SureSelect^{QXT} Whole Genome Library Prep for Illumina Multiplexed Sequencing

Featuring Transposase-Based Library Prep Technology

Protocol

Version E0, April 2018

SureSelect platform manufactured with Agilent SurePrint Technology

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed whole-genome library preparation using the SureSelect^{QXT} system.

If you wish to prepare target-enriched libraries using the SureSelect^{QXT} system, instead see publication part number G9681-90000 at www.genomics.agilent.com.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps to prepare dual-indexed gDNA sequencing libraries for the Illumina platform.

3 Reference

This chapter contains reference information, including component kit contents, index sequences, and dual index usage guidelines.

What’s New in Version E0

- Support for replacement of SureSelect^{QXT} Reagent Kits p/n G9682A/G9682B with p/n G9684A/G9684B for use with Illumina’s HiSeq and MiSeq platforms (see Table 1 on page 12, Table 17 on page 40, and Table 19 on page 41 including footnotes for each table)
- Support for use of Illumina’s HiSeq 3000 and HiSeq 4000 platforms for downstream sequencing steps (see page 31 through page 33)
- Updates to sequencing kit selection and seeding concentration guidelines (see page 31)
- Updates to custom sequencing primer dilution instructions (See page 32 to page 34)
- Update to sequencing run setup recommendations (see“HiSeq or NextSeq 500 platform sequencing run setup and adaptor trimming guidelines" on page 36)
- Updates to dual index multiplexing guidelines (see Table 24 on page 45)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see page 13)
- Updates to supplier name for materials purchased from Thermo Fisher Scientific (see Table 1 on page 12 and Table 2 on page 13)
- Addition of product guarantee and support statement (see *Note* on page 9)
- Updates to Technical Support contact information (see page 2)

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1
Before You Begin

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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE

Agilent guarantees performance and provides technical support for the SureSelect reagents required for this workflow only when used as directed in this Protocol.

Procedural Notes

- The SureSelect^{QXT} system requires high-quality DNA samples for optimal performance. Use best practices for verifying DNA sample quality before initiating the workflow. For best practice, store diluted DNA solutions at 4°C to avoid repeated freeze-thaw cycles, which may compromise DNA quality.
- Performance of the SureSelect^{QXT} library preparation protocol is very sensitive to variations in amounts of DNA sample and other reaction components. It is important to quantify and dilute DNA samples as described on page 17. Carefully measure volumes for all reaction components, and combine components as described on page 17. Use best-practices for liquid handling, including regular pipette calibration, to ensure precise volume measurement.
- Use care in handling the SureSelect QXT Enzyme Mix. After removing the vial from storage at –20°C, keep on ice or in a cold block while in use. Return the vial to storage at –20°C promptly after use.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of domed caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.

- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for SureSelect^{QXT} Library Preparation

Description	Vendor and part number
SureSelect^{QXT} Library Prep Kit (for Illumina HiSeq, MiSeq, and NextSeq platforms)*	Agilent
16 Samples	p/n G9684A
96 Samples	p/n G9684B
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit dsDNA HS Assay Kit *or*	Thermo Fisher Scientific p/n Q32851
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* This Protocol also supports use of Agilent p/n G9682A (16 samples) and p/n G9682B (96 samples), which were retired in May 2018 but remain supported until the expiration date listed on kit Certificates of Analysis. Going forward, users of the retired G9682A/B products should order Agilent p/n G9684A (16 samples) and p/n G9684B (96 samples), which now support sequencing library preparation for Illumina's HiSeq, MiSeq, and NextSeq platforms.

Required Equipment

Table 2 Required Equipment for SureSelect^{QXT} Library Preparation

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware: 96-well plates OR 8-well strip tubes	 Agilent p/n 410088 Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
DNA Analysis Platform and Consumables Agilent 2100 Bioanalyzer Instrument Agilent 2100 Expert SW Laptop Bundle (optional) High Sensitivity DNA Kit	 Agilent p/n G2939BA Agilent p/n G2953CA Agilent p/n 5067-4626
Qubit Fluorometer	Thermo Fisher Scientific p/n Q32857
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent

Table 2 Required Equipment for SureSelect^{QXT} Library Preparation

Description	Vendor and part number
Magnetic separator*	Thermo Fisher Scientific p/n 12331D or equivalent
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

* Select a magnetic separator configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

2
Sample Preparation

Step 1. Fragment and adaptor-tag the genomic DNA samples 16
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This section contains instructions for genomic DNA sequencing library preparation for Illumina platforms.

Step 1. Fragment and adaptor-tag the genomic DNA samples

In this step, the gDNA is enzymatically fragmented and adaptors are added to ends of the fragments in a single reaction. This step uses the SureSelect^{QXT} Library Prep Kit components listed in Table 3 in addition to some reagents obtained from other suppliers (see Table 1 on page 12).

Table 3 Reagents for DNA fragmentation and adaptor-tagging

Kit Component	Storage Location	Where Used
SureSelect QXT Stop Solution	SureSelect QXT Library Prep Kit Box 1, Room Temperature	page 16 (below)
SureSelect QXT Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 18
SureSelect QXT Enzyme Mix ILM	SureSelect QXT Library Prep Kit Box 2, –20°C	page 18

Before you begin, remove the SureSelect QXT Enzyme Mix ILM and the SureSelect QXT Buffer tubes from storage at –20°C and place on ice. Vortex each reagent vigorously to mix before use. Remove the AMPure XP beads from storage at 4°C and allow to warm up to room temperature.

NOTE While obtaining components for this step, also remove the DMSO vial from the SureSelect QXT Library Prep Kit Box 2 in –20°C storage. Leave the DMSO vial at room temperature in preparation for use on page 24.

For each DNA sample to be sequenced, prepare 1 library.

1 Verify that the SureSelect QXT Stop Solution contains 25% ethanol, by referring to the container label and the instructions below.

   Before the first use of a fresh container, add 1.5 mL of ethanol to the provided bottle containing 4.5 mL of stop solution, for a final ethanol concentration of 25%. Seal the bottle then vortex well to mix. After adding the ethanol, be sure to mark the label for reference by later users.

   Keep the prepared 1X SureSelect QXT Stop Solution at room temperature, tightly sealed, until it is used on page 19.

2 Prepare reagents for the purification protocols on page 20 and page 25.

  a Verify that the AMPure XP beads are being held at room temperature. The beads should be held at room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*

  b Prepare 800 µL of fresh 70% ethanol per sample, plus excess, for use in the purification steps. The 70% ethanol may be used for multiple steps done on the same day, when stored in a sealed container.

3 Quantify and dilute gDNA samples using two serial fluorometric assays:

  a Use the Qubit dsDNA BR Assay or Qubit dsDNA HS Assay to determine the initial concentration of each gDNA sample. Follow the manufacturer's instructions for the specific assay kit and the Qubit instrument. This step is critical for successful preparation of input DNA at the required concentration to ensure optimal fragmentation.

  b Dilute each gDNA sample with nuclease-free water to a final concentration of 50 ng/µL in a 1.5-mL LoBind tube.

  c Carefully measure the DNA concentration of each of the 50 ng/µL dilutions using a second Qubit dsDNA BR or HS Assay.

  d Adjust each gDNA sample with nuclease-free water to a final concentration of 25 ng/µL in a 1.5-mL LoBind tube.

CAUTION The duration and temperature of incubation for DNA fragmentation must be precisely controlled for optimal results. Make sure to preprogram the thermal cycler as directed in step 4 before setting up the fragmentation reactions. Do not exceed 10 minutes at 45°C, as indicated in Table 4.

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4 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) with the program in Table 4. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the fragmentation reactions.

Table 4 Thermal cycler program for DNA fragmentation

Step	Temperature	Time
Step 1	45°C	10 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

5 Before use, vortex the SureSelect QXT Buffer and SureSelect QXT Enzyme Mix ILM tubes vigorously at high speed. Note that the SSEL QXT Buffer is viscous and thorough and vigorous mixing is critical for optimal fragmentation.

   These components are in liquid form when removed from –20°C storage and should be returned to –20°C storage promptly after use in step 6.

CAUTION

Minor variations in volumes of the solutions combined in step 6 below may result in DNA fragment size variation.

The SureSelect QXT Buffer and Enzyme Mix solutions are highly viscous. Be sure to follow the dispensing and mixing instructions in the steps below. Thorough mixing of the reagents and reactions is critical for optimal performance.

6 Set up the fragmentation reactions on ice using a PCR plate or strip tube. Components must be added in the order listed below. Do not pre-mix the SureSelect QXT Buffer and Enzyme Mix.

   a To each sample well, add 17 µL of SureSelect QXT Buffer.

   b Add 2 µL of each DNA sample to its assigned sample well. While dispensing the DNA, be sure to place the pipette tip at the bottom of the well.

   c Add 1 µL of SureSelect QXT Enzyme Mix, ILM to each sample well. While dispensing the enzyme mixture, place the pipette tip at the bottom of the well. After dispensing of the 1 µL of enzyme mix, pipette up and down 8 to 10 times to ensure complete transfer of the viscous solution to the well.

7 Seal the wells, briefly spin, then mix thoroughly by vortexing the plate or strip tube at high speed for 20 seconds.

8 Briefly spin the samples, then immediately place the plate or strip tube in the SureCycler 8800 thermal cycler. Press the *Play* button to resume the thermal cycling program in Table 4.

9 During the 10-minute incubation of samples in the SureCycler, vigorously vortex the AMPure XP beads at high speed to ensure homogeneous distribution of beads throughout the solution so that the beads are ready for use on page 20.

10 When the thermal cycler has completed the 1-minute incubation at 4°C, immediately place the samples on ice and proceed to step 11.

11 Add 32 µL of 1X SureSelect QXT Stop Solution (containing 25% ethanol) to each fragmentation reaction. Seal the wells with fresh caps, then vortex at high speed for 5 seconds. Briefly spin the plate or strip tube to collect the liquid.

Incubate the samples at room temperature for 1 minute. Proceed directly to the purification protocol on page 20.

Step 2. Purify the adaptor-tagged library using AMPure XP beads

Before you begin, verify that the AMPure XP beads have been incubated at room temperature for at least 30 minutes and that fresh 70% ethanol has been prepared for use in step 6.

1. Verify that the AMPure XP bead suspension has been well mixed and appears homogeneous and consistent in color.
2. Add 52 µL of the homogeneous bead suspension to each sample well containing the 52-µL DNA samples. Seal the wells, then vortex for 5 seconds. Briefly spin the samples to collect the liquid, without pelleting the beads.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
3. Incubate samples for 5 minutes at room temperature.
4. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
5. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
6. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
7. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
8. Repeat step 6 and step 7 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
9. Dry the samples on the thermal cycler (with lid open) at 37°C for 1 to 3 minutes. Do not overdry the samples.
10. Add 11 µL of nuclease-free water to each sample well.
11. Seal the sample wells, then mix well on a vortex mixer and briefly spin the plate or tubes to collect the liquid.
12. Incubate for 2 minutes at room temperature.
13. Put the plate or tubes in the magnetic stand and leave for 2 minutes or until the solution in each well is clear.

14 Remove each cleared supernatant (approximately 10 µL) to wells of a fresh plate or strip tube and keep on ice. You can discard the beads at this time.

Step 3. Amplify and index the adaptor-tagged DNA library

In this step, the adaptor-tagged DNA libraries are PCR amplified using an appropriate pair of dual indexing primers.

CAUTION To avoid cross-contaminating libraries, set up PCR reactions in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Before you begin, thaw then vortex to mix the reagents listed in Table 5. Keep all reagents except DMSO on ice.

Table 5 Reagents for PCR amplification and indexing

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 23
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 23
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 23
SureSelect QXT P7 and P5 dual indexing primers	SureSelect QXT Library Prep Kit Box 2, –20°C	page 23
DMSO	Transferred to Room Temperature storage on page 16	page 23

Prepare one indexing amplification reaction for each DNA library.

1 Determine the appropriate index assignments for each sample. See the Reference section for sequences of the index portion of the P7 and P5 indexing primers used to amplify the DNA libraries in this step. For the HiSeq 2000/2500 or MiSeq platform see Table 20 and Table 21. For the HiSeq 3000/4000 or NextSeq platform see Table 20 and Table 22.

   Use a different indexing primer combination for each sample to be sequenced in the same lane.

NOTE For sample multiplexing, Agilent recommends maximizing index diversity on both P7 and P5 primers as required for color balance. For example, when 8-plexing, use eight different P7 index primers with two P5 index primers. See Table 23 on page 44 and Table 24 on page 45 for additional details.

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 6, on ice. Mix well on a vortex mixer and keep on ice.

Table 6 Preparation of PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
Nuclease-free water	22 µL	374 µL
Herculase II 5× Reaction Buffer	10 µL	170 µL
100 mM dNTP Mix (25 mM each dNTP)	0.5 µL	8.5 µL
DMSO	2.5 µL	42.5 µL
Herculase II Fusion DNA Polymerase	1 µL	17 µL
Total	**36 µL**	**612 µL**

3 Add 36 µL of the PCR reaction mixture from step 2 to each 10-µL purified library DNA sample held on ice from step 14 on page 21.

4 Add 2 µL of the appropriate P7 dual indexing primer (P7 i1 to P7 i12) to each PCR reaction well. Add only one of the twelve possible P7 primers to each reaction well.

5 Add 2 µL of the appropriate P5 dual indexing primer (P5 i13 to P5 i20) to each PCR reaction well. Add only one of the eight possible P5 primers to each reaction well.

6 Seal the wells and mix by vortexing gently for 5 seconds. Spin samples briefly to collect the liquid.

7 Incubate the plate or strip tube in the thermal cycler (with the heated lid ON) and run the program in Table 7.

Table 7 Thermal cycler program for PCR indexing

Segment Number	Number of Cycles	Temperature	Time
1	1	68°C	2 minutes
2	1	98°C	30 seconds
3	5	98°C	30 seconds
		56°C	30 seconds
		72°C	1 minute
4	1	4°C	Hold

Step 4. Purify the amplified library with AMPure XP beads

Before you begin, verify that the AMPure XP beads have been kept at room temperature for at least 30 minutes and that fresh 70% ethanol has been prepared for use in step 6.

1 Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.

2 Transfer the samples to room temperature, then add 35 µL of the homogeneous bead suspension to each sample well containing the 50-µL amplified DNA samples. Seal the wells, then vortex for 5 seconds. Briefly spin the samples to collect the liquid.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.

3 Incubate samples for 5 minutes at room temperature.

4 Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).

5 While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.

6 Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.

7 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

8 Repeat step 6 and step 7 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.

9 Dry the samples on the thermal cycler (with lid open) at 37°C for 1 to 3 minutes. Do not overdry the samples.

10 Add 30 µL of nuclease-free water to each sample well.

11 Seal the sample wells, then mix well on a vortex mixer and briefly spin the plate or tubes to collect the liquid.

12 Incubate for 2 minutes at room temperature.

13 Put the plate or tubes in the magnetic stand and leave for 2 minutes or until the solution in each well is clear.

14 Remove each cleared supernatant (approximately 29 µL) to a fresh LoBind tube. You can discard the beads at this time.

15 Remove a 1-µL sample of each amplified library for analysis in "Step 5. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay" on page 27. Dilute each of the 1-µL samples with 9 µL of nuclease-free water prior to analysis.

Stopping Point If you do not continue to the next step, store the libraries at 4°C short term or at –20°C for long term storage.

Step 5. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified indexed DNA. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

NOTE

Do not use Agilent's DNA 1000 Assay to analyze the whole genome samples. The expected distribution of whole genome library fragment sizes is not compatible with the DNA 1000 Assay. See Figure 1, below, for a sample electropherogram.

The presence of magnetic beads in the samples may adversely impact the Bioanalyzer results. If you supspect bead contamination in the samples, place the plate or strip tube on the magnetic rack before withdrawing samples for analysis.

1 Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of a 1:10 dilution of each sample for the analysis. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
3 Analyze the results, using the guidelines below:
   - Typical whole genome library electropherograms show a broad distribution of DNA fragments. A sample electropherogram is shown in Figure 1.
   - Check the Average Size [bp] of DNA fragments reported in the Bioanalyzer results. Sequencing data may be acquired from libraries with a broad range of average fragment sizes. The protocol has been optimized, however, to produce whole genome libraries with average DNA fragment sizes between approximately 600 and 1000 bp.

NOTE

An average fragment size significantly less than 600 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, libraries with an unusually large average fragment size may indicate too much gDNA in the fragmentation reaction and may require higher DNA concentrations for optimal cluster density in the sequencing reaction.

4 Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at –20°C for prolonged storage.

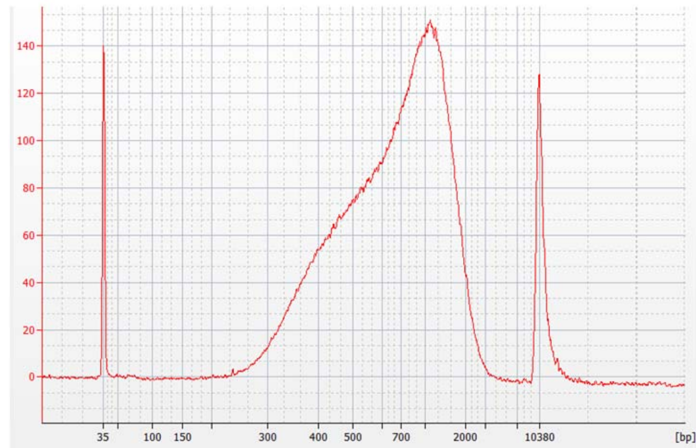


Figure 1 Analysis of amplified library DNA using a High-Sensitivity DNA Assay.

Step 6. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Guidelines for optimal low-level multiplexing of samples indexed using the SureSelect^{QXT} dual indexes are provided on page 44.

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of indexed sample to use.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where *V(f)* is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool

is the number of indexes, and

C(i) is the initial concentration of each indexed sample.

Table 8 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 8 Example of indexed sample volume calculation for total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

Step 7. Prepare sequencing samples

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 9 for kit configurations compatible with the recommended read length plus reads for the SureSelect^{QXT} 8-bp dual indexes.

The optimal seeding concentration for SureSelect^{QXT} whole genome libraries varies according to sequencing platform, run type and Illumina kit version. See Table 9 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality.

To do this step, refer to the manufacturer's instructions, using the modifications described on page 32 for use of the SureSelect^{QXT} Read Primers with the Illumina Paired-End Cluster Generation Kits. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 9 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length*	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	14–20 pM
HiSeq 2500	High Output	2 × 100 bp	4 x 50 Cycle Kit†	v3	14–20 pM
HiSeq 2500	High Output	2 × 125 bp	250 Cycle Kit	v4	14–20 pM
HiSeq 2000	All Runs	2 × 100 bp	4 x 50 Cycle Kit†	v3	14–20 pM
HiSeq 2000	All Runs	2 × 100 bp	250 Cycle Kit†	v4	14–20 pM
MiSeq	All Runs	2 × 100 bp	300 Cycle Kit	v2	14–20 pM
MiSeq	All Runs	2 × 300 bp	600 Cycle Kit	v3	14–20 pM
NextSeq 500/550	All Runs	2 × 150 bp	300 Cycle Kit	v2	2 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	200–220 pM

* If your application requires a different read length, verify that you have sufficient sequencing reagents to complete Reads 1 and 2 in addition to the dual 8-bp index reads.

† A single 200-cycle kit does not include enough reagents to complete Reads 1 and 2 in addition to the dual 8-bp index reads in this format. If preferred, the additional reads may be supported by using one 200-cycle kit plus one 50-cycle kit.

Using the SureSelect^{QXT} Read Primers with Illumina's Paired-End Cluster Generation Kits

To sequence the SureSelect^{QXT} libraries on Illumina's sequencing platforms, you need to use the following custom sequencing primers, provided in SureSelect QXT Library Prep Kit Box 2:

- **SureSelect QXT Read Primer 1**
- **SureSelect QXT Read Primer 2**
- **SureSelect QXT Index 1 Read Primer** (may also be labeled as *SureSelect QXT Index Read Primer*)
- **SureSelect QXT Index 2 Read Primer** (may also be labeled as *SureSelect QXT Index 2 Read Primer NSQ*; this primer is used for HiSeq 3000, HiSeq 4000, and NextSeq platforms only)

These SureSelect^{QXT} custom sequencing primers are provided at 100 µM and must be diluted in the corresponding Illumina primer solution, using the platform-specific instructions below:

HiSeq 2000 or HiSeq 2500 platform, combine the primers as shown in Table 10 or Table 11 on page 33.

For the HiSeq 3000 or HiSeq 4000 platform, combine the primers as shown in Table 12 on page 33.

For the MiSeq platform, combine the primers as shown in Table 13 on page 34.

For the NextSeq platform, combine the primers as shown in Table 14 or Table 15 on page 34.

NOTE

It is important to combine the primers precisely in the indicated ratios. Carefully follow the instructions indicated in Table 10 to Table 15. Where specified, add the custom primer volume directly to the solution already in cBot reagent plate wells. Otherwise, combine measured volumes of each solution; do not rely on volumes reported on vial labels or in Illumina literature. Vortex each mixture vigorously to ensure homogeneity for proper detection of the indexes using the custom read primers.

Table 10 HiSeq2000 and HiSeq 2500 High Output custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	6 µl SureSelect QXT Read Primer 1 (brown cap)	1194 µl HP6 or HP10	1.2 ml*
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	2985 µl HP8 or HP12	3 ml
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP7 or HP11	3 ml

* Aliquot the mixture as directed for HP6 or HP10 in Illumina's cluster generation protocol.

Table 11 HiSeq 2500 Rapid Mode custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	8.8 µl SureSelect QXT Read Primer 1 (brown cap)	1741.2 µl HP10	1.75 ml
Index	8.8 µl SureSelect QXT Index 1 Read Primer (clear cap)	1741.2 µl HP12	1.75 ml
Read 2	8.8 µl SureSelect QXT Read Primer 2 (black cap)	1741.2 µl HP11	1.75 ml

Table 12 HiSeq 3000 and HiSeq 4000 custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume	Reagent Rack Position
Read 1	1.5 µl SureSelect QXT Read Primer 1 (brown cap)	298.5 µl HP10*	0.3 ml per well	cBot Column 11
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP11	3 ml	16
Index 1+ Index 2	22.5 µl SureSelect QXT Index 1 Read Primer (clear cap) + 22.5 µl SureSelect QXT Index 2 Read Primer (purple cap)	4455 µl HP14	4.5 ml	17

* Use cBot recipe *HiSeq_3000_4000_HD_Exclusion_Amp_v1.0*. Add 1.5 µl SureSelect QXT Read Primer 1 to the 298.5 µl of HP10 in each well of column 11 in the cBot reagent plate.

Table 13 MiSeq platform custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume	Final Cartridge Position
Read 1	3 µl SureSelect QXT Read Primer 1 (brown cap)	597 µl HP10 (well 12)	0.6 ml	well 18
Index	3 µl SureSelect QXT Index 1 Read Primer (clear cap)	597 µl HP12 (well 13)	0.6 ml	well 19
Read 2	3 µl SureSelect QXT Read Primer 2 (black cap)	597 µl HP11 (well 14)	0.6 ml	well 20

Table 14 NextSeq 500/550 High-Output v2 Kit custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	3.9 µl SureSelect QXT Read Primer 1 (brown cap)	1296.1 µl BP10 (from well 20)	1.3 ml	well 7
Read 2	4.2 µl SureSelect QXT Read Primer 2 (black cap)	1395.8 µl BP11 (from well 21)	1.4 ml	well 8
Index 1+ Index 2	6 µl SureSelect QXT Index 1 Read Primer (clear cap) + 6 µl SureSelect QXT Index 2 Read Primer (purple cap)	1988 µl BP14 (from well 22)	2 ml	well 9

Table 15 NextSeq 500/550 Mid-Output v2 Kit custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	2.7 µl SureSelect QXT Read Primer 1 (brown cap)	897.3 µl BP10 (from well 20)	0.9 ml	well 7
Read 2	3.3 µl SureSelect QXT Read Primer 2 (black cap)	1096.7 µl BP11 (from well 21)	1.1 ml	well 8
Index 1+ Index 2	4.8 µl SureSelect QXT Index 1Read Primer (clear cap) + 4.8 µl SureSelect QXT Index 2 Read Primer (purple cap)	1590.4 µl BP14 (from well 22)	1.6 ml	well 9

Step 8. Set up the sequencing run and trim adaptors from the reads

Refer to Illumina protocols to set up custom sequencing primer runs, using the additional guidelines outlined below.

For SureSelect^{QXT} dual index sequence information, see tables on page 42.

Before aligning reads to the reference genome, SureSelect^{QXT} adaptor sequences must be trimmed from the reads. You can use SureCall, Agilent's NGS data analysis software, to perform adaptor trimming, alignment of reads and variant calling of sequencing data generated from either the HiSeq or the MiSeq platform. To download SureCall free-of-charge and for additional information, including tutorials on this software, visit the SureCall page at www.genomics.agilent.com.

SureCall is compatible with FASTQ files generated by HiSeq, MiSeq, or NextSeq platform. To use SureCall to analyze SureSelect^{QXT}-generated data, you first need to define an analysis workflow. This analysis workflow identifies the libraries as SureSelect^{QXT} libraries and enables automated adaptor trimming. The trimmed FASTQ files can then be used for alignment to generate BAMs for downstream analysis.

To create the analysis workflow, refer to Figure 2 on page 36. Upon starting SureCall, click the **Analysis Workflow** tab. Choose the appropriate analysis type (single sample, paired, or trio analysis), and then click the **Import Unaligned Files** button. Within the *Select Unaligned Sample Files* window, specify your read 1 and read 2 files using the **Add** buttons. Using the menus near the bottom of the screen, select **Default SureSelect QXT Method** from the *Analysis Method* menu, choose the appropriate design description from the *Design* menu, and select **Illumina** from the *Platform* menu. Once done, refer to the SureCall guide for next steps on alignment and variant calling.

If using another pipeline for alignment and downstream analysis, refer to the platform-specific guidelines starting on page 36.

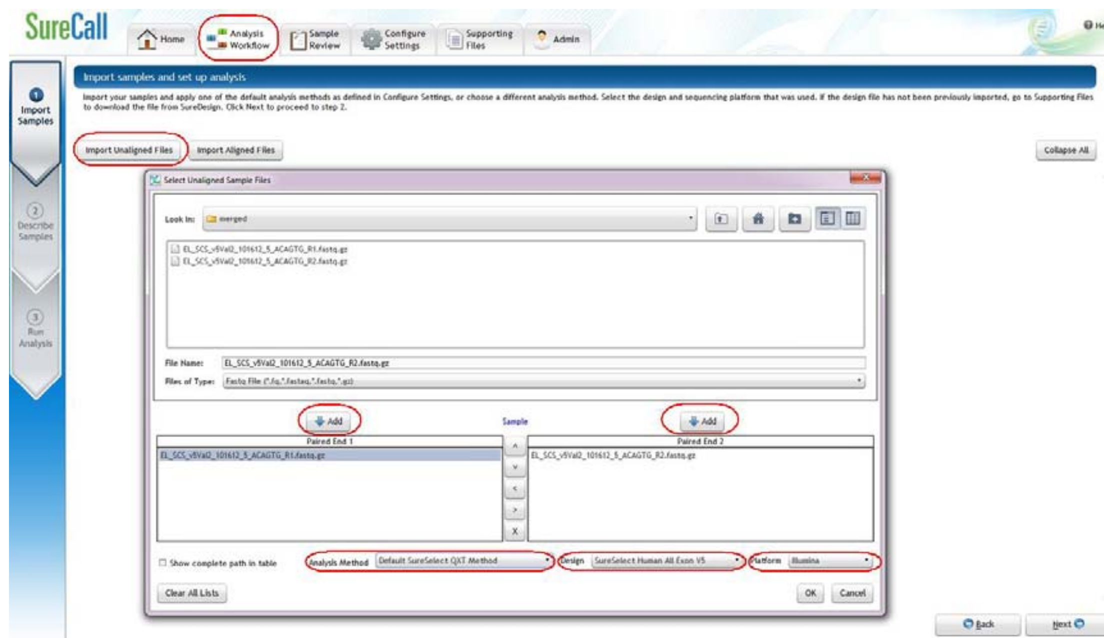


Figure 2 Analysis workflow setup in SureCall.

MiSeq platform sequencing run setup and adaptor trimming guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom primer Sample Sheet.

Set up the run to include adapter trimming using the IEM Sample Sheet Wizard. When prompted by the wizard, select the *Use Adapter Trimming* option, and specify **CTGTCTCTTGATCACA** as the adapter sequence. This enables the MiSeq Reporter software to identify the adaptor sequence and trim the adaptor from reads.

HiSeq or NextSeq 500 platform sequencing run setup and adaptor trimming guidelines

Set up sequencing runs using the settings shown in Table 16. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface. Since custom primers are spiked

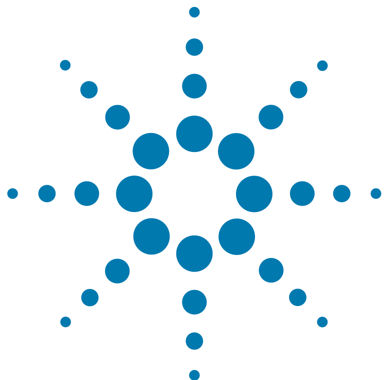
into the standard sequencing primer tubes, no additional specialized settings are required to accommodate the use of custom primers in the run.

For the NextSeq platform, Cycle Number and custom sequencing primer settings can be specified on the *Run Configuration* screen of the instrument control software interface.

Table 16 Run Configuration screen Cycle Number settings

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	100

After the sequencing run is complete, generate demultiplexed FASTQ data following Illumina's instructions and then trim adaptor sequences from the reads using Agilent's Read Trimmer tool. This tool takes in data in FASTQ format and removes the adaptor sequence from the ends of the sequencing reads, generating trimmed FASTQ data as output. To download the Read Trimmer tool free-of-charge and for additional information on this resource, visit www.agilent.com/genomics.



3
Reference

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This chapter contains reference information, including component kit contents and reference information for use during the downstream sample sequencing steps.

Kit Contents

SureSelect^{QXT} Library Prep Kits contain the following component kits:

Table 17 SureSelect^{QXT} Library Prep Kit Contents

Component Kits	Storage Condition	G9684A 16 Samples	G9684B 96 Samples	G9682A* 16 Samples	G9682B* 96 Samples
SureSelect QXT Library Prep Kit, Box 1	Room Temperature	5500-0119	5500-0119	5500-0119	5500-0119
SureSelect QXT Library Prep Kit, Box 2	–20°C	5500-0126	5500-0127	5500-0120	5500-0121

* SureSelect^{QXT} Reagent Kits p/n G9682A (16 samples) and p/n G9682B (96 samples) were retired in May, 2018 and replaced with p/n G9684A (16 samples) and p/n G9684B (96 samples).

The contents of each of the component kits listed in Table 17 are described in Table 18 and Table 19.

Table 18 SureSelect QXT Library Prep Kit, Box 1 Content

Kit Component	16 Reactions	96 Reactions
SureSelect QXT Stop Solution	bottle	bottle

Table 19 SureSelect QXT Library Prep Kit Box 2 Content

Kit Component	16 Reactions		96 Reactions	
	p/n 5500-0126	p/n 5500-0120*	p/n 5500-0127	p/n 5500-0121*
SureSelect QXT Buffer	tube with white cap	tube with white cap	bottle	bottle
SureSelect QXT Enzyme Mix ILM	tube with orange cap	tube with orange cap	tube with orange cap	tube with orange cap
Herculase II Fusion DNA Polymerase	tube with red cap	tube with red cap	tube with red cap	tube with red cap
Herculase II 5× Reaction Buffer	tube with clear cap	tube with clear cap	tube with clear cap	tube with clear cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap	tube with green cap	tube with green cap	tube with green cap
DMSO	tube with green cap	tube with green cap	tube with green cap	tube with green cap
SureSelect QXT Read Primer 1	tube with amber cap	tube with amber cap	tube with amber cap	tube with amber cap
SureSelect QXT Read Primer 2	tube with black cap	tube with black cap	tube with black cap	tube with black cap
SureSelect QXT Index 1 Read Primer	tube with clear cap	tube with clear cap (labeled as SureSelect QXT Index Read Primer)	tube with clear cap	tube with clear cap (labeled as SureSelect QXT Index Read Primer)
SureSelect QXT Index 2 Read Primer	tube with purple cap	Not provided	tube with purple cap	Not provided
SureSelect QXT P7 dual indexing primers	P7 i1 through P7 i8 provided in 8 tubes with yellow caps (one tube per primer)	P7 i1 through P7 i8 provided in 8 tubes with yellow caps (one tube per primer)	P7 i1 through P7 i12 provided in 12 tubes with yellow caps (one tube per primer)	P7 i1 through P7 i12 provided in 12 tubes with yellow caps (one tube per primer)
SureSelect QXT P5 dual indexing primers	P5 i13 and P5 i14 provided in 2 tubes with blue caps (one tube per primer)	P5 i13 and P5 i14 provided in 2 tubes with blue caps (one tube per primer)	P5 i13 through P5 i20 provided in 8 tubes with blue caps (one tube per primer)	P5 i13 through P5 i20 provided in 8 tubes with blue caps (one tube per primer)

* This p/n was retired in May, 2018 but remains supported until the expiration date listed on the Certificate of Analysis.

Nucleotide Sequences of SureSelect QXT Dual Indexes

The nucleotide sequence of each SureSelect QXT index is provided in the tables below.

Note that some index number assignments of the SureSelect QXT P5 and P7 indexes differ from the index numbers assignments used by Illumina for indexes of similar or identical sequence.

Each index is 8 bases in length. Refer to Illumina's sequencing run setup instructions for sequencing libraries using 8-base indexes.

Table 20 SureSelect QXT P7 Indexes 1 to 12

Index Name with Number	Sequence
P7 Index 1 (P7 i1)	TAAGGCGA
P7 Index 2 (P7 i2)	CGTACTAG
P7 Index 3 (P7 i3)	AGGCAGAA
P7 Index 4 (P7 i4)	TCCTGAGC
P7 Index 5 (P7 i5)	GTAGAGGA
P7 Index 6 (P7 i6)	TAGGCATG
P7 Index 7 (P7 i7)	CTCTCTAC
P7 Index 8 (P7 i8)	CAGAGAGG
P7 Index 9 (P7 i9)	GCTACGCT
P7 Index 10 (P7 i10)	CGAGGCTG
P7 Index 11 (P7 i11)	AAGAGGCA
P7 Index 12 (P7 i12)	GGACTCCT

Table 21 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 2000/2500 or MiSeq platform

Index Number	Sequence
P5 Index 13 (P5 i13)	TAGATCGC
P5 Index 14 (P5 i14)	CTCTCTAT
P5 Index 15 (P5 i15)	TATCCTCT
P5 Index 16 (P5 i16)	AGAGTAGA
P5 Index 17 (P5 i17)	GTAAGGAG
P5 Index 18 (P5 i18)	ACTGCATA
P5 Index 19 (P5 i19)	AAGGAGTA
P5 Index 20 (P5 i20)	CTAAGCCT

Table 22 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 3000/4000 or NextSeq platform*

Index Number	Sequence
P5 Index 13 (P5 i13)	GCGATCTA
P5 Index 14 (P5 i14)	ATAGAGAG
P5 Index 15 (P5 i15)	AGAGGATA
P5 Index 16 (P5 i16)	TCTACTCT
P5 Index 17 (P5 i17)	CTCCTTAC
P5 Index 18 (P5 i18)	TATGCAGT
P5 Index 19 (P5 i19)	TACTCCTT
P5 Index 20 (P5 i20)	AGGCTTAG

* When doing HiSeq 3000/4000 or NextSeq runs through BaseSpace, use the reverse complement sequences provided in Table 21.

Guidelines for Multiplexing with Dual-Indexed Samples

Agilent recommends following the dual index sample pooling guidelines shown in Table 23 for 16 reaction kits and shown in Table 24 for 96 reaction kits. These are designed to maintain color balance at each cycle of the index reads on both ends. They also provide flexibility of demultiplexing as single or dual indexed samples in low-plexity experiments. One-base mismatches should be allowed during demultiplexing.

Table 23 Dual index sample pooling guidelines for 16 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect^{QXT} P7 Indexes	Recommended SureSelect^{QXT} P5 Indexes
1-plex	Any P7 index (i1 to i8)	Either P5 index (i13 or i14)
2-plex	P7 i1 and P7 i2 OR P7 i2 and P7 i4	P5 i13 and P5 i14
3-plex	P7 i1, P7 i2 and P7 i4 OR P7 i3, P7 i4 and P7 i6 OR P7 i5, P7 i7 and P7 i8	P5 i13 and P5 i14 (as needed)
4- or 5-plex	P7 i1, P7 i2, P7 i4 and any additional P7 index(es) OR P7 i3, P7 i4, P7 i6 and any additional P7 index(es) OR P7 i5, P7 i7, P7 i8 and any additional P7 index(es)	P5 i13 and P5 i14 (as needed)
6- to 8-plex	Any combination of 6, 7, or 8 different P7 indexes	P5 i13 and P5 i14 (as needed)
9-to 16-plex	All eight P7 indexes (i1 to i8)	P5 i13 and P5 i14 (as needed)

Table 24 Dual index sample pooling guidelines for 96 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect^{QXT} P7 Indexes	Recommended SureSelect^{QXT} P5 Indexes
1-plex	Any P7 index i1 to i12	Any P5 index (i13 to i20)
2-plex	P7 i1 and P7 i2 OR P7 i2 and P7 i4	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18
3-plex	P7 i1, P7 i2 and P7 i4 OR P7 i3, P7 i4 and P7 i6 OR P7 i5, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
4-plex	P7 i1, P7 i2, P7 i3* and P7 i4 OR P7 i3, P7 i4, P7 i5* and P7 i6 OR P7 i5, P7 i6*, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
5-plex	P7 i1, P7 i2, P7 i3*, P7 i4 and P7 i5* OR P7 i3, P7 i4, P7 i5*, P7 i6 and p7 i7* OR P7 i5, P7 i6*, P7 i7, P7 i8 and p7 i9*	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
6- to 12-plex	Any combination of P7 indexes i1 to i12 using each index only once	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
13-to 96-plex	All twelve P7 indexes (i1 to i12)	P5 i13 and P5 i14 and any other P5 index OR P5 i15 and P5 i16 and any other P5 index OR P5 i17 and P5 i18 and any other P5 index (as needed)

* The indicated indexes may be substituted with another index, as long as the substitute index differs from all others used in the sample pool.

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In This Book

This guide contains information to run the SureSelect^{QXT} Whole Genome Library Prep protocol for Illumina paired-end multiplexed sequencing.

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