



CleanPlex[®] SARS-CoV-2 FLEX Research and Surveillance Panels User Guide

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

- CleanPlex[®] SARS-CoV-2 FLEX Research and Surveillance Panel
- SARS-CoV-2 Emerging Variants Panel Add-on v2

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

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Revision History

Document	Date	Description of Change
UG4004-01	October 2020	<ul style="list-style-type: none">Initial version
UG4004-02	November 2020	<ul style="list-style-type: none">Updated the Applications sectionAdded the Terminology sectionUpdated data analysis guide suggestionsUpdated the mPCR cycling conditions
UG4004-03	January 2021	<ul style="list-style-type: none">Updated index information to reference additional document provided in Product Document page.Added minor clarification to indexing steps for plated indexes.Updated sample input RNA requirement section for clarity and removed nonapplicable lines that were causing confusion.
UG4004-04	April 2021	<ul style="list-style-type: none">Emerging Variants Panel Add-on description, application information, and use information were updated.Document number error was corrected.Total reads per sample information was added for clarity.
UG4004-05	October 2021	<ul style="list-style-type: none">Updated language to reflect most recent language for clarity of procedures. No major changes.Added Ion Torrent compatibility information
UG4004-06	February 2022	<ul style="list-style-type: none">Emerging Variants Panel Add-on v2 description, updated details from v1, and application information.

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Overview

Product Information

CleanPlex® SARS-CoV-2 FLEX Research and Surveillance Panels are a fast, robust, and versatile solution for target enrichment and library preparation for next-generation sequencing (NGS) on Illumina® or DNBSEQ™ sequencing platforms. The CleanPlex SARS-CoV-2 FLEX panel generates highly accurate data from minimal RNA input using a fast and simple workflow. The CleanPlex SARS-CoV-2 FLEX Panel is as compared to the original SARS-CoV-2 panel has improved performance for some lower performing amplicons and a more robust design to maintain high coverage as the SARS-CoV-2 virus mutates with time. The CleanPlex SARS-CoV-2 FLEX Panel is preemptively created with expertly designed degenerate primers added to the existing optimized primers for potentially more comprehensive coverage of polymorphic regions. This enables researchers to generate valuable insights in the identification of viral infection and, active and robust surveillance of acquired genetic variability through the high mutation rate of the virus.

CleanPlex SARS-CoV-2 FLEX Panel is powered by Paragon Genomics' proprietary CleanPlex Technology, which combines an advanced primer design algorithm and an innovative background cleaning chemistry to generate best-in-class target enrichment performance and efficient use of sequencing reads. The patented CleanPlex background cleaning chemistry effectively removes nonspecific PCR products to enable ultra-high multiplexing of amplicons. More than 20,000 amplicons can be multiplexed per primer pool to build large NGS panels that can interrogate megabase-size genomic regions to unlock new applications using a streamlined workflow.

CleanPlex SARS-CoV-2 FLEX product line offers two panel designs tailored specifically to your sequencing needs, with an Illumina/Ion Torrent version and an MGI version. These two designs vary in the adapter sequences used to make them compatible with either platform, enabling flexibility regarding your sequencing strategy.

CleanPlex SARS-CoV-2 FLEX Panels feature a fast and simple workflow that can be completed in less than 6 hours from extracted RNA to indexed NGS libraries ready for sequencing. The workflow involves just 3 (MGI version) or 4 (Illumina/Ion Torrent version) steps, each consisting of a thermal-cycling or incubation reaction followed by a library purification using magnetic beads. CleanPlex SARS-CoV-2 Panels have minimal tube-to-tube transfer, offering many benefits of a single-tube workflow such as preservation of nucleotide material and prevention of handling errors and sample mix-up.

The first step of the CleanPlex SARS-CoV-2 FLEX workflow is cDNA synthesis and purification from purified RNA sample. The second step is a multiplex PCR reaction that uses target-specific primers to amplify targets of interest, thus covering the entire SARS-CoV-2 genome with the 2-pool design. Supplied additionally is an internal human (host) housekeeping RNA control primer pair to be added to the mPCR mix. This amplicon visualizes at 470bp separate from the library at 300bp. The third step is a digestion reaction that performs background cleaning by removing nonspecific PCR products. The last step is a PCR reaction that uses CleanPlex Indexed PCR Primers for the sequencer of choice to amplify

and add sample-level indexes to the generated libraries. CleanMag® Magnetic Beads are recommended for library purification. See the Workflow section for a detailed depiction of the CleanPlex workflow.



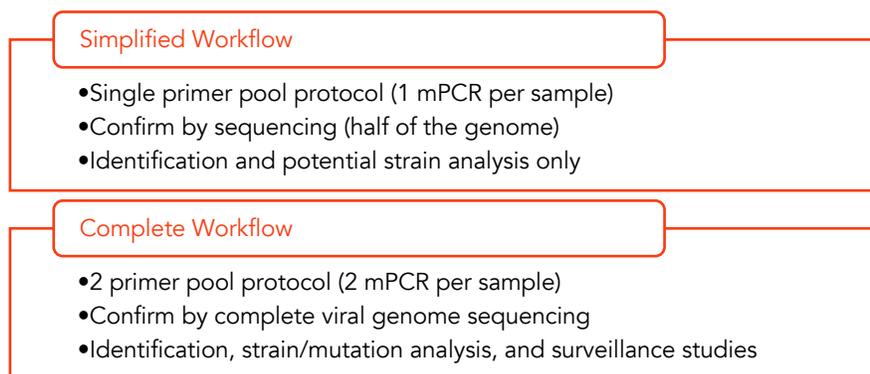
CleanPlex Target Enrichment and Library Preparation Workflow for Illumina Sequencing

5.5 hours of total assay time, 60 minutes of hands-on time

Applications

The CleanPlex SARS-CoV-2 FLEX Research and Surveillance Panels can be used to effectively amplify and detect SARS-CoV-2 RNA in samples using our multiplex PCR technology, enabling reliable results from even low copy numbers. Our assay produces clean characteristic target peaks of defined sizes, which allows for direct detection of the presence of viral genome from the *Coronaviridae* family by electrophoresis. Sequencing is then used to provide confirmation of the species as well as phylogenetic information for specific strain discrimination. The primer design was specifically directed against SARS-CoV-2 but can amplify conserved regions of other coronaviruses in a sample, giving additional insight. These multiplex PCR based panels with degenerate primers are mutation tolerant to allow pinpointing sequence variability due to evolution within the SARS-CoV-2 genome, therefore providing a powerful solution for more in-depth research and surveillance purposes.

The FLEX panel is a two-pool design to cover the entire SARS-CoV-2 genome. However, for applications that do not require such in-depth study of the entire genome, just one of the two pools can be used for faster identification and to save on sequencing space. The single-pool application allows a straight forward one mPCR workflow, allowing for faster turn around and reduced magnetic bead use.



Organisms	Sample Types	Applications
<ul style="list-style-type: none"> • Virus 	<ul style="list-style-type: none"> • Purified RNA from bronchoalveolar lavage, tracheal aspirate, sputum, NP/OP swab, nasopharyngeal wash/aspirate or nasal aspirate 	<ul style="list-style-type: none"> • Identify viral RNA from the <i>Coronaviridae</i> family • Confirm specific SARS-CoV-2 strain(s) present in samples through sequencing • Phylogenetic / Surveillance studies through in-depth sequence data analysis

- High-sensitivity detection
-

FLEX Panel Positive control

The provided control human housekeeping primer pair is only necessary in one of the two mPCR pools. This control allows visual (via fragment analysis) confirmation of sample quality and library preparation success- especially for confident negative sample calling. The human RNA amplicon is ~470 bp whereas the SARS-CoV-2 amplicons are ~350bp, allowing an easy sequencing free confirmation. For samples with high viral loads, the human amplicon may not be present in a fragment analysis trace due to the overwhelming amplification of the SARS-CoV-2 product. However, this is not an issue as the control is meant for more confident negative sample calling only, by removing concerns about library preparation or sample input concerns.

The use of the control human housekeeping primer in the mPCR step is optional, but suggested for applications that commonly run negative samples.

Emerging Variants Panel Add-On v2 Primers

The SARS-CoV-2 Emerging Variant Panel Add-on v2 (EVA v2) is designed to be used with the existing CleanPlex SARS-CoV-2 products to maintain high coverage for variant calling and identification as the virus evolves. As recent variants of interest and concern exhibit multiple mutations in the receptor binding domain locations of the spike protein, Paragon Genomics has updated its initial Emerging Variant Panel Add-on product to consistently maintain high coverage of the virus.

Based on a consensus from world health organization regarding the need to survey and monitor emerging variants, EVA v2 was developed to help characterize variants and enable efficient control measures. Critical mutations for the Beta (B.1.351), Delta (B.1.617.2, AY), Mu (B.1.621, B1.621.1), and Omicron (B.1.1.529) lineages were evaluated for the design and optimization of the Emerging Variant Panel Add-on v2 primers and added onto the original primer design. Defining variant characteristics for the sub-lineages for omicron (BA.1, BA.2, and BA.3) can all be identified with EVA v2. For mutations that fall on a priming region, additional primers were created when appropriate to upkeep the amplification efficiency for potentially affected amplicons. Paragon's advanced primer design and single-tube workflow allows for robust genomic coverage despite novel variants as "super amplicons", which are made from the forward and reverse primers of adjacent primer pairs, maintain robust genomic coverage despite potential mutations.

The Emerging Variant Panel Add-on v2 primers can be easily implemented into any CleanPlex SARS-CoV-2 library preparation workflow. The two primer pools from the Add-on kit are added to the master mixes during the multiplex PCR step with no other changes to the overall workflow. This flexibility allows current CleanPlex users to access the most recent updates without having to replace any existing inventory. If additional adjustments are necessary in the future, the Add-on primer pools can be easily revised to include additional primers for superior coverage and performance, as required for your leading research projects.

Product Information	
Description and Reaction Size	SKU
SARS-CoV-2 Emerging Variant Panel Add-on v2 (8 rxn)	918022
SARS-CoV-2 Emerging Variant Panel Add-on v2 (96 rxn)	918023
SARS-CoV-2 Emerging Variant Panel Add-on v2 (384 rxn)	918024

Other Infectious Disease Applications

For additional SARS-CoV-2 research, we offer a variety of ready to use panels to support host susceptibility studies and co-Infections detection of other respiratory viruses.

Please visit the infectious diseases application page for additional information:

<https://www.paragongenomics.com/applications/infectious-disease/>

All panels can be found under Infectious Diseases Product Category:

<https://www.paragongenomics.com/product-category/products/ready-to-use-panels/infectious-disease/>

SARS-CoV-2 Related Ready to Use Panels		
Panel	SKU	Description
CleanPlex SARS-CoV-2 Research and Surveillance Panel	918011	Original SARS-CoV-2 full viral genome sequencing panel
CleanPlex SARS-CoV-2 FLEX Research and Surveillance Panel	918014	FLEX panel for frontline SARS-CoV-2 mutation research
CleanPlex Respiratory Research Panel	918301	Respiratory Panel contains additional viral content for concurrent Influenza and SARS-CoV-2 detection and flu subtyping
CleanPlex ACE2& TMPRSS2 Panel	916121	Host (human) germline panel for mutation analysis of <i>ACE2</i> and <i>TMPRSS2</i> and associated eQTL for expression level related research, and susceptibility investigations.

Other Applications

CleanPlex SARS-CoV-2 FLEX Research and Surveillance NGS Panel User Guide

For other targeted sequencing applications, Paragon Genomics also offer a while variety of both ready to use panel and custom panels for oncology, drug discovery, and more.

Visit our applications page for additional information: <https://www.paragongenomics.com/applications/>

Other Ready to Use Panels			
Panel	SKU	Application	Description
CleanPlex OncoZoom Cancer HotSpot Panel	916002	Oncology	Target 2,900+ hotspots in 65 genes with known cancer associations.
CleanPlex BRCA1 & BRCA2 Panel v3	916006	Oncology	Target the entire coding region including 20 bases of padding around all targeted coding exons in <i>BRCA1&2</i>
CleanPlex TP53 Panel	916009	Oncology	Target the entire coding region and flanking intronic sequences of <i>TP53</i> for somatic mutations detection low as 1%
CleanPlex UMI Lung Cancer Panel	916066	Oncology	Interrogate 23 genes containing variants that are frequently mutated in lung cancer using a hotspot panel designed to work with cell-free DNA (cfDNA). Detect low-frequency variants below 0.5% MAF.
CleanPlex TMB 500 Panel	916074	Oncology	Interrogate 516 genes associated with the major solid tumor types to assess tumor mutational burden and characterize mutation profile.
OmniFusion™ RNA Lung Cancer Panel	917101	Oncology	Interrogate >530 known RNA fusion and additional novel RNA fusion using a single-primer method.
AccuFusion™ RNA Lung Cancer Panel	917104	Oncology	Interrogate ~280 known RNA fusions using a target specific dual-primer multiplex PCR method.
CleanPlex Mitochondrial Disease Panel	916063	Genetic Disease	Detect and investigate important variants with complete coverage of the entire human mitochondrial genome.
CleanPlex Hereditary Cancer Panel v2	916115	Genetic Disease	Interrogate 37 genes associated with cancers of the breast, ovary, uterus, skin, prostate, and gastrointestinal system, including rs12516 and rs8176318 in <i>BRCA1</i> and Boland inversion in <i>MSH2*</i> .
CleanPlex CFTR Panel	916117	Genetic Disease	Target the entire exon and include 20 bases of padding around all targeted coding exons for <i>CFTR</i> .

CleanPlex Custom NGS Panels	---	Variable	One-on-one service to design a custom panel specific to your applications and needs.
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Compatible Sequencing Instruments

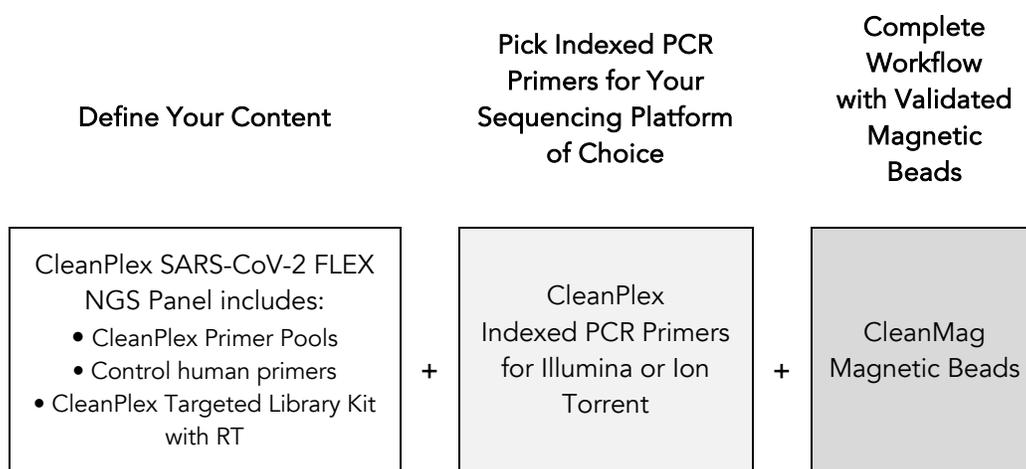
CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries that are compatible with Illumina sequencing platforms, including NovaSeq[®] 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq[®] Series, MiSeq[®] System, MiniSeq[®] System, and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

CleanPlex Indexed PCR Primers for Ion Torrent are used to generate CleanPlex target-enriched NGS libraries that are compatible all Ion Torrent sequencing platforms, including Ion PGM System, Ion Proton System, and Ion GeneStudio S5 Series.

Kit Contents

The protocol outlined in the CleanPlex SARS-CoV-2 FLEX Panel User Guide requires the following components, which need to be ordered separately:

- CleanPlex SARS-CoV-2 FLEX Panel + control human housekeeping primers
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)



Panel Specifications

Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Average Amplicon Length	Average Library Length
CleanPlex SARS-CoV-2 FLEX Panel	2	10X	354	149 bp	285 bp
Emerging Variants Panel Add-on v2*	2	10X	N/A*	N/A	N/A

* When EVA v2 is used with FLEX panel, expect a total of 361 amplicons.

CleanPlex SARS-CoV-2 FLEX Panel— Kit Contents, Store at –20°C						
Component	Configuration		2-Pool			
	Cap Color	SKU	Size	8 Rxns	96 Rxns	384 Rxns
			918013	918014	918015	
RT Primer Mix DP	Purple Striped		24 µl	288 µl	1152 µl	
RT Enzyme	Blue Striped		8 µl	96 µl	384 µl	
100X Control Human Primers	Lavender		8 µl	25 µl	50 µl	
10x SARS-CoV-2 Flex Primer Pool 1	--		8 µl	96 µl	384 µl	
10x SARS-CoV-2 Flex Primer Pool 2	--		8 µl	96 µl	384 µl	
RT Buffer DP	Green Striped		40 µl	480 µl	1920 µl	
5X mPCR Mix	Green		32 µl	384 µl	1536 µl	
CP Reagent Buffer	White		16 µl	192 µl	768 µl	
CP Digestion Reagent	Yellow		8 µl	96 µl	384 µl	
Stop Buffer	Red		64 µl	768 µl	3072 µl	
5X 2nd PCR Mix	Blue		64 µl	768 µl	3072 µl	
TE Buffer	Clear		500 µl	4 ml	16 ml	
Water (DNase and RNase-free)	Clear		500 µl	4 ml	16 ml	

SARS-CoV-2 Emerging Variant Panel Add-on v2 — Kit Contents, Store at –20°C						
Component	Configuration		2-Pool			
	Cap Color	SKU	Size	8 Rxns	96 Rxns	384 Rxns
			918022	918023	918024	
10X SARS-CoV-2 Panel Addon v2 Pool 1	--		8 µl	96 µl	384 µl	
10X SARS-CoV-2 Panel Addon v2 Pool 2	--		8 µl	96 µl	384 µl	

Required Materials and Equipment Not Included

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

CleanPlex Dual-Indexed PCR Primers for Illumina — Store at –20°C			
	SKU	Size (Reactions)	Format
CleanPlex Dual-Indexed PCR Primers for Illumina Set T	716021	9	Random 3 i7 indexes + 3 i5 indexes (9 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set A	716006 716017	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set B	716018 716019	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set A	716033 716045	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set B	716034 716046	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set C	716035 716047	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set D	716036 716048	96 384	Prepooled i5 and i7 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set C	716037	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set D	716038	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set E	716039	96	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Unique Dual-Indexed PCR Primers for Illumina Set F	716040	96	96 i7 indexes + i5 indexes (96-well plate)

CleanPlex Single-Indexed PCR Primers for Ion Torrent — Store at –20°C

	SKU	Size (Reactions)	Format
CleanPlex® Single-Indexed PCR Primers v2 for Ion Torrent Set T	716016	8	8 tubes
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set C	716053	192	96 indexes, (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set D	716054	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set E	716055	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set F	716056	192	96 indexes (96-well plate)

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

CleanMag Magnetic Beads — Store at 2–8°C

	SKU	Size (Volume)	Reactions 2-Pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~5
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~25
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~100
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~300
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~2,250

- For PCR tubes or strips, CleanMag Magnetic Rack (SKU 719001) or equivalent magnetic racks designed for PCR strip workflows
- For 96-well PCR plates, CleanMag Magnetic Plate (SKU 719002) or equivalent magnetic plates designed for PCR plate workflows

CleanMag Magnetic Rack & Plate

	SKU	Compatibility
CleanMag Magnetic Rack	719001	2 rows of 12 tubes each for PCR strip tubes
CleanMag Magnetic Plate	719002	96-well PCR plates, full or semi- skirted compatible

- 70% ethanol (freshly prepared)
- Nuclease-free water
- Nuclease-free, low bind, thin-wall PCR strip tubes with attached caps, or 96-well PCR plate with adhesive film
- Pipettors and low-retention filtered pipette tips
- Thermal cycler
- Mini-centrifuge, or 96-well plate centrifuge
- Qubit® Fluorometer, RNA HS (High Sensitivity) and dsDNA HS Assay Kits, or equivalent
- Agilent® 2100 Bioanalyzer® Instrument and Agilent® High Sensitivity DNA Kit, or equivalent

Storage, Handling, and Usage

CleanPlex SARS-CoV-2 FLEX Panels and CleanPlex Indexed PCR Primers are shipped on blue ice (ice packs). Upon receipt, immediately store both at -20°C in a constant-temperature freezer. Do not store in a freezer with auto-defrost or frost-free features. Do not store at -80°C.

CleanMag Magnetic 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 in a constant-temperature refrigerator. Do not freeze. Do not store at -20°C with other reagents.

Always ensure that all frozen components such as primer pool and CP Digestion Buffer are fully thawed and have been vortexed and spun down to bring all liquids to the bottom of the tubes prior to use.

The 100x Human Control primers are best stored in aliquots to prevent frequent freeze thawing as the Human controls are at a much lower concentration than primer pools, which is not sensitive to reasonable numbers of freeze thaw cycles.

The components containing enzymes are viscous (10X SARS-CoV-2 pools, RT Enzyme Mix, 5X mPCR Mix, CP Digestion Reagent, 5X 2nd PCR Mix) and 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 SARS-CoV-2 FLEX 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.

CleanPlex SARS-CoV-2 FLEX Research and Surveillance NGS Panel User Guide

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

Workflow

The following diagram illustrates CleanPlex NGS Panel's targeted NGS library preparation workflow.



Protocol

General 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 (10X SARS-CoV-2 FLEX Pool(s), RT Enzyme Mix, 5X mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) on ice during their respective procedures. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spun-down before use.
- Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination between samples, multiplex PCR (mPCR) primers, or indexed PCR primers. Always change pipette tips between samples and change gloves frequently. Clean all workstations and tools with 10% bleach followed by water, then alcohol at the end of each work day.
- Use a calibrated PCR thermal cycler as specified by the manufacturer's user guide. Validated thermal cyclers include Bio-Rad C1000, Eppendorf Mastercycler series, and Applied Biosystems GeneAmp PCR System 9700. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second and higher, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed, or use the default setting (no ramp adjustment).
- To ensure accurate assembly of reactions, withdraw viscous solutions (such as 10X SARS-CoV-2 FLEX Pools, RT Enzyme Mix, 5X mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly mix each assembly to ensure solutions are homogenous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.
- Always keep working solutions and PCR products on ice until needed. Combine PCR mixes just immediately prior to use and do not prolong storage of combined PCR mixes and PCR products.
- The protocol is designed to minimize the number of tube-to-tube transfer in order to avoid or reduce sample loss. Except for the RT reaction step and pooling of mPCR products, the entire protocol is performed following a single-tube workflow, with no tube-to-tube transfers.
- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross-contamination between samples.
- Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for a successful bead purification.

- Always pre-warm thermal cyclers, pre-warm a water bath or heat block to 65°C or 37°C as described in the protocol.
- Assign sample indexes to specific samples before starting the protocol.
- Always prepare a master mix of reagents when working with multiplex reactions. Prepare ~5-10% 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/

RNA Best Practices

- RNA is a single-stranded polynucleotide that is incredibly susceptible to degradation by base- or enzyme-catalyzed hydrolysis. This means that working with RNA can be more demanding than its double-stranded counterpart, DNA, due to the chemical instability of RNA and because of the pervasive presence of RNases. Furthermore, unlike DNases, RNases have no requirement for metal ion co-factors, and can remain active even after prolonged boiling or autoclaving.
- When working with RNA, wear gloves at all times. After putting on gloves, avoid touching contaminated surfaces and equipment with the gloved hands. Even if all the reagents have been decontaminated, RNases can be reintroduced by contact with ungloved hands or with unfiltered air.
- Use sterile, disposable plasticware whenever possible. These require no treatment and are considered to be RNase-free.
Note: *Autoclaving without DEPC/DMPC treatment is insufficient for inactivating RNases.*
- If possible, designate a special area for RNA work only. Treat surfaces of benches, glassware, and pipettes with commercially available RNase-inactivating agents such as RNase Zap™.
- In addition, wipe benches with a three-wash method, using 10% bleach, followed by water, and finish with 70-100% ethanol **each time** prior to use, in order to rid the area of microorganisms.
- Whenever possible, purchase reagents that are free of RNases. Be sure to separate reagents used for RNA work from "general use reagents" in the laboratory, and use only for RNA processing.
- Prior to processing, store RNA at -70 to -80°C, as aliquots in ethanol, isopropanol or lysis buffer as indicated by extraction methods. Most RNA is relatively stable at this temperature. Centrifuge the RNA and resuspend in an appropriate RNase-free buffer before use. Use autoclaved DEPC water to elute and resuspend RNA or alternatively use molecular grade water certified as RNase- and DNase-free.
- Although DNA is relatively stable at elevated temperatures (+100°C), most RNA is not. Therefore, avoid high temperatures (above +65°C) since these can affect the integrity of RNA. This means it is critical to handle solutions of RNA on ice when using, including for thawing and processing RNA samples and related reactions.

Input RNA Requirements

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

Panel	RNA Input Range	Recommended RNA Input
CleanPlex SARS-CoV-2 FLEX Panel	5-11 μ L of extracted RNA	11 μ L of extracted RNA

- The maximum volume of RNA input per RT Reaction is 11 μ L. An input of at least 5 μ L of extracted and purified RNA is recommended per pool. When working with low viral load samples or unknown viral load samples, use the full 11 μ L RNA input for best chances of detection.
- This kit is compatible with RNA extraction kits for downstream PCR applications. Some recommended RNA extractions kits include: QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit, RNeasy® Mini Kit (QIAGEN), EZ1 DSP Virus Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit.
- If total nucleic acid kits were used, DNase treatment is not necessary but can be used.
- Use freshly extracted RNA when possible. If storage is necessary, aliquot into single use volumes and store at -80 °C for prolonged storage. Avoid freeze-thawing RNA samples to prevent degradation.
- Compatible RNA buffering systems are Tris HCl or TE.

Set up and Preparations

- When working with multiple samples, prepare a master mix of RT Primer Annealing Reaction Mixture for RT. Mix well, aliquot into individual tubes, then add the recommended RNA input to each reaction.
- Bring magnetic bead solution to room temperature for at least 30 minutes before use. Replace in 2-8 °C storage at the end of the day.
- Freshly prepare 12mL (per 8 reactions) of 70% ethanol at volume ratios of 7 to 3 respectively. Do not top off one liquid with the other in a volumetric container because the volumetric ratio will not be accurate. When water and ethanol are mixed, the final volume will be less than the sum of individual volumes. Lower concentration of ethanol will affect final library yield.
- For all samples to be sequenced together, assign a specific index combination (CleanPlex Dual-Indexed PCR Primers for Illumina) to each sample.
- **Note:** Use the 1-Pool method for quick detection, reduced hands-on time, and reduced total sequencing reads. However, for complete genome sequencing use the 2-Pool method for in-depth analysis.
- **Note:** There are safe stopping points after Post-RT Purification, Post-mPCR Purification, and Post-2nd PCR Purification. After starting Digestion Step, samples must be carried through to the 2nd PCR reaction without stopping. Plan accordingly.

1A. Reverse Transcription (RT) Reaction

- 1A.1. Thaw RT-Primer Mix and RT Buffer, vortex vigorously to mix, quick spin to collect liquid, then store on ice. Keep RT enzyme on ice when not in use. Using thin-wall PCR strip tubes (or a 96-well PCR plate), prepare the Reverse Transcription Annealing Reaction Mixture by adding components in the following order on ice or a cold block.

Note: When working with multiple samples, prepare a master mix of RT Primer annealing reaction Mixture. Mix well, aliquot into individual tubes, then add RNA samples to each reaction.

Note: Pre-warm a thermal cycler or heating unit to 65°C before beginning the RT Primer Annealing Reaction.

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



Important! Use RNA Best Practices by performing all reactions on ice or a cold block, and diligently avoid contamination and introduction of RNases until RT Reaction (1A) and RT Purification (1B) are complete.

RT Primer Annealing Reaction Mixture

Reagent	Cap Color	Volume per reaction
DEPC Treated Nuclease-Free Water	Clear	11 – X μ l
RT Primer Mix DP	Purple Striped	3 μ l
Purified RNA	—	X μ l
Total Volume per reaction		14 μl

- 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 or by vortexing vigorously for at least 5 seconds until homogenous. While ensuring thorough mixing, avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds 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. Preheat the thermocycler to 65 °C (with heated lid at 105°C) then load the tubes/plate in the thermal cycler and run the following thermal cycling protocol to anneal RT Primers.

RT Primer Annealing Reaction Incubation Protocol			
Step	Temperature	Time	Ramping*
Incubation	65 °C	5 min	-
Hold	4 °C	∞	-

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



Important! Do not stop and store samples. Immediately after RT primer annealing, continue to the Reverse Transcription Reaction.

- 1A.4. Place the tubes/plate on ice or cold block immediately after incubation. Open the tubes or carefully remove the adhesive film from the PCR plate. Prepare the Reverse Transcription (RT) Reaction Mixture by adding components in the following order on ice or a cold block.

Note: When working with multiple samples, prepare a master mix of RT Reaction Mixture. Mix well and aliquot into individual tubes.

RT Reaction Mixture		
Reagent	Cap Color	Volume per reaction
RT Buffer DP	Green Striped	5 µl
RT Enzyme	Blue Striped	1 µl
Reaction from Step 1A.3	—	14 µl
Total Volume per reaction		20 µl

- 1A.5. 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. While ensuring thorough mixing, avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds 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: Avoid over vortexing, RNA is single-stranded and susceptible to manual shearing and excessive fragmentation.

- 1A.6. Prechill the thermocycler to 8 °C, then load the tubes/plate in the thermal cycler and run the following thermal cycling protocol.

Step	Temperature	Time	Ramping*
Equilibration	8 °C	10 min	-
Incubation	42 °C	80 min	-
Hold	4 °C	∞	

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

- 1A.7. When thermocycling is complete, immediately add 2 µl of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is now approximately 22 µl.



Important! Do not stop and store samples after the RT Reaction. Add Stop buffer then proceed to Step 1B, Post-RT Purification immediately.

1B. Post-RT Purification



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



Important! Use freshly prepared 70% ethanol. Lower concentration ethanol may result in lower yields.

- 1B.1. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **2.2 X** bead-based purification by adding 48 μ l of magnetic beads to each sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogenous.

Post RT Purification — 2.2 X Beads-to-Sample Volume Ratio	
Reagent	Volume per reaction
Reverse Transcription Product	22 μ l
Magnetic Beads to add	48 μ l



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

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

- 1B.2. Incubate the mixture for **10 minutes** at room temperature.
- 1B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/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.4. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.



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

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

- 1B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.
- 1B.6. Repeat step 1B.5.
- 1B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnet rack to gather the beads. Carefully remove the residual ethanol in each tube/well. Keeping the tubes/plate open on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over- or under-dry.**

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

- 1B.8. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The cDNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.



From here on in, magnetic bead is retained throughout the entire workflow. The magnetic beads do accumulate and can be carried over to next steps without any issues. There is no need to remove the beads by transferring eluted products to new tubes. This action will only decrease your final yield, and increase the total hands-on time, leading to more sample-to-sample variability and decreased efficiency.

1B.9. Proceed to Step 2A. Multiplex PCR Reaction.



Safe Stopping Point. Purified products may be stored at 10°C for up to 1 hour or at –20°C for up to 24 hours.

2A. Multiplex PCR (mPCR) Reaction



Important: Each primer pool requires one mPCR and 5 μ l of the purified RT reaction product. When working with a 1-pool workflow, the remaining 5 μ l of the purified RT reaction can be stored at -20°C for future use or discarded. When working with the 2-pool workflow, split the 10 μ l of the purified RT reaction into two 5 μ l tubes (1 for each pool), beads included.

Important: With first use, it is best to aliquot and store 100x Human Control Primers to minimize freeze thaw cycles. The controls are provided at 100x for better stability. Before each use, perform a 1:10 dilution of the 100x Controls for ease of pipetting. If a large master mix is made, one can also pipette directly from 100x Control if the pipetting volume is manageable. Make up the volume difference in water to maintain 5 μ L per reaction master mix volume.

Important: Add the diluted 10X control human primer to only one mPCR tube, irrespective of doing the one or two-pool workflow. Adjust the volume of Nuclease-Free Water to make final volume of mPCR Reaction Master Mix to 5 μ l. **See Applications section on page 6 and 7 for additional information on Control Human Primer use.**

- 2A.1. Prepare the mPCR Reaction Mixture by adding components in the following order on ice or a cold block. Mix the RT reaction product from 1B. 8 well and add 5 μ l to each mPCR reaction.

Note: Use the 1-Pool method for quick detection and reduced turnaround time. However, if in-depth sequencing is desired use the 2-Pool method for a more complete coverage. See application section for additional information.

Note: When working with multiple reactions, prepare a master mix. Mix well, then add 5 μ l of master mix to each sample. 2-pool workflow requires 2 mPCR reactions per sample.



SARS-CoV-2 Emerging Variants Primer Add-On v2 (SKU 918022, 918023, 918024) is a product intended to be used with The SARS-CoV-2 FLEX panel for improved uniformity of coverage. If using this product, the primer pools are to be added into the mPCR reactions of respective FLEX primer pool master mixes. Please see Applications section on page 7 for additional information.

If these primer pools are not used, ignore the primers in the follow table, and proceed accordingly with nuclease free water for 5 μ L reaction volume per pool.

mPCR Reaction Master Mix per pool		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	As needed to make up 5uL total reaction volume
(OPTIONAL) 10X diluted Control Human Primer	Lavender	(1 µl)
(OPTIONAL) 10X Emerging Variants Panel Add-on v2 Primer Pool 1 or Pool 2	Red and Yellow Striped	(1uL)
5X mPCR Mix	Green	2 µl
10X SARS-CoV-2 FLEX Primer Pool 1 or Pool 2	Red and Green	1 µl
Total Volume per reaction		5 µl



Important! 5X mPCR Mix and 10X Primer Pool(s) are viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution when handling these reagents.

mPCR Reaction Mixture	
Reagent	Volume per reaction per pool
Purified RT sample	5 µl
mPCR Master mix for Pool 1 or Pool 2	5 µl
Total Volume per reaction	10 µl

- 2A.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 vortexing for at least 5 seconds until homogenous. Avoid prolonged vortexing. Spin briefly for 3 seconds 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.

- 2A.3. Load the tubes/plate in the thermal cycler with a heated lid at 105 °C and run the following thermal cycling protocol.

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles**
Initial Denaturation	95 °C	10 min	-	-
Denaturation	98 °C	15 sec	3 °C/s	10
Annealing/Extension	60 °C	5 min	2 °C/s	
Hold	10 °C	∞		

* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is $\leq 5^{\circ}\text{C}/\text{S}$.

** for applications strictly detecting low viral counts (<10 copies/rxn) or highly degraded samples, an additional 2-4 mPCR cycles can be added for increased sensitivity.

- 2A.4. Immediately add 2 μl of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing.

Note: Do not allow samples to hold at 10 °C for longer than 30 minutes before adding Stop Buffer.

- 2A.5. Proceed to Step 2B. Post-mPCR Purification.



Important! Do not stop and store samples after the mPCR Reaction. Add Stop buffer then proceed to Step 2B, Post-mPCR Purification immediately.

2B. Post-mPCR Purification



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



Important! Use freshly prepared 70% ethanol. Lower concentration ethanol may result in lower yields.

- 2B.1. Open the tubes or carefully remove the adhesive film from the PCR plate.
- When using 1-pool workflow, add 10 μl of TE Buffer to each sample for a total of 22 μl volume.
 - When using 2-pool workflow, combine the 12 μl multiplex PCR products for each sample for a total of 24 μl volume.
- 2B.2. Quick spin the tubes/plate to collect the liquid. Open the tubes or carefully remove the adhesive film from the PCR plate.
- 2B.2. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1.3X** bead-based purification by adding magnetic beads to each sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogenous.

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

Reagent	1-Pool workflow	2-Pool workflow
Total Sample Volume	22 μl (12 μl of sample + 10 μl of TE)	24 μl
Magnetic Beads to add	29 μl	31 μl



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

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

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

- 2B.5. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.



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

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

- 2B.6. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex**. Carefully remove and discard the supernatant without disturbing the beads.
- 2B.7. Repeat step 2B.6
- 2B.8. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnet rack to gather the beads. Carefully remove the residual ethanol in each tube/well. Keeping the tubes/plate open on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry**.

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

2B.9. Remove tubes/wells from the magnetic rack and add 10 μ l TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

2B.10. Proceed to Step 3A. Digestion Reaction.



Safe Stopping Point. Purified products may be stored at 10°C for up to 1 hour or at –20°C for up to 24 hours.

3A. Digestion Reaction

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

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

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

- 3A.1. Prepare Digestion Reaction Master Mix and add to each purified sample from Step 2B. The magnetic beads in the sample mixture do not affect the Digestion Reaction.

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

Digestion Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	7 µl
CP Reagent Buffer	White	2 µl
CP Digestion Reagent	Yellow	1 µl
Total Volume per reaction		10 µl

Digestion Reaction Mixture	
Reagent	Volume per reaction
Digestion Reaction Master Mix	10 µl
Purified Sample from Step 2B	10 µl
Total Volume per reaction	20 µl

- 3A.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 for 3 seconds to collect the liquid.

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

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

- 3A.3. Incubate at 37°C for exactly 10 minutes. **Do not** incubate shorter or longer than 10 minutes.
- 3A.4. Immediately add 2 µl of Stop Buffer (red cap) to each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is approximately 22µl.
- 3A.5. Proceed to Step 3B. Post-Digestion Purification immediately.



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

3B. Post-Digestion Purification

Note: Remember to assign a specific index or unique combination of dual index to each sample before starting this step.

- 3B.1. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1.3X** bead-based purification by adding **29 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogenous.

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



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

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

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



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

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

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

3B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate open 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.*

3B.8. Remove tubes/wells from the magnetic rack and add 10 μ l TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

3B.9. Proceed to Step 4A. Second PCR Reaction immediately.



Important! Do not stop and store samples after Post-Digestion Purification. Proceed to Step 4A. Second PCR Reaction immediately.

4A. Second PCR Reaction

Note: Remember to assign a specific index or unique combination of dual indexes to each sample before starting this step based on your sequencing needs. Either combinatorial or Unique Dual-Index (UDI) primers can be used for these libraries.

- 4A.1 Thaw Indexed PCR Primers, vortex thoroughly, then spin briefly to collect the liquid. Prepare Second PCR Reaction Master Mix and add to each purified sample from Step 3B. Then add a unique (combination of) Indexed PCR Primer(s) to each sample.

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

Second PCR Reaction Master Mix

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

Second PCR Reaction Mixture

Reagent	Illumina Volume per reaction	Ion Torrent Volume per reaction
Second PCR Reaction Master Mix	26 μ l	26 μ l
Purified Sample from Step 3B	10 μ l	10 μ l
i5 Dual-Indexed PCR Primer for Illumina	2 μ l*	—
i7 Dual-Indexed PCR Primer for Illumina	2 μ l*	—
Single-Indexed PCR Primer for Ion Torrent	—	4 μ l
Total Volume per reaction	40 μl	40 μl

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

Note: For Illumina indexes in plates, the i5 and i7 index sequence information (plate maps) can be downloaded from [Product Document Page](#) under Indexes and Useful Tools.



Important! When handling Dual-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.

- 4A.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 for 3 seconds 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.

- 4A.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. Use the three tables below for thermal cycling protocol.

Note: The Second PCR thermal cycling protocol depends on the starting RNA input amount and RNA quality. Generally, lower quality RNA, lower RNA input amount, or fewer amplicons in a panel require more PCR cycles.

Second PCR Reaction — Thermal Cycling Protocol

Step	Temperature	Time	Ramping*	Cycles**
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	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 SARS-CoV-2 FLEX NGS Panels — Second PCR Cycle Number

Number of Amplicons	Cycles
1-Pool workflow	25
2-Pool workflow	24

Note: For optimal yield, the SARS-CoV-2 panels may benefit from adjustment to Second PCR cycle numbers based on the sample quality, viral input (when known), and application.

****** For applications that specialize in very low viral count detection based on the Ct values we can make the following changes:

For example: to accommodate the increased 2 - 4 mPCR cycles to a total of 12 -14 cycles we can reduce the 2nd PCR cycle number by 2 - 4 cycles i.e. reduce from 24 cycles to 20 - 22 cycles.

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



Important! Do not stop and store samples after the Second PCR Reaction. Proceed to Step 4B. Post-Second PCR Purification immediately.

4B. Post-Second PCR Purification

- 4B.1. Vortex the magnetic beads suspension vigorously until homogenous. Perform a **1X** bead-based purification by adding **40 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogenous.

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



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

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

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



Important! Removing all residual supernatant from the 2nd 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.

- 4B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the

magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

4B.6. Repeat step 4B.5.

4B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate open on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tubes/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-drying and under-drying the beads can lead to reduced yield.*

4B.8. Remove tubes/wells from the magnetic rack and add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads. At this point the library is complete and can be stored with beads at –20°C.

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



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

Quality Control Prior to Sequencing

After library preparation, amplification of viral RNA can be visualized via electrophoresis. This can be accomplished with high sensitivity fragment analyzers such as Agilent® 2100 Bioanalyzer® Instrument and Agilent® High Sensitivity DNA Kit (Agilent, Part. No. 5067-4626). The presence of a peak at ~275 bp indicates successful amplification of the targeted regions. With sequencing, it can be confirmed that the viral RNA amplified is from the SARS-CoV-2 genome. The human housekeeping RNA control amplicon visualizes at ~470bp separate from the library (~275 bp) and allows visual confirmation of sample quality and library preparation - especially for negative samples.

Note: When viral copies are high in a sample, this control target is not always visible. However, this should not be an issue as the purpose of this control is to provide confident negative sample calling. This is not a sample control, but rather a library preparation control.

In addition to qualitative confirmation of amplified product, fragment analysis allows confirmation of the quality of the library produced. It is highly recommended that a high sensitivity fragment analyzer is used to visualize the peak shape, size, and potential background concentration to ensure library preparation was performed correctly prior to sequencing.

If qualitative confirmation is not required prior to sequencing, a fragment analysis is not necessary for high-throughput workflows after ensuring that high quality libraries with little nonspecific product can be consistently produced. The final library can also be quantified using a fluorometer such as Qubit™ with dsDNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) or using a qPCR method. However, these methods will only give you the absolute yield and not differentiate potential background from the actual library.

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

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

Recommended Sequencing Length and Depth

All CleanPlex Ready-to-Use NGS Panels are designed to be compatible with PE 150 bp reads (2x150 bp).

Recommended Sequencing Depth		
Panel	Application	Average Read Depth
CleanPlex SARS-CoV-2 FLEX Panel	detection	50-200X
	variant identification	200X-1000X
	co-infection, strain analysis, other in-depth applications	1,000X or more

Terminology

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

When calculating how many samples can be sequenced in one chip, it is Important to refer to the correct total read capacity of the chip. For amplicon based sequencing PhiX is needed to improve the diversity of the run for improved read quality. Refer to Illumina suggested PhiX concentrations for each sequencer and allocate enough reads to it when calculating the total sample multiplexing ability.

For example:

For **1000x** coverage of this panel with **354** amplicons, it requires a total of 354,000 cluster reads or 708,000 paired end reads per sample. With a **Miseq V2 kit** (~15 million single reads or 30 paired end reads) you can sequence approximately **42** samples per run.

For recommended sample multiplexing on various Illumina NGS instruments, refer to the panel-specific product sheet available for download on the product document page:

https://www.paragongenomics.com/product_documents/

A sample multiplexing calculator also be download from the product document page.

Supporting Information

Dual-Indexed PCR Primers for Illumina

Primer Sequences

Each sample is indexed by a pair of Indexed PCR Primers for sequencing on Illumina platforms. **XXXXXXXX** denotes the 8-nucleotide index region of the primer. Index sequences are listed below.

i5 Indexed Primer

5' -AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

i7 Indexed Primer

5' -CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

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

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

Index Sequences



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

CleanPlex Dual-Indexed PCR Primers for Illumina Set T

3 x 3 indexes, 9 reactions (SKU 716021)

A selection of three i5 Indexes and three i7 Indexes from Illumina Set A to create 9 unique index combinations. Index selection may vary from kit to kit.

CleanPlex Dual-Indexed PCR Primers for Illumina Set A

12 x 8 indexes in tubes, 96 reactions (SKU 716006)

12 x 8 indexes in tubes, 384 reactions (SKU 716017)

i7 Index	Index Sequence	17 Bases for Sample Sheet All Illumina Systems	i5 Index	Index Sequence	i5 Bases for Sample Sheet	
					MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
A701	GTCGTGAT	ATCACGAC	A501	TGAACCTT	TGAACCTT	AAGGTTC A
A702	ACCACTGT	ACAGTGGT	A502	TGCTAAGT	TGCTAAGT	ACTTAGCA
A703	TGGATCTG	CAGATCCA	A503	TGTTCTCT	TGTTCTCT	AGAGAACA
A704	CCGTTTGT	ACAAACGG	A504	TAAGACAC	TAAGACAC	GTGTCTTA
A705	TGCTGGGT	ACCCAGCA	A505	CTAATCGA	CTAATCGA	TCGATTAG
A706	GAGGGGTT	AACCCCTC	A506	CTAGAACA	CTAGAACA	TGTTCTAG
A707	AGGTTGGG	CCCAACCT	A507	TAAGTTCC	TAAGTTCC	GGAACCTA
A708	GTGTGGTG	CACCACAC	A508	TAGACCTA	TAGACCTA	TAGGTCTA
A709	TGGGTTTC	GAAACCCA				
A710	TGGTCACA	TGTGACCA				
A711	TTGACCCT	AGGGTCAA				
A712	CCACTCCT	AGGAGTGG				

CleanPlex Dual-Indexed PCR Primers for Illumina Set B

12 x 8 indexes in tubes, 96 reactions (SKU 716018)

12 x 8 indexes in tubes, 384 reactions (SKU 716019)

i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems	i5 Index	Index Sequence	i5 Bases for Sample Sheet	
					MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
Q7005	ATATTCAC	GTGAATAT	Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q7006	GCGCCTGT	ACAGGCGC	Q5002	GATATCGA	GATATCGA	TCGATATC
Q7007	ACTCTATG	CATAGAGT	Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q7008	GTCTCGCA	TGCGAGAC	Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q7015	AGTAGAGA	TCTCTACT	Q5009	CCAACAGA	CCAACAGA	TCTGTTGG
Q7016	GACGAGAG	CTCTCGTC	Q5010	TTGGTGAG	TTGGTGAG	CTCACCAA
Q7017	AGACTTGG	CCAAGTCT	Q5013	AACCGCGG	AACCGCGG	CCGCGGTT
Q7018	GAGTCCAA	TTGGA CTC	Q5014	GGTTATAA	GGTTATAA	TTATAACC
Q7023	AATTCTGC	GCAGAATT				
Q7024	GGCCTCAT	ATGAGGCC				
Q7025	ATCTTAGT	ACTAAGAT				
Q7026	GCTCCGAC	GTCGGAGC				

Single-Indexed PCR Primers for Ion Torrent

Primer Sequences

Each sample is indexed by uniquely designed Indexed PCR Primers for sequencing on Ion Torrent platforms. Only CleanPlex Single-Indexed PCR Primers for Ion Torrent are compatible with CleanPlex workflow for generating libraries intended for Ion Torrent sequencing.

Index Sequences

CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent Sets C, D, E, and F contain index sequences identical to the sequences of the equivalent IonCode™ barcodes. These indexed PCR primers are provided in sealed 96-well PCR plates and arrange in ascending order in columns.



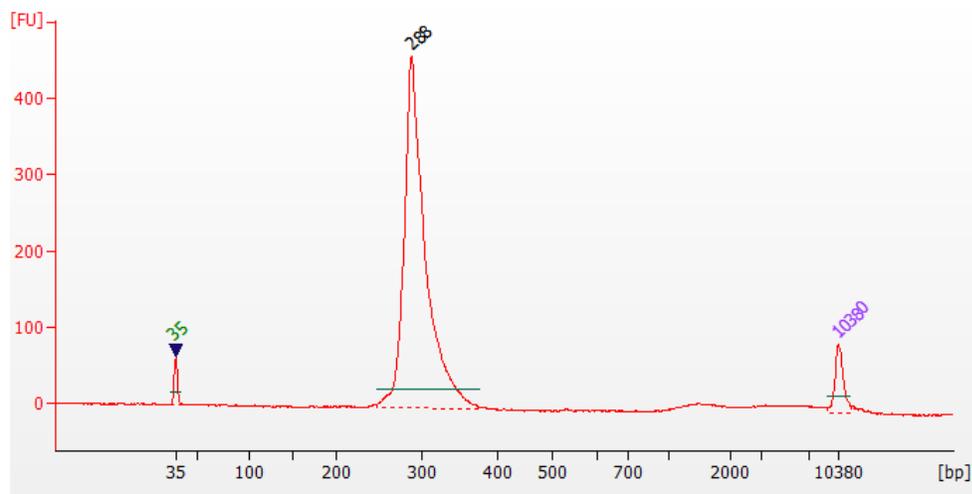
For plated indexes v2 sets C, D, E, and F, plate map and sequences information can be downloaded from [Product Document Page](#) under Indexes.

Troubleshooting Guide

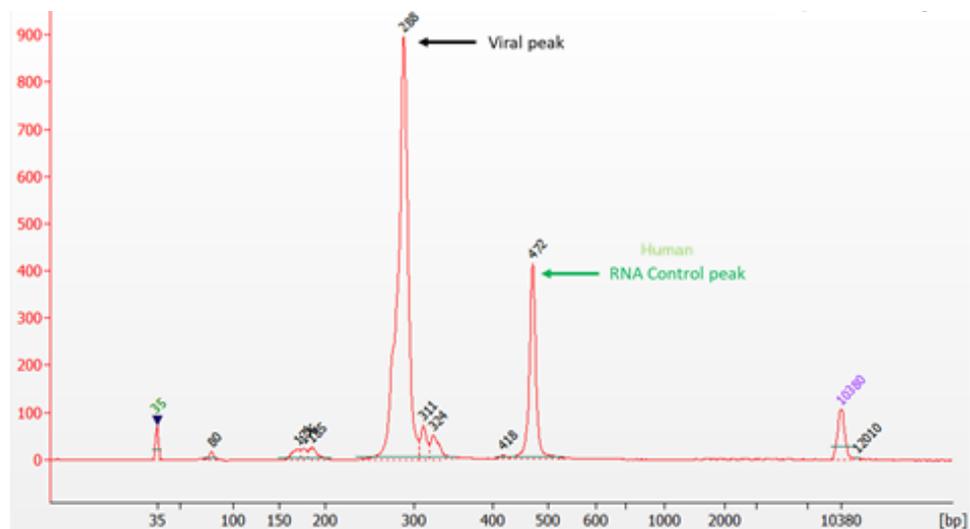
Examples of Libraries Prepared with CleanPlex SARS-CoV-2 FLEX Panel

Depending on the workflow and fragment analyzer used, the CleanPlex SARS-CoV-2 FLEX Panel library peak should be between 250 and 350 bp. Below is a representative Agilent® 2100 Bioanalyzer® trace generated using SARS-CoV-2 FLEX Panel.

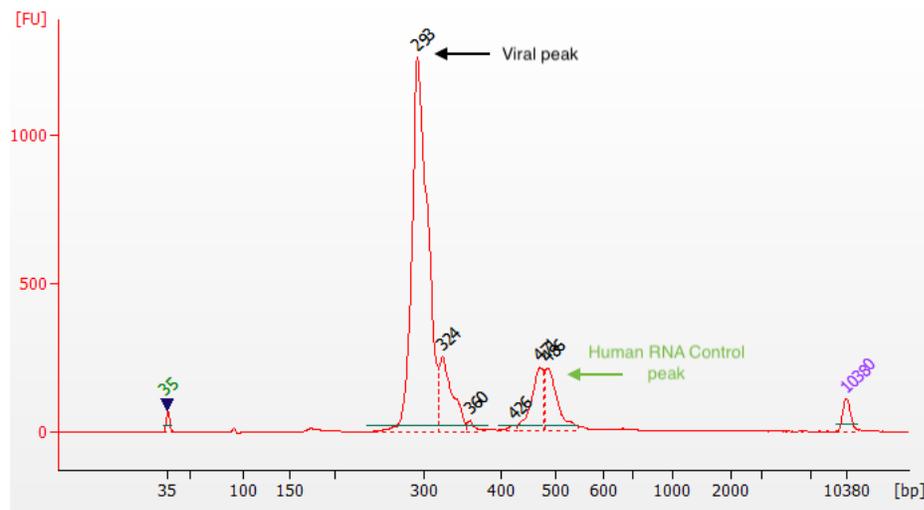
CleanPlex SARS-CoV-2 FLEX Panel



CleanPlex SARS-CoV-2 Panel with Control Human Primer



CleanPlex SARS-CoV-2 FLEX Panel with Add-on v2 & Control Human Primers



Potential Causes for Extra Peaks and Suggested Solutions

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

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

Peaks around 150–190 bp are residues of digested nonspecific amplification products and adapter dimers. They come from incomplete removal of low molecular weight DNA fragments during the Post-Digestion Purification or nonspecific products formed during or after the Multiplex PCR (mPCR) Reaction due to deviation from protocol or high concentration of panel. The digestion reagent degrades 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 mPCR products to sit for too long (and form nonspecific products) before purification.
- CleanPlex Custom NGS Panels may require panel titration for optimal performance. Often dimers will decrease by reducing the panel concentration in the mPCR Reaction Mixture.
- Incorrect annealing time, specifically unnecessarily longer annealing time.
- Cross contamination of Pool 1 and Pool 2 primers in mPCR reactions.

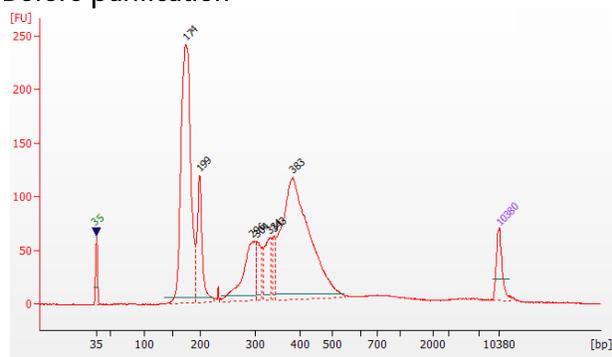
Broad peaks spread across 600 – 10,000 bp range are nonspecific products due to overamplification. Double check that the correct mPCR and Second PCR cycle numbers were used. PCR cycles are determined based on the amplicon count per pool of your panel, RNA input amount, and RNA quality.

Try reducing the Second PCR cycles by 2-3 cycles if issue persists. If you're working with a custom panel for organisms with a much smaller genome than human's, consider reducing the RNA input and Second PCR cycles accordingly.

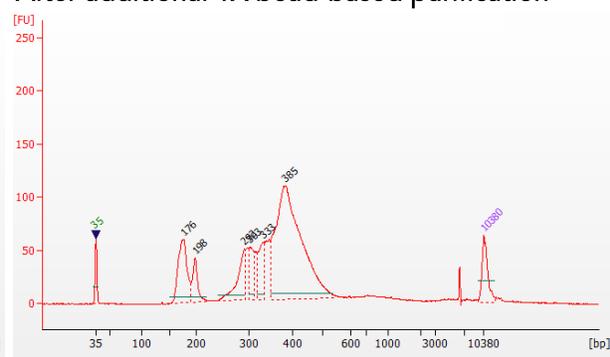
Removing nonspecific products from final libraries

If short nonspecific products described above are present in significant amounts, they can be reduced by pooling indexed libraries (that will be sequenced in the same lane) and performing one additional round of 1X magnetic bead-based purification if the pooled library **concentration is >3,000 pM** and the **total volume is >20 µL**. Low volume and low concentration libraries should not be bead-purified again as it will lead to significant loss of the library of interest. In the figures below, the Agilent® 2100 Bioanalyzer® trace on the left shows a CleanPlex Hereditary Cancer NGS library that contains significant adapter dimers due to poor library preparation. The Bioanalyzer trace on the right shows the same library after an additional 1X magnetic bead-based purification. The bead purification can be repeated once more if volume and concentration still meet the criteria above. We recommend keeping short adapter dimer peaks to less than 5% for best results. When possible it's always better to repeat the library preparation with the correct steps so nonspecific products do not form in the first place.

Before purification



After additional 1X bead-based purification



Potential Reasons for No Library Peaks and No In-Sample Positive Control Peak

- 30% ethanol instead of 70% ethanol was used in DNA or cDNA purification with magnetic beads.
- Magnetic beads were not added for one or more of the purification steps.
- Stop Buffer was not added or was added too late after Digestion Reaction, resulting in over-digestion of the samples. This may happen when handling a large number of samples.
- RNA input was too low due to incorrect quantification, try using more input RNA.
- RNA quality is extremely degraded. Try using more input RNA.
- Incompatible indexed PCR primers were using in the Second PCR Reaction. Only use CleanPlex Indexed PCR Primers for Illumina with CleanPlex NGS Panels for Illumina.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do **not** use magnetic racks designed for 1.5 ml tubes.

Additional Resources

Please visit <https://www.paragongenomics.com/faq-items/> for additional troubleshooting resources.

Data Analysis Recommendations for Illumina

Paragon Genomics offers this panel of 343 amplicons covering 99.7% of the SARS-CoV-2 genome (MN908947/NC_045512.2) with 92 bases uncovered at the ends of the genome and 1 control amplicon covering the TATA Binding protein (TBP) housekeeping gene of the human (host) control. The 343 amplicons covering the SARS-CoV-2 genome are distributed into two pools with amplicon size (without index and adapter) range from 116-196bp (median 149bp), the panel can be used for Illumina 2 X 150. Below are some notes on data processing.

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

1. Adapter Trimming.

With amplicon size ranges from 116 to 196bp, it is recommended to trim leftover adapter sequences for 2X150 sequencing before read mapping.

Following is an example command with open source software cutadapt (<https://cutadapt.readthedocs.io/en/stable/>).

```
cutadapt -g CCTACACGACGCTCTTCCGATCT \  
-a AGATCGGAAGAGCACACGTCTGAA \  
-A AGATCGGAAGAGCGTCGTGTAGG \  
-G TTCAGACGTGTGCTCTTCCGATCT \  
-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
```

2. Map reads to reference genome.

The design was based on MN908947/NC_045512.2 and it is recommended to perform read mapping against the reference sequence of NC_045512.2. Bwa mem is recommended for read mapping and de-duplication procedure shall be skipped.

3. Trim primer sequences.

Before construction of a consensus genome sequence, it is recommended to remove primer sequences. Software package *fgbio* is recommended. The trimming should be based primarily on reads' mapping starts/ends positions and primers 3' ends' coordinates in *amplinsert.bed* files. We recommend using the DNA amplicon workflow on Illumina instruments for trimming primers and calling variants. An alternative approach for primer trimming is using the software package *fgbio*. It requires primer genomic coordinates in a tab delimited file which can be provided by Paragon Genomics to customers.

Following is an example command.

```
java -jar fgbio-1.2.0-e7ac607-SNAPSHOT.jar TrimPrimers -i input.bam -o  
output.primerTrim.bam -p primer_info.tab -H true
```

4. Calculate QC metrics.

In order to assess the 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.

5. Downstream analysis.

Paragon Genomics could provide any additional design files to be used in virus detection or strain identification. SOPHiA Genomic also provides [analysis support](#) for the CleanPlex SARS-CoV-2 Panel. For suggestions or more information on analysis solutions, please contact sales@paragongenomics or techsupport@paragongenomics.com.

Data Analysis Recommendations for Ion Torrent

We recommend customers to use analysis software provided by Ion Torrent. A few suggestions specific to Paragon Genomics panels are given below.

1. **BED Files.** Visit <https://www.paragongenomics.com/my-account/downloads/> to download panel-specific amplicon BED files and amplicon insert BED files. For CleanPlex Custom Panels, the BED files are available for download via the associated account through our design portal: <https://www.paragongenomics.com/panel-design-my-design-requests/>
The amplicon insert BED file should be used for variant calling on Torrent Suite (TS) and Ion Reporter (IR).
2. **Extra Trimming settings.** When planning or reanalyzing a sequencing run with CleanPlex library on TS, user should add these options to both Pre-BaseCaller Args and BaseCaller Args : --extra-trim-left 24 --extra-trim-right 24 , to avoid misestimate of base quality.

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

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

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



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

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

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

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

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

Phone: 650-822-7545

Email: techsupport@paragongenomics.com

Website: www.paragongenomics.com

Paragon Genomics

5020 Brandin Court, FL 2

Fremont, CA 94538 USA

1-510-363-9918

contact@paragongenomics.com

www.paragongenomics.com

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