



S I N G U L A R
G E N O M I C S



PARAGON
G E N O M I C S

CleanPlex[®] NGS Panel Addendum

User Guide for Sequencing on the G4[®] Platform

This is an addendum to the CleanPlex NGS Panel User Guide that describes the processes for generating libraries compatible with Singular Genomics G4 Sequencing Platform. All CleanPlex Ready to Use and Custom Targeted Library Preparation can be adjusted accordingly.

For other User Guides, see our product document page.

Get the latest user guide at: www.paragongenomics.com/product_documents/

This document and its contents are proprietary to Paragon Genomics, Inc., and are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Paragon Genomics. Paragon Genomics does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The contents of this document are provided "AS IS." This document may be updated or changed without notice at any time. This information could contain technical inaccuracies, typographical errors and out-of-date information. Use of the information is therefore at your own risk. In no event shall PARAGON GENOMICS, INC. be liable for any special, indirect, incidental or consequential damages resulting from or related to the use of this document. A non-exhaustive list of Paragon Genomics' marks, many of which are registered in the United States and other countries can be viewed at: www.paragongenomics.com/company/legal/. Paragon Genomics may refer to the products or services offered by other companies by their brand name or company name solely for clarity, and does not claim any rights in those third party marks or names. Paragon Genomics' products may be covered by one or more of the patents as indicated at: www.paragongenomics.com/company/legal/.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s). FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY, AND WILL VOID ANY WARRANTY APPLICABLE TO THE PRODUCT(S).

PARAGON GENOMICS DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN.

CleanPlex[®] NGS Panels are intended for Research Use Only. It may not be used for any other purposes including, but not limited to, use in diagnostics, therapeutics, or in humans. CleanPlex NGS Panels may not be transferred to third parties, resold, modified for resale or used to manufacture commercial products without prior written approval of Paragon Genomics, Inc.

All trademarks are the property of Paragon Genomics, Inc. or their respective owners. Singular Genomics, G4, and the Singular Genomics logo are trademarks for Singular Genomics Systems, Inc.

©2023 Paragon Genomics, Inc. All Rights Reserved.

Revision History

Document	Date	Description of Change
UG7002-01	December 2023	<ul style="list-style-type: none">• Initial Release

Table of Contents

Overview	4
Addendum Scope	4
Compatible Sequencing Instruments	4
Singular Genomics Index Offerings	4
Workflow	5
Protocol	6
Resources	6
Best Practices for Singular Genomics Index Materials	6
Procedures Prior to Second PCR Reaction	6
3A. Singular Genomics Specific Second PCR Reaction for G4 Compatible libraries	6
Quality Control Prior to Sequencing	10
Sequencing on the G4 Platform	11
Recommended Sequencing Length and Depth	11
Supporting Information	13
Best Practices and Primer Sequences	13
Additional Resources	13
Data Analysis Recommendations	14
Technical Support	16

Overview

Addendum Scope

This User Guide Addendum is meant to be used in conjunction with other CleanPlex, CleanPlex UMI, OmniFusion, Accufusion, AgriType User Guides to generate Singular Genomics® G4® compatible and ready to sequence targeted libraries for sequencing. This document will also detail associated G4 specific sample index information, sequencing guidance, and analysis suggestions and options.

For detailed information on Paragon Genomics technology, applications, kit content, other sequencer compatible workflow, and general troubleshooting, please refer to the respective User Guides for the technology and panel.

For additional questions and support, please contact support@paragongenomics.com.

Compatible Sequencing Instruments

Singular Unique Dual Indexed Primers are used to generate CleanPlex target-enriched NGS libraries that are compatible with the G4 sequencing platform.

Singular Genomics Index Offerings

The Singular Genomics G4 Platform requires S1 and S2 sequences for the formation of clusters on the flow cell. The SP1 and SP2 tags are positioned directly adjacent to the insert. When constructing libraries for multiplexed reads, index 1 and index 2 sequences are placed in between S1 and SP1, and S2 and SP2 respectively. For more information about Singular Genomics primers and adapters, see the [Adapters and Indices for the G4 Sequencing Platform Reference Guide](#).

Singular Genomics provides two types of primers that can be directly used in the standard 2nd PCR reactions for Paragon Genomics technologies:

1. SG UDI

The Singular Genomics Unique Dual Indices (UDIs) provide primers designed to support the preparation of up to 96 uniquely indexed libraries, that can be sequenced together in one flow cell. For more information such as available sets and associated part number see [Singular Genomics UDI Overview](#).

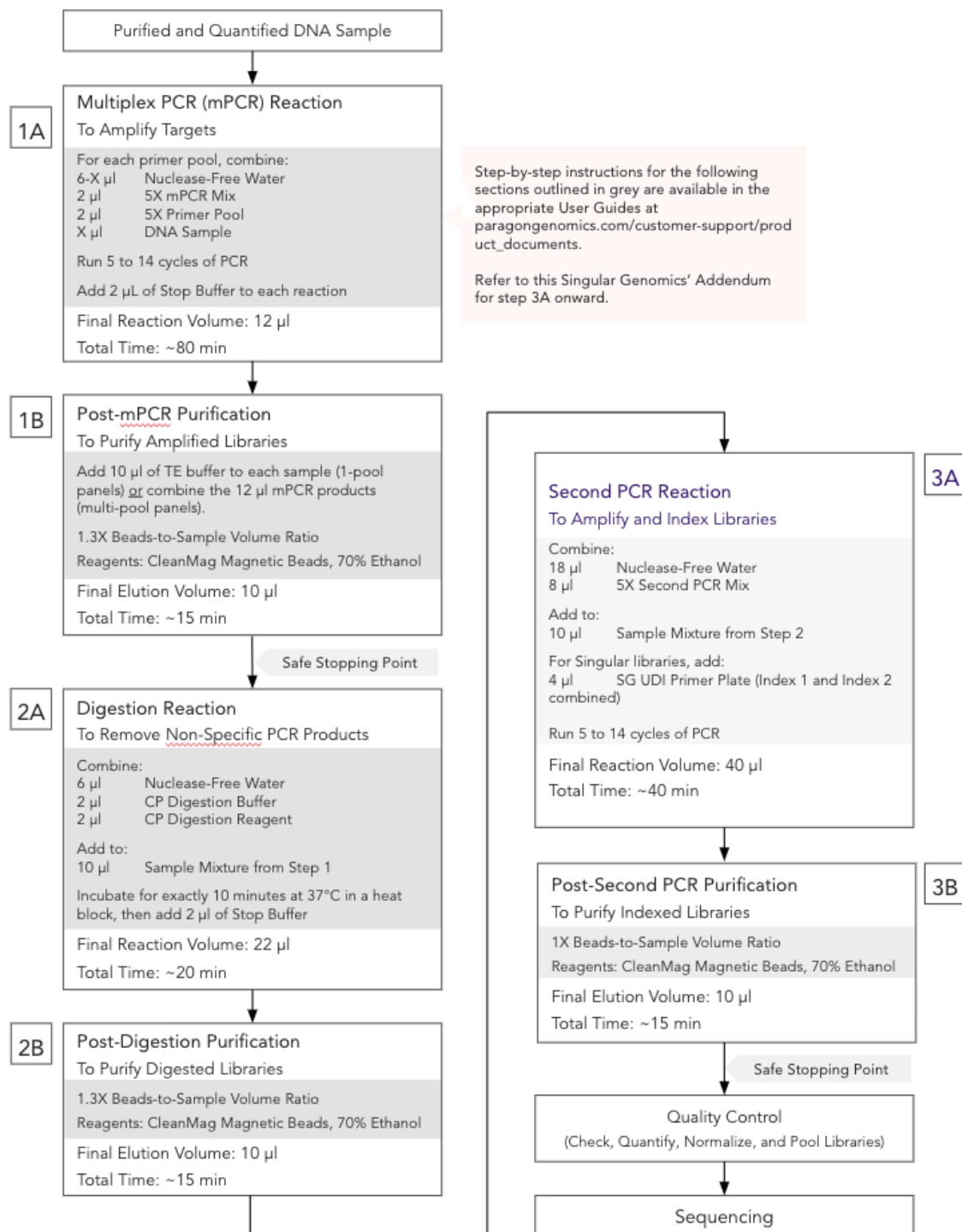
2. SG Non-indexed Library Prep Primers

The G4 Non-Indexed Library Prep Primers provide primers designed to support the preparation of non-indexed libraries. Libraries prepared with Non-indexed Primers cannot be pooled prior to sequencing. These primers can also be used to convert existing libraries containing SP1 and SP2 sequences compatible for sequencing on the G4 Platform.

For more information such as part numbers, see [G4 Non-Indexed Primers Overview](#).

Workflow

Please refer to the [appropriate Paragon Genomics' User Guide](#) for specific instructions from Step 1A – 2B, 3B as indicated in the workflow below in grey. This addendum covers specific instructions for Step 3A.



Protocol

Resources

- Refer to the G4 Support Hub: <https://singulargenomics.com/g4/support/> to download Singular Genomics UDI or G4 Non-Indexed Primers Overview for best practices in handling index materials.
- A list of all Paragon Genomics technology and panel user guides can be found in the [product documents page](#) located in the support tab of paragongenomics.com:

Best Practices for Singular Genomics Index Materials

- Always handle indexes with care by regularly changing tips and gloves and spinning down plates to minimize cross contamination.

Procedures Prior to Second PCR Reaction

Please refer to the [appropriate Paragon Genomics User Guide](#) starting from the beginning up until right before the Second PCR Reaction. Proceed with the following amplification and Singular Genomics index incorporation steps.

3A. Singular Genomics Specific Second PCR Reaction for G4 Compatible libraries

Note: Remember to assign a specific index to each sample before starting this step.

1. Thaw and prepare Singular Genomics Indexed Primers according to best practices detailed in the Singular Genomics UDI or Nonindexed Primer Overviews, available for download or upon request at the [G4 Support Hub](#) website.
2. Prepare Second PCR Reaction Master Mix according to the table below and add the mix to each purified sample from the previous step. Then add 4 μ l of one unique indexed primer 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 μ l

Second PCR Reaction Mixture	
Reagent	Volume per reaction
Second PCR Reaction Master Mix	26 μ l
Purified Sample from Step 2B	10 μ l
Singular UDI or Non-indexed Primers	4 μ l
Total Volume per reaction	40 μ l



Important! When handling Indexed Primers, take extra care to prevent cross contamination by handling plates carefully and changing pipette tips and gloves as necessary. Avoid touching the materials with anything non-disposable.

- Replace the cap for the reaction tube or adhesive film seal on the sample plate. 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 cross contamination during vortexing or evaporation during thermal cycling.

- Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. Refer to the [Second PCR Reaction section of the appropriate Paragon Genomics panel User Guide](#) to determine the suggested PCR cycles for your reaction.

Notes:

1. The Second PCR thermal cycling protocol depends on the starting DNA input amount and DNA quality. Generally, lower quality DNA, lower DNA input amount, or fewer amplicons in a panel requires more PCR cycles.

2. Use the per primer pool DNA input to determine the correct cycles.

3. Nanogram input suggestions are based on Human Genome DNA 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. 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

CleanPlex Custom NGS Panels — Second PCR Cycle Number

Total Number of Amplicons* in Panel	10 ng of High Quality gDNA per Pool	10 ng of Low Quality DNA per Pool (eg. FFPE DNA)
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

Second PCR Reaction — Input Specific Cycle Number	
Input DNA per Pool	Change in Cycle Number
0.1 ng	+ 7
1 ng	+ 3 to + 4
5 ng	+ 1
10 ng	-
20 ng	- 1
40 ng	- 2
Low Quality DNA	+ 1 to + 2

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 2 nd PCR tables in the respective User Guide for cycle numbers
Annealing/Extension	60 °C	75 sec	2 °C/s	
Hold	10 °C	∞		

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

- Refer to the [appropriate Paragon Genomics User Guide](#) and proceed to the next step, 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 the next step, Post-Second PCR purification immediately.

Quality Control Prior to Sequencing

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

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

Expected library peak size and shapes for your panel can be found in the troubleshooting guide at the end of the [appropriate Paragon Genomics User Guide](#). The expected amplicon size distribution for Custom Panels can be found in the design files folder, available for download via your account through our website: Custom panels [ParagonDesigner Portal](#) [View Designs](#).

Please see the troubleshooting guide towards the end of the [appropriate Paragon Genomics User Guide](#) should nonspecific peaks or unexpected yield be observed.

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.

Sequencing on the G4 Platform

Please refer to the [G4 User Guide](#) and the [PhiX Control Overview](#) available for download at Singular's [Support Hub](#). Please contact your Singular Support representative or care@singulargenomics.com if you have additional questions about the sequencing process.

Additional calculation sheets and tools can be found on the Singular Genomics website:

- [G4 Sequencing Platform Quick Reference Card](#)
- [G4 Loading Concentration Calculator](#)
- [Sample Data Sheet Generator](#)

Recommended Sequencing Length and Depth

All CleanPlex Ready-to-Use Panels are designed to be compatible with PE 150 bp reads (2x150 bp) on the G4 Sequencing Platform. CleanPlex Custom NGS Panels are also typically designed for 2x150 bp sequencing unless otherwise decided and communicated between the customer and the Paragon Genomics design team. It is recommended to follow and incorporate suggestions from Singular Genomics for PhiX concentration into the final sample multiplexing calculation.

For detecting germline mutations, the recommended average sequencing read depth is 500X. For detecting somatic mutations down to 1% minor allele frequency, the recommended average sequencing read depth is 5,000X. Based on results, depth can be further reduced to maintain sufficient coverage and increase sample multiplexing efficiency.

Recommended Sequencing Depth		
Panel	Application	Suggested Average Coverage Depth
CleanPlex OncoZoom Cancer HotSpot Panel	Somatic Mutations	2,000-5,000X
CleanPlex BRCA1 & BRCA2 Panel	Somatic Mutations	2,000-5,000X
	Germline Mutations	100X-500X
CleanPlex TP53 Panel	Somatic Mutations	2,000-5,000X
CleanPlex Mitochondrial Disease Panel	Germline Mutations	100X-500X
CleanPlex CFTR Panel	Germline Mutations	100X-500X
CleanPlex ACE2 & TMPRSS2 Panel	Germline Mutations	100X-500X

Terminology

Coverage	Also known as depth of coverage, is achieved with one set of paired end reads that cover the entire length of the amplicon.
----------	---

Read	One continuous act of sequencing. For example, 1 million clusters on a flow cell would produce 1 million single reads and 2 million paired-end reads. Paired end reads count as two individual reads, and required for 1X coverage.
------	---

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

For example:

For 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.

Supporting Information

Best Practices and Primer Sequences

- Refer to the G4 [Support Hub](#) to download Singular Genomics UDI or G4 Non-Indexed Primers Overview for best practices to handle index materials and the primer sequences.
- Always handle indexes with care by regularly changing tips and gloves and spinning down plates to minimize cross contamination.

Additional Resources

Please visit www.paragongenomics.com/product/faq/ for additional troubleshooting resources related to Paragon Genomics processes and products.

For supplemental visual aids, please refer to the CleanPlex workflow video tutorials in the Support tab of the website: <https://www.paragongenomics.com/customer-support/videos/>

Please visit <https://singulargenomics.com/faqs/> for additional troubleshooting resources related to G4 processes and products.

Data Analysis Recommendations

Paragon Genomics libraries sequenced on G4 Sequencing Platform can be analyzed the same way as libraries sequenced from other major sequencing platforms.

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

Please refer to the following recommended steps for analyzing CleanPlex NGS libraries sequenced on the G4 Sequencing Platform.

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 2 x 150 sequencing before read mapping.

Trimming adapters from Singular Genomics data can be done using common NGS bioinformatic tools such as cutadapt5 or BBDuk6. Singular Genomics recommends using at least 12 bp of the adapter sequence to trim. For standard runs using the SP1 and SP2 sequencing primers, the sequences that can be used to trim R1 and R2 are listed below, and in the [Adapters and Indices for the G4 Sequencing Platform Reference Guide](#).

```
SP1 Read 1 AGATCGGAAGAGCACCA
SP2 Read 2 AGATCGGAAGAGCGTC
```

(<https://cutadapt.readthedocs.io/en/stable/>).

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. This can be accomplished with the software package fgbio. It requires primer genomic coordinates in a tab delimited file which can be provided by Paragon Genomics to customers as requested.

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.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

Phone: 650-822-7545

Email: support@paragongenomics.com

Website: www.paragongenomics.com

Paragon Genomics
5020 Brandin Court, 2nd Floor
Fremont, CA 94538-3140, USA
1-510-363-9918
contact@paragongenomics.com
www.paragongenomics.com

© 2023 Paragon Genomics, Inc. All rights reserved.