



CleanPlex[®] UMI NGS Panel

User Guide

This user guide is for the following products for sequencing Illumina[®] platforms:

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

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Revision History

Document	Date	Description of Change
UG2001-01	September 2018	<ul style="list-style-type: none">● Initial version
UG2001-02	February 2019	<ul style="list-style-type: none">● Included additional tips under Best Practices
UG2001-03	May 2021	<ul style="list-style-type: none">● Updated workflow with stop buffer addition after mPCR step for more robust workflow, especially for high throughput workflows● Included additional information for custom UMI panels and two pool workflow.
UG2001-04	September 2023	<ul style="list-style-type: none">● Corrected minor issues to reflect consistent naming of 5X Barcoding Reagent● Updated address
UG2001-05	January 2024	<ul style="list-style-type: none">● Added transfer of tube after post-barcoding and post-resolving purification final elution steps.● Adjusted bead purification ratios.
UG2001-06	April 2024	<ul style="list-style-type: none">● Update workflow diagram (Pg. 12) to align bead purification concentration outlined in the Post-Barcoding Purification and Post-Resolving Purification protocol.
UG2001-07	October 2024	<ul style="list-style-type: none">● Updated data analysis step for trimming padding sequences

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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 ensure 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
<ul style="list-style-type: none"> • Genomic DNA from tissues and circulating cells • Degraded DNA from FFPE tissues • Cell-free DNA (cfDNA) from plasma and other biofluids 	<ul style="list-style-type: none"> • Tumor profiling • Variant detection and discovery • Clonal evolution analysis

Compatible Sequencing Instruments

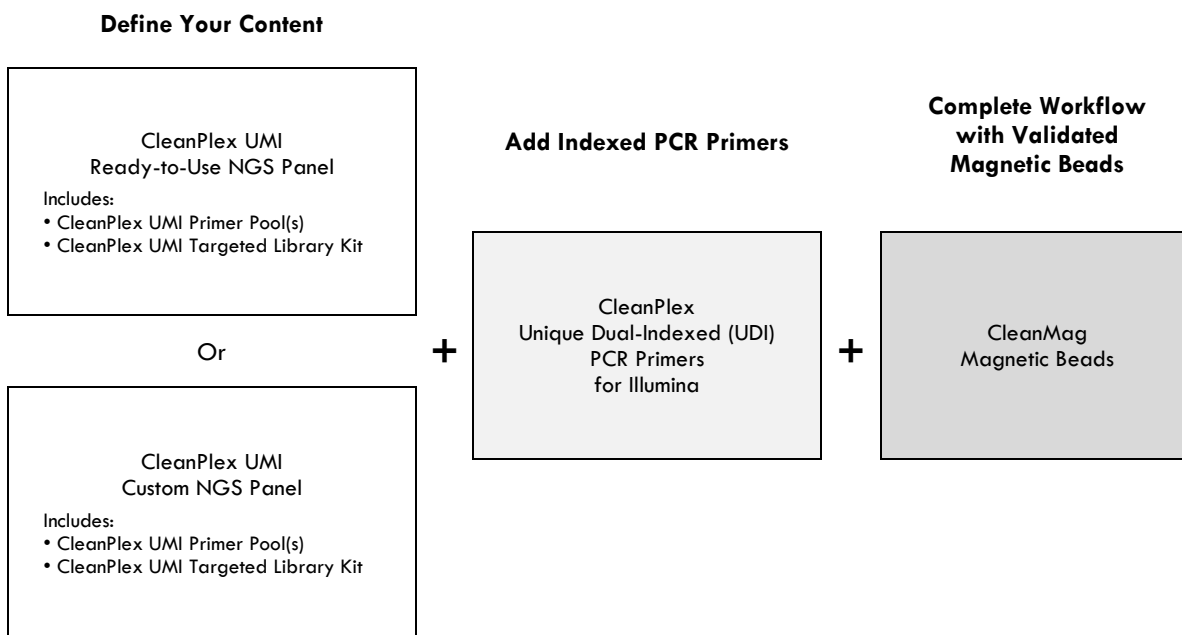
CleanPlex® Unique Dual-Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, MiSeq® System, MiniSeq® System, and iSeq®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

For compatibility on other sequencing platforms outside of what is the scope of this User Guide, please contact support@paragongenomics.com for separate User Guide, addendums, and other updates.

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		
			Primer Pool 1	Primer Pool 2	CleanPlex UMI Targeted Library Kit
CleanPlex UMI Lung Cancer Panel	916064	8	16 µl	---	1-Pool, 8 Rxns
	916065	32	64 µl	---	1-Pool, 32 Rxns
	916066	96	192 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 UMI 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 Striped		64 µl	256 µl	768 µl	128 µl	512 µl	1,536 µl
Stop Buffer	Red		32 µl	128 µl	384 µl	64 µl	256 µl	768 µl
Resolving Buffer	Orange Striped		16 µl	64 µl	192 µl	32 µl	128 µl	384 µl
Resolving Reagent	Yellow Striped		16 µl	64 µl	192 µl	32 µl	128 µl	384 µl
5X Amplifying Reagent	Blue Striped		64 µl	256 µl	768 µl	64 µl	256 µl	768 µl
TE Buffer	Clear		250 µl	1.0 ml	3.0 ml	500 µl	2 ml	5 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)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set C	716037	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set D	716038	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set E	716039	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated 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 $2 - 8^{\circ}\text{C}$				
	SKU	Size (Volume)	Reactions 1 pool panels	Reactions 2 pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~ 8	~5
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~40	~25
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~160	~100
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~500	~300
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3800	~2200

- 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 & 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
- 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, and CleanPlex Unique Dual-Indexed PCR Primers are shipped on blue ice (ice packs). Upon receipt, immediately store CleanPlex UMI NGS Panels and CleanPlex Unique Dual-Indexed (UDI) 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 solution is shipped at room temperature or on blue ice (ice packs). **CleanMag Magnetic beads may be frozen during transit.** This single freeze thaw will not affect the performance of the beads. Upon receipt, immediately store CleanMag Magnetic Beads at 2°C to 8°C . Do not store at

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.

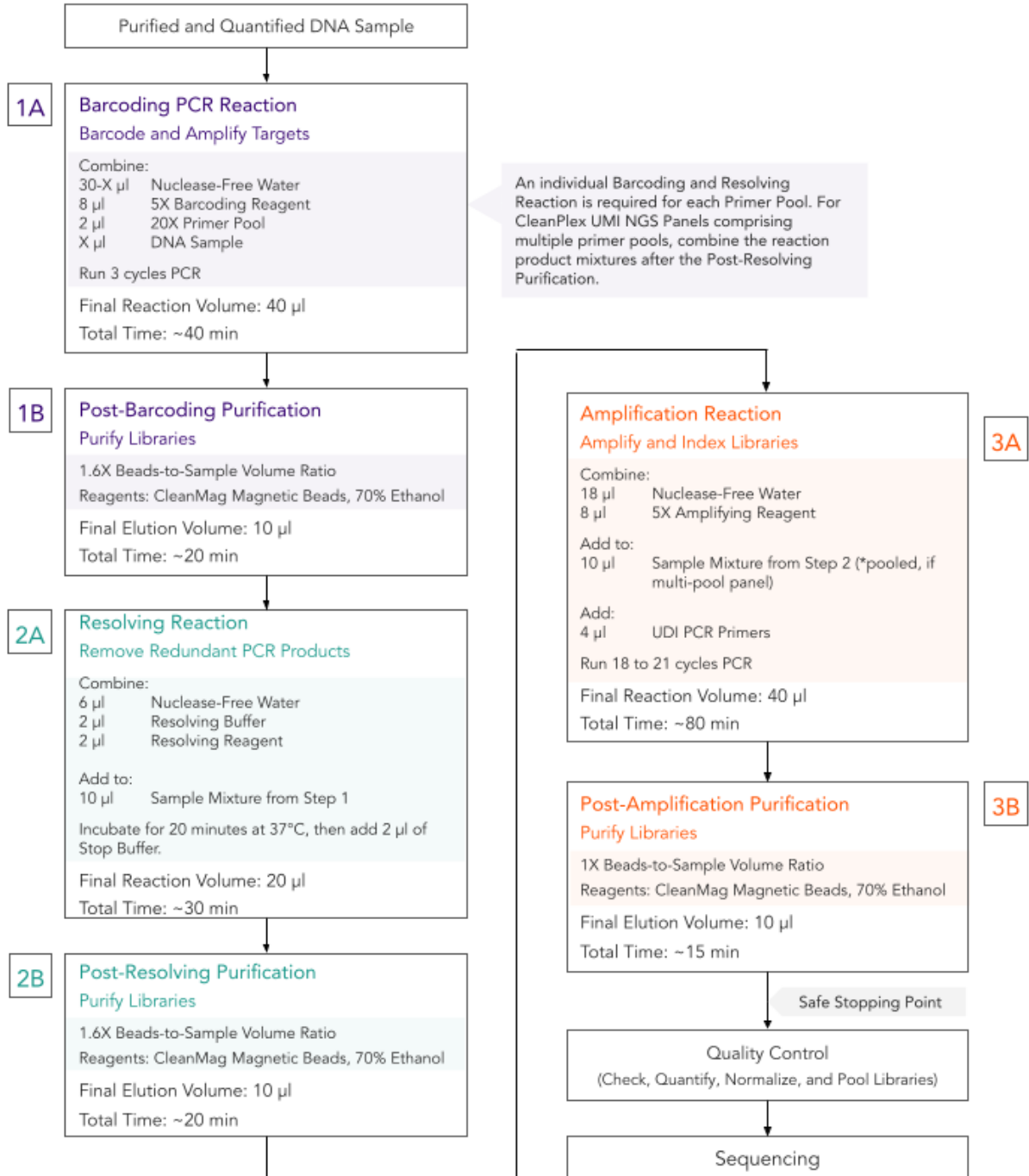
The components containing enzymes (5X Barcoding Reagent, Resolving Reagent, and 5X Amplifying Reagent) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C .

CleanPlex UMI NGS Panels are developed, designed and sold exclusively for research use only. None of 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 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 workday.
- 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.
- 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.
- Always keep working solutions and PCR products on ice until needed. Combine PCR mixes for multipool panels 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 single-pool UMI Panels, the entire protocol is performed following a single-tube workflow, with no tube-to-tube transfers. For multi-pool UMI Panels, the individual barcoding products from the primer pool-specific reactions are combined into one tube, and the remaining protocol is carried out using a single-tube workflow.
- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross contamination between samples.
- Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for a successful bead purification.
- Always pre-warm thermal 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.

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	50 – 80 ng
CleanPlex UMI Custom Panel	Variable	Variable	Variable

- 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.
- For down to 0.1% low allele frequency detection of known variants, use 50 ng or more DNA input for best performance.
- The maximum volume of DNA input per Barcoding PCR Reaction is 30 μ l.
- 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.
- For panels specific to organisms other than humans, DNA input needs will vary based on the genome size of the organism. For example, significantly less DNA input is needed for bacteria panels as each nanogram of DNA contains many more copies of the target DNA. An input titration can be done to determine minimum DNA input. Please reach out to support@paragongenomics.com for additional clarification on input for custom UMI panels.
- CleanPlex UMI panels are supplied as 20X concentrated primer pools, or unless as labeled.

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. **For UMI Panels with multiple primer pools, prepare individual reaction for each primer pool.**

Note: When working with multiple samples, normalize all DNA samples to the same concentration, and prepare a mPCR master mix of Barcoding Reaction Mixture for each primer pool. Mix well, aliquot into individual tubes, then add the recommended DNA input to each reaction. For UMI Panels with multiple primer pools, prepare a separate Barcoding Reaction Mixture for each primer pool per sample.

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



Important! Barcoding Reagent and 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 at least 5 seconds until homogenous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

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

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

- 1A.3.** Load the tubes/plate in the thermal cycler with a heated lid at 105°C 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, the default setting can be used if max ramping speed is ≤5°C/S.

- 1A.4.** Immediately after the thermal cycling ends, 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 now is approximately 42 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-Barcoding Purification immediately.



Important! Do not stop and store PCR products 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. Open the tubes or carefully remove the adhesive film from the PCR plate. **For CleanPlex UMI NGS Panels with multiple primer pools, perform this step separately for each pool.**

1B.2. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1.6X** bead-based purification by adding magnetic beads to each reaction. 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 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.

Note: For example, for a 2-pool workflow, please process each pool separately per the volumes specified below at this step.

Post-Barcoding Purification — 1.6X Beads-to-Sample Volume Ratio	
Reagent	Per Pool
Volume of Sample per reaction	42 µl
Volume of Magnetic Beads per reaction	67.2 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. 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, the magnet is low performing. Please consider getting a different magnetic 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.



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

Note: Especially when working with 96-well PCR plates, a strong compatible magnetic rack is essential. If your magnetic rack 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. 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 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.

- 1B.9.** Add 10 µl TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads completely. The DNA will be immediately released from the beads (there is no need to remove the beads). Spin briefly to collect the liquid. **Transfer the liquid of each reaction to a new tube.** Some magnetic beads may be transferred together with the liquid. These magnetic beads do not affect the following reaction.
- 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: After starting the Resolving Reaction, the samples cannot be stored and must continue to Post-Resolving Purification and Second PCR Reaction. Plan accordingly.

Note: When thawing Resolving Buffer, ensure all visible precipitate has dissolved. Bring to room temperature and vortex to dissolve the precipitate as needed.

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

- 2A.1.** Prepare Resolving Reaction Master Mix and add to each purified reaction from **Step 1B**. **For multipool panels, proceed with Resolving Reaction step for each pool separately.**

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

Resolving Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	6 µl
Resolving Buffer	Orange Striped	2 µl
Resolving Reagent	Yellow Striped	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.2.** Mix by pipetting up and down at least 5 times or vortexing vigorously for at least 5 seconds until homogenous. Avoid unnecessarily prolonged vortexing. Spin briefly to collect the liquid.

Note: It is crucial that the reaction mixture is homogenous prior to incubation. Incomplete mixing can cause incomplete resolution of the barcodes in the library and incomplete removal 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 20 minutes. **Do not** incubate longer than 20 minutes.

- 2A.4** Immediately after the thermal incubation ends, 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 now is approximately 22 µl.

2A.3. Proceed to **Step 2B.** Post-Resolving Purification immediately.



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 **1.6X** bead-based purification by adding **35.2 µl** of magnetic beads to each reaction. **For CleanPlex UMI NGS Panels with multiple primer pools, perform this step separately for each pool.** Mix by pipetting up and down at least 5 times or vortex vigorously at least 5 seconds 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, especially when working with 96-well PCR plates.

Post-Resolving Purification 1.6X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Resolving Reaction Product	22 µl
Magnetic Beads	35.2 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 a full 10 minutes allows for the efficient recovery of barcoded products and avoids the residual activity of the Resolving Reagent.

- 2B.3.** Briefly spin the tubes/plate. 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 the tube, remove the pipette tip, and 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.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: *Extreme over-drying and under-drying of the beads can lead to reduced yield.*

2B.8. Add 10µl of TE buffer to each tube/well. Briefly spin and vortex to resuspend the beads completely. The DNA will be immediately released from the beads. Spin briefly to collect the liquid. **Transfer the liquid of each reaction to a new tube.** Some of the magnetic beads may be transferred together with the liquid. These magnetic beads do not affect the following reaction.

2B.9. Proceed to the **Step 3A** Amplification 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.

For multipool panels, combine the purified product from each pool into a single tube and proceed with Amplification Reaction on the combined pool.

- 3A.1.** Thaw CleanPlex Unique Dual-Indexed (UDI) PCR Primers for Illumina, vortex thoroughly, then briefly spin 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 or 16 μl of master mix to each sample.

Amplification Reaction Master Mix

Reagent	Cap Color	Volume per reaction	
		1 Pool Panel	2 Pool Panel
Nuclease-Free Water	—	18 μl	8 μl
5X Amplifying Reagent	Blue Striped	8 μl	8 μl
Total Volume per reaction		26 μl	16 μl

Amplification Reaction Mixture

Reagent	Volume per reaction	
	1 Pool Panel	2 Pool Panel
Amplification Reaction Master Mix	26 μl	16 μl
(Pooled) Purified Sample from Step 2B	10 μl	20 μl
Unique Dual-Indexed PCR Primer for Illumina	4 μl	4 μl
Total Volume per reaction	40 μl	40 μl



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

Important! When handling plated Indexed PCR Primers take extra care to prevent cross contamination by spinning down the plate before pipetting from each well.

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

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

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

- 3A.3.** Load the tubes/plate in the thermal cycler with a heated lid at 105°C 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 and quantity. Generally, lower quality DNA, lower DNA input amount, or fewer amplicons in a panel require more PCR cycles. See tables below for thermal cycling protocol and cycle number suggestions.

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 50 ng of DNA was added to each of the two primer pools of a CleanPlex UMI Custom NGS Panel, use 50 ng to determine the Amplification Reaction cycle number.

Note: Use the number of amplicons per pool to determine the correct Amplification Reaction cycles for CleanPlex UMI Custom NGS Panels. Typically, a custom multi-pool panel from a single design will evenly split the total amplicons into each primer pool. Please refer to the “ampinsert” file of your custom design to determine the amplicon count per pool or contact Technical Support if you have any questions.

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, the default setting can be used if max ramping speed is ≤5°C/S.

**CleanPlex UMI Lung Cancer Panel Amplification Reaction —
PCR Cycle Number**

cfDNA Input Amount	gDNA/FFPE DNA Input Amount	PCR Cycle Number
30 – 60 ng	20 – 30 ng	20
61 – 80 ng	31 – 60 ng	19
--	61 – 80 ng	18

CleanPlex UMI Custom NGS panels Amplification Reaction- Total Amplicon Specific Cycle Number	
Total Number of Amplicons in Panel	Change in Cycle Number
7 – 25	+ 2
26 – 50	+ 1
51 – 75	—
76 – 300	- 1
301 – 500	- 2

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

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.** Briefly spin the tubes/plate. 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 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 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 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: Excessive over-drying and under-drying of 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 to separate the beads until the liquid is clear. 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

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

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

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

Custom panels → ParagonDesigner Portal → View Designs.

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

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

Recommended Sequencing Length and Depth

All CleanPlex Ready-to-Use UMI NGS Panels are designed to be compatible with PE 150 bp reads (2x150 bp). CleanPlex Custom UMI NGS Panels also have standard 2x150 bp designs unless otherwise decided and communicated between the customer and the Paragon Genomics panel design team.

For detecting known somatic mutations down to 0.1% minor allele frequency, the recommended average sequencing depth is 7,500 paired end reads (or 3750X coverage) per amplicon per nanogram of input DNA.

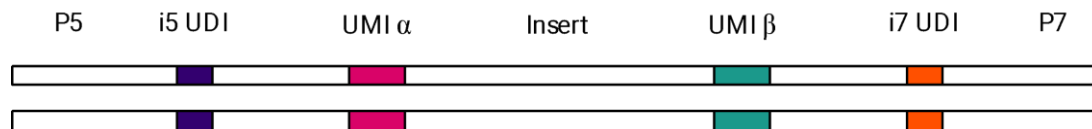
Total PE reads per sample = # amplicons x # ng DNA input x 7500 PE reads.

UMI libraries require much deeper sequencing than standard NGS panels without UMI because UMI generates additional resolution that must be supported by additional sequencing needs. The total reads are directly proportional to the number of templates (DNA input) because each template is uniquely barcoded in the library preparation process.

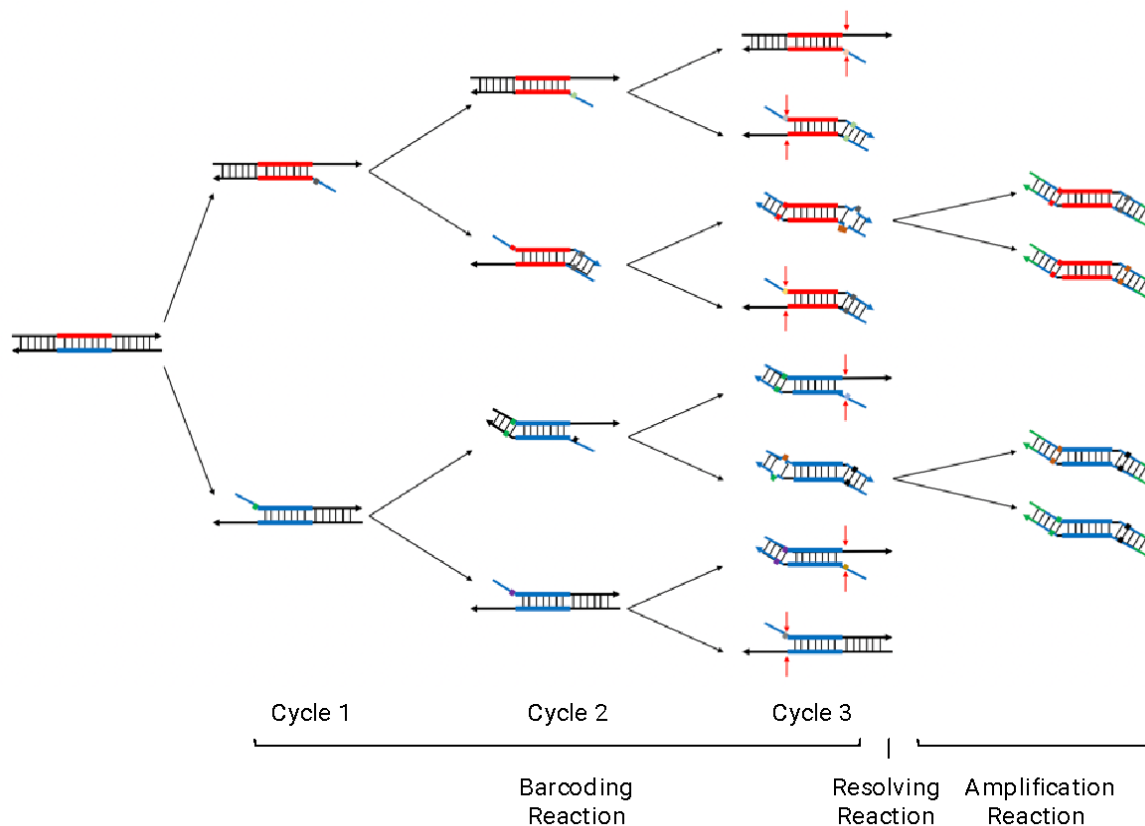
Supporting Information

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 1 and 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 Indexed PCR Primers for sequencing on Illumina platforms.

[index sequence] denotes the 8 or 10-nucleotide index region of the primer. Index sequences are listed below.

i5 Indexed Primer

5' AATGATACGGCGACCACCGAGATCTACAC [index sequence] ACACTCTTCCCTACACGACGCTCTCCGATCT

i7 Indexed Primer

5' CAAGCAGAAGACGGCATAACGAGAT [index sequence] GTGACTGGAGTTCAGACGTGTGCTCTCCGATCT

Set A and Set B have mutually exclusive sequences and allow use together to form a total of 32 index pairs for sequencing up to 32 samples on one Illumina flow cell. Sets A and B have 8 nucleotide indexes and can be sequenced with any other CleanPlex 8 nucleotide indexes such as the Dual Primer Indexes for Illumina, which uses combinatorial indexes.

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

CleanPlex Unique Dual-Indexed (UDI) PCR Primers for Illumina Set A (in tubes)

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	iSeq, 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	GCTTGTC A	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 (UDI) PCR Primers for Illumina Set B (in tubes)

16 indexes, 32 reactions (SKU 716013)

16 indexes, 96 reactions (SKU 716014)

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	iSeq, MiniSeq, NextSeq, HiSeq 3000/4000
17	CTTCACGG	CCGTGAAG	GCGTTGGA	GCGTTGGA	TCCAACGC
18	TCCTGTAA	TTACAGGA	ATACCAAG	ATACCAAG	CTTGGTAT
19	AGAAATGCC	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	TTGGACTION	TTGGACTION	AAGTCCAA
32	GACGCTCC	GGAGCGTC	CCAAGTCC	CCAAGTCC	GGACTIONGG

Plated Index Sequences

Additional sequence and Sample sheet information such as plate maps for plated indexes can be downloaded from [Product Document Page](#) under Indexes and Useful Tools.

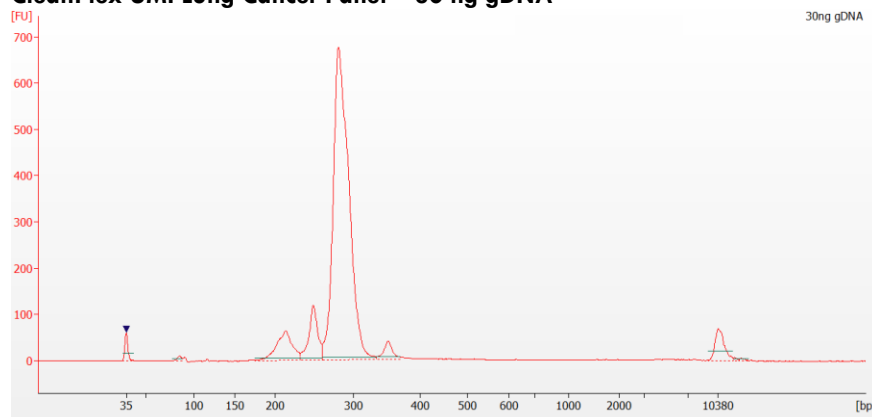
CleanPlex Plated Unique Dual primer indexes for Illumina are 10-nucleotide indexes. They cannot be sequenced with libraries generated with 8nt indexes, as it will interfere with proper sample demultiplexing.

Troubleshooting Guide

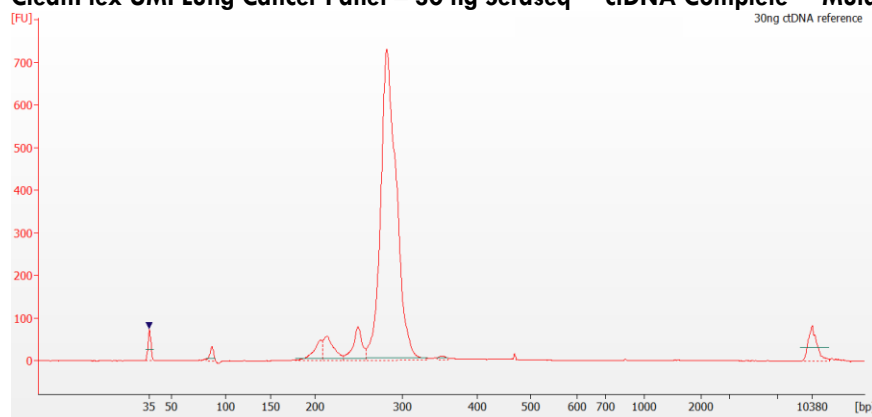
Examples of Libraries Prepared with CleanPlex UMI NGS Panels

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 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 Amplification 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 Resolving Purification or nonspecific products formed during or after the Barcoding Reaction due to deviation from protocol or high concentration of panel. The resolving reagent degrades nonspecific 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 barcoding products to sit for too long (and form nonspecific products) before purification.
- CleanPlex Custom UMI NGS Panels may require panel titration for optimal performance. Often dimers will decrease by reducing the panel concentration in the Barcoding Reaction Mixture. However, this needs to be confirmed by sequencing to ensure the data quality is maintained or improves.
- Incorrect annealing time, specifically unnecessarily longer annealing time.

Broad peaks spread across 500 – 10,000 bp range are nonspecific products due to overamplification. Double check that the correct mPCR and Second PCR cycle numbers were used. PCR cycles are determined based on the amplicon count per pool of your panel, DNA input amount, and DNA quality. Try reducing the Second PCR cycles by 2-3 cycles if issue persists. If you are 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.
- Magnetic bead volume was incorrect.
- The Resolving Reaction Mixture was incubated for significantly longer 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 used in the Amplification Reaction. Only use CleanPlex Unique Dual-Indexed PCR Primers with CleanPlex UMI NGS panels.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do **not** use magnetic racks designed for 1.5 ml tubes.

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

Data Analysis Recommendations for Illumina Paired-end Sequencing using CleanPlex UMI Panels.

Paragon Genomics offers analysis service for our CleanPlex UMI. Please contact your sales or support representative for additional information. For customers who would like to analyze sequencing data of CleanPlex UMI panels using their own analysis pipeline, we provide the following recommendations.

1. Trim padding sequences.

As shown in the diagram of library structure below, 16-base UMI-1 is added right upstream of the amplicon and 16-base UMI-2 is added right downstream of it. If the amplicon size is less than 105bp, then beyond both UMIs, the 15-base padding sequences (PAD-1 and PAD-2) are added to accommodate chemistry requirements of library construction. Addition of padding sequences is optional.



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

```
cutadapt -g GTTATTGGACGTTTA \
-a TTACCTTTTATGAAG \
-A TAAACGTCCAATAAC \
-G CTTCATAAAAGGTAA \
-e 0.1 -O 9 -m 20 -n 2 \
-o R1_out.fq.gz -p R2_out.fq.gz R1_in.fq.gz R2_in.fq.gz \
> cutadapt_report.output.txt
```

The above command would trim leftover adapter sequences as well. Primer sequences are not recommended to be trimmed from reads.

2. Map reads to reference genome.

It is recommended to perform read mapping, indel-realignment, and base quality score recalibration according to GATK guidance. De-duplication procedure shall be skipped. Sub-sampling number shall be set to be higher than maximum read depth.

3. Calculate QC metrics.

In order to assess quality of the sequencing results, it is recommended to assign mapped reads to amplicons based on mapping position. Subsequently, the following metrics can be used to measure general performance of the panel.

- **Mapping Rate:** Percentage of reads mapped to reference genome. It assesses primer-dimers and other PCR artifacts.
- **On-Target Rate:** Percentage of mapped reads that aligned to the targeted regions. It assesses binding/amplification specificity of designed primers.
- **Coverage Uniformity:** Percentage of amplicons with read depth equal to or greater than 20% of mean read depth of all amplicons in the panel. It measures performance uniformity of amplicons in the panel.

To accommodate the calculation, a file in BED format listing amplicon start and end coordinates will be provided to customers. The BED file can be downloaded from <https://www.paragongenomics.com/my-account/downloads/> using the associated order account after the purchase has been shipped.

For CleanPlex UMI custom panels, the file (or other files mentioned in below sessions) is available for download via your account through our website:

Custom panels → ParagonDesigner Portal → View Designs → Design Files

4. Build consensus reads.

Mapped reads assigned to individual amplicons can be further grouped by barcode sequences (UMI-1 for R1 reads and UMI-2 for R2 reads). Reads with the same UMI sequence can be grouped together and a consensus read can be generated for each UMI group.

5. Call variants.

Variants can be called from consensus reads. Only calls within amplicon insert region (excluding primer sequences) are to be reported. To accommodate that, a file in BED format listing amplicon insert start and end coordinates will be provided to customers as described in session 3.

See next page for an annotated wrapper detailing the steps for CleanPlex UMI Panel consensus building and variant calling.

Alternatively, customers may use other variant calling software as appropriate, or contact your sales representative for third party option suggestions.

UMI Panels 12 step analysis for building consensus sequences

```
#CleanPlex UMI 12 step analysis v.2
```

```
Consensus_Build-Variant_call.sh
```

```
##Trim trailing adapters in the R1 and R2 reads using cutadapt
```

```
cutadapt -g CCTACACGACGCTCTTCCGATCT \  
-a AGATCGGAAGAGCACACGTCTGAA \  
-A AGATCGGAAGAGCGTCGTGTAGG \  
-G TTCAGACGTGTGCTCTTCCGATCT \  
-o SampleID.1.fastq -p SampleID.2.fastq SampleID.rawreads.1.fastq  
SampleID.rawreads.2.fastq -e 0.1 -O 9 -m 20 -n 2
```

```
##Only when the panel is CleanPlex UMI Lung Cancer , cutadapt needs to be run  
for a second time
```

```
cutadapt -g GTTATTGGACGTTTA \  
-a TTACCTTTTATGAAG \  
-A TAAACGTCCAATAAC \  
-G CTTCATAAAAGGTAA \  
-e 0.1 -O 9 -m 20 -n 2 \  
-o SampleID.trimmed.1.fastq -p SampleID.trimmed.1.fastq SampleID.1.fastq  
SampleID.2.fastq
```

```
##Step 1: Convert the trimmed fastq reads to unmapped BAM
```

```
java -jar picard.jar FastqToSam F1=SampleID.trimmed.1.fastq  
F2=SampleID.trimmed.2.fastq SM=SampleID O=SampleID.step1-unmapped.bam
```

```
##Step 2: Extract the UMI information to store as a tag
```

```
java -jar fgbio.jar ExtractUmisFromBam --input=SampleID.step1-unmapped.bam --  
output=SampleID.step2-unmapped-umi.bam --read-structure=16M+T 16M+T --single-  
tag=RX --molecular-index-tags=ZA ZB
```

```
##Step 3: Convert the template read to fastq
```

```
java -jar picard.jar SamToFastq I=SampleID.step2-unmapped-umi.bam  
F=SampleID.step3-R1Template.fastq F2=SampleID.step3-R2Template.fastq
```

```
##Step 4: Map the template reads against the human genome
```

```
bwa mem -t No_threads_computation -M hg19.fa SampleID.step3-R1Template.fastq  
SampleID.step3-R2Template.fastq | samtools view -bh -F 256 - >  
SampleID.step4-MappedTemplate.bam
```

```
##Step 5: Merge the mapped reads with the UMI info
```

```
java -jar picard.jar MergeBamAlignment UNMAPPED=SampleID.step2-unmapped-  
umi.bam ALIGNED=SampleID.step4-MappedTemplate.bam R=hg19.fa O=SampleID.step5-  
Mapped-UMI.bam
```

##Step 6: Group reads by UMI

```
java -jar fgbio.jar GroupReadsByUmi --input=SampleID.step5-Mapped-UMI.bam --
output=SampleID.step6-Mapped-GroupedByUMI.bam --strategy=adjacency --edits=1
--min-map=10
```

##Step 7: Call consensus on grouped reads

```
java -jar fgbio.jar CallMolecularConsensusReads --input=SampleID.step6-
Mapped-GroupedByUMI.bam --output=SampleID.step7-ConsensusReads-Unmapped.bam -
-min-reads=3
```

##Step 8: Convert the consensus reads to fastq

```
java -jar picard.jar SamToFastq I=SampleID.step7-ConsensusReads-Unmapped.bam
F=SampleID.step8-R1Consensus.fastq F2=SampleID.step8-R2Consensus.fastq
```

##Step 9: Map the consensus reads against the human genome

```
bwa mem -t No_threads_computation -M hg19.fa SampleID.step8-R1Consensus.fastq
SampleID.step8-R2Consensus.fastq > SampleID.step9-Mapped-Consensus.bam
```

##Step 10: Merge the unmapped and the mapped consensus files

```
java -jar picard.jar MergeBamAlignment UNMAPPED=SampleID.step7-
ConsensusReads-Unmapped.bam ALIGNED=SampleID.step9-Mapped-Consensus.bam
R=$Ref O=SampleID.step10-Mapped-Consensus-UMI.bam
```

##Step 11: Create index

```
samtools index -b SampleID.step10-Mapped-Consensus-UMI.bam SampleID.step10-
Mapped-Consensus-UMI.bai
```

##Step 12: Call variant caller

```
VarDict -G hg19.fa -N SampleID -b SampleID.step10-Mapped-Consensus-UMI.bam -f
Allele_Freq -q 11 -r 2 -k 1 -I 50 -c 1 -S 2 -E 3 -g 4 PanelName.ampInsert.bed
| teststrandbias.R | var2vcf_valid.pl -N $tumor_group -E -f $af >
SampleID_AF_consensus vardict.vcf
```

Variables

```
# No_threads_computation : No. Of threads to be used for computation
# Allele_Freq : Allele frequency, 1% would be depicted as 0.01
```

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

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