



CleanPlex[®] TMB 500 Panel

User Guide

This user guide is for the following product:

- CleanPlex[®] TMB 500 Panel

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Revision History

Document	Date	Description of Change
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Overview

Product Information

The CleanPlex TMB 500 Panel is a rapid next-generation sequencing (NGS) assay for detecting somatic variants and assessing tumor mutational burden (TMB) in tumor samples. The panel generates highly accurate data from as little as 20 ng of DNA (10 ng per pool for each of the two primer pools) using a fast and simple workflow that is compatible with the Illumina® sequencing platform. The CleanPlex TMB 500 Panel is expertly optimized and analyzes 516 cancer-relevant genes with 1.64 Mb of genomics content (1.19 Mb coding sequencing content) to generate valuable insights for oncology research.

The CleanPlex TMB 500 Panel is powered by Paragon Genomics' proprietary CleanPlex Technology, which combines an advanced primer design algorithm and an innovative background cleaning chemistry to generate best-in-class target enrichment performance and efficient use of sequencing reads. The patented CleanPlex background cleaning chemistry effectively removes non-specific PCR products to enable ultra-high multiplexing of amplicons. More than 20,000 amplicons can be multiplexed per primer pool to build large NGS panels that can interrogate megabase-size genomic regions to unlock new applications using a streamlined workflow.

The CleanPlex TMB 500 Panel features a fast and simple workflow that can be completed in less than 6 hours from input DNA to indexed and amplified NGS libraries. The workflow involves just 3 steps, each consisting of a thermal-cycling or incubation reaction followed by a library purification using magnetic beads. Tube-to-tube transfer is minimized throughout the protocol to preserve DNA material and prevent handling errors and sample mix-up.

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



CleanPlex TMB 500 Target Enrichment and Library Preparation Workflow

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

Applications

The CleanPlex TMB 500 Panel can be used to detect germline and somatic mutations (down to 5% MAF) in a variety of applications including tumor mutation burden analysis.

The table below shows some of the sample types and applications of the CleanPlex TMB 500 Panel. This is not meant to be an exhaustive or restrictive list.

Organism	Sample Types	Applications
<ul style="list-style-type: none"> Human 	<ul style="list-style-type: none"> Degraded DNA from FFPE tissues. Note: highly degrade FFPE DNA will likely produce low-quality libraries. Genomic DNA from whole blood, tissues, biofluids, buccal swab, dried blood spots (DBSs), feces, circulating cells, and cultured cells 	<ul style="list-style-type: none"> Tumor mutation burden analysis Tumor profiling Variant detection

Compatible Sequencing Instruments

CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex TMB 500 libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, and MiSeq® System. Choose a sequencing instrument that will meet the sequencing and sample multiplexing requirements of the experiment.

Kit Contents

The protocol outlined in the CleanPlex TMB 500 Panel User Guide requires the following components, which need to be ordered separately:

- CleanPlex TMB 500 Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)

Panel Specifications

Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Amplicon Length	Average Library Length
CleanPlex TMB 500 Panel	2	5X	27,296	105-120 bp (average 113 bp)	249 bp

CleanPlex TMB 500 Panel — Kit Contents, Store at –20°C

Panel	SKU	Size (Reactions)	Components		
			Primer Pool 1	Primer Pool 2	CleanPlex Targeted Library Kit
CleanPlex TMB 500 Panel	916073	6	12 µl	12 µl	2-pool, 6 rxns
	916074	96	192 µl	192 µl	2-pool, 96 rxns

A CleanPlex Targeted Library Kit is included in every CleanPlex TMB 500 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)

Component	Cap Color	SKU	Configuration	2-Pool
			Size	6 Rxns
5X Ultra mPCR Mix	Green		24 µl	384 µl
CP Reagent Buffer	White		12 µl	192 µl
CP Digestion Reagent	Yellow		12 µl	192 µl
Stop Buffer	Red		36 µl	576 µl
5X 2nd PCR Mix	Blue		48 µl	768 µl
TE Buffer	Clear		500 µl	6 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)

- CleanMag Magnetic Beads, or equivalent — eg. Agencourt™ AMPure™ XP Kit (Beckman Coulter, A63880, A63881, or A63882)

CleanMag Magnetic Beads — Store at 2–8°C

	SKU	Size (Volume)	2 Pool Panel Reactions
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~7
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~40
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~490
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,700

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

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

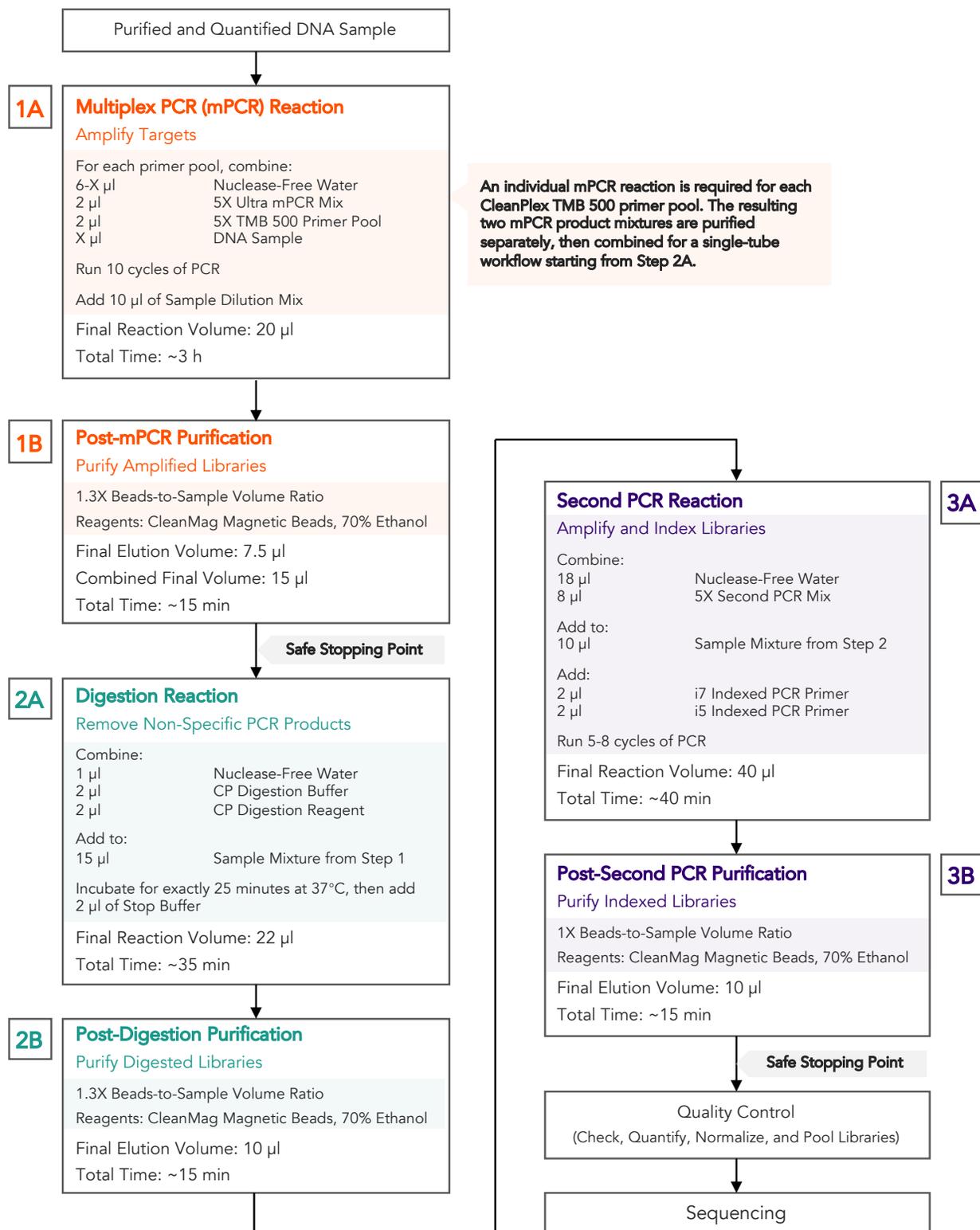
The components containing enzymes (5X Ultra mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C .

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

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

Workflow

The following diagram illustrates CleanPlex TMB 500 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 Ultra mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) on ice during their respective procedures. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spun-down before use.
- Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination between samples, multiplex PCR (mPCR) primers, or indexed PCR primers. Always change pipette tips between samples and change gloves frequently. Clean all workstations and tools with 10% bleach followed by water, then alcohol at the end of each work day.
- Use a calibrated PCR thermal cycler as specified by the manufacturer's user guide. Validated thermal cyclers include Bio-Rad C1000, Eppendorf Mastercycler series, and Applied Biosystems GeneAmp PCR System 9700. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second and higher, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed, or use the default setting (no ramp adjustment).
- To ensure accurate assembly of reactions, withdraw viscous solution (such as 5X Ultra mPCR Mix, CP Digestion Reagent, and 5X 2nd PCR Mix) slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly mix each assembly to ensure solutions are homogeneous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.
- Always keep working solutions, PCR tubes and plates, and PCR products on ice until needed. Combine PCR mixes just immediately prior to use and do not prolong storage of combined PCR mixes and PCR products.
- The protocol is designed to minimize the number of tube-to-tube transfer in order to avoid or reduce sample loss. For the CleanPlex TMB 500 Panel, the individual mPCR products from the two primer pool-specific reactions are purified **separately** then 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 TMB 500 Panel	10–40 ng	10 ng	20 ng

- The maximum volume of DNA input per Multiplex PCR Reaction is 6 μ l. For the CleanPlex TMB 500 Panel, which has 2 primer pools, an individual Multiplex PCR Reaction is required for each primer pool.
- Qubit dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) or an equivalent fluorometric method is recommended for measuring DNA concentration. UV spectrophotometry methods (e.g. NanoDrop™ spectrophotometer) are not recommended because it can significantly overestimate the DNA concentration.
- 10–40 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 5% frequency.
- When DNA quality is low or unknown (such as DNA from FFPE tissues), higher DNA input can be used to produce better library quality and reach lower limits of detection.
- Avoid diluting DNA samples to < 10 ng/ μ l for prolonged storage as lower concentrations of DNA are less stable in solution. Avoid freeze-thawing dilute DNA samples when possible, and measure sample concentrations immediately prior to use to avoid inaccurate sample input.

1A. Multiplex PCR (mPCR) Reaction

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

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

Note: When working with FFPE DNA, a good standard practice is to evaluate the quality of the FFPE DNA prior to library preparation. Highly degrade FFPE DNA will likely produce low-quality libraries. When using FFPE DNA, it is recommended to use 1 μ L of the 5X CleanPlex TMB 500 Primer Pool instead of 2 μ L for each reaction.

mPCR Reaction Mixture			
Reagent	Cap Color	Volume per reaction (gDNA)	Volume per reaction (FFPE DNA)
Nuclease-Free Water	—	6 – X μ l	7 – X μ l
5X Ultra mPCR Mix	Green	2 μ l	2 μ l
5X CleanPlex TMB 500 Primer Pool	Varies	2 μ l	1 μ l
DNA Sample	—	X μ l	X μ l
Total Volume per reaction		10 μl	10 μl



Important! 5X Ultra mPCR Mix and 5X CleanPlex TMB 500 Primer Pools are viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution when handling these reagents.

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

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

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

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

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	98 °C	30 sec	-	1
Denaturation	98 °C	15 sec	3 °C/s	10
Annealing/Extension	60 °C	16 min	2 °C/s	
Hold	10 °C	∞		

* For thermal cyclers without adjustable ramp speed, use the default settings.

- 1A.4. Do NOT allow samples to sit at 10 °C hold for longer than 30 minutes.
- 1A.5. Prior to removing the samples from the thermocycler, prepare Sample Dilution Mix by adding the components in the table shown below. After the mPCR cycles are complete, add 10 µL of this the Sample Dilution Mix into each mPCR product.

Sample Dilution Mix		
Reagent	Cap Color	Volume per reaction
TE Buffer	Clear	8 µl
Stop Buffer	Red	2 µl

- 1A.6. Proceed to Step 1B. Post-mPCR Purification immediately for separate purification of the mPCR product mixtures from Pool 1 and Pool 2.



Important. Do not stop and store PCR products after multiplex PCR reaction. Proceed to Step 1B, Post-mPCR Purification immediately.

1B. Post-mPCR Purification



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



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

- 1B.1. Spin the tubes/plate for a few seconds to collect the liquid. Open the tubes or carefully remove the adhesive film from the PCR plate. Do **not** combine the mPCR product mixtures from Pool 1 and Pool 2. The two mixtures must be individually purified.
- 1B.2. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a **1.3X** bead-based purification by adding magnetic beads to **each** mPCR product mixture as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

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

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

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

	Pool 1	Pool 2
Volume of mPCR Product Mixture per reaction	20 μ l (10 μ l of mPCR Product + 10 μ l of Sample Dilution Mix)	20 μ l (10 μ l of mPCR Product + 10 μ l of Sample Dilution Mix)
Volume of Magnetic Beads per reaction	26 μ l	26 μ l

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

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

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

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

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

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

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

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

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

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

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

- 1B.9. Add 7.5 μ l TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads. The DNA will be immediately released from the beads (there is no need to remove the beads). Spin briefly to collect the liquid and the beads.
- 1B.10. For each sample, combine all of the liquid and beads from Pool 1 with all of liquid and beads from the corresponding Pool 2. The final volume for each sample should be 15 μ l
- 1B.11. Proceed to Step 2A. Digestion Reaction.



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

2A. Digestion Reaction

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

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

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

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

Note: When working with multiple reactions, prepare a master mix. Mix well, then add 5 µl of master mix to each sample.

Digestion Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	1 µl
CP Reagent Buffer	White	2 µl
CP Digestion Reagent	Yellow	2 µl
Total Volume per reaction		5 µl

Digestion Reaction Mixture	
Reagent	Volume per reaction
Digestion Reaction Master Mix	5 µl
Purified Sample from Step 1B	15 µl
Total Volume per reaction	20 µl

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

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

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

- 2A.3. Incubate at 37°C for exactly 25 minutes. **Do not** incubate shorter or longer than 25 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 least 5 times or vortex vigorously at least 5 seconds until homogeneous.

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

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

Post-Digestion Purification — 1.3X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Digestion Reaction Product	22 µl
Magnetic Beads	29 µl

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

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

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

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

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

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



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

3A. Second PCR Reaction

Note: Remember to assign an index combination (CleanPlex Dual-Indexed PCR Primers for Illumina) to each sample before starting this step.

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

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

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

Reagent	Illumina Volume per reaction
Second PCR Reaction Master Mix	26 μ l
Purified Sample from Step 2B	10 μ l
i5 Indexed PCR Primer for Illumina	2 μ l
i7 Indexed PCR Primer for Illumina	2 μ l
Total Volume per reaction	40 μl



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

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

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

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

- 3A.3. Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. The Second PCR thermal cycling protocol depends on the starting DNA input amount and DNA quality. Generally, lower quality DNA or lower DNA input amount requires more PCR cycles. See tables below for thermal cycling protocol and cycle number suggestions for CleanPlex TMB 500 Panel.

Note: Use the amount of DNA input per primer pool to determine the correct cycles, not the combined DNA input from all pools. For example, if 10 ng of DNA was added to each of the two primer pools of the CleanPlex TMB 500 Panel, use 10 ng to determine the Second PCR cycle number.

Second PCR Reaction — Thermal Cycling Protocol

Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	6**
Annealing/Extension	60 °C	75 sec	2 °C/s	
Hold	10 °C	∞		

* For thermal cyclers without adjustable ramp speed, use the default settings.

** When using 10 ng of high quality gDNA.

Second PCR Reaction — Input Specific Cycle Number

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

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



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

3B. Post-Second PCR Purification

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

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

Note: Ensure the solution is thoroughly mixed before proceeding.

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

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

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

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

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

Note: *To perform QC and sequencing, place the tubes/plate on the magnetic rack to separate the beads. Avoid disturbing and aspirating the beads when transferring the clear supernatant for QC or sequencing.*



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

Quality Control Prior to Sequencing

Check library quality and concentration using an Agilent 2100 BioAnalyzer Instrument and Agilent High Sensitivity DNA Kit, or a qPCR-based method. The final library can also be measured using a Qubit Fluorometer; however, this method will 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.

Using the concentrations of the library peak(s) only, normalize and pool samples of different index combinations for sequencing.

After pooling indexed libraries (that will be sequenced in the same lane), perform one additional round of 1.2X magnetic bead-based purification to remove excess primer dimer and adapter dimer.

Ensure the total library pool is **>3,000 pM** and the **total volume is >5 uL**. Low volume and low concentration libraries should not be bead purified again as it will lead to significant loss of the library of interest.

Note: Due to the small size of the CleanPlex TMB 500 library, the additional round of 1.2X magnetic bead-based purification could reduce the library concentration by about half.

Refer to the Troubleshooting Guide at the end of this document for additional information about primer dimers and adapter dimers.

Recommended Sequencing Length and Depth

CleanPlex TMB 500 Panel is designed to be compatible with PE 150 bp reads (2x150 bp). For detecting somatic mutations down to 5% minor allele frequency, the recommended average sequencing read depth is at least 1,000X (or 2,000X paired-end reads)

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' -AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3'

i7 Indexed Primer

5' -CAAGCAGAAGACGGCATAACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*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	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	17 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)

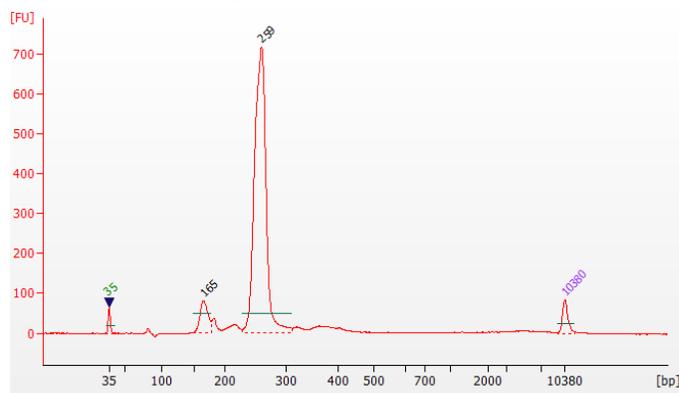
i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems	i5 Index	Index Sequence	i5 Bases for Sample Sheet	
					MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
Q7005	ATATTCAC	GTGAATAT	Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q7006	GCGCCTGT	ACAGGCGC	Q5002	GATATCGA	GATATCGA	TCGATATC
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				

Troubleshooting Guide

Examples of Libraries Prepared with CleanPlex TMB 500 Panel

The library peak should be between about 220 bp and 300 bp. Below is a representative Agilent Bioanalyzer traces generated for CleanPlex TMB 500 Panel using 10 ng of gDNA as input. The presence of adapter-dimer is not unusual due to the high multiplicity of the reaction.

CleanPlex TMB 500 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.

Due to the high multiplicity of the CleanPlex TMB 500 panel, there is a high likelihood of nonspecific products (primer dimer and adapter dimer) present in the libraries. After pooling indexed libraries (for sequencing in the same lane), if nonspecific products are present in significant amounts (>5%), they can be removed by performing one round of 1.2X magnetic bead-based purification, as instructed in the Quality Control Prior to Sequencing section.

Note: Due to the small size of the CleanPlex TMB 500 library, the additional round of 1.2X magnetic bead-based purification could reduce the library concentration by about half.

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 magnetic bead-based purification if the pooled library **concentrations is >3,000pM** and the **total volume is >5 uL**. Low volume and low concentration libraries should not be bead purified again as it will lead to significant loss of the library of interest. Try to keep short adapter dimer peaks less than 5% for best results when possible. When possible, it is always better to repeat the library prep with corrected steps so the nonspecific products do not form in the first place.

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.

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 during the 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.
- Poor DNA quality, especially for FFPE DNA. Higher inputs are recommended.
- Incompatible index primers were using in the Second PCR Reaction
- 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.
- A low-resolution fragment analysis chip (i.e. DNA 1000 Agilent chip) was used.
- An incompatible thermal cycler was used. Try with another model of thermal cycler.
- An incorrect number of second PCR cycles or mPCR cycles was used, causing the product to under-amplify.

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.
 1. **On-Target Rate:** Percentage of mapped reads aligned to the targeted regions.
 2. **Coverage Uniformity:** Percentage of amplicons with read depth equal to or greater than 20% of mean read depth of all amplicons in the panel.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

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