

# ADAPTING LIBRARIES FOR THE G4™ –RETAINING ORIGINAL INDICES

## Introduction

This library preparation guide provides the information for adapting an indexed third-party library for compatibility with the G4™ Sequencing Platform while retaining indices. The protocol can be used for un-pooled or pooled libraries.

The guide contains the following information:

- An explanation of the workflow.
- Information about the SG Library Compatibility Kit from Singular Genomics™, needed for the adapting libraries while retaining indices workflow.
- Input requirements, required consumables, and required equipment.
- The PCR-based protocol for adapting libraries, cleanup, and quality control.
- Considerations for demultiplexing.

## 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.

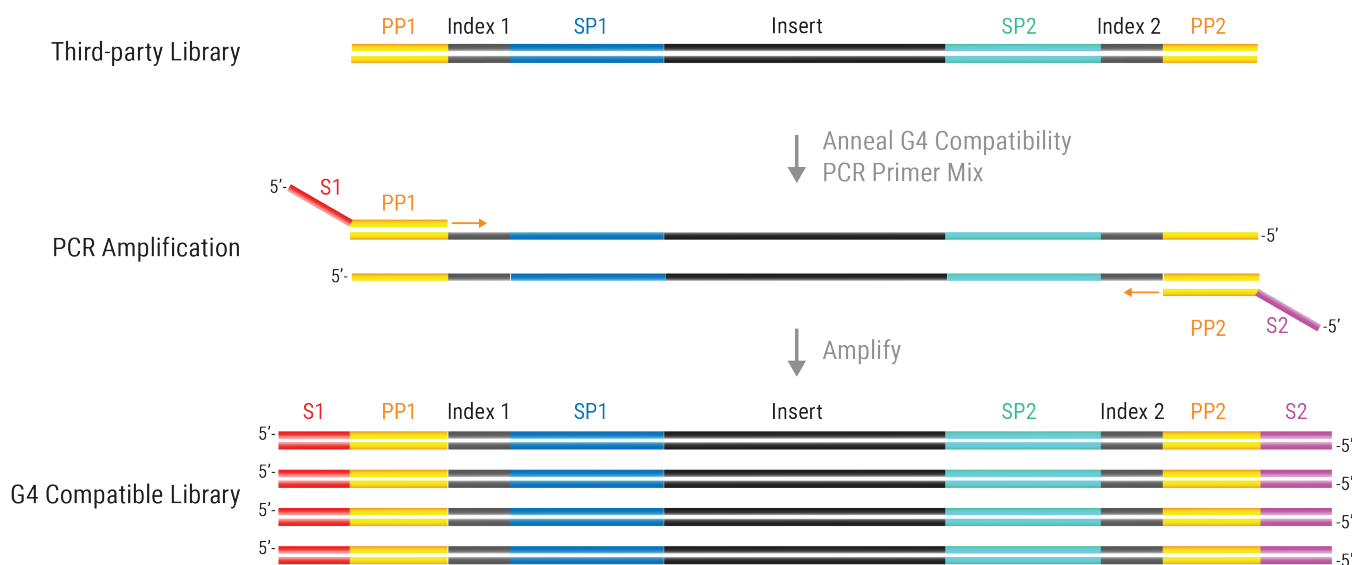
For more information, see the [Adapters and Indices for the G4 Sequencing Platform Reference Guide](#).

## Adapting Libraries Retaining Original Indices

Adapting indexed third-party libraries for compatibility with the G4 Sequencing Platform is straightforward. Common third-party libraries rely on specific platform primers to introduce anchoring sites. These sites (designated PP1 and PP2 in Figure 2) are adjacent to the indices on the outside of the fragment. In addition, many third-party platforms employ SP1 and SP2 sequences as sequencing primer binding sites, located on the inside of the indices (see Figure 2). SP1 and SP2 sequences are used as primer binding sites in Singular Genomics sequencing too. Furthermore, this workflow is also compatible with other sequencing primer binding sites, as long as the corresponding custom sequencing primer is used.

In this workflow, perform a PCR using the provided SG Compatibility PCR Primer Mix from the SG Library Compatibility Kit. The resulting G4 compatible library retains sequences present within the PP1 and PP2 sequences of the indexed third-party library, including the indices, insert, and SP1 and SP2 sites (Figure 2). After this, clean up the product with a PCR purification kit and follow the regular workflow for clustering on the G4.

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**Figure 2** Strategy for Adapting Third-party Libraries to Singular Sequencing Retaining Indices. Note that Index 1 is SP1 adjacent and Index 2 is SP2 adjacent.

## Sequencing Primers

S1-PP1, S2-PP2, SP1, and SP2 sequences provide binding sites for primers to sequence the indices and inserts, respectively. Sequencing of G4 compatible library in the adapting libraries while retaining indices workflow is done with the primers listed in the following order:

Sequence Order	Sequenced Segment	Location	Sequencing Primer
1	Index 1	SP1 adjacent	S1-PP1 Index Sequencing Primer
2	Insert Read 1	From SP1 inward	SP1
3	Index 2	SP2 adjacent	S2-PP2 Index Sequencing Primer
4	Insert Read 2	From SP2 inward	SP2

Make sure to use the custom index sequencing primers S1-PP1 Index Sequencing Primer and S2-PP2 Index Sequencing Primer for index sequencing. The custom index sequencing primers come with the SG Library Compatibility Kit and need to be added to the custom index primer wells in the reagent cartridge. See the G4 Sequencing Platform User Guide for instructions on how to use them. Use the provided 5X PAB to dilute the custom index sequencing primers to 1  $\mu$ M final concentration and load 2 mL per custom index primer well.

The SP1 and SP2 sequencing primers for the insert are the regular G4 sequencing primers that are included in the reagent cartridge.

### NOTE

Make sure to account for differences in index sequencing between G4 and third-party sequencing platforms when demultiplexing the samples. See *Index Differences in G4 and Third-party Sample Data Sheets* on page 8 for more information.

## 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 while retaining indices.

### Supported Product

Use the following product for adapting libraries while retaining indices:

Product Name	Ordering #	Quantity
SG Library Compatibility Kit (24 Rxns), containing: <ul style="list-style-type: none"> <li>• SG Compatibility PCR Primer Mix (24 Rxns; 5 <math>\mu</math>M each)</li> <li>• S1-PP1 Index Sequencing Primer (24 Rxns; 50 <math>\mu</math>M)</li> <li>• S2-PP2 Index Sequencing Primer (24 Rxns; 50 <math>\mu</math>M)</li> <li>• 5X PAB (Primer Annealing Buffer; for diluting custom index sequencing primers)</li> </ul>	#700,141	24 reactions

### Input DNA Requirements

Starting material is a sequencing library or pooled libraries with PP1 and PP2 sequences flanking the indices. Library concentration should be measured using a Qubit before proceeding.

### Laboratory Consumables

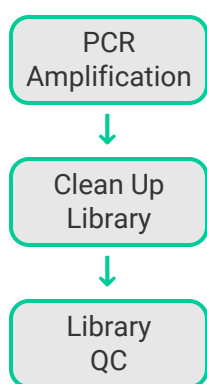
Consumable	Supplier	Order Number	Purpose
sparQ HiFi PCR Master Mix. Using a different PCR master mix that has not been properly tested may not produce reliable results.	QuantaBio	95192	PCR amplification
Ethanol 200 proof (absolute) for molecular biology	General lab supplier		Washing beads
Molecular-grade nuclease-free water	General lab supplier		Various dilutions
One of these magnetic bead cleanup kits: <ul style="list-style-type: none"> <li>• SparQ PureMag</li> <li>• SPRIselect</li> <li>• AMPure XP</li> </ul>	From one of suppliers: <ul style="list-style-type: none"> <li>• QuantaBio</li> <li>• Beckman Coulter</li> <li>• Beckman Coulter</li> </ul>	These order numbers: <ul style="list-style-type: none"> <li>• 95196</li> <li>• B23319, B23317, or B23318</li> <li>• A63880, A63881, or A63882</li> </ul>	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	<a href="#">Thermo Fisher Scientific</a>	<a href="#">Q33238</a> , or similar	Measure DNA concentration
BioAnalyzer, TapeStation, or similar	<a href="#">Agilent</a> 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 while retaining indices 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 or library pool to G4 sequencing in a PCR reaction the following way:

1. Dilute library or library pool 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
<b>Total</b>	<b>50 μL</b>

**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		57°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
<b>TOTAL</b>	<b>95 µL</b>

### 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.

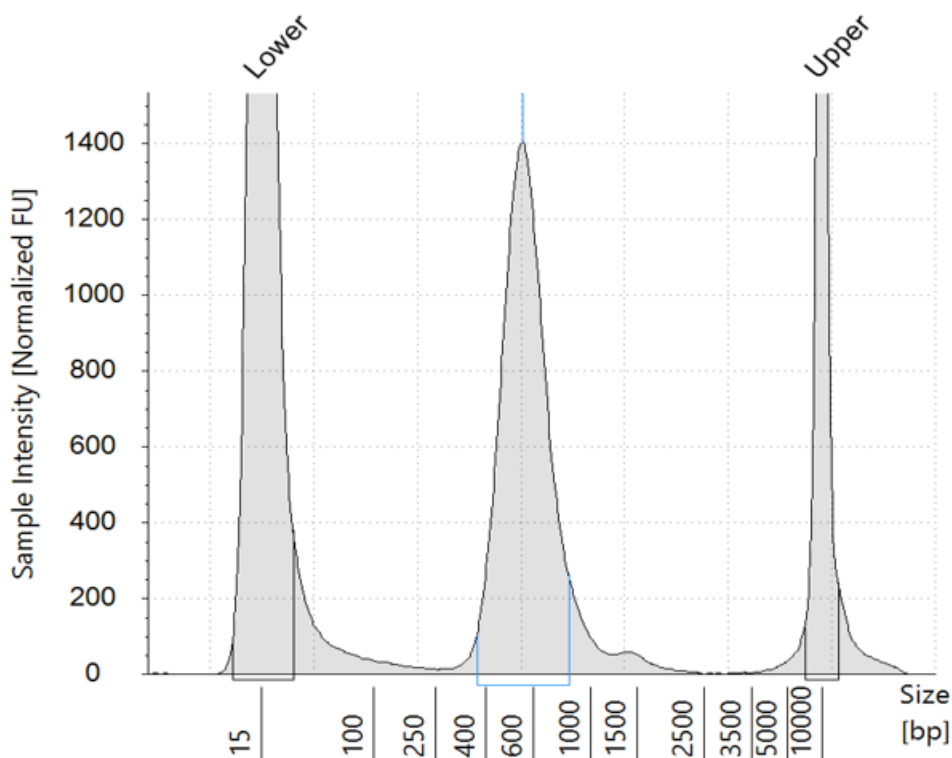
16. Transfer 16  $\mu\text{L}$  of supernatant to new PCR tube or plate without disturbing the beads.  
Store the samples at  $-20^{\circ}\text{C}$  or proceed with library QC.

## 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 ( $\text{ng}/\mu\text{L}$ ) of each library.
3. Check the quality and determine the average fragment size (bp) of the library by running 1  $\mu\text{L}$  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).

To calculate expected library size, take the initial expected total library size and add 48 bp.



**Figure 3** Example of a clean TapeStation trace

### NOTE

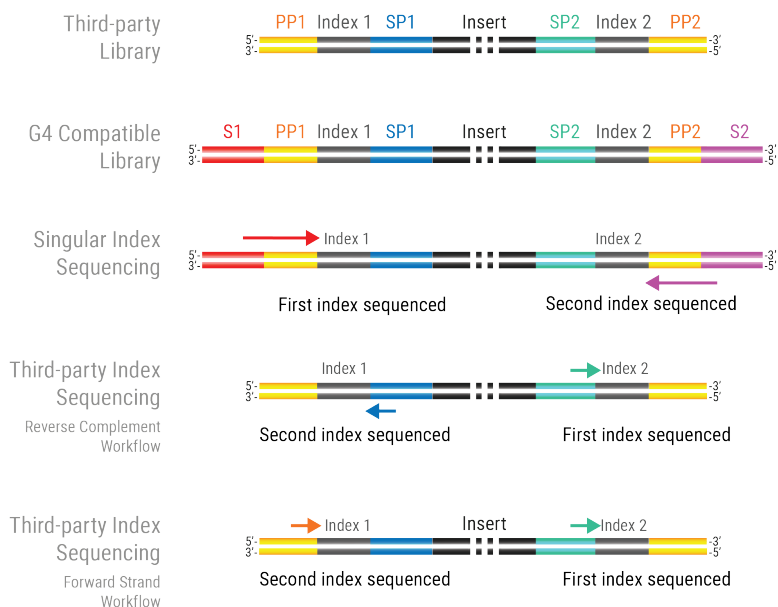
If there are unexpected peaks compared to the expected library size and weight markers (such as primer dimers), remove them with a more stringent magnetic bead purification. See the Quality Control section in the [G4™ Best Practices and Quality Control Guide](#) or the magnetic bead manufacturer's documentation 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  $\mu\text{L}$  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  $\text{ng}/\mu\text{L}$ ] \* 1,000,000) / (660 \* Size in bp)

## Index Differences in G4 and Third-party Sample Data Sheets

The order and direction of index sequencing is different between G4 and third-party platforms. In addition, the direction of index sequencing varies for the third-party *Reverse Complement Workflow* and *Forward Strand Workflow*. This affects how sample data sheets should be set up. Figure 4 illustrates the differences and similarities in index sequencing.



**Figure 4** Index Sequencing Directions and Order for G4 Sequencing and Examples for Third-party Sequencing

## Index Nomenclature in This Topic

Third-Party indices can have various names. In this topic, we use the following nomenclature:

- **Index 1 = SP1-adjacent index.** We refer to the third-party index that is located next to SP1 as the SP1-adjacent index. This corresponds to Index 1 for the G4 Sequencing Platform.
- **Index 2 = SP2-adjacent index.** We refer to the third-party index that is located next to SP2 as the SP2-adjacent index. This corresponds to Index 2 for the G4 Sequencing Platform.

## Order of Index Sequencing

The comparison of the order of index sequencing is as follows:

	First Index Sequenced	Second Index Sequenced
G4 index sequencing	Index 1	Index 2
Third-party Platform Reverse Complement Workflow	SP2-adjacent Index	SP1-adjacent Index
Third-party Platform Forward Strand Workflow	SP2-adjacent Index	SP1-adjacent Index



## Direction of Index Sequencing

The comparison of the direction of index sequencing is as follows:

	Index 1 SP1-adjacent Index	Index 2 SP2-adjacent Index
G4 index sequencing	Outside in, towards the insert	Outside in, towards the insert
Third-party Platform Reverse Complement Workflow	Inside out, towards the fragment ends	Inside out, towards the fragment ends
Third-party Platform Forward Strand Workflow	Outside in, towards the insert	Inside out, towards the fragment ends

## Differences in Sample Data Sheet Index Entry

This affects how to demultiplex the sample, which is specified in the sample data sheet. The table below summarizes how to set up the G4 sample data sheet, compared to the entries in a sample sheet for third-party platforms.

G4 index sequencing	Third-party Platform, Reverse Complement Workflow	Third-party Platform, Forward Strand Workflow
Index 1	Reverse complement of SP1-adjacent Index, second index sequenced	Sequence of SP1-adjacent Index, second index sequenced
Index 2	Reverse complement of SP2-adjacent Index, first index sequenced	Reverse complement of SP2-adjacent Index, first index sequenced

### NOTE

If there is an error in the index settings in the sample data sheet, the samples will not demultiplex properly. To redo demultiplexing, correct the sample data sheet and follow the directions in *Fixing Improper Demultiplexing* in the *G4 Sequencing Platform User Guide*.

## Revision History

Document #	Revision	Release Date	Description of Change
600025	Rev. 0	May 2023	Initial release.

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