCAGCCATATC	GGTGAT	324	TTCCTCTAATTC	GGTGAT
301	CCTTACTCATC	GGTGAT	325	CTTCCATTCGAC	GGTGAT
302	TCCTTCCACTTC	GGTGAT	326	CCTACAAGATTC	GGTGAT
303	TGAACCATTGAC	GGTGAT	327	TCTTGAAGATGC	GGTGAT
304	TTAGGATCATTC	GGTGAT	328	TGAAGCCATCTC	GGTGAT
305	TTCCAATTCCAC	GGTGAT	329	CTAAGCTTGGTC	GGTGAT
306	TTCTGGTTCTTC	GGTGAT	330	CTTGGATAAC	GGTGAT
307	TTCTGTCCGC	GGTGAT	331	CAAGCCACCGTC	GGTGAT
308	TCCGAAGAGATC	GGTGAT	332	CCTAGGTCTTC	GGTGAT
309	TTACACGGAC	GGTGAT	333	TTGACTTCCGGC	GGTGAT
310	TAAGTCCAATTC	GGTGAT	334	CCAGACCGAAC	GGTGAT
311	TAAGATTCGGC	GGTGAT	335	TCTAGCCATCGC	GGTGAT
312	AGGCCTAATTC	GGTGAT	336	CGCCAAGAATC	GGTGAT

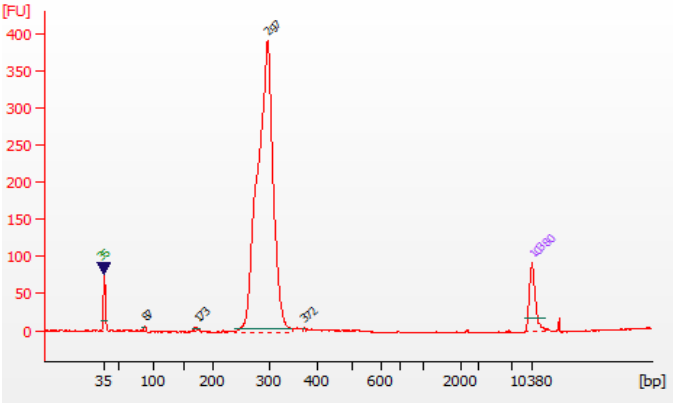
CleanPlex Single-Indexed PCR Primers for Ion Torrent Set F (Continued)					
Index	Index Sequence	Adapter	Index	Index Sequence	Adapter
337	TGAGGCATGGTC	GGTGAT	361	TTCATTCCGTTC	GGTGAT
338	CGAGATTCGGAC	GGTGAT	362	CTGGCATCGGAC	GGTGAT
339	TCAACCTGATC	GGTGAT	363	CTTGACCTGGTC	GGTGAT
340	TCCTGAGGTATC	GGTGAT	364	TTCTACCTCAAC	GGTGAT
341	AAGATGAATC	GGTGAT	365	AAGGAATCGTC	GGTGAT
342	CCTGGAAGACGC	GGTGAT	366	CCAACATTATC	GGTGAT
343	TCTAAGACTTC	GGTGAT	367	TCAAGAAGTTC	GGTGAT
344	CGAACATATTC	GGTGAT	368	CCGAACACTTC	GGTGAT
345	CGAGGCAATGAC	GGTGAT	369	TACATCCATC	GGTGAT
346	TTCGCCAACAC	GGTGAT	370	TCCGCCATGC	GGTGAT
347	TCGGCACGAATC	GGTGAT	371	TGCCTGGATC	GGTGAT
348	TCCGTTCGGTC	GGTGAT	372	TGGAGATTGGTC	GGTGAT
349	CCTTAGGATGGC	GGTGAT	373	CTAAGATCCGC	GGTGAT
350	CACCACCAATTC	GGTGAT	374	CTAGCCAATGAC	GGTGAT
351	TTGAAGCCAGGC	GGTGAT	375	CAAGAATAATTC	GGTGAT
352	CCTCCAATCGGC	GGTGAT	376	CTTGAGAATTAC	GGTGAT
353	TTACAATGAATC	GGTGAT	377	CACCATCCGGTC	GGTGAT
354	TCCTGCATGATC	GGTGAT	378	CTTGAAGACGAC	GGTGAT
355	CGCTTCCAAC	GGTGAT	379	TTCTACAATATC	GGTGAT
356	TGACAACTTC	GGTGAT	380	CGGAACCTTGC	GGTGAT
357	CTTGGCCAACTC	GGTGAT	381	AACAATTCGAAC	GGTGAT
358	TCTTGGCAATGC	GGTGAT	382	TTGCACCGTTC	GGTGAT
359	AAGGCATCCAAC	GGTGAT	383	TTGGACTTATTC	GGTGAT
360	CCGACCGGATTC	GGTGAT	384	TCGAGATTAATC	GGTGAT

Troubleshooting Guide

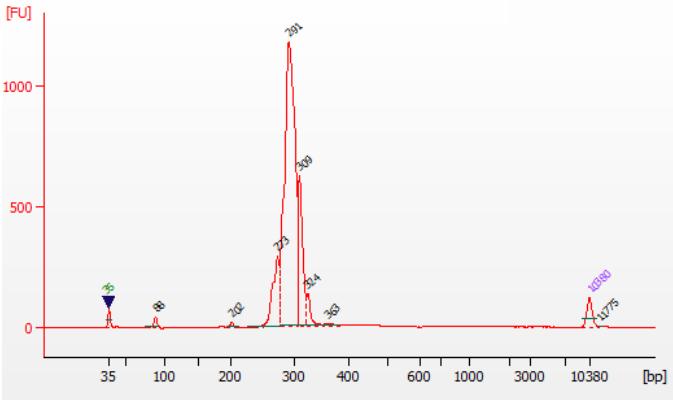
Examples of Libraries Prepared with CleanPlex NGS Panels

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.

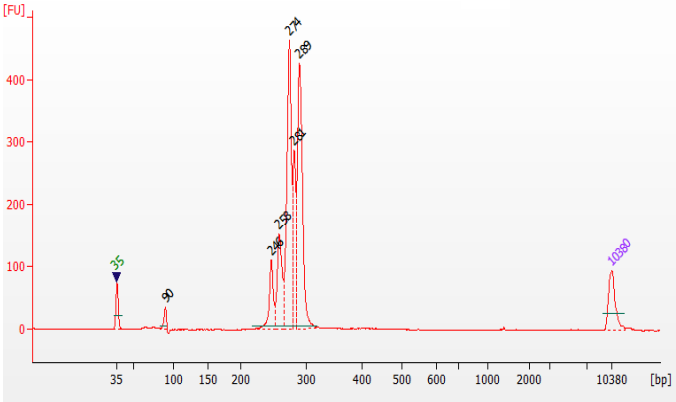
CleanPlex OncoZoom Cancer HotSpot Panel



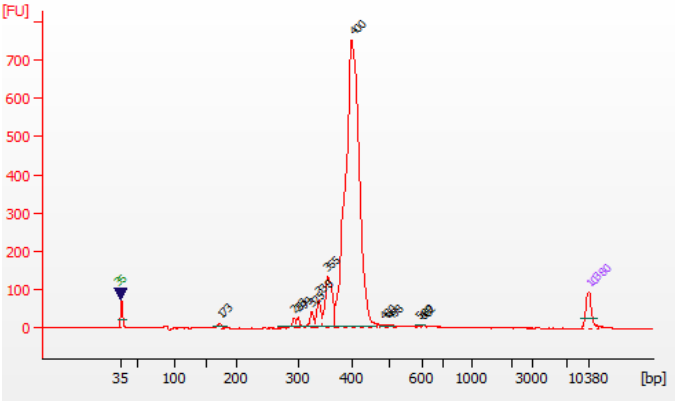
CleanPlex BRCA1 & BRCA2 Panel



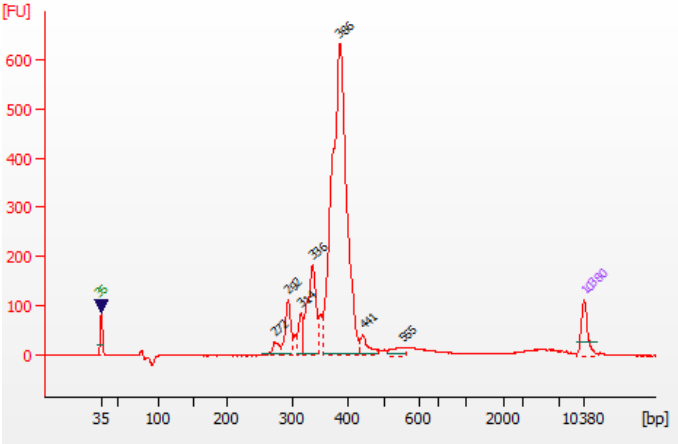
CleanPlex TP53 Panel



CleanPlex Mitochondrial Disease Panel



CleanPlex CFTR Panel



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 specified amount of indexed primers to Second PCR Reaction Primer.

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 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:

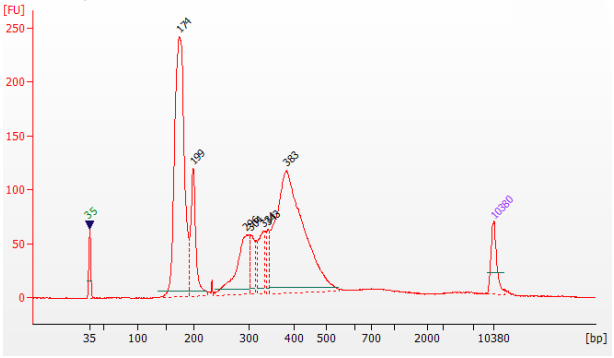
- Inaccurate pipetting of magnetic bead volume.
- Insufficient mixing of reaction solutions or bead and sample mixture.
- Insufficient removal of supernatant and ethanol washes during purification.
- Allowing mPCR products to sit for too long (and form nonspecific products) before purification.
- CleanPlex 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.

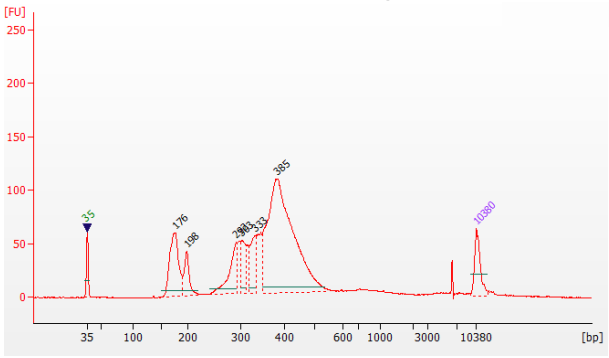
Removing nonspecific products from final libraries

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 >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 using in the Second PCR Reaction. Only use CleanPlex for MGI Indexed PCR Primers with CleanPlex for MGI 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 the Broad Institute's GATK Best Practice (https://software.broadinstitute.org/gatk/best-practices/) as general guiding principles for sequencing data analysis.

Please refer to the following recommended steps for analyzing CleanPlex NGS libraries sequenced on Illumina platforms.

1. **Adapter Trimming.** Trim Illumina adapter sequences using a sequence trimming software such as cutadapt (https://cutadapt.readthedocs.io/en/stable/). Following are the adapter sequences to be trimmed from 3' of the reads.

   R1 reads: AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
   R2 reads: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

2. **Suggested Workflow.** Use the following workflow to process and analyze the sequencing data:

   - Perform read mapping WITHOUT trimming primer sequences.
   - Omit de-duplication step.
   - Set sub-sampling number to be higher than maximum read depth.

3. **BED Files.** Visit www.paragongenomics.com/product_documents/ to download panel-specific amplicon BED files and amplicon insert BED files. For CleanPlex Custom Panels, contact techsupport@paragongenomics.com.

4. **Read Assignment and Variant Calling.** For gene panels, for which amplicons are distributed in two or more separate pools, we recommend assigning reads to amplicons based on the mapping position, and then separating them according to amplicon pool assignment. Subsequent variant calling would be performed separately for reads from pool 1 and pool 2, and so on. The variant calling results would then be summarized for final report. Alternatively, primer sequences can be trimmed from reads before mapping and variant calling.

5. **Performance Metrics.** The following metrics may be used to measure the performance of a CleanPlex NGS Panel:
   - **Mapping Rate:** Percentage of reads mapped to reference genome. (Typically, ~98% when using high quality gDNA with CleanPlex Ready-to-Use NGS Panels.)
   - **On-Target Rate:** Percentage of mapped reads aligned to the targeted regions. (Typically, ~98% when using high quality gDNA with CleanPlex Ready-to-Use NGS Panels.)
   - **Coverage Uniformity:** Percentage of amplicons with read depth equal to or greater than 20% of mean read depth of all amplicons in the panel. (Typically, ~98% when using high quality gDNA with CleanPlex Ready-to-Use NGS Panels.)

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. **Trim tag sequences.** 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 commend would trim left-over adapter sequences as well.

2. **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. Paragon Genomics provide primer sequences to facilitate that.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

Phone: 650-822-7545
Email: techsupport@paragongenomics.com
Website: www.paragongenomics.com

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