

# CleanPlex<sup>®</sup> RNA Custom NGS Panel

## User Guide

This user guide is for the following products for sequencing on Illumina<sup>®</sup> and Ion Torrent<sup>™</sup> NGS platforms:

- CleanPlex<sup>®</sup> RNA Custom NGS Panel

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
UG3003-01	June 2023	<ul style="list-style-type: none"><li>Initial version</li></ul>

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# Overview

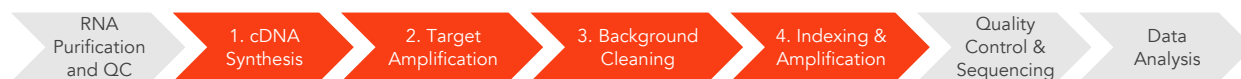
## Product Information

CleanPlex® Targeted RNA sequencing technology is a fast, robust, and versatile solution for target enrichment and library preparation for next-generation sequencing (NGS) on Illumina® and Ion Torrent™ sequencing platforms. CleanPlex NGS RNA Panels generate highly accurate data from as little as 1 ng of input using a fast and simple workflow. CleanPlex Custom NGS Panels are made-to-order to target user-defined genomic regions of interest.

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

CleanPlex NGS RNA Panels feature a fast and simple workflow that can be completed in about 6 hours from RNA to indexed NGS libraries. The workflow involves just 4 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 RNA & DNA material and prevent handling errors and sample mix-up.

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



### CleanPlex RNA Target Enrichment and Library Preparation Workflow

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

## Applications

CleanPlex Custom RNA NGS Panels can be built from 7 to 20,000 amplicons per primer pool to target RNA transcript regions of your choice. Amplicons can be designed with size ranging from 80 to 500 bp to accommodate different sample types and the needs of specific applications.

The table below shows some of the organisms, sample types, and applications that users have studied using CleanPlex RNA NGS Panels. This is not meant to be an exhaustive or restrictive list.

Organisms	Sample Types	Applications
<ul style="list-style-type: none"> <li>• Human</li> <li>• Mammals</li> <li>• Fish</li> <li>• Insects</li> <li>• Plants</li> <li>• <b>Pathogens/</b> Microorganisms</li> </ul>	<ul style="list-style-type: none"> <li>• Genomic RNA</li> <li>• Degraded RNA from FFPE tissues</li> <li>• Cell-free RNA (cfRNA) from plasma and other biofluids</li> <li>• single cell RNA</li> </ul>	<ul style="list-style-type: none"> <li>• Gene expression profiling and analysis</li> <li>• Variant Detection</li> <li>• Fusion detection</li> <li>• Splice variant analysis</li> <li>• Biomarker discovery</li> <li>• Pathway analysis</li> <li>• RNA pathogen detection</li> </ul>

## Other Applications

For other targeted sequencing applications, Paragon Genomics also offer a while variety of both ready to use panel and custom panels for infectious disease, 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
<b>OmniFusion™ RNA Lung Cancer Panel</b>	917101	Oncology	Interrogate >530 known RNA fusion and additional novel RNA fusion using a single-primer method.
<b>AccuFusion™ RNA Lung Cancer Panel</b>	917104	Oncology	Interrogate ~280 known RNA fusions using a target specific dual-primer multiplex PCR method.
<b>CleanPlex SARS-CoV-2 FLEX Research and Surveillance Panel</b>	918014	Infectious Disease	FLEX panel for frontline SARS-CoV-2 mutation research
<b>CleanPlex Respiratory Research Panel</b>	918301	Infectious Disease	Respiratory Panel contains additional viral content for concurrent Influenza and SARS-CoV-2 detection and flu subtyping

## 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.

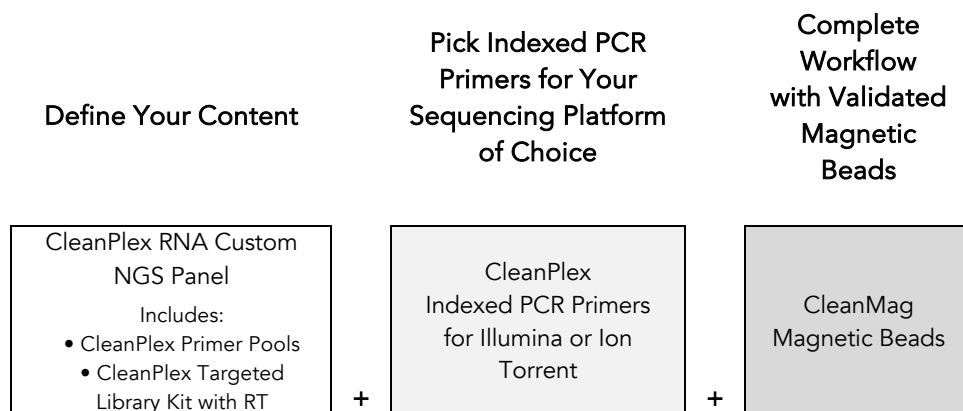
CleanPlex Indexed PCR Primers for Ion Torrent are used to generate CleanPlex target-enriched NGS libraries that are compatible all Ion Torrent sequencing platforms, including Ion PGM System, Ion Proton System, and Ion GeneStudio S5 Series.

Please contact [support@paragongenomics.com](mailto:support@paragongenomics.com) for additional information regarding compatibility with the other sequencing platforms such as Singular Genomics and Oxford Nanopore.

## Kit Contents

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

- CleanPlex RNA Custom NGS Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)



### Panel Specifications

Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Average Amplicon Length	Average Library Length
CleanPlex RNA Custom NGS Panels	Varies	5X	Varies	Varies	Varies

A CleanPlex Targeted Library Kit is included in every CleanPlex RNA Custom NGS Panel. Please note that the CleanPlex Targeted Library Kit is **not** sold separately.

### CleanPlex® Targeted RNA Custom Panel — Kit Contents, Store at –20°C

Configuration	1-Pool		2-Pool			
	Size	96 Rxns	384 Rxns	96 Rxns	384 Rxns	
Component	Cap Color	SKU	937400	937401	937402	937403
RT Primer Mix DP	Purple Striped	288 µl	1152 µl	288 µl	1152 µl	
RT Enzyme	Blue Striped	96 µl	384 µl	96 µl	384 µl	
RT Buffer DP	Green Striped	480 µl	1920 µl	480 µl	1920 µl	
5X mPCR Mix	Green	192 µl	768 µl	384 µl	1536 µl	
CP Reagent Buffer	White	192 µl	768 µl	384 µl	1536 µl	



CP Digestion Reagent	Yellow	192 µl	768 µl	384 µl	1536 µl
Stop Buffer	Red	384 µl	1536 µl	576 µl	2304 µl
5X 2nd PCR Mix	Blue	768 µl	3072 µl	768 µl	3072 µl
TE Buffer	Clear	500 µl	4 ml	500 µl	4 ml

## Required Materials and Equipment Not Included

- CleanPlex Indexed PCR Primers (visit [www.paragongenomics.com/store](http://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 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 Plated Unique Dual-Indexed PCR Primers for Illumina Set C	716037 716049	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set D	716038 716050	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set E	716039 716051	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set F	716040 716052	96 384	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 1-pool panels	Reactions 2-pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~6	~5
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~33	~25
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~130	~100
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~400	~300
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,000	~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. We recommend twin.tec PCR Plate 96, semi-skirted, colorless (Catalog #: 951020303).

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 ( i.e. Axygen Aluminum Sealing Film (REF: PCR-AS-600)
- Pipettors and low-retention filtered pipette tips
- Thermal cycler
- Mini-centrifuge, 96-well plate centrifuge
- Qubit® Fluorometer and dsDNA HS (high sensitivity) Assay Kit, or equivalent
- Agilent® 2100 Bioanalyzer® Instrument and Agilent High Sensitivity DNA Kit, or equivalent

## Storage, Handling, and Usage

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

CleanMag Magnetic Bead solution is shipped at room temperature or on blue ice (ice packs). **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 are fully thawed and have been vortexed and spun down to bring all liquids to the bottom of the tubes prior to use.

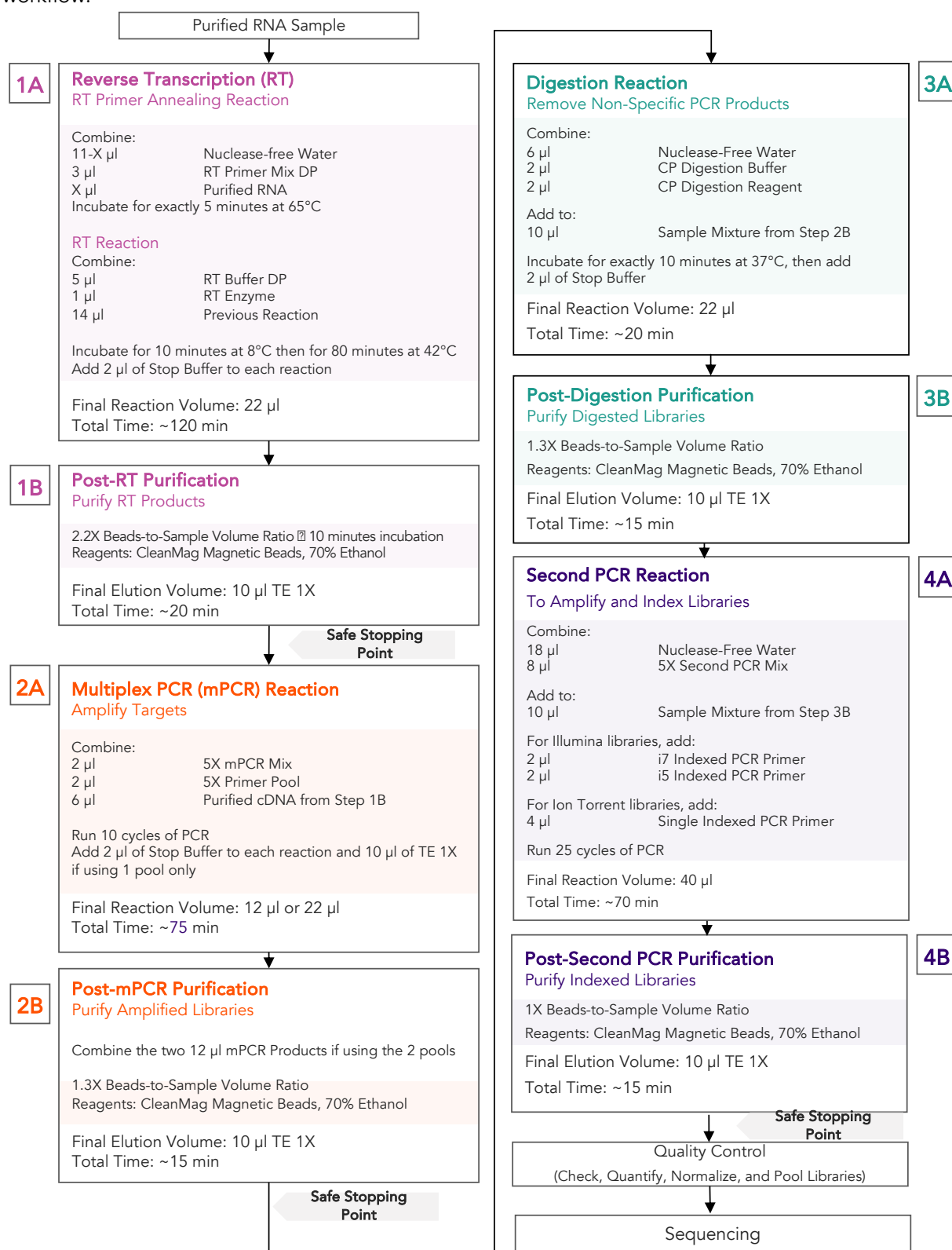
The components containing enzymes (RT Enzyme Mix, 5X mPCR Mix, CP Digestion Reagent and 5X 2nd PCR Mix) are specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below -23°C.

CleanPlex RNA 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 RNA Custom 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 (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 Primer 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 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/](http://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 (per pool)	Recommended RNA Input (per pool)	Recommended Low Quality RNA input ( per pool)
CleanPlex RNA Custom NGS Panels	10-100 ng	10 ng	20 ng

- The maximum volume of RNA input per RT Reaction is 11  $\mu$ l, and half is carried over to mPCR and final library generation. Adjust the RNA input based on the application: for example, for gene expression analysis, depending on the relative expression levels of the RNA targets, you may want to ensure the lowest expressing targets are well represented in the reaction.
- For multipool panels, start with enough RNA such that each mPCR has sufficient cDNA input.
- This kit is compatible with RNA extraction kits for PCR applications. Choose an RNA extraction method that is compatible with your application. Some compatible RNA extractions kits include: QIAamp® Viral RNA Mini Kit, QIAamp® MinElute Virus Spin Kit, RNeasy® Mini Kit (QIAGEN), Roche MagNA Pure Compact RNA Isolation Kit, Roche MagNA Pure Compact Nucleic Acid Isolation Kit, Roche MagNA Pure 96 DNA
- 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 70% ethanol by combining 100% ethanol and nuclease-free water 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: There are safe stopping points after Post-RT Purification, Post-mPCR Purification, and Post-2<sup>nd</sup> PCR Purification. After starting Digestion Step, samples must be carried through to the 2<sup>nd</sup> PCR reaction without stopping. Plan accordingly.



## 1A. Reverse Transcription (RT) Reaction: To Generate cDNA

- 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 $\mu$ l
RT Primer Mix DP	Purple Striped	3 $\mu$ l
Purified RNA	—	X $\mu$ l
<b>Total Volume per reaction</b>		<b>14 <math>\mu</math>l</b>

- 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 incubation 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 add into individual reactions.

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
<b>Total Volume per reaction</b>		<b>20 µl</b>

- 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: To Purify cDNA



**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  $\mu$ 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	
	Volume per reaction
Reverse Transcription Product	22 $\mu$ l
Magnetic Beads to add	48 $\mu$ 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  $\mu$ l 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  $\mu$ 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.
- 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  $\mu$ l 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: To Amplify Targets

- 2A.1. Using thin-wall PCR strip tubes (or a 96-well PCR plate), prepare the mPCR Reaction Mixture by adding components in the following order on ice or a cold block. For CleanPlex Custom NGS Panels with multiple primer pools, prepare individual reaction for each primer pool.

**Note:** When working with multi-pool panels, the 10uL of cDNA will be divided equally between pools.

mPCR Reaction Mixture ( 1 Pool panel)		
Reagent	Cap Color	Volume per reaction
5X mPCR Mix	Green	2 $\mu$ l
5X Primer Pool	Varies	2 $\mu$ l
cDNA from 1B	—	6 uL or less*
<b>Total Volume per reaction</b>		<b>10 <math>\mu</math>l</b>

\*Out of the total available 10uL cDNA, use a max 6 uL of cDNA for single pool panels. The remaining purified RT sample volume (4 uL) can be stored at -20C for future use if needed. For multipool panels, equally divide the the cDNA into each mPCR reaction.



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

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

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

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

- 2A.3. Load the tubes/plate in the thermal cycler with a heated lid at 105 °C and run the following thermal cycling protocol to amplify target DNA regions. Use the table below to determine the mPCR thermal cycling conditions for specific CleanPlex NGS Panels.

mPCR Thermal Cycling Protocol				
Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	1
Denaturation	98 °C	15 sec	3 °C/s	Refer to table below for cycle numbers
Annealing/Extension	60 °C	See table below	2 °C/s	
Hold	10 °C	∞		

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

**Note:** For CleanPlex Custom NGS panels, use per pool amplicon number to determine the appropriate annealing/extension times using the table below. *Typically multi-pool panels have evenly split amplicons. 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.*

Panel Specific mPCR Thermal Cycling Conditions		
Panel	Annealing/Extension Time	mPCR Cycle Number
CleanPlex Custom NGS Panels	5 min for 7–2,000 amplicons per pool 8 min for 2,000–5,000 amplicons per pool 16 min for 5,000–20,000 amplicons per pool	10

- 2A.4. Immediately add 2  $\mu\text{l}$  of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing. Spin down again briefly to collect the liquid. The volume of each sample is now approximately 12  $\mu\text{l}$ .

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

Proceed to Step 1B. Post-mPCR Purification.



**Important.** Do not stop and store PCR products after multiplex PCR reaction. Add Stop Buffer then proceed to Step 2B, Post-mPCR Purification immediately.

## 2B. Post-mPCR Purification: To Purify Amplified Libraries



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



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

- 2B.1. Open the tubes or carefully remove the adhesive film from the PCR plate.
- For CleanPlex NGS Panels comprising of one primer pool, add 10  $\mu\text{l}$  of TE buffer to each sample.
  - For CleanPlex NGS Panels with multiple primer pools, combine the 12  $\mu\text{l}$  multiplex PCR products for each pool for each sample into one tube and perform purification on the combined product. See table below for examples.
- 2B.2. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a **1.3X** bead-based purification by adding magnetic beads to the sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-mPCR Purification — 1.3X Beads-to-Sample Volume Ratio				
	1-Pool	2-Pool	3-Pool	4-Pool
Volume of Combined Sample per reaction	22 $\mu\text{l}$ (12 $\mu\text{l}$ of sample + 10 $\mu\text{l}$ of TE Buffer)	24 $\mu\text{l}$	36 $\mu\text{l}$	48 $\mu\text{l}$
Volume of Magnetic Beads per reaction	29 $\mu\text{l}$	31 $\mu\text{l}$	47 $\mu\text{l}$	62 $\mu\text{l}$



**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.

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



- 2B.4. Briefly spin down 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 down 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 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 1B.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  $\mu$ l TE buffer directly onto the pellet for each tube/well. Briefly spin down 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 2-8°C for up to 1 hour or at -20°C for up to 24 hours.

### 3A. Digestion Reaction: To Remove Non-Specific PCR Products

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

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

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

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

3A.1. Prepare Digestion Reaction Master Mix and add directly into the same wells of each purified sample from Step 1B. No tube-to-tube transfer is needed and the magnetic beads from the previous reaction are carried onto the next steps and do not affect future reactions.

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

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

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

- 3A.2. Mix by pipetting up and down at least 5 times or vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin down briefly to collect the liquid.

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

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

- 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 down again briefly to collect the liquid. The volume of each sample is approximately 22 µl.
- 3A.5. Proceed to Step 2B. Post-Digestion Purification immediately.



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

### 3B. Post-Digestion Purification: To Purify Digested Libraries



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



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

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

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

Reagent	Volume per reaction
Digestion Reaction Product	22 µl
Magnetic Beads	29 µl



**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 down 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 down step ensures complete removal of supernatant.

- 3B.5. Add 180  $\mu$ 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 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  $\mu$ l TE buffer directly onto the pellet for each tube/well. Briefly spin down 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: To Amplify and Index Libraries

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

- 4A.1. Thaw Indexed PCR Primers, vortex thoroughly, then spin down briefly to collect the liquid. Prepare Second PCR Reaction Master Mix and add to each purified sample from Step 2B. Then add a unique (combination of) Indexed PCR Primer(s) to each sample.

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

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

Second PCR Reaction Master Mix		
Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	18 $\mu$ l
5X 2nd PCR Mix	Blue	8 $\mu$ l
<b>Total Volume per reaction</b>		<b>26 <math>\mu</math>l</b>

Second PCR Reaction Mixture			
Reagent		Illumina Volume per reaction	Ion Torrent Volume per reaction
Second PCR Reaction Master Mix		26 $\mu$ l	26 $\mu$ l
Purified Sample from Step 2B		10 $\mu$ l	10 $\mu$ l
i5 Indexed PCR Primer for Illumina		2 $\mu$ l	—
i7 Indexed PCR Primer for Illumina		2 $\mu$ l	—
Single-Indexed PCR Primer for Ion Torrent		—	4 $\mu$ l
<b>Total Volume per reaction</b>		<b>40 <math>\mu</math>l</b>	<b>40 <math>\mu</math>l</b>

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



**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.

- 4A.2. Close the caps of the PCR tubes or seal the PCR plate with adhesive film. Spin down briefly to collect the liquid, then mix thoroughly by pipetting up and down at least 5 times or vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin down briefly to collect the liquid.

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

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

- 4A.3. Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. Use the three tables below for thermal cycling protocol and cycle number suggestions for CleanPlex Ready-to-Use NGS Panels and CleanPlex Custom NGS Panels.

**Notes:**

1. 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 requires more PCR cycles.
2. Use the per primer pool RNA input to determine the correct cycles in the tables below.
3. Nanogram input suggestions below are based on Human Genome RNA samples. For custom panels amplifying target regions of other organisms, use the genome size vs human to calculate nanogram equivalent as needed.
4. For CleanPlex Custom NGS Panels, use the total number of amplicons to determine the appropriate Second 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	Refer to table below for cycle numbers
Annealing/Extension	Illumina 60 °C	Ion Torrent 68 °C	75 sec	2 °C/s	
Hold	10 °C		∞		

\* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/Ss..



CleanPlex Custom NGS Panels — Second PCR Cycle Number		
Total Number of Amplicons* in Panel	10 ng of High Quality RNA per Pool	10 ng of Low Quality RNA per Pool (eg. FFPE cDNA)
7 - 100	13	14
101 - 200	11	12
201 - 500	10	11
501 – 1,000	9	10
1,001 – 2,000	8	9
2,001 – 5,000	7	8
5,001 – 12,000	6	7
12,001 – 20,000	5	6

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

Second PCR Reaction — Input Specific Cycle Number	
Input RNA per Pool	Change in Cycle Number
5 ng	+ 1
10 ng	-
50 ng	- 2
Low quality RNA	+ 3 to 5

**Note:** For optimal yield, the CleanPlex RNA Custom Panels may benefit from adjustment to Second PCR cycle numbers based on the sample quality, viral input (when known), and application. Please contact [support@paragongenomics.com](mailto:support@paragongenomics.com) for additional suggestions.

- 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 PCR product after Second PCR. Proceed to 3B, Post-Second PCR purification immediately.

## 4B. Post-Second PCR Purification: To Purify Indexed Libraries

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

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



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

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

- 4B.2. Incubate the mixture for 5 minutes at room temperature.
- 4B.3. Briefly spin down 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 to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.



**Important!** Removing all residual supernatant from the Second PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin down 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 down the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tubes/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

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

4B.8. Remove tubes/wells from the magnetic rack and add 10  $\mu$ l TE buffer directly onto the pellet for each tube/well. Briefly spin down 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 down 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  $-20^{\circ}\text{C}$ .

**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  $-20^{\circ}\text{C}$  until ready to sequence. When taking the library out for QC and sequencing, vortex briefly and place the tubes or plate on a magnetic rack to pull the beads to one side. Avoid pipetting the beads, which will affect QC and sequencing.

## Quality Control Prior to Sequencing

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

CleanPlex Ready to Use typically have a yield of 6 nM to 30nM, depending on RNA sample quantity/quality and cycle numbers. Custom panel yield might be more variable. Library quantity is not indicative of library quality. Additional input or 2<sup>nd</sup> PCR cycles can be adjusted accordingly for optimal yield for your downstream processing or sequencing needs.

The expected amplicon size distribution or peak shape 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 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 RNA Custom NGS Panels are designed to be compatible with Illumina PE 150bp reads (2 x 150bp) unless otherwise decided and communicated between the customer and the Paragon Genomics panel design team. It is recommended to follow Illumina's suggestions for PhiX concentration according to sequencer used and incorporate that calculate in the final sample multiplexing calculation.

## Terminology

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

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

**For example:**

For **1000X** coverage of a panel with **100** amplicons, it requires a total of 100,000 cluster reads or 200,000 paired end reads per sample. With a **Miseq V2 kit** (~15 million single reads or 30 million paired end reads) you can sequence about **150** samples per run.

For a sample multiplexing calculator and recommended sample multiplexing on various Illumina NGS instruments, please refer to tools section and panel-specific product sheets at [www.paragongenomics.com/product\\_documents/](http://www.paragongenomics.com/product_documents/).

# Supporting Information

## Dual-Indexed PCR Primers for Illumina

### Best Practices

- Employ best laboratory practices to avoid cross contamination of indexed primers. Upon removing the primers from -20C storage, centrifuge the plate at room temperature (e.g. for 1min at 280 x g), ensure ALL the liquid is collected in the bottom of the wells, then use accordingly. We do not recommend vortexing the adapter plates as it could result in cross-contamination of the indexed primers. Pipette mix to resuspend individual adapters prior to use.
- The foil cover is pierceable and peelable. If peeling the cover off, take care to slowly remove the foil cover to avoid cross-contamination and to remove the foil evenly.
- Upon first use, carefully remove the foil cover to avoid cross-contamination. Discard the original foil cover and do not reuse. If only using a subset of the adapters, partial use of the foil is not recommended. Please replace the seal entirely once it has been opened.

### Primer Sequences

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

i5 Indexed Primer

5' -AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

i7 Indexed Primer

5' -CAAGCAGAAGACGGCATAACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

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

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

### Index Sequences



Additional sequence and Sample sheet information can be downloaded from [Product Document Page](#) under Indexes and Useful Tools.

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**CleanPlex Dual-Indexed PCR Primers for Illumina Set T**  
3 x 3 indexes, 9 reactions (SKU 716021)

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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.

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**CleanPlex Dual-Indexed PCR Primers for Illumina Set A**

12 x 8 indexes, 96 reactions (SKU 716006)

12 x 8 indexes, 384 reactions (SKU 716017)

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



## CleanPlex Dual-Indexed PCR Primers for Illumina Set B

12 x 8 indexes, 96 reactions (SKU 716018)

12 x 8 indexes, 384 reactions (SKU 716019)

i7 Index	Index Sequence	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
Q7005	ATATTCAC	GTGAATAT	Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q7006	GCGCCTGT	ACAGGCGC	Q5002	GATATCGA	GATATCGA	TCGATATC
Q7007	ACTCTATG	CATAGAGT	Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q7008	GTCTCGCA	TGCGAGAC	Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q7015	AGTAGAGA	TCTCTACT	Q5009	CCAACAGA	CCAACAGA	TCTGTTGG
Q7016	GACGAGAG	CTCTCGTC	Q5010	TTGGTGAG	TTGGTGAG	CTCACCAA
Q7017	AGACTTGG	CCAAGTCT	Q5013	AACCGCGG	AACCGCGG	CCGCGGTT
Q7018	GAGTCCAA	TTGGACTC	Q5014	GGTTATAA	GGTTATAA	TTATAACC
Q7023	AATTCTGC	GCAGAATT				
Q7024	GGCCTCAT	ATGAGGCC				
Q7025	ATCTTAGT	ACTAAGAT				
Q7026	GCTCCGAC	GTCGGAGC				

## 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 NGS Panels

Depending on the CleanPlex NGS Panel used, library peak(s) should be between 200 and 400 bp, unless otherwise designed to be larger.

### Potential Causes for Extra Peaks and Suggested Solutions

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

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

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

- Inaccurate pipetting of magnetic bead volume.
- Insufficient mixing of reaction solutions or bead and sample mixture.
- Insufficient removal of supernatant and ethanol washes during purification.
- Allowing mPCR products to sit for too long (and form nonspecific products) before purification.
- 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.
- Insufficient RNA/cDNA input.

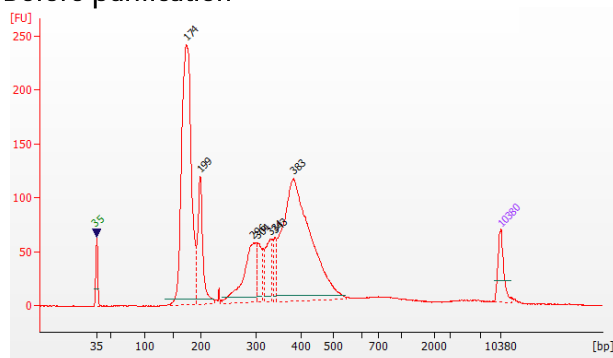
**Broad peaks spread across 500 – 10,000 bp range** are nonspecific products due to overamplification. Double check that the correct mPCR and Second PCR cycle numbers were used. PCR cycles are determined based on the amplicon count per pool of your panel, RNA input amount, and RNA quality. Try reducing the Second PCR cycles by 2-3 cycles if this 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.

Broad peaks can also be due to genomic DNA carryover or nonspecific amplification due to prime/template imbalance.

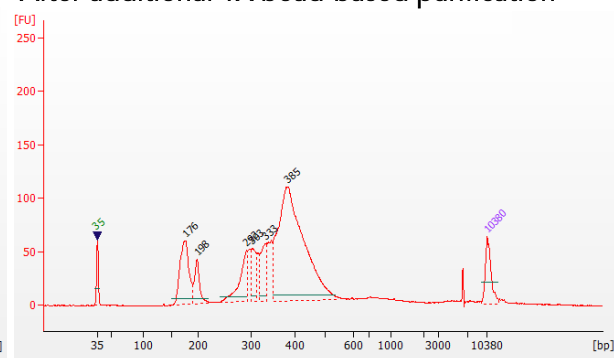
### Removing nonspecific products from final libraries

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 **concentrations is >3,000 pM** and the **total volume is >20  $\mu$ L**. 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 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 corrected steps so nonspecific products do not form in the first place.

### Before purification



### After additional 1X bead-based purification



## Potential Reasons for No Peaks

- 30% ethanol instead of 70% ethanol was used in 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/cDNA quantification was inaccurate, especially if using spectrophotometric methods, such as the NanoDrop instrument. Try using more input RNA.
- RNA quality is extremely degraded. Try using more input RNA.
- Incompatible indexed PCR primers were used in the Second PCR Reaction. Only use CleanPlex Index primers with CleanPlex library prep. Use the appropriate index primers for the sequencer of choice.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do **not** use magnetic racks designed for 1.5 ml tubes.

## Additional Resources

Please visit [www.paragongenomics.com/product/faq/](http://www.paragongenomics.com/product/faq/) for additional troubleshooting resources.

## Data Analysis Recommendations for Illumina

We recommend on-instrument analysis using Illumina's DNA Amplicon Module for the simplest and most streamlined experience for QC. However, downstream analysis like differential expression analysis or fusion detection are outside the scope of the module- we recommend using another platform or pipeline to perform these functions. Please follow Illumina's latest user guide for step-by-step instructions, which is available in their support center. Manifest files are automatically provided for hg19 designs (in DesignDetails.zip) and upon request, Paragon Genomics can provide the manifest file for other genomes.

As an alternative, the Broad Institute's GATK Best Practice (<https://software.broadinstitute.org/gatk/best-practices/>) offers general guiding principles for sequencing data analysis.

For off-instrument analysis, please refer to the following recommended steps for analyzing CleanPlex NGS libraries sequenced on Illumina platforms.

1. **BED Files.** Visit <https://www.paragongenomics.com/my-account/downloads/> to download panel-specific amplicon insert BED file. For CleanPlex Custom Panels, the BED file is available for download via the associated order account through our design portal: <https://www.paragongenomics.com/panel-design-my-design-requests/>

### 2. Adapter Trimming.

For panel with amplicon inserts that fall less than 150bp lengths, 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
```

### 3. Map reads to reference genome.

Ready to use panels are designed against GRCh37 (hg19) reference genome. For customer panels, please refer to your specified reference genome. Bwa mem is recommended for read mapping and de-duplication procedure shall be skipped.

### 4. Trim primer sequences.

Before construction of a consensus genome sequence, it is recommended to remove primer sequences. The trimming should be based primarily on reads' mapping starts/ends positions and primers 3' ends' coordinates in ampInsert.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
```

### 5. 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.

## 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 insert BED file. For CleanPlex Custom Panels, the BED file is available for download via the associated order account through our design portal: <https://www.paragongenomics.com/panel-design-my-design-requests/>
2. **Extra Trimming settings.** When working in the Ion environment, such as the Torrent Suite, Ion Reporter, or VariantCaller Plugin, discard any custom argument and revert to default arguments for your chip type and application combination, then try adding to both Pre-BaseCaller Args and BaseCaller Args : `--extra-trim-left 24 --extra-trim-right 24` , to avoid misassignment of read quality.

Setting	Description
Pre Basecaller args for calibration	<code>--extra-trim-left 24 --extra-trim-right 24</code>
Basecaller args	<code>--extra-trim-left 24 --extra-trim-right 24</code>

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. The trimming should be based primarily on reads' mapping starts/ends positions and primers 3' ends' coordinates in ampInert bed files. We recommend using the Torrent Variant Caller plugin for trimming primers and calling variants. For other applications that require actual primer sequences, please contact Paragon Genomics to acquire them.



## Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

**Phone:** 650-822-7545

**Email:** [support@paragongenomics.com](mailto:support@paragongenomics.com)

**Website:** [www.paragongenomics.com](http://www.paragongenomics.com)

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