CleanPlex® OncoZoom NGS Panel for Single Cell

User Guide

This user guide is for the following products for Single Cell DNA sequencing on Illumina® NGS platforms:

- CleanPlex® OncoZoom® NGS Panel

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

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UG1006-01
September 2021

Revision History

Document	Date	Description of Change
UG1006-01	September 2021	• Initial version

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Overview

Product Information

CleanPlex® OncoZoom NGS Panel for Single Cell is a fast, robust, and versatile solution for target enrichment and library preparation from single cells for next-generation sequencing (NGS) on Illumina® sequencing platforms. CleanPlex® OncoZoom NGS Panel for Single Cell generates highly accurate data from DNA input from a single cell using a fast and simple workflow. CleanPlex® OncoZoom NGS Panel for Single Cell is expertly optimized with predesigned primers to generate valuable insights in key cancer research areas.

CleanPlex® OncoZoom NGS Panel for Single Cell 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.

CleanPlex NGS Panels feature a fast and simple workflow that can be completed in about 5 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® OncoZoom NGS Panel for Single Cell, 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
5 hours of total assay time, 75 minutes of hands-on time

Applications

The CleanPlex® OncoZoom NGS Panel for Single Cell can be used for rapid detection of somatic mutations across 2900+ hotspot regions of 65 oncogenes and tumor suppressor genes. This kit has direct applications in cancer monitoring and characterization, which in turn, has critical translational applications.
The CleanPlex® OncoZoom NGS Panel allows for accurate genomic profiling of single CTCs (circulating tumor cells) from blood to other body fluids like pleural effusions, cerebrospinal fluid, etc. This can facilitate cancer subtyping and is of critical importance for accurate and personalized therapeutic purposes.

Other Applications

For other bulk (non-single cell) targeted sequencing applications, Paragon Genomics also offers a wide variety of both Ready-To-Use and custom panels for oncology, drug discovery, and more.
Visit our applications page for additional information: https://www.paragongenomics.com/applications/

Compatible Sequencing Instruments

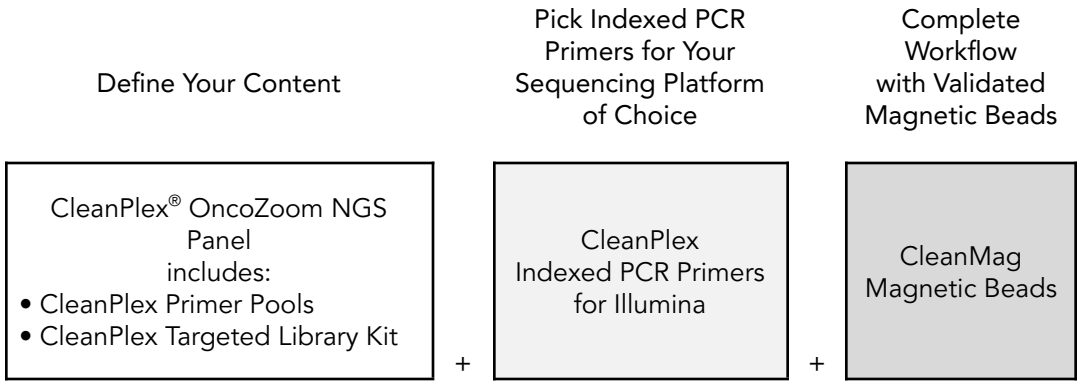
CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, MiSeq® System, MiniSeq® System, and iSeq®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

CleanPlex Indexed PCR Primers for Ion Torrent are used to generate CleanPlex target-enriched NGS libraries that are compatible with 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® OncoZoom NGS Panel for Single Cell User Guide requires the following components, which need to be ordered separately:

- CleanPlex® OncoZoom 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 NGS Panel	1	5X*	601	146 bp	282 bp

* The panel is labeled as 5X for standard bulk sample prep. For a single cell workflow, the working concentration is 0.25X.

CleanPlex NGS Panel — Kit Contents, Store at –20°C				
Panel	SKU	Size (Reactions)	Components	
			Primer Pool 1	CleanPlex Targeted Library Kit
CleanPlex® OncoZoom NGS Panel	916001	8	16 µl	1-pool, 8 rxns
	916002	96	192 µl	1-pool, 96 rxns

A CleanPlex Targeted Library Kit is included in the CleanPlex® OncoZoom NGS Panel for Single Cell. Please note that the CleanPlex Targeted Library Kit is **not** sold separately.

	CleanPlex® OncoZoom NGS Panel for Single Cell — Kit Contents, Store at –20°C (not sold separately)		
	Configuration	1-Pool	
	Size	8 Rxns	96 Rxns
Component	Cap Color	916001	916002
5X mPCR Mix	Green	16 µl	192 µl
CP Reagent Buffer	White	16 µl	192 µl
CP Digestion Reagent	Yellow	16 µl	192 µl
Stop Buffer	Red	32 µl	384 µl
5X 2nd PCR Mix	Blue	64 µl	768 µl
TE Buffer	Clear	500 µl	4 ml

Required Materials and Equipment Not Included

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

CleanPlex Dual-Indexed PCR Primers for Illumina _ Store at –20°C			
	SKU	Size (Reactions)	Format
CleanPlex Dual-Indexed PCR Primers for Illumina Set T	716021	9	Random 3 i7 indexes + 3 i5 indexes (9 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set A	716006 716017	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set B	716018 716019	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set C	716037	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set D	716038	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set E	716039	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set F	716040	96	96 i7 indexes + i5 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
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~8
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~40
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~165
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~500
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,750

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

CleanMag Magnetic Bead solution is shipped at room temperature or on blue ice (ice packs). Magnetic beads may be frozen during transit and upon arrival. This single freeze thaw will not affect the performance of the beads. Upon receipt, immediately store CleanMag Magnetic Beads at 2°C to 8°C in a constant-temperature refrigerator. Do not freeze again. Do not store at -20°C with other reagents. Always ensure that all frozen components are fully thawed and have been vortexed and spun down to bring all liquids to the bottom of the tubes prior to use.

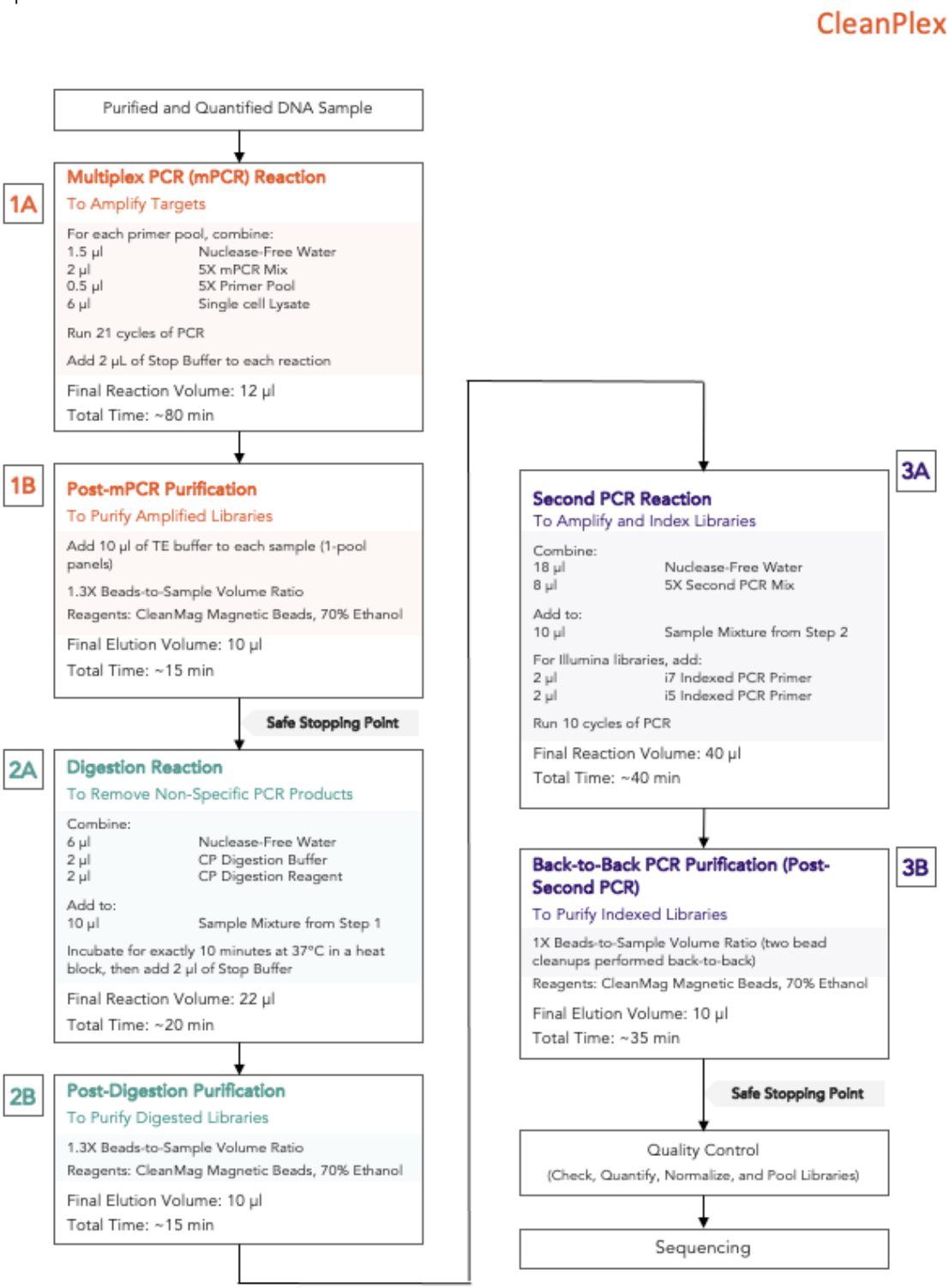
The components containing enzymes are viscous and (5X mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

CleanPlex® OncoZoom NGS Panel for Single Cell is 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 the CleanPlex NGS Panel for Single Cell 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 the tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly pipette or vortex mix each assembly to ensure solutions are homogeneous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.

- Always keep working solutions and PCR products on ice until needed. Combine PCR mixes just immediately prior to use and do not prolong storage of combined PCR mixes and PCR products.

- The protocol is designed to minimize the number of tube-to-tube transfers in order to avoid or reduce sample loss. For this single-pool CleanPlex® OncoZoom NGS Panel for Single Cell, the entire protocol is performed following a single-tube workflow, with no tube-to-tube transfers.

- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross contamination between samples.

- Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for a successful bead purification

- Always pre-warm thermal cyclers, pre-warm a water bath or heat block to 37°C.

- Assign sample indexes to specific samples before starting the protocol.

- Always prepare a master mix of reagents when working with multiplex reactions. Prepare ~5% excess of each master mix to allow for pipetting losses.

Input DNA Requirements

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

Panel	DNA Input Range
CleanPlex® OncoZoom NGS Panel for Single Cell	1 - 10 cells

- The maximum volume of DNA input per Multiplex PCR Reaction is 7 µl.
- Perform cell lysis using a single cell Lysis kit. Make sure to resuspend final cell lysate in a maximum of 7ul of cell lysis buffer.
- Recommended cell lysis kit: ThermoFisher's Single Cell Lysis Kit (Cat. No. 4458235) or an equivalent method. To concentrate the lysate for downstream processes, add 5 ul of cell lysis buffer without DNase and incubate 1hr at RT. Then add 0.5ul of Stop Buffer and incubate 2 mins at RT. Use the entire volume (~6ul) in the mPCR reaction.
- Compatible DNA buffering systems are Tris HCl or TE.
- For any application using more than 10 cells, please decrease the mPCR and 2nd PCR cycles accordingly to minimize over amplification.

Set up and Preparations

- When working with multiple samples, it is recommended to prepare a master mix of mPCR Reaction Mixture. 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.
- For every 8 reactions, freshly prepare 10mL of 70% Ethanol by combining 7mL of 100% ethanol and 3mL 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. Lower concentration of ethanol will affect final library yield.
- For all samples to be sequenced together, assign a specific 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 the Digestion Step, samples must be carried through to 2nd PCR purification without stopping. Plan accordingly.
- Upon initial receipt, Stop Buffer can be thawed and stored continuously at room temperature.

1A. Multiplex PCR (mPCR) Reaction

1A.1. Using thin-wall PCR strip tubes (or a 96-well PCR plate), prepare the mPCR Reaction Mixture by adding components in the following order on ice or a cold block.

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

mPCR Reaction Mixture		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	1.5 µl
5X mPCR Mix	Green	2 µl
5X mPCR Primer Pool*	Varies	0.5 µl
Single Cell Lysate	—	6 µl
Total Volume per reaction		10 µl

*Note that the panel is labeled at 5X for bulk input workflows. Working concentration for Single Cell workflow is 0.25X.

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Important! 5X mPCR Mix and 5X mPCR Primer Pool(s) are viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution when handling these reagents.

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

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

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

1A.3. Load the tubes/plate in the thermal cycler with a heated lid at 105 °C and run the following thermal cycling protocol to amplify target DNA regions.

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	21 cycles
Annealing/Extension	60 °C	5 min	2 °C/s	
Hold	10 °C	∞		
* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/S.				

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.

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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

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

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

1B.1. Open the tubes or carefully remove the adhesive film from the PCR plate. Add 10 µl of TE buffer to each sample.

1B.2. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a 1.3X bead-based purification by adding magnetic beads to the sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-mPCR Purification — 1.3X Beads-to-Sample Volume Ratio	
	1-Pool
Volume of Combined Sample per reaction	22 µl (12 µl of sample + 10 µl of TE Buffer)
Volume of Magnetic Beads per reaction	29 µl

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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.

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

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

1B.6. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. Do not vortex. Carefully remove and discard the supernatant without disturbing the beads.

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

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

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

1B.9. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

1B.10. Proceed to Step 2A. Digestion Reaction.

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

2A. Digestion Reaction

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

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

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

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

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

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

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

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

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

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

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

2A.3. Incubate at 37°C for exactly 10 minutes. Do not incubate shorter or longer than 10 minutes.

2A.4. Immediately add 2 µl of Stop Buffer (red cap) to each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is approximately 22 µl.

2A.5. Proceed to Step 2B. Post-Digestion Purification immediately.

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

2B. Post-Digestion Purification

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

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

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

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

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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.

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

2B.5. Add 180 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 open on the magnetic rack, air-dry the beads at room temperature for 5 minutes. Do not over or under dry.

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

2B.8. Remove tubes/wells from the magnetic rack and add 10 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.9. Proceed to Step 3A. Second PCR Reaction immediately.

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

3A. Second PCR Reaction

Note: *Remember to assign a specific index or unique combination of dual index to each sample before starting this step. Either combinatorial or Unique Dual-Index primers can be used for any CleanPlex libraries.*

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

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

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

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

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

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

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

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

3A.3. Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. Use the three tables below for thermal cycling protocol and cycle number suggestions for CleanPlex Ready-to-Use NGS Panels and CleanPlex Custom NGS Panels.

Note*: The Second PCR thermal cycling protocol depends on the starting DNA input amount and DNA quality. Generally, lower quality DNA, lower DNA input amount, or fewer amplicons in a panel requires more PCR cycles. 10 cycles are recommended for a starting input of 1-10 cells. To adapt the assay with more cells (11-20 cells), please reduce the number of cycles accordingly.*

Second PCR Reaction — Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	10
Annealing/Extension	60 °C	75 sec	2 °C/s	
Hold	10 °C	∞		
* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/Ss..				

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.

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

3B. Back-to-Back PCR Purification (Post-Second PCR)

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.

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

⚠

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

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

3B.2. Incubate the mixture for 5 minutes at room temperature.

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

3B.4. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

⚠

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

3B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

3B.6. Repeat step 3B.5.

3B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each 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-drying and under-drying the beads can lead to reduced yield.*

3B.8. Remove tubes/wells from the magnetic rack and add 20 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.

Suggestion! If you are working with high throughput samples, pool all of the samples in equimolar concentrations BEFORE performing the second back-to-back bead purification step.

3B.9. Vortex the magnetic beads suspension to disperse beads. Perform a 1X bead-based purification by adding 20 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.

2nd Back-to-Back PCR Purification — 1X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Magnetic Beads	20 µl
Second PCR Reaction Product	20 µl

3B.10. Repeat steps 3B.2 - 3B.7.

3B.11. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads. At this point the library is complete and can be stored with beads at –20°C.

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

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

Quality Control Prior to Sequencing

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

CleanPlex Ready to Use typically have a yield of 6 nM to 30nM. Library quantity is not indicative of library quality. Sufficient quantity is important for downstream pooling and sequencing.

Expected library peak size and shapes can be found in the troubleshooting guide at the end of this user guide.

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

The CleanPlex®OncoZoom NGS Panel for Single Cell is designed to be compatible with PE 150 bp reads (2x150 bp).

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

Recommended Sequencing Depth		
Panel	Application	Suggested Average Read Depth (Paired End Reads)
CleanPlex®OncoZoom NGS Panel for Single Cell	Somatic Mutations	500X

Terminology

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

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

For example:
For 500X coverage of this panel with 601 amplicons, it requires a total of 300,500 cluster reads or 601,000 paired end reads per sample. With a Miseq V2 kit (15 million single reads or 30 million paired end reads) you can sequence about 50 samples (30 M PE reads/0.6M PE reads) per run.

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

Supporting Information

Dual-Indexed PCR Primers for Illumina

Primer Sequences

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

i5 Indexed Primer
5'-AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**-3'**

i7 Indexed Primer
5'-CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**-3'**

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

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

Index Sequences

⚠

Additional sequence and Sample sheet information can be downloaded from Product Document Page under Indexes and Useful Tools.

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

CleanPlex Dual-Indexed PCR Primers for Illumina Set A
12 x 8 indexes, 96 reactions (SKU 716006)
12 x 8 indexes, 384 reactions (SKU 716017)

i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems
A701	GTCGTGAT	ATCACGAC
A702	ACCACTGT	ACAGTGGT
A703	TGGATCTG	CAGATCCA
A704	CCGTTTGT	ACAAACGG
A705	TGCTGGGT	ACCCAGCA
A706	GAGGGGTT	AACCCCTC
A707	AGGTTGGG	CCCAACCT
A708	GTGTGGTG	CACCACAC
A709	TGGGTTTC	GAAACCCA
A710	TGGTCACA	TGTGACCA
A711	TTGACCCT	AGGGTCAA
A712	CCACTCCT	AGGAGTGG

i5 Index	Index Sequence	i5 Bases for Sample Sheet	
		MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
A501	TGAACCTT	TGAACCTT	AAGGTTCA
A502	TGCTAAGT	TGCTAAGT	ACTTAGCA
A503	TGTTCTCT	TGTTCTCT	AGAGAACA
A504	TAAGACAC	TAAGACAC	GTGTCTTA
A505	CTAATCGA	CTAATCGA	TCGATTAG
A506	CTAGAACA	CTAGAACA	TGTTCTAG
A507	TAAGTTCC	TAAGTTCC	GGAACTTA
A508	TAGACCTA	TAGACCTA	TAGGTCTA

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
A711	TTGACCCT	AGGGTCAA
A712	CCACTCCT	AGGAGTGG

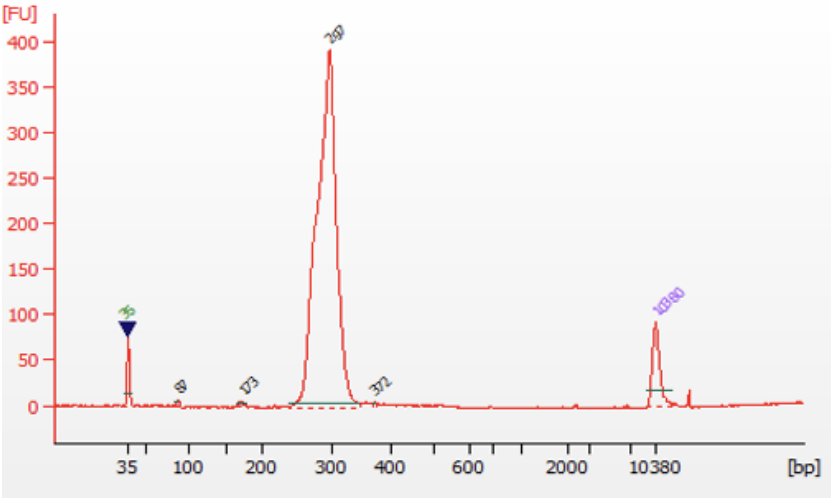
i5 Index	Index Sequence	i5 Bases for Sample Sheet MiSeq, NovaSeq, HiSeq 2000/2500	i5 Bases for Sample Sheet MiniSeq, NextSeq, HiSeq 3000/4000
Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q5002	GATATCGA	GATATCGA	TCGATATC
Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q5009	CCAACAGA	CCAACAGA	TCTGTTGG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

Troubleshooting Guide

Examples of Libraries Prepared with Single Cell CleanPlex OncoZoom NGS Panel

The library peak should be around 300bp. Below is a representative Agilent Bioanalyzer trace for reference.

CleanPlex Single Cell OncoZoom Cancer HotSpot Panel



Potential Causes for Extra Peaks and Suggested Solutions

Peaks around 70–90 bp are index primer dimers from the Second PCR Reaction and result from incomplete removal of low molecular weight DNA fragments during the final magnetic bead purification (Post-Second PCR Purification). When these peaks are high, it usually indicates one or more of the following:

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

Peaks around 150–190 bp are residues of digested non-specific amplification products and adapter dimers. They come from incomplete removal of low molecular weight DNA fragments during the Post-Digestion Purification or nonspecific products formed during or after the Multiplex PCR (mPCR) Reaction due to deviation from protocol or high concentration of the panel. The digestion reagent degrades non-specific amplification products into small pieces, which are then removed during magnetic bead purification. These peaks are usually caused by one or more of the following:

- Inaccurate pipetting of magnetic bead volume.
- Insufficient mixing of reaction solutions or bead and sample mixture.
- Insufficient removal of supernatant and ethanol washes during purification.
- Allowing mPCR products to sit for too long (and form nonspecific products) before purification.
- Incorrect annealing time, specifically unnecessarily longer annealing time.
- DNA input issues, DNA material not present.

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 for the starting number of cells. 10 cycles during the Second PCR is recommended for 1-10 cells and a reduction in cycles is recommended for 11-20 cells.

Potential Reasons for No Peaks

- No cells were present in the upstream steps, particularly if the process started with a single isolated cell instead of a cluster.
- Cell lysis or DNA extraction system is inhibitory to PCR.
- 30% ethanol instead of 70% ethanol was used in DNA purification with magnetic beads.
- Magnetic beads were not added or at an incorrect ratio 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.
- Incompatible indexed PCR primers were used in the Second PCR Reaction. Only use CleanPlex Index primers with CleanPlex library prep. Use the appropriate index primers for the sequencer of choice.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do not use magnetic racks designed for 1.5 ml tubes.

Additional Resources

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

Data Analysis Recommendations for Illumina

We recommend the Broad Institute's GATK Best Practice (https://software.broadinstitute.org/gatk/best-practices/) as general guiding principles for sequencing data analysis.

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 https://www.paragongenomics.com/my-account/downloads/ to download panel-specific amplicon BED files and amplicon insert BED files. For CleanPlex Custom Panels, the BED files are available for download via your account through our website:
   Custom panels ParagonDesigner Portal View Designs Design Files

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. [II1] [YF2]

1. BED Files. Visit https://www.paragongenomics.com/my-account/downloads/ to download panel-specific amplicon BED files and amplicon insert BED files. For CleanPlex Custom Panels, the BED files are available for download via the associated account through our design portal:

   https://www.paragongenomics.com/panel-design-my-design-requests/

   The amplicon insert BED file should be used for variant calling on Torrent Suite (TS) and Ion Reporter (IR).

2. Extra Trimming settings. When planning or reanalyzing a sequencing run with CleanPlex library on TS, user should add these optionsto both Pre-BaseCaller Args and BaseCaller Args : --extra-trim-left 24 --extra-trim-right 24 , to avoid misestimate of base quality.

Setting	Description
Pre Basecaller args for calibration	--extra-trim-left 24 --extra-trim-right 24
Basecaller args	--extra-trim-left 24 --extra-trim-right 24

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

   As shown in the diagram of library structure below, a short tag sequence (TAG-1 and TAG-2 respectively) is attached to both ends of an amplicon. The tag sequences are added during primer synthesis and they serve as priming site in second PCR reaction where Ion Torrent adapters are added. It is the best to trim those sequences from sequencing read before read mapping. [II3] [YF4]

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

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

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

4. Trim primer sequences. For a single-pool panel, we don't recommend to trim primer sequences from sequencing reads. For a two-pool panel, however, trimming primer sequences with a software is recommended to avoid false positive calls from primer binding sites. Paragon Genomics provide primer coordinates or sequences in the BED files to facilitate that. For example, the variantCaller plugin in Torrent Suite can perform primer trimming and produce a *ptrim.bam file for downstream use.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

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