SINGULAR GENOMICS

ADAPTING LIBRARIES FOR THE G4™—INSERT ONLY

Introduction

This library preparation guide provides the information for adapting a library for compatibility with the G4™ Sequencing Platform using the SP1 and SP2 sequences flanking the insert of the library. This preparation workflow is suitable to adapt individual libraries. To adapt pooled libraries, refer to the *Adapting Libraries for the G4™ Retaining Indices* guide.

The guide contains the following information:

- An explanation of the workflow.
- Supported Singular Genomics™ kits, input requirements, required consumables, and required equipment.
- The PCR-based protocol for adapting libraries, cleanup, and quality control.

G4 Sequencing Nucleotide Tags

Libraries for sequencing on the G4 Sequencing Platform need several functional nucleotide tags at each end of the inserts (Figure 1). At the 5' ends, Singular proprietary platform sequences S1 and S2 are attached as anchors for the formation of clusters on the flow cell. The SP1 and SP2 tags, which are identical to the SP1 and SP2 sequencing primers used in many existing applications, 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.

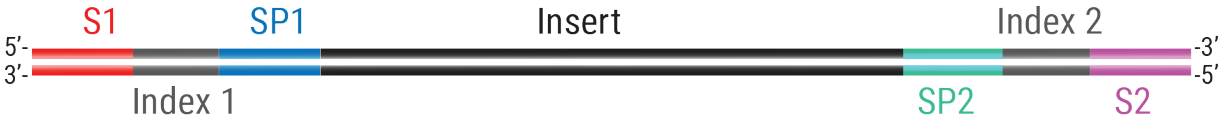

Figure 1 Structure of a Singular library.

For more information, see the Adapters and Indices for the G4 Sequencing Platform Reference Guide.

Adapting Libraries Using SP1 and SP2

Adapting existing libraries for compatibility with the G4 Sequencing Platform is often straightforward. Many libraries already contain SP1 and SP2 sequences adjacent to the insert. Do a PCR using either S1-index1-SP1 and S2-index2-SP2 primers, or, if no index is required, S1-SP1 and S2-SP2 primers (Figure 2). After this, clean up the product with a PCR purification kit and follow the regular workflow for clustering on the G4. This will retain the insert in the final G4 compatible library.

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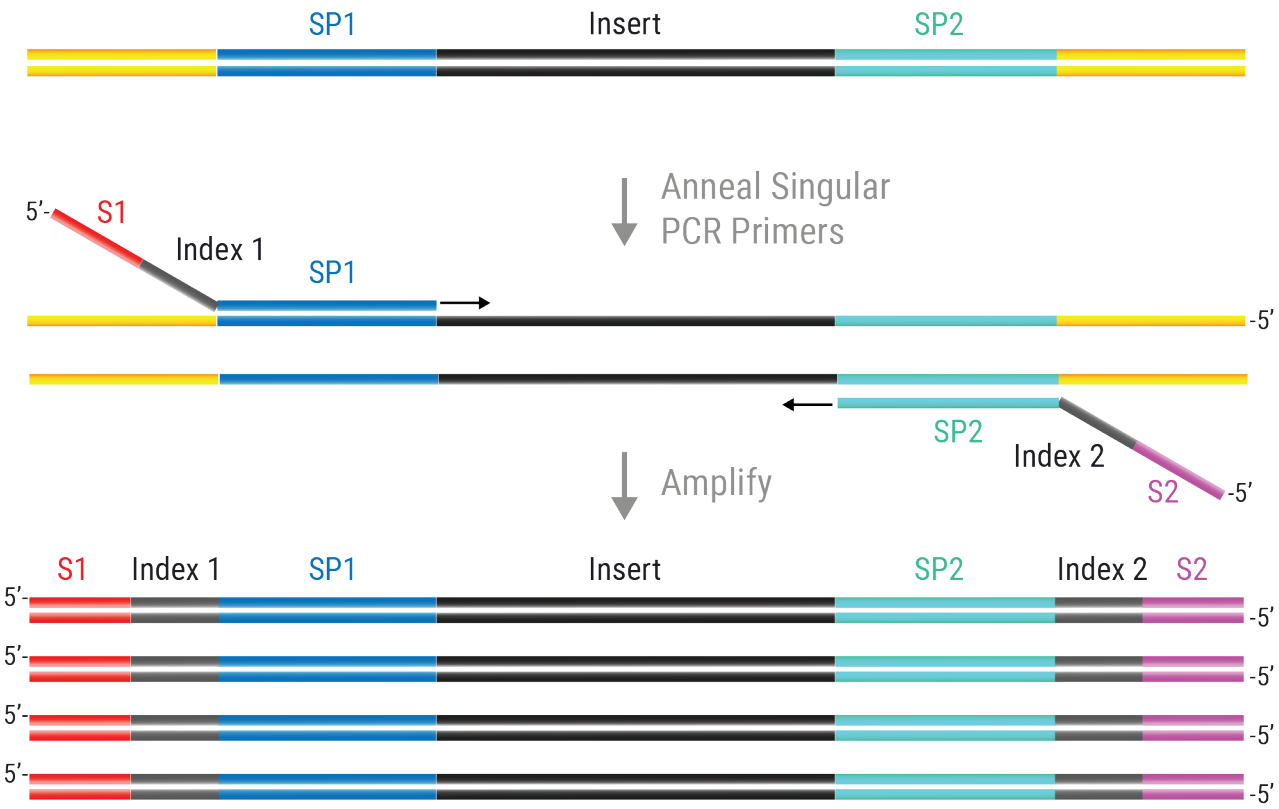


Figure 2 Strategy for Adapting Existing Libraries to Singular Sequencing Using SP1 and SP2 Sequences.

NOTE
Index sequences outside the SP1 and SP2 tags are not retained using this method.

Sequencing Primers

The S1, S2, SP1, and SP2 nucleotides tags are used as binding sites for primers to sequence the inserts and indices in the following way:

Sequence Order	Sequenced Segment	Sequencing Primer
1	Index 1	S1
2	Insert Read 1	SP1
3	Index 2	S2
4	Insert Read 2	SP2

Supported Kits, Input, Consumables, and Equipment

The section below provides information for the supported kits, input requirements, required consumables, and required equipment for adapting libraries using SP1 and SP2.

Supported Products

You can perform the protocol for adapting libraries using SP1 and SP2 with either non-indexed or indexed primers. Use one of the following products:

Product Name	Ordering #	Quantity
SG Non-Indexed Library Prep Primers (24 Rxns)	#700,119	24 reactions
Singular Genomics Unique Dual Indices - Set of 96	#700,134	96 reactions
Singular Genomics Unique Dual Indices - Set of 24 [Set A]	#700,135	24 reactions
Singular Genomics Unique Dual Indices - Set of 24 [Set B]	#700,136	24 reactions

Input DNA Requirements

Starting material is a sequencing library with SP1 and SP2 sequences flanking the insert. Library concentration should have been measured using a Qubit.

Laboratory Consumables

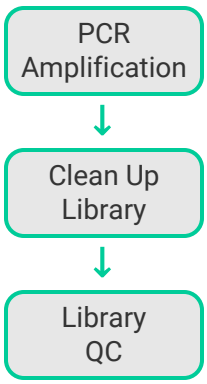
Consumable	Supplier	Order Number	Purpose
sparQ HiFi PCR Master Mix. Using a different PCR master mix that has not been properly tested may not produce reliable results.	QuantaBio	95192	PCR amplification
Ethanol 200 proof (absolute) for molecular biology	General lab supplier		Washing beads
Molecular-grade nuclease-free water	General lab supplier		Various dilutions
One of these magnetic bead cleanup kits: • SparQ PureMag • SPRIselect • AMPure XP	From one of suppliers: • QuantaBio • Beckman Coulter • Beckman Coulter	These order numbers: • 95196 • B23319, B23317, or B23318 • A63880, A63881, or A63882	Reaction cleanup and size selection
Qubit™ dsDNA HS and BR Assay Kits	Thermo Fisher Scientific	Q32851 or Q32854	Measure DNA concentration
0.2 mL thin-walled PCR tube or PCR plate	General lab supplier	General lab supplier	Reactions, cleanup, dilutions.
Disposable gloves, powder-free	General lab supplier	General lab supplier	General purpose
Ice	Lab	Lab	General purpose

Laboratory Equipment

Equipment	Supplier	Order Number	Purpose
Thermocycler	General lab supplier		Sample library preparation
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Q33238, or similar	Measure DNA concentration
BioAnalyzer, TapeStation, or similar	Agilent or general lab supplier		Check DNA quality and size
Vortex	General lab supplier		Sample library preparation
Benchtop Microcentrifuge	General lab supplier		Sample library preparation
Calibrated pipettes (at least P2, P10, P20, P200, and P1000)	General lab supplier		Sample library preparation
Magnetic beads stand or plate	Magnetic beads supplier		Sample library preparation
Microcentrifuge tube racks	General lab supplier		Sample library preparation
Freezer, frost-free -25°C to -15°C	General lab supplier		Storing reagents
Refrigerator 2°C to 8°C	General lab supplier		Storing flow cells and buffers

Protocol for Adapting Libraries

The workflow for adapting libraries using SP1 and SP2 consists of the following three steps:



Step	Estimated Total time
Adapt library with PCR.	30 minutes
Clean up and size select PCR-amplified library.	30 minutes
Determine yield and quality of library.	30 minutes

These steps are described below.

PCR Amplification

Adapt 2 ng per library to G4 sequencing in a PCR reaction the following way:

1. Dilute library down to 1 ng/µL with water.

   NOTE
   If the library concentration is below 1 ng/µL, use the library undiluted, but adjust the library and water volumes in step 3 accordingly to achieve 2 ng of library in the reaction.

2. Thaw the sparQ HiFi PCR Master Mix.on ice if needed and keep on ice before use.
3. Add the PCR reagents to a new tube or plate:

Reagent	Volume
Molecular-grade Water	20 µL
PCR Primers (5 µM each)	3 µL
sparQ HiFi PCR Master Mix (2X)	25 µL
Library (1 ng/µL)	2 µL
Total	**50 µL**

   NOTE
   If setting up multiple reactions, consider preparing a master mix on ice with PCR Master Mix, water, and primers. Multiply the volumes of these reagents by the number of reactions and make sure to add 10% to the volumes to account for pipetting inaccuracies and dead volume. Then add 48 µL of the mix to the 2 µL library in a new tube or plate.

4. Mix by pipetting up and down 10 times and spin down briefly. Make sure all bubbles are removed.

5. Program a thermal cycler with the parameters listed in the table below. Set the instrument's heated lid to 105°C. When the thermal cycler block reaches 98°C, pause the program.

Step	Cycles	Temperature	Time
Initialization	1	98°C	2 min.
Denaturation	7	98°C	20 sec.
Annealing		60°C	30 sec.
Extension		72°C	30 sec.
Final Extension	1	72°C	1 min.
Final Hold	1	4°C	Hold

6. Place tubes or plate in the thermal cycler, close the lid, and resume the program.

After the samples have cooled in the final hold step, proceed to the cleanup step.

Post-PCR Cleanup

After the PCR amplification, perform a cleanup with magnetic beads. The following protocol can be done using SparQ, SPRI, or AMPure magnetic beads.

1. Equilibrate magnetic beads to room temperature for 15-30 minutes prior to use.
2. Vortex magnetic beads for 30 seconds until fully resuspended.
3. Add magnetic beads to each tube or well with PCR-amplified sample:

	Input DNA ≥ 100 ng
Sample	50 µL
Magnetic beads	45 µL
TOTAL	**95 µL**

NOTE
Purification ratio between sample and magnetic beads should be adjusted depending on the size of the library. See the documentation provided by the magnetic bead manufacturer for recommended purification ratio.

4. Mix well by pipetting up and down 10 times or vortexing.
5. Incubate at room temperature for 5 minutes.
6. Place samples on magnetic plate or rack, wait until solution is clear, about 3 minutes.
7. Carefully aspirate and discard the supernatant using a P200 pipette without disturbing the beads. Do not remove the plate or tubes from the magnetic plate or rack.
8. Add 200 µL freshly prepared 80% ethanol to the beads on the magnetic plate or rack.
9. Incubate for 1 minute.
10. Repeat step 7–9 for a total of 2 washes, while keeping the samples on the magnetic plate or rack.
11. Remove supernatant using a P200 pipette, then remove all remaining ethanol with a P20 or P10 pipette without disturbing the beads.

    NOTE
    Do not dry the beads.

12. Take tubes or plate off magnet and add 17 µL of molecular-grade water to each tube.
13. Mix well by pipetting up and down 10 times or vortex and spin down briefly.
14. Incubate samples for 2 minutes.
15. Return to magnet plate or rack and wait until solution is clear, about 3 minutes.

16. Transfer 16 µL of supernatant to new PCR tube or plate without disturbing the beads.

Store the samples at -20°C or proceed with library QC.

Library Quality Control

After cleanup, check the quality and concentration of the library.

1. Equilibrate the Qubit reagents to room temperature for 15-30 minutes prior to use.
2. Using the Qubit 1X dsDNA HS kit, determine the concentration (ng/µL) of each library.
3. Check the quality and determine the average fragment size (bp) of the library by running 1 µL of the cleaned library on a BioAnalyzer, TapeStation, or equivalent method to visualize size distribution. There should be no peaks of higher or lower molecular weight than the expected library size, apart from the weight markers peaks (see Figure 3).

   Note that the non-indexed primers are 62 bp long, while the indexed primers are 75 bp long. To calculate expected library size, take the expected insert size and add 124 bp for non-indexed runs or 150 bp for indexed runs.

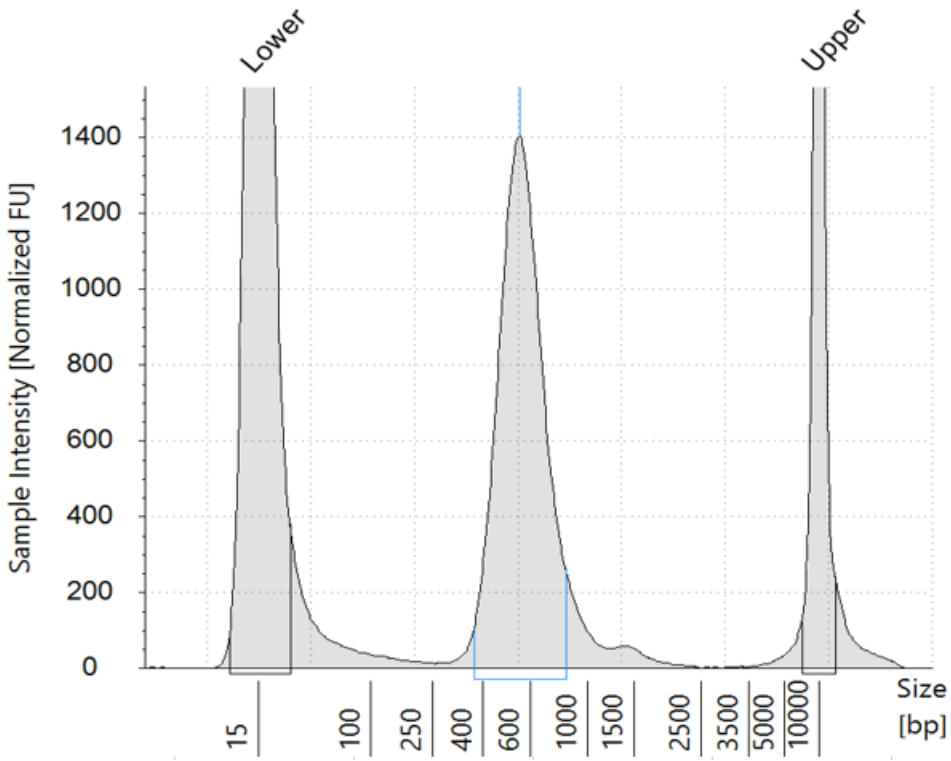


Figure 3 Example of a clean TapeStation trace

NOTE

If there are unexpected peaks compared to the expected library size and weight markers (such as primer dimers), remove them with a more stringent magnetic bead purification. See the Quality Control section in the G4™ Best Practices and Quality Control Guide or the magnetic bead manufacturer's documentation for suggestions.

Optional: Dilute each library in water to a standard concentration (in nM) for pooling (if required) and loading on the G4 Sequencing Platform. We recommend storing at least 10 µL of libraries at a minimum concentration of 10 nM in a 0.2 mL PCR tube or PCR plate.

Note that Concentration [in nM] = (Concentration [in ng/µL] * 1,000,000) / (660 * Size in bp)

Revision History

Document #	Revision	Release Date	Description of Change
600024	Rev. 0	May 2023	Initial release.

Customer Care

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Website	singulargenomics.com
Email	care@singulargenomics.com
Telephone	442.SG.CARES(442.742.2737)

Singular Genomics Systems, Inc.

3010 Science Park Rd

San Diego, CA 92121 U.S.A.