CleanPlex® UMI NGS Panel

User Guide

This user guide is for the following products:

- CleanPlex® UMI Lung Cancer Panel
- CleanPlex® UMI Custom NGS Panel

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UG2001-02
February 2019
For Research Use Only. Not for use in diagnostic procedures.

Revision History

Document	Date	Description of Change
UG2001-01	September 2018	• Initial version
UG2001-02	February 2019	• Included additional tips under Best Practices

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Overview

Product Information

CleanPlex® UMI NGS Panels are a powerful and ultrafast solution designed for confident detection of low-frequency variants, such as those in circulating tumor DNA (ctDNA) in liquid biopsy samples. Input DNA is molecular barcoded with unique molecular identifiers (UMIs), target enriched, and converted into next-generation sequencing (NGS) libraries for sequencing on Illumina® platforms using a simple and rapid workflow that can be completed in just half a day. The incorporation of UMIs allows amplification and sequencing errors to be bioinformatically removed to enable confident detection of ultralow-frequency variants in cell-free DNA (cfDNA), FFPE DNA, and other clinically-relevant samples. CleanPlex UMI Ready-to-Use NGS Panels are expertly optimized with predesigned primers to generate valuable insights in key cancer and disease research areas. CleanPlex UMI Custom NGS Panels are made-to-order to target user-defined genomic regions of interest.

CleanPlex UMI NGS Panels are powered by Paragon Genomics' proprietary CleanPlex UMI Technology, which combines an advanced primer design algorithm and an innovative molecular barcoding chemistry to generate best-in-class performance for detecting ultralow-frequency variants through deep sequencing. The patented CleanPlex UMI molecular barcoding chemistry labels each DNA molecule with a degenerate UMI and allow the two strands of DNA to be bioinformatically distinguished to increase the specificity of variant detection.

CleanPlex NGS Panels feature a rapid and simple workflow that can be completed in about 3.5 hours from input DNA to molecular barcoded and target enriched 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 UMI NGS Panel, such as the CleanPlex UMI Lung Cancer Panel, has no tube-to-tube transfer and thus offers the many benefits of a single-tube workflow.

The first step of the CleanPlex UMI workflow is a multiplex PCR reaction that uses UMI-labeled target-specific primers to barcode and amplify targets of interest. The second step is a biochemical reaction that resolves the correct UMIs by removing PCR products carrying redundant and partial UMIs. The last step is an amplification reaction that uses CleanPlex Unique Dual-Indexed PCR Primers, which ensures sample demultiplexing with high accuracy, 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 UMI workflow.

CleanPlex UMI Molecular Barcoding, Target Enrichment, and Library Preparation Workflow

~3.5 hours of total assay time, ~85 minutes of hands-on time

Applications

CleanPlex UMI NGS Panels are suitable for detecting DNA present in the minor fraction of heterogeneous samples, such as circulating tumor DNA (ctDNA) in cell-free DNA (cfDNA) samples, fetal DNA in maternal cfDNA samples, tumor DNA in FFPE samples, and tumor cell subpopulation DNA in DNA of circulating cells.

The table below shows some of the suitable applications for CleanPlex UMI NGS Panels. This is not meant to be an exhaustive or restrictive list.

Sample Types	Applications
• Genomic DNA from tissues and circulating cells	• Tumor profiling
• Degraded DNA from FFPE tissues	• Variant detection and discovery
• Cell-free DNA (cfDNA) from plasma and other biofluids	• Clonal evolution analysis

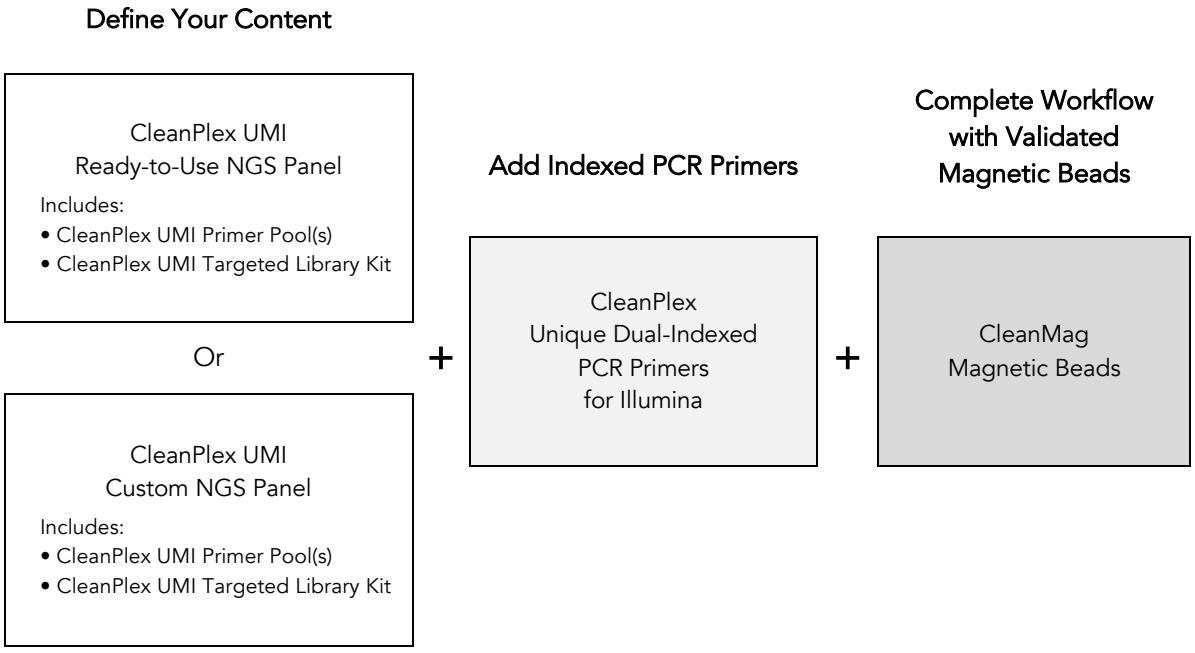
Compatible Sequencing Instruments

CleanPlex Unique Dual-Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries 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.

Kit Contents

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

- CleanPlex UMI Ready-to-Use NGS Panel or CleanPlex UMI Custom NGS Panel
- CleanPlex Unique Dual-Indexed (UDI) PCR Primers for Illumina
- 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 UMI Lung Cancer Panel	1	20X	53	82 bp	288 bp
CleanPlex UMI Custom NGS Panels	Varies	20X	Varies	Varies	Varies

CleanPlex UMI NGS Panels Kit Contents — Store at –20°C					
Panel	SKU	Size (Reactions)	Components		
			Prime Pool 1	Primer Pool 2	CleanPlex Targeted Library Kit
CleanPlex UMI Lung Cancer Panel	916064	8	32 µl	---	1-Pool, 8 Rxns
	916065	32	128 µl	---	1-Pool, 32 Rxns
	916066	96	384 µl	---	1-Pool, 96 Rxns
CleanPlex UMI Custom NGS Panels	---	96+	Varies		Varies

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

CleanPlex Targeted Library Kit Contents — Store at –20°C (not sold separately)								
	Configuration		1-Pool			2-Pool		
		Size	8 Rxns	32 Rxns	96 Rxns	8 Rxns	32 Rxns	96 Rxns
Component	Cap Color	SKU	816015	816025	816016	816017	816026	816018
5X Barcoding Reagent	Green		64 µl	256 µl	768 µl	128 µl	512 µl	1,536 µl
Resolving Buffer	Orange		16 µl	64 µl	192 µl	16 µl	64 µl	192 µl
Resolving Reagent	Yellow		16 µl	64 µl	192 µl	16 µl	64 µl	192 µl
5X Amplifying Reagent	Blue		64 µl	256 µl	768 µl	64 µl	256 µl	768 µl
TE Buffer	Clear		500 µl	2 ml	4 ml	500 µl	2 ml	4 ml

Required Materials and Equipment Not Included

- CleanPlex Unique Dual-Indexed (UDI) PCR Primers for Illumina

CleanPlex Unique Dual-Indexed PCR Primers for Illumina — Store at –20°C			
	SKU	Size (Reactions)	Format
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set A	716011 716012	32 96	16 indexes (16 tubes)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set B	716013 716014	32 96	16 indexes (16 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)	Reactions
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~9
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~45
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~540
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~4,050

- For PCR tubes or strips, CleanMag Magnetic Rack, for 0.2 ml 8/12-tube PCR Strip (SKU 719001) or equivalent magnetic racks designed for PCR strip workflows
- For 96-well PCR plates, CleanMag Magnetic Plate, for 96-well PCR plates (SKU 719002) or equivalent magnetic plates designed for PCR plate workflows
- CleanMag Magnetic Rack, for 0.2 mL 8/12 tube PCR Strip (SKU 791001), or equivalent
- 70% ethanol (freshly prepared)
- Nuclease-free water
- 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® 3.0 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 UMI NGS Panels, CleanPlex Unique Dual-Indexed PCR Primers, and CleanMag Magnetic Beads are shipped on dry ice or blue ice, depending on the country of destination. Upon receipt, store CleanPlex UMI NGS Panels and CleanPlex Unique Dual-Indexed PCR Primers at –20°C in a constant-temperature freezer. Do not store in a freezer with auto-defrost or frost-free features. Store CleanMag Magnetic Beads at 2–8°C (**do not freeze**).

When stored under these conditions and handled correctly, all kit components will retain full activity for one year from the date of receipt.

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.

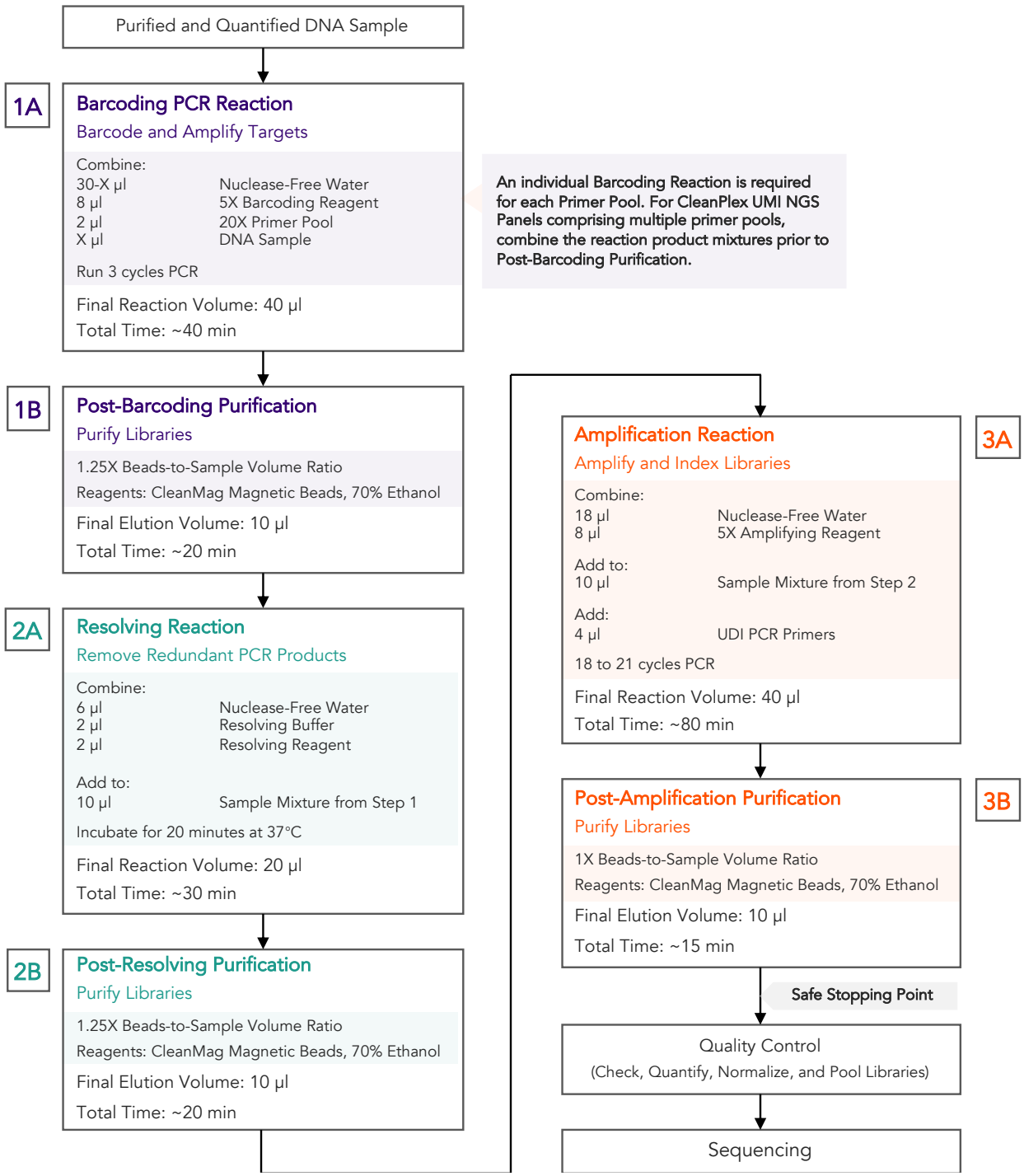
Note that the components containing enzymes (5X Barcoding Reagent, Resolving Reagent, and 5X Amplifying Reagent) are specially formulated for storage at –20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

CleanPlex UMI NGS Panels are developed, designed and sold exclusively for research use only. None of products or their individual the 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 UMI NGS Panel's targeted NGS library preparation workflow.



Protocol

Best Practices

- When using the kit for the first time, briefly vortex and spin the tubes in the kit to bring the liquid to the bottom of the tubes. Store the tubes containing enzymes (5X Barcoding Reagent, Resolving Reagent, 5X Amplifying Reagent) on ice during procedure. 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 in 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, use the recommended ramp speed in the user guide, or use the default setting (no ramp adjustment).

- To ensure accurate assembly of reactions, withdraw viscous solution (such as 5X Barcoding Reagent, Resolving Reagent, Resolving Buffer, and 5X Amplifying Reagent) slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly mix each assembly to ensure solutions are homogenous prior to PCR and incubations. Remember to briefly spin the tubes or 96-well plate after mixing.

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

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

- Always pre-warm thermal cycler, 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	gDNA Input Range (per pool)	FFPE DNA Input Range (per pool)	cfDNA Input Range (per pool)
CleanPlex UMI Lung Cancer Panel	20–80 ng	30–80 ng	30–80 ng

- When possible, use as much input DNA as available to obtain the highest quality data and achieve the lowest limit of detection. Do **not** use more than 100 ng of input DNA per reaction as this is outside the operating range of the chemistry and library quality could be adversely affected.
- The maximum volume of DNA input per Barcoding PCR Reaction is 30 µl.
- Qubit dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) is recommended for measuring DNA concentration. UV spectrophotometry methods (e.g. NanoDrop™ spectrophotometer) are not recommended because it can significantly overestimate the DNA concentration
- CleanPlex UMI panels are supplied as 20X concentrated primer pools.

1A. Barcoding Reaction

1A.1. Using thin-wall PCR tubes strips (or 96-well PCR plate for multiple samples), add the components in the following order on ice or a cold block.

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.

Barcoding Reaction Mixture		
Reagent	**Cap Color**	**Volume per reaction**
Nuclease-Free Water	—	30 – X µl
5X Barcoding Reagent	Green	8 µl
20X Barcoding Primer Pool	Varies	2 µl
DNA Sample	—	X µl
Total Volume per reaction		**40 µl**

Important! Barcoding Reagent and Barcoding Primer Pool(s) are viscous. Pipette slowly and 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 ~3 seconds or until homogenous. Avoid prolonged vortexing. Spin briefly to collect the liquid.

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

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

1A.3. Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and barcode target DNA regions.

Barcoding Reaction Thermal Cycling Protocol				
Step	**Temperature**	**Time**	**Ramping***	**Cycles**
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	3 Cycles
Annealing/Extension	60 °C	4 min	0.4 °C/s	
Hold	10 °C	∞		

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

1A.4. Proceed to Step 1B. Post-Barcoding Purification immediately.

Important! Do not stop and store PCR product after the Barcoding PCR Reaction. Proceed to Step 1B. Post-Barcoding Purification immediately.

1B. Post-Barcoding Purification

Important! Bring magnetic beads to room temperature and vortex thoroughly to disperse the beads before 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 2 seconds. Open the tubes or carefully remove the adhesive film from the 96-well plate.

1B.2. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1.25X** bead-based purification by adding **50 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or vortex vigorously for ~5 seconds or until homogenous.

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.*

Post-Barcoding Purification — 1.25X Beads-to-Sample Volume Ratio

Reagent	Volume per reaction
Magnetic Beads	50 µl
Barcoding PCR Reaction Product	40 µl

1B.3. Incubate the mixture for **10 minutes** at room temperature.

Note*: Incubation for a full 10 minutes is crucial for collecting all barcoded PCR products.*

1B.4. Spin the tubes/plate for 2 seconds. Place the tubes/plate on a magnetic rack and incubate for **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 spin again for 2 seconds 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 Barcoding PCR Reaction prior to washing is critical to obtaining a clean, high-quality library.*

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 (the side without beads) against the magnet to move the beads from one side of the tube 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 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.*

1B.9. Add 10 µl 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.

1B.10. Proceed to Step 2A. Resolving Reaction immediately.

Important! Do not stop and store PCR product after Post-Barcoding PCR Purification. Proceed to Step 2A. Resolving Reaction immediately.

2A. Resolving Reaction

Note: *Set a water bath/heat block to 37°C, or pre-warm a thermal cycler to 37°C before beginning removal of redundant barcodes.*

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

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

Resolving Reaction Master Mix		
Reagent	**Cap Color**	**Volume per reaction**
Nuclease-Free Water	—	6 µl
Resolving Buffer	Orange	2 µl
Resolving Reagent	Yellow	2 µl
Total Volume per reaction		**10 µl**

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

2A.1. Close the tubes or seal the plate with adhesive film, vortex vigorously for 3 seconds (avoid prolonged vortexing), and spin briefly to collect the liquid.

2A.2. Incubate at 37°C for exactly 20 minutes. **Do not** incubate longer than 20 minutes.

2A.3. Proceed to the next step immediately. Do not store samples at room temperature or in a refrigerator/freezer.

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

2B. Post-Resolving Purification

2B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1.25X** bead-based purification by adding **25 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or vortex vigorously for ~5 seconds or until homogenous.

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.*

Post-Resolving Purification — 1.25X Beads-to-Sample Volume Ratio	
Reagent	**Volume per reaction**
Magnetic Beads	25 µl
Resolving Reaction Product	20 µl

2B.2. Incubate the mixture for **10 minutes** at room temperature. Immediately go to the next step. Do not store the reaction at room temperature or in refrigerator/freezer.

Note: Incubation with beads for full 10 minutes allows for the efficient recovery of barcoded products and avoids the residual activity of Resolving Reagent.

2B.3. Spin the tubes/plate for 2 seconds. Place the tubes/plate on a magnetic rack and incubate for **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.

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.*

2B.4. Cap/seal the tubes/plate, and spin again for 2 seconds 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 to obtaining a clean, high-quality library.*

2B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Remove the tubes 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.1. 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.*

2B.2. Add 10 µl TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads and elute DNA from the beads (there is no need to remove the beads). Spin briefly to collect the liquid.

2B.7. Proceed to the Step 3A Purification Reaction immediately.

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

3A. Amplification Reaction

Note: *Remember to assign a specific index (CleanPlex Unique Dual-Indexed PCR Primers for Illumina) to each sample before starting this step.*

3A.1. Thaw CleanPlex Unique Dual-Indexed PCR Primers for Illumina, vortex for 5 seconds, then spin for 2 seconds to collect the liquid. Prepare Amplification Reaction Master Mix and add to each purified sample from Step 2B. Then add a Unique Dual-Indexed PCR Primer 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.*

Amplification Reaction Master Mix		
Reagent	**Cap Color**	**Volume per reaction**
Nuclease-Free Water	—	18 µl
5X Amplifying Reagent	Blue	8 µl
Total Volume per reaction		**26 µl**

Amplification Reaction Mixture	
Reagent	**Volume per reaction**
Amplification Reaction Master Mix	26 µl
Purified Sample from Step 2B	10 µl
Unique Dual-Indexed PCR Primer for Illumina	4 µl
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 vortexing vigorously for ~3 seconds or until homogenous. Avoid prolonged vortexing. Spin briefly to collect the liquid.

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

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

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

Amplification Reaction — Thermal Cycling Protocol				
Step	**Temperature**	**Time**	**Ramping***	**Cycles**
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	See table below
Annealing/Extension	60 °C	75 sec	2 °C/s	
Hold	10 °C	∞		

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

Amplification Reaction — PCR Cycle Number		
cfDNA Input Amount	**gDNA/FFPE DNA Input Amount**	**PCR Cycle Number**
30–60 ng	20–30 ng	20
60–80 ng	30–60 ng	19
–	60–80 ng	18

Note*: The optimal PCR cycle number may require additional optimization for each specific input DNA sample type.*

3A.4. Proceed to Step 3B. Post-Amplification Purification immediately.

Important! Do not stop and store PCR product after the Amplification Reaction. Proceed to Step 3B. Post-Amplification Purification immediately.

3B. Post-Amplification 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 ~5 seconds or until homogenous.

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-Amplification Purification — 1X Beads-to-Sample Volume Ratio	
Reagent	**Volume per reaction**
Magnetic Beads	40 µl
Amplification Reaction Product	40 µl

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

3B.3. Spin the tubes/plate for 2 seconds. Place the tubes/plate on a magnetic rack and incubate for **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 spin again for 2 seconds 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 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 µl 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 and incubate until the liquid is clear to separate the beads. Avoid disturbing 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

Measure library quality and concentration using an Agilent 2100 BioAnalyzer Instrument and Agilent High Sensitivity dsDNA Kit. or a qPCR-based method. The final library can also be measured using a Qubit 3.0 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 fragment analyzer is used to visualize the peak shape, size, and potential background concentration. The optimal library concentration is 8,000-16,000 pM.

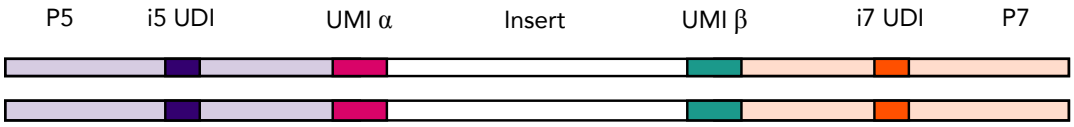
Recommended Minimum Sequencing Depth

The recommended minimum sequencing depth for detection mutation at 0.1% minor allele frequency is 7,500 paired-end reads per amplicon per nanogram of input DNA.

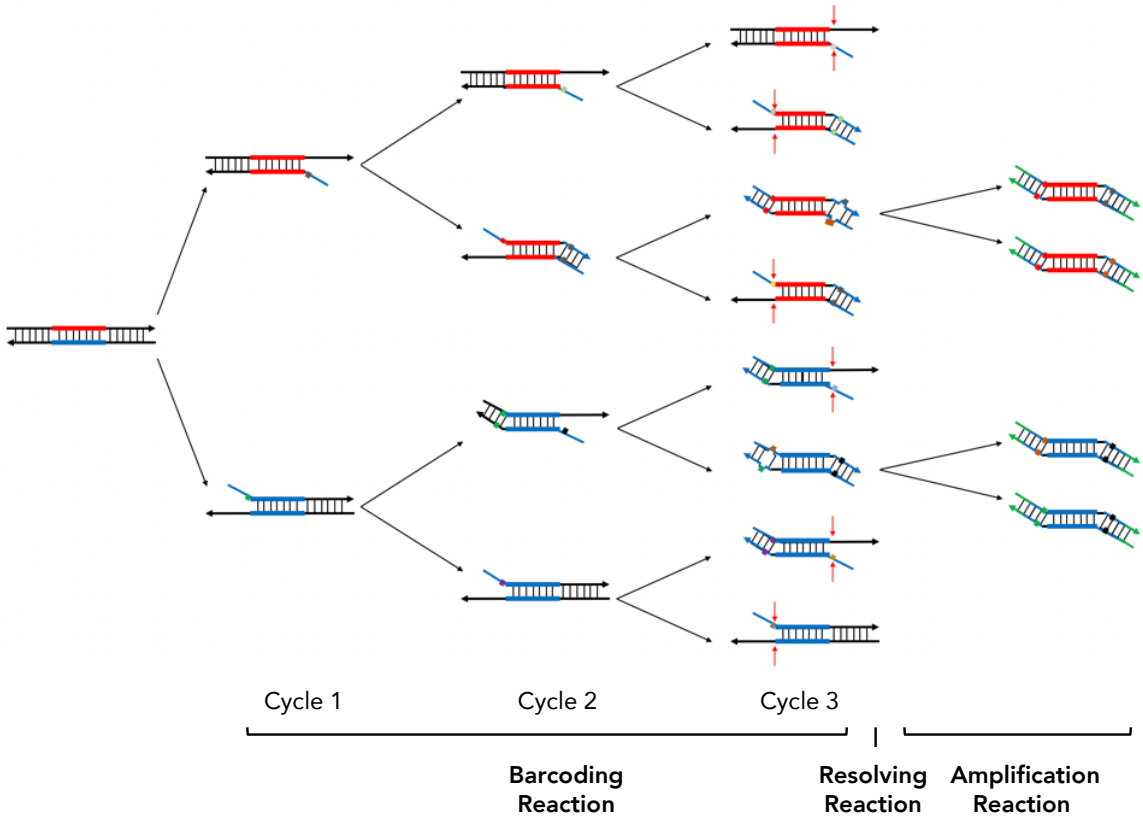
Supporting Information

CleanPlex UMI Library Structure

Molecular-barcoded and target-enriched libraries prepared using CleanPlex UMI NGS Panel contain two 16-nucleotide degenerate unique molecular identifiers (UMIs), one on each end of the insert. They are positioned to be read at the start of Read 1and Read 2 on Illumina NGS platforms. The libraries are also barcoded at the sample level with unique dual indexes (UDIs) to ensure sample demultiplexing with high accuracy.



CleanPlex UMI targeted library preparation result in two pairs of library products, each pair originates from a single strand of DNA. Between each pair of library products, they share the same UMI on one side of the insert, but not on the other, allowing the pair to be grouped and compared against each other to obtain more accurate consensus sequences.



Unique Dual-Indexed (UDI) PCR Primers for Illumina

Primer Sequences

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

i5 Indexed Primer
5'AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

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

Index Sequences

CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set A
16 indexes, 32 reactions (SKU 716011)
16 indexes, 96 reactions (SKU 716012)

UDI	i7 Index Sequence	i7 Bases for Sample Sheet All Illumina Systems	i5 Index Sequence	i5 Bases for Sample Sheet	
				MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
1	CCAAGTCC	GGACTTGG	GACGCTCC	GACGCTCC	GGAGCGTC
2	TTGGACTT	AAGTCCAA	AGTATCTT	AGTATCTT	AAGATACT
3	CAGTGGAT	ATCCACTG	GCTTGCGC	GCTTGCGC	GCGCAAGC
4	TGACAAGC	GCTTGTCA	ATCCATAT	ATCCATAT	ATATGGAT
5	CTAGCTTG	CAAGCTAG	GTTGTCCG	GTTGTCCG	CGGACAAC
6	TCGATCCA	TGGATCGA	ACCACTTA	ACCACTTA	TAAGTGGT
7	CCTGAACT	AGTTCAGG	TGTCGTAG	TGTCGTAG	CTACGACA
8	TTCAGGTC	GACCTGAA	CACTACGA	CACTACGA	TCGTAGTG
9	CTTAAGCC	GGCTTAAG	GCAGAATC	GCAGAATC	GATTCTGC
10	TCCGGATT	AATCCGGA	ATGAGGCT	ATGAGGCT	AGCCTCAT
11	CTGTATTA	TAATACAG	TTCTAACG	TTCTAACG	CGTTAGAA
12	TCACGCCG	CGGCGTGA	CCTCGGTA	CCTCGGTA	TACCGAGG
13	ACTTACAT	ATGTAAGT	GAGGCATT	GAGGCATT	AATGCCTC
14	GTCCGTGC	GCACGGAC	AGAATGCC	AGAATGCC	GGCATTCT
15	ATACCAAG	CTTGGTAT	TCCTGTAA	TCCTGTAA	TTACAGGA
16	GCGTTGGA	TCCAACGC	CTTCACGG	CTTCACGG	CCGTGAAG

CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set B
16 indexes, 32 reactions (SKU 716013)
16 indexes, 96 reactions (SKU 716013)

UDI	i7 Index Sequence	i7 Bases for Sample Sheet All Illumina Systems	i5 Index Sequence	i5 Bases for Sample Sheet	
				MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
17	CTTCACGG	CCGTGAAG	GCGTTGGA	GCGTTGGA	TCCAACGC
18	TCCTGTAA	TTACAGGA	ATACCAAG	ATACCAAG	CTTGGTAT
19	AGAATGCC	GGCATTCT	GTCCGTGC	GTCCGTGC	GCACGGAC
20	GAGGCATT	AATGCCTC	ACTTACAT	ACTTACAT	ATGTAAGT
21	CCTCGGTA	TACCGAGG	TCACGCCG	TCACGCCG	CGGCGTGA
22	TTCTAACG	CGTTAGAA	CTGTATTA	CTGTATTA	TAATACAG
23	ATGAGGCT	AGCCTCAT	TCCGGATT	TCCGGATT	AATCCGGA
24	GCAGAATC	GATTCTGC	CTTAAGCC	CTTAAGCC	GGCTTAAG
25	CACTACGA	TCGTAGTG	TTCAGGTC	TTCAGGTC	GACCTGAA
26	TGTCGTAG	CTACGACA	CCTGAACT	CCTGAACT	AGTTCAGG
27	ACCACTTA	TAAGTGGT	TCGATCCA	TCGATCCA	TGGATCGA
28	GTTGTCCG	CGGACAAC	CTAGCTTG	CTAGCTTG	CAAGCTAG
29	ATCCATAT	ATATGGAT	TGACAAGC	TGACAAGC	GCTTGTCA
30	GCTTGCGC	GCGCAAGC	CAGTGGAT	CAGTGGAT	ATCCACTG
31	AGTATCTT	AAGATACT	TTGGACTT	TTGGACTT	AAGTCCAA
32	GACGCTCC	GGAGCGTC	CCAAGTCC	CCAAGTCC	GGACTTGG

Data Analysis Recommendations for Illumina

For questions and assistance regarding data analysis, please contact Paragon Genomics Technical Support.

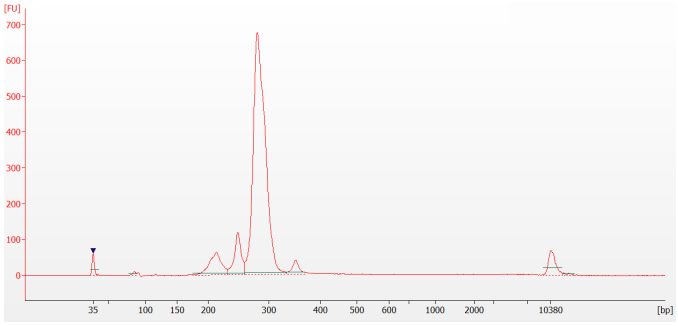
Phone: 650-822-7545
Email: techsupport@paragongenomics.com
Website: www.paragongenomics.com

Troubleshooting Guide

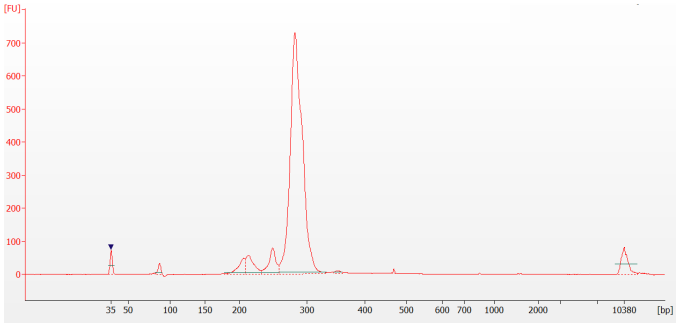
Examples of Libraries Prepared with CleanPlex UMI NGS Panels

Depending on the CleanPlex UMI NGS Panel used, library peak(s) should be between 200 and 400 bp. Below are representative Agilent Bioanalyzer traces generated for the CleanPlex UMI Lung Cancer Panel.

CleanPlex UMI Lung Cancer Panel – 30 ng gDNA



CleanPlex UMI Lung Cancer Panel – 30 ng Seraseq™ ctDNA Complete™ Mutation Mix



Potential Causes for Extra Peaks and Suggested Solutions

Peaks around 70–90 bp are primer dimers from the Amplification Reaction and result from incomplete removal of low molecular weight DNA fragments during the final magnetic bead purification (Post-Amplification Purification). When these peaks are high, it usually indicates inaccurate pipetting of magnetic beads when making a large number of libraries in a short period of time or insufficient removal of supernatant and/or ethanol washes during the last purification.

Potential Reasons for No Peaks

- 30% ethanol instead of 70% ethanol was used in DNA purification with magnetic beads.
- Magnetic beads were not added for one or more of the purification steps.
- The Resolving Reaction Mixture was incubated for more than 20 minutes. This may happen when handling a large number of samples.
- DNA quantification was inaccurate, especially if using spectrophotometric methods, such as the NanoDrop instrument.
- Incompatible index primers were using in the Amplification Reaction
- A weak or incompatible magnetic rack was used to perform magnetic bead purification. Do **not** use magnetic racks designed for 1.5 ml tubes.

Please visit www.paragongenomics.com/faqs/ for additional troubleshooting resources.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

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