AgriPrep™
Library Prep Kit

for Illumina® Sequencing Platforms

Catalog numbers:

301721: AgriPrep Library Prep Kit, 4x96 Reactions - Set 1000
301722: AgriPrep Library Prep Kit, 4x96 Reactions - Set 2000
301723: AgriPrep Library Prep Kit, 4x96 Reactions - Set 3000
301724: AgriPrep Library Prep Kit, 4x96 Reactions - Set 4000
301729: AgriPrep Library Prep Kit, 4x96 Reactions - Any Index
301737: AgriPrep Library Prep Kit, 1x96 Reactions - Any Index

User Guide

v20260106

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Introduction

The AgriPrep Library Prep kit uses the patent-pending ExpressPlex™ workflow featuring seqWell's high performance engineered TnX™ transposase. AgriPrep has been optimized for low-coverage whole genome sequencing (lcWGS) of plant and animal genomes for agrigenomics applications such as genotyping-by-sequencing (GBS) and enables rapid variant discovery, genomic selection, and population-scale analysis. AgriPrep Library Prep kits provide a streamlined, one-step multiplexed library preparation workflow that is compatible with a 20-fold range of DNA input,and automatically normalizes read output per sample across a 5-fold range, while minimizing labor and consumable costs. Using the AgriPrep kit, a 96-plex library can be prepared for library QC and sequencing in 100 minutes, with less than 30 minutes of hands-on time.

AgriPrep Library Prep Kits utilize a proprietary mixture of enzymes to tag input DNA with full length indexed adapters and amplify libraries all in a single reaction. The kit employs combinatorial dual indexing to enable high levels of multiplexing, where 96 different i7 sample indexes are combined with a shared i5 plate index (Workflow Diagram and Appendix C). Within each plate, sample pool sizes are flexible and can be between 8 - 96 samples per pool. Each 4 x 96 kit provides four different indexing plates and each plate contains a unique i5 plate index, enabling barcoding with up to 384 distinct i7 + i5 index combinations which can be pooled together in a single sequencing run. In addition, there are four different sets of 384 index combinations available (each sold separately) enabling a total of 1,536* available barcode combinations that can be loaded on a single sequencing run when combining samples prepared from all four sets.

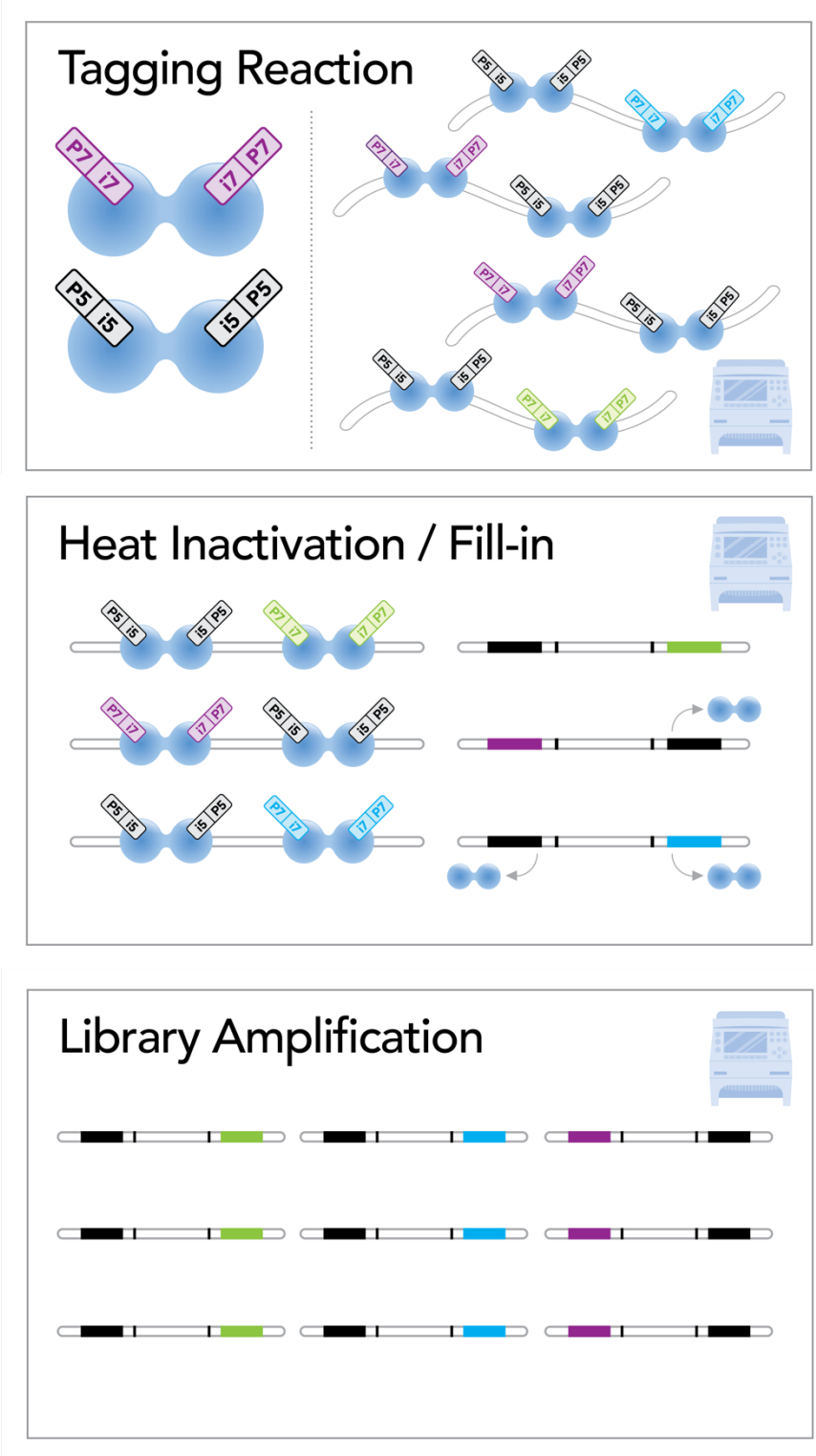
AgriPrep is optimized to generate libraries from a working range of 10–200 ng of plant or animal genomic DNA per 16 µl reaction, and automatically normalizes read output per sample and insert size for DNA inputs from 20–100 ng. AgriPrep typically generates fragment lengths from 300 - 1200 bp depending on the input DNA quality, input DNA size, and the magnetic bead cleanup ratio used. High-quality DNA input (DIN ≥ 7) is recommended for optimal results. The resulting libraries are compatible with the Illumina MiSeq™, MiSeq™ i100, NextSeq™, iSeq™, and NovaSeq™ sequencing platforms. For running with Illumina XLEAP-SBS chemistry, guidelines for optimal batching can be found on our website in the Technical Data Sheets category on our resources page. Please also refer to Illumina's guidelines for optimal color balancing for different sequencing chemistries.

Each AgriPrep kit comes with a vial of PhiRx™ Indexed Control, which is a dual-indexed control library made from phiX174 genomic DNA and optimized for Illumina sequencing platforms, particularly two-color systems like XLEAP SBS chemistry on NextSeq 1000/2000, MiSeq i100 and NovaSeq X. By

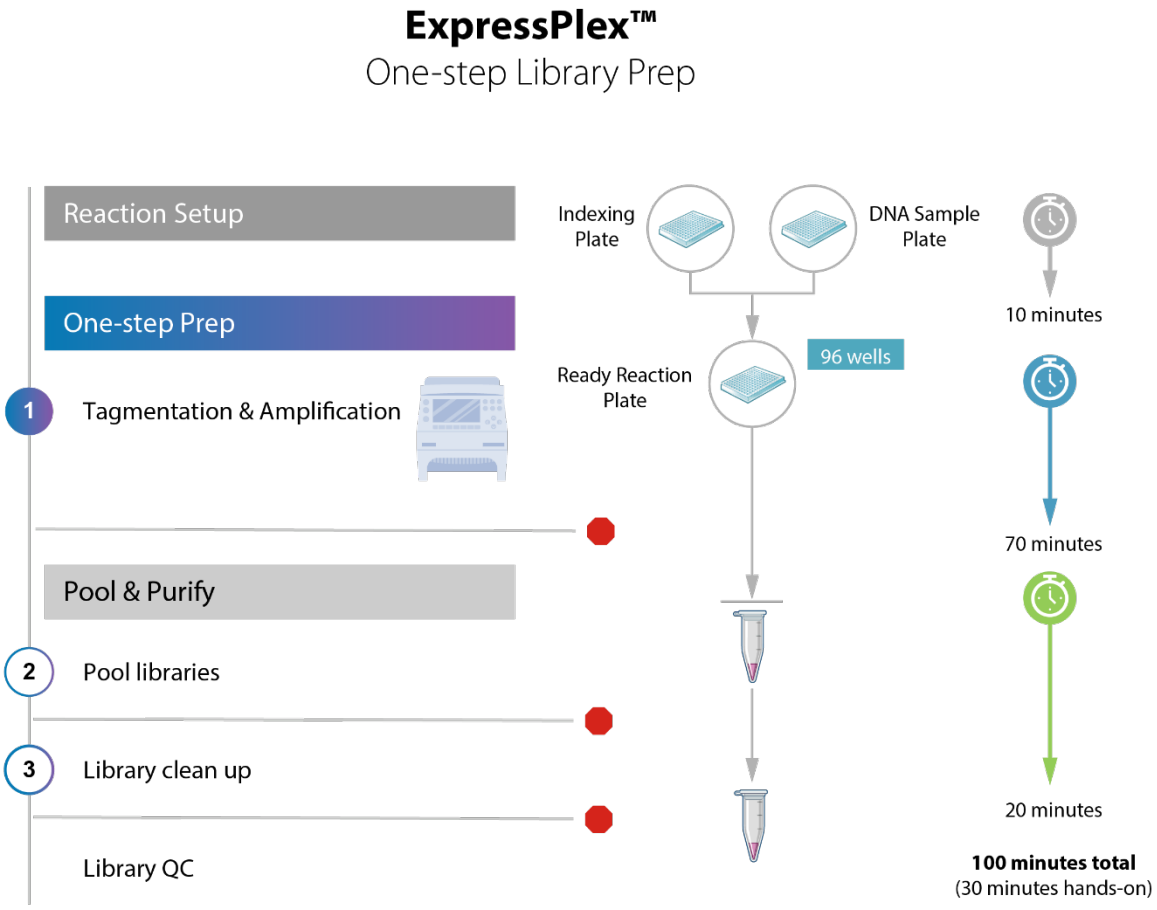
* Up to 3,072 unique combinations are available via custom ordering.

improving color balancing for combinatorial indexed AgriPrep libraries (where there are only 1-4 i5 indexes used), our PhiRx Indexed Control ensures cleaner, more accurate sequencing results and can be used as a 1:1 substitution for Illumina's standard PhiX Control Library. Please see Appendix D for PhiRx usage information.

AgriPrep Molecular Diagram

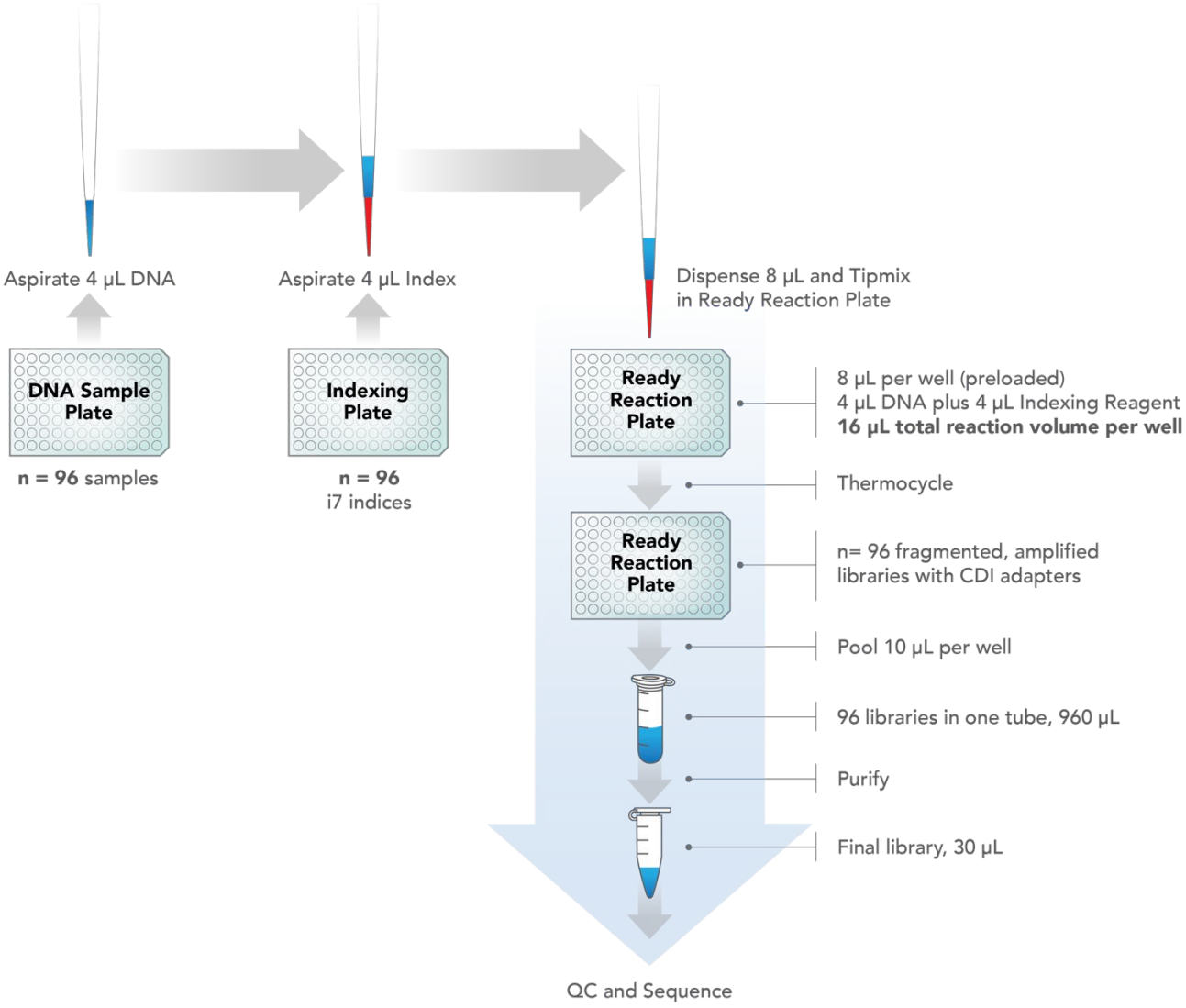


AgriPrep Workflow Diagram



AgriPrep

EP Green Automation Workflow Diagram



Kit Components

AgriPrep Library Prep Kit Components (4X96 Reactions)

AgriPrep Library Prep Kit, 4X96 - Set 1000

Catalog No. 301721

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4x96 Indexing Reagent Plates - 1001, 1002, 1003, 1004	301701	Indexing Plates (96-well) in white fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
2	Box 2 - 4x96 Ready Reaction Plates	301708	Ready Reaction Plates (96-well) in green fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

AgriPrep Library Prep Kit, 4X96 - Set 2000

Catalog No. 301722

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4x96 Indexing Reagent Plates - 2001, 2002, 2003, 2004	301702	Indexing Plates (96-well) in white fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
2	Box 2 - 4x96 Ready Reaction Plates	301708	Ready Reaction Plates (96-well) in green fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

AgriPrep Library Prep Kit, 4X96 - Set 3000

Catalog No. 301723

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4x96 Indexing Reagent Plates - 3001, 3002, 3003, 3004	301703	Indexing Plates (96-well) in white fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
2	Box 2 - 4x96 Ready Reaction Plates	301708	Ready Reaction Plates (96-well) in green fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

* Larger volumes (15 ml) of MAGwise paramagnetic beads are also available to purchase separately (REF: 101002).

AgriPrep Library Prep Kit, 4X96 - Set 4000
Catalog No. 301724

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4x96 Indexing Reagent Plates - 4001, 4002, 4003, 4004	301704	Indexing Plates (96-well) in white fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
2	Box 2 - 4x96 Ready Reaction Plates	301708	Ready Reaction Plates (96-well) in green fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

AgriPrep Library Prep Kit, 4X96 - Any Index
Catalog No. 301729

Item	Component	REF	Description	Storage	Qty
1	Box 1 - 4x96 Indexing Reagent Plates (any index)	Any 1 of: 301701 301702 301703 301704	Indexing Plates (96-well) in white fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
2	Box 2 - 4x96 Ready Reaction Plates	301708	Ready Reaction Plates (96-well) in green fully-skirted PCR plates	-25° to -15°C	1 Box of 4 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

* Larger volumes (15 ml) of MAGwise paramagnetic beads are also available to purchase separately (REF: 101002).

AgriPrep Library Prep Kit Components (1X96 Reactions)

AgriPrep Library Prep Kit, 1X96 - Any Index

Catalog No. 301737

Item	Component	REF	Description	Storage	Qty
1	Box: Indexing Reagent Plate (any index); Ready Reaction Plate	301719	Indexing Plates (96-well) in white fully-skirted PCR plates Ready Reaction Plate (96-well) in fully-skirted, green PCR plate	-25° to -15°C	1 Box of 2 Plates
3	MAGwise paramagnetic beads*	101003	5 ml of MAGwise beads in a 10 ml screwcap tube	2° to 8°C	1
4	PhiRx™ Indexed Control	301184	10 µL of 10nM PhiRx Indexed Control solution in a screwcap tube	-25° to -15°C	1

* Larger volumes (15 ml) of MAGwise paramagnetic beads are also available to purchase separately (REF: 101002).

User-Supplied Reagents, Equipment, Consumables, and Thermal Cycler Program

Reagents

- 100% Ethanol (molecular biology grade)
- Tris-HCl, pH 8.0
- Ultrapure Water (PCR grade)
- Quant-iT™ PicoGreen™ dsDNA Assay Kits (ThermoFisher P/N: P7589), or other validated dsDNA quantification assay
- Electrophoretic analysis reagents - Agilent TapeStation (High Sensitivity D5000 or D5000 kits), Bioanalyzer (High Sensitivity DNA or DNA7500 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474)

Equipment & Consumables

- Single-channel pipettors (1-20 µl, 20-200 µl, 100-1,000 µl)
- Multi-channel pipettors (1-10 µl, 10-200 µl)
- Pipette tips (low-retention barrier tips)
- Eppendorf Tubes® (1.5 ml and 2 ml, DNA LoBind Tubes)
- PCR plate seals (must be evaporation-resistant)
- 96-well thermal cycler (compatible with fully skirted PCR plates and 8-tube PCR strips)
- Magnetic stand for 1.5 ml tubes
- 0.2 ml 8-tube PCR strips and caps/seals
- Benchtop centrifuge to pulse-spin tubes and 8-tube PCR strips
- Plate centrifuge
- Vortex mixer
- Electrophoretic analysis equipment - Agilent TapeStation, Bioanalyzer,or Fragment Analyzer
- Fluorometer for dsDNA quantification assay

Thermal Cycler Program <u>AGRIPREP</u> (with lid-heating set to 105°C):

15	min	55°C	Tagging
5	min	75°C	Fill-in/Heat-kill
3	min	68°C	
5	min	79°C	
3	min	68°C	
5	min	83°C	
1	min	98°C	Initial denaturation
15	sec	98°C	Amplification (PCR) **8 PCR cycles**
30	sec	64°C	
1	min	72°C	
5	min	72°C	Final extension
Hold		4°C	

Before starting the procedure:

Review FAQs. FAQs can be found here: https://seqwell.com/resource-category/faqs/. Review these prior to your first run.

Measure and adjust input DNA concentration. Assay the DNA concentration of each sample to be processed using PicoGreen or other validated dsDNA assay. Globally adjust the average concentration of input DNA across each plate to 10 ng/µl in 10 mM Tris-HCl, pH 8.0. Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity. *Refer to Appendix A for more detailed information on globally adjusting the average input DNA concentration.*

Program the thermal cycler. For convenience, set up the thermal cycler program described in the protocol before starting.

Pulse-spin kit components. Liquids can condense or shift locations inside containers during shipment or storage. Before using the reagent plates and tubes, pulse-spin in a suitable centrifuge to gather the reagents at the bottom of the well or tube. If the kit components freeze during shipment or storage, thaw, mix and pulse-spin before use.

Equilibrate MAGwise Paramagnetic Beads to room temperature. MAGwise beads can be stored for up to 2 weeks at room temperature or for longer periods at 2° - 8°C. If stored cold, warm at room temperature for 30 minutes before use. Vortex to thoroughly resuspend beads prior to use. To transfer volumes accurately, pipette slowly and do not pre-wet pipette tips.

Prepare 80% ethanol fresh daily.

Prepare 10 mM Tris-HCl, pH 8.0. Prepare 10 mM Tris-HCl, pH 8.0 from a concentrated stock solution diluted with ultrapure water (both molecular-biology grade). Do not use EDTA-containing solutions (e.g., TE).

Safe stopping points are indicated in the protocol. For optimal results, proceed directly to the next step unless a safe stopping point is indicated.

Procedure

Key Items Before Starting:

- Refer to **Appendix A** for instructions to globally adjust the average input DNA concentration.
- AgriPrep reactions can be set up at room temperature.
- If processing more than one plate, multiple reaction plates may be set up and thermal cycled at the same time before proceeding to subsequent steps.
- If manually preparing 96 samples at a time (full plate), proceed to Section 1A below.
- If manually preparing fewer than 96 samples at a time, proceed to Section 1B on page 15.
- If using automation for reaction set up, proceed to Section 1C on page 16.

1A. AgriPrep Reaction Setup (Manual, full plate)

If preparing libraries from 96 samples at a time (full plate), complete the setup and thermal cycle directly in the Ready Reaction Plate as follows (see section 1B on next page for alternate instructions on preparing <96 samples in a batch**):**

a. Pre-label each **Ready Reaction Plate** with the index set ID and any other relevant information to allow easy identification of your samples.

b. Pulse-spin one **Indexing Reagent Plate** and one **Ready Reaction Plate** in a tabletop centrifuge; then remove the heat seals carefully.

   Do not try to puncture seals with pipette tips; seals must be removed by peeling.

c. Carefully transfer 4 µl of **Indexing Reagent** to each corresponding well of the **Ready Reaction Plate** with a multichannel pipette, using new tips for each transfer.

d. Next, transfer 4 µl of input DNA (at approximately 5 - 25 ng/µl) to each well (one sample per well) of the **Ready Reaction Plate**. Mix thoroughly by pipetting up and down ten times at 4 µl, being careful not to introduce excessive bubbles. Use clean tips for addition of each DNA sample.

   Optional*:* If excessive bubbles are present after reaction set-up, use a tabletop centrifuge to collapse the bubbles prior to proceeding.

e. Seal the **Ready Reaction Plate**, and proceed directly to Section 2: Thermal Cycling on Page 17.

1B. AgriPrep Reaction Setup (Manual, partial plate)

If preparing fewer than 96 samples (batches of 16 – 88 samples), complete the set up in a separate PCR-plate or strip tube(s) as follows:

NOTE: **Batches of <16 samples may be processed ONLY IF an experienced user is comfortable with a final elution volume of 15 µl.**

a. Pulse-spin one **Indexing Reagent Plate** and one **Ready Reaction Plate** in a tabletop centrifuge.

b. Only cut and peel the heat seal from the wells of both the **Indexing Reagent Plate** and **Ready Reaction Plate** corresponding to the total number of samples that will be processed. This may be done by ***carefully*** using a razor blade to cut the seal.

   The **Indexing Reagent Plate** and **Ready Reaction Plate** can be thawed and refrozen up to 12 times without impacting performance.

c. Carefully transfer 4 µl of **Indexing Reagent** from the unsealed wells to each **corresponding unsealed well** of **Ready Reaction Plate** with a multichannel pipette. Use new tips for each column transfer.

d. Next, transfer 4 µl of input DNA (at approximately 5 - 25 ng/µl) to each well (one sample per well) of the **Ready Reaction Plate**. Mix thoroughly by pipetting up and down ten times at 4 µl, being careful not to introduce excessive bubbles. Use clean tips for addition of each DNA sample.

   Optional*:* If excessive bubbles are present after reaction set-up, use a tabletop centrifuge to collapse the bubbles prior to proceeding.

e. After mixing all the reaction components and DNA together in the **Reaction Ready Plate**, transfer all the contents (16 µl) to a clean 8-tube PCR strip(s) or a clean PCR plate.

f. Seal tubes or the plate and spin down briefly.

g. After verifying that the seals on the unused portion of the **Indexing Reagent Plate** and **Ready Reaction Plate** are still intact, cover the used wells to prevent contamination of the unused reagents when the plates are handled again. Then return the sealed/resealed plates to the freezer for later use.

h. Proceed directly to Section 2: Thermal Cycling on Page 17.

1C. AgriPrep Reaction Setup (Using Automated liquid handlers)

AgriPrep reactions can be set up at room temperature directly in the **Ready Reaction Plate** using a 96-channel pipetting head, an 8-channel pipetting head, or even an automated single-channel pipetting device. By using the same pipette tips to aspirate indexing reagents and the DNA samples, also known as the *EP Green Method,* the plastic consumables and time required for setup can be dramatically reduced. Optimal throughput is achieved using 96-channel instruments.

a. Pre-label each **Ready Reaction Plate** with the number from the **Indexing Reagent Plate** and a name to allow easy identification of your samples.

b. Pulse-spin one **Indexing Reagent Plate** and one **Ready Reaction Plate** in a tabletop centrifuge and remove the heat seals carefully.

c. Aspirate 4 µl of approximately 5 - 25 ng/µl input DNA into all channels of the pipette. Using the same tips, aspirate 4 µl of **Indexing Reagent** into all channels of the pipette.

   ***NOTE**: Sufficient overage is provided to reliably aspirate 4 µl of **Indexing Reagent**, however each well of the **Indexing Reagent Plate** is intended for one use only.*

d. Dispense 8 µl from the tips into the **Ready Reaction Plate.**

e. Mix thoroughly by aspirating and dispensing an 8 µl volume ten times, being careful not to introduce bubbles. If excessive bubbles are present after reaction setup, use a tabletop centrifuge to collapse the bubbles prior to proceeding.

f. Seal the **Ready Reaction Plate**, and proceed directly to Section 2: Thermal Cycling on Page 17.

2. Thermal Cycling

a. Transfer the plate or strip-tube(s) containing the assembled reactions to a thermal cycler, and run the **AGRIPREP** thermal cycling program below, with lid-heating set to 105°C:

15	min	55°C	Tagging
5	min	75°C	Fill-in/Heat-kill
3	min	68°C	
5	min	79°C	
3	min	68°C	
5	min	83°C	
1	min	98°C	Initial denaturation
15	sec	98°C	Amplification (PCR) **8 PCR cycles**
30	sec	64°C	
1	min	72°C	
5	min	72°C	Final extension
Hold		4°C	

SAFE STOPPING POINT

Proceed immediately to the next step

or store the amplified libraries at -20°C.

3. Library Pooling

a. After library amplification, pulse-spin the **Ready Reaction Plate (or for <96 samples, the strip tube(s) or reaction plate)** and then open the seal/cap.

b. Using a multichannel pipette or an automated 8-channel pipetting head, pool 10 µl of each amplified library from each column into a single prelabeled 8-tube PCR strip. The same pipette tips may be used for pooling multiple reactions.

c. After mixing by pipetting, transfer the entire volume from each well of the 8-tube PCR strip into a prelabeled 2 ml LoBind tube.

   If excessive bubbles are present after pooling, use a tabletop centrifuge to collapse the bubbles prior to proceeding. User may freeze any unpurified amplified libraries remaining in the **Ready Reaction Plate**, providing an option to purify more libraries later if any sample(s) should require additional sequencing depth.

4. Library Pool Purification

a. Ensure MAGwise beads have been equilibrated to room temperature for at least 30 minutes before use.

b. Vortex the *room temperature* MAGwise to ensure that the beads are fully resuspended before use.

c. To the pool of AgriPrep libraries made in the previous section, add 0.75X volumetric equivalent of MAGwise**.** To determine this volume, measure the volume of the pooled library via reverse pipetting, and multiply that volume by 0.75.

d. Add MAGwise and mix thoroughly by pipetting up and down ≥10X. Incubate on the bench for 5 minutes to allow the DNA to bind.

***NOTE**: Refer to Library Quantification and QC section for optimization of library size using different bead clean up ratio.*

e. Place the tube on a magnetic stand and let the bead pellet form on one side of the tube and wait until the supernatant appears completely clear (5 minutes or less).

f. Remove and discard the supernatant with a pipette, without disturbing the bead pellet.

g. Wash beads with 80% ethanol.

   i. With the tube in the magnetic stand, add sufficient 80% ethanol to cover the bead pellet, without disturbing the bead pellet.

   ii. After ≥30 seconds, remove and discard the supernatant, without disturbing the bead pellet.

h. Repeat the previous step for a total of 2 washes with 80% ethanol. [Do not air dry the bead pellets---proceed immediately to the next step]

Useful tip: After using a large pipette tip to remove the waste ethanol from the second wash, briefly pulse-spin, return to the magnet, and then use a smaller pipette tip to remove any residual volume, if visible.

i. Immediately remove the tube from the magnetic stand and pipette 30 µl of 10 mM Tris-HCl, pH 8 on top of the bead pellet. Pipette the solution along the inner wall of the tube multiple times to thoroughly resuspend the bead pellet.

***Optional**:* Lower elution volumes (<30 µl) can be used to increase the concentration of libraries prepared from 16 – 88 samples. Fewer than 16 samples may be processed ONLY IF an experienced user is comfortable with a final elution volume of 15 µl.

j. Incubate the tube on the bench for at least 5 minutes to elute the libraries from the beads.

k. Return tube to magnetic stand and allow the bead pellet to reform on the inner wall of the tube (~2 minutes).

l. When the supernatant has cleared completely, carefully transfer the DNA eluate to a clean 1.5 ml LoBind tube. The transferred eluate contains the purified pooled library.

SAFE STOPPING POINT

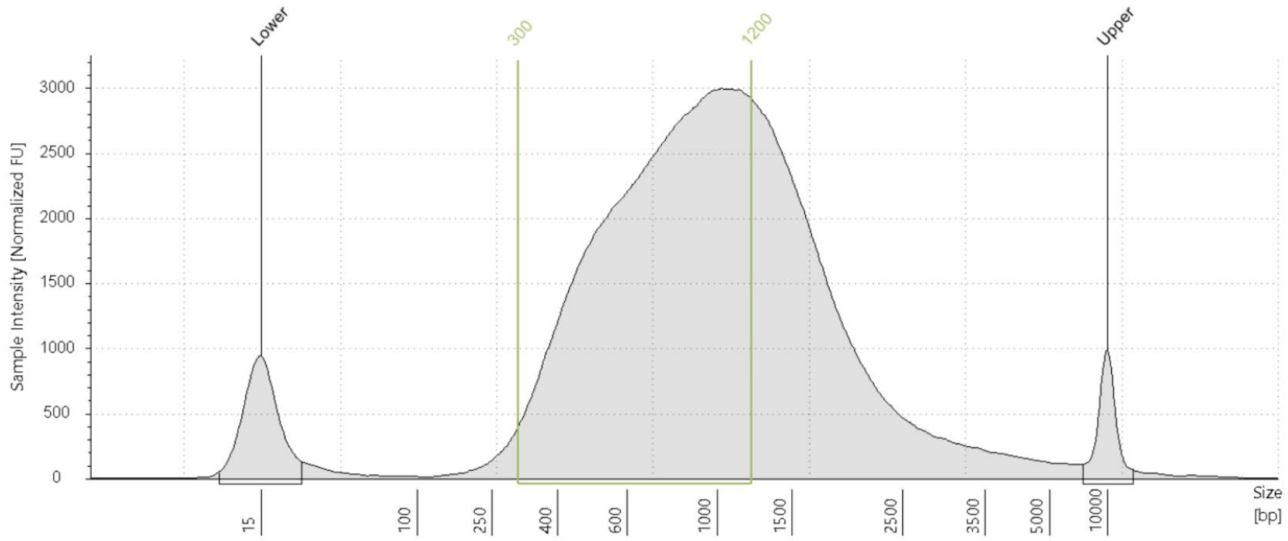
Proceed immediately to Library Quantification and QC
or store the amplified libraries at -20°C

Library Quantification and QC

Library quantification and QC with electrophoretic analysis: Quantify the pooled, purified AgriPrep library using Quant-iT™ PicoGreen™ dsDNA Assay, Qubit™ dsDNA HS Assay (see below) or other validated dsDNA quantification assay. Alternatively, if using electrophoretic analysis for library quantification, use the region analysis function and calculate the library concentration from the clusterable fragment region; however, this calculation may not be the most accurate. For a more accurate measurement of the concentration of clusterable library fragments, use qPCR (see below).

Assay the fragment size using Agilent Tapestation (High Sensitivity D5000 or D5000 kits), Bioanalyzer (High Sensitivity DNA or DNA7500 kits), or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit, DNF-474) following the manufacturer's instructions for these instruments. For optimal sequencing results with AgriPrep libraries, use a region analysis for fragments of **300 - 1,200 bp** to determine the average clusterable fragment length for size adjustment.

Refer to the figures below for representative traces for pooled, purified libraries run on the Tapestation.



Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total
762	5940	13600	62.78

Figure 1. Representative Tapestation (High Sensitivity D5000) electropherogram of a pooled, purified 96-plex AgriPrep library with human DNA input (diluted 20-fold prior to electrophoresis). Region (green) shows the range of clusterable fragments. Region analysis table shows example of library quantification results.

Library quantification by TapeStation **ONLY** (Library diluted 20-fold prior to electrophoresis):

- Clusterable region average library size: 717 [bp]
- Clusterable region library concentration: 1200 [pg/µl] × 20 = 24.0 [ng/µl]
- Estimated library concentration: $\frac{24.0\left[\frac{ng}{\mu l}\right]}{660\left[\frac{g}{mol}\right]\times 717\ [bp]} \times 10^6 = 50.7\ [nM]$

Library quantification with dsDNA specific fluorometric method: AgriPrep libraries are double stranded. Use 2 µL to quantify the pooled, purified library using the Qubit™ dsDNA assay, Quant-iT™ PicoGreen™ dsDNA assay or other validated dsDNA quantification assay. Follow the manufacturers' instructions for the appropriate dilutions. Use the average fragment size as determined by electrophoresis to calculate the library concentration and the dilution factor required for loading your sequencing system.

Library quantification with qPCR assay: qPCR is a very sensitive method of measuring library fragments that have both adaptor sequences on either end which will subsequently form clusters on a flow cell. Use 2 µl of the purified, multiplexed AgriPrep library for qPCR analysis with Illumina qPCR primer 1.1/2.1. Follow kit and instrument instructions for appropriate conditions and dilutions.

Conversion from ng/µl to nM: Use the following formula to convert library concentration from ng/µl to nM:

$$Library\ concentration\ [nM] = \frac{Library\ concentration\ \left[\frac{ng}{\mu L}\right]}{660\left[\frac{g}{mol}\right] \times Average\ fragment\ size\ [bp]} \times 10^6$$

Sequencing on Illumina platforms

Read configuration: AgriPrep Library Prep kit libraries are dual indexed with 10 base indices. Index lists can be found in Appendix C. AgriPrep libraries are sequenced using the same primers as standard Illumina libraries, and consequently, custom sequencing primers are not needed. Longer reads deliver greater read depth and read lengths of 2 x 150 or greater are recommended for *de novo* assembly. The i7 and i5 index reads must be 10 bases long for AgriPrep libraries, although the index reads and non-index reads can be adjusted for different sequencing kits, speed, or read depth requirements. For example, the sequencing run can be demultiplexed using only the unique i7 barcodes when running 96 or fewer AgriPrep libraries on a sequencer.

Library dilution, denaturation and sequencing: Follow Illumina's provided guidelines for appropriate dilution and loading procedures specific to your sequencing system. Not all DNA fragments can efficiently generate clusters. Longer library fragments generally do not cluster as efficiently on the flow cell. It is essential to optimize the loading concentration based on your sample type, library QC methods and library fragment distribution. Try adjusting loading concentration over subsequent sequencing runs to optimize cluster density and sequencing run performance.

Add PhiRx Indexed Control to library pool. See Appendix D for instructions.

Appendix A: Adjusting the Input Sample Concentration

AgriPrep Library Prep Kits perform optimally with 10 - 200 ng of total dsDNA input per 16 µl reaction, and normalize from 20-100ng. Individual adjustment of each sample concentration is not necessary.

If the method used to purify DNA upstream of library prep is well-characterized and generates relatively consistent concentrations of DNA per sample, it may be sufficient to only assay several samples from a 96-well plate (i.e., spot-check the dsDNA concentration using Qubit dsDNA assay or Quant-iT PicoGreen to estimate the average DNA concentration across all samples).

If all the samples already fall within the 2.5 – 50 ng/µl range, no adjustment is required. If, however, the average concentration of all the samples exceeds 25 ng/µl, calculate the global dilution factor using the formula below:

$$\text{Global dilution factor (X)} = \frac{\text{Average assayed dsDNA concentration (ng/µl)}}{\text{10 ng/µl}}$$

The global dilution factor is applied to the input samples in a 96-well plate so that the average DNA concentration across all samples will be approximately 10 ng/µl (*i.e.,* resulting in an average of 40 ng of input being added per full AgriPrep reaction volume).

NOTE*: If the DNA concentration of the input samples is not easily confined to an approximately 20-fold range (maximum concentration divided by minimum concentration), or, if an average sample concentration of ≥2.5 ng/µl cannot be routinely achieved, consider optimizing the method used to generate input DNA.*

Important Reminder: Do not use EDTA-containing solutions (*e.g.*, TE buffer) to dissolve or dilute input DNA because EDTA can suppress enzymatic activity.

Appendix B: Sample Sheet and Sequencer Guidelines

Illumina sequencing systems and chemistries differ in their use of sample sheets, availability of on-instrument demultiplexing, optimal color balancing, and run setup methods. If you have questions regarding your specific sequencer, contact Illumina tech support for the best guidance on setting up your run using your sequencing platform.

AgriPrep libraries are similar to the Nextera adapter sequences and are dual-indexed using 10 base indices for both the i7 and i5 index sequences. AgriPrep libraries do NOT require custom sequencing primers.

All Illumina sequencers read the i7 index in the forward direction (as listed in Appendix C). The i5 index, however, is read differently on different sequencers depending on the version/chemistry of the sequencing kits. In this case, if using an Illumina sample sheet template, enter the i5 index in the forward direction as the sample sheet will auto-generate the reverse complement if needed. If demultiplexing using bcl2fastq and using a sequencer that reads the reverse complement of the i5 index sequence, enter the reverse complement of the i5 index shown in Appendix C.

Please refer to Illumina's website for the most up-to-date index sequencing guidelines: https://support.illumina.com/downloads/indexed-sequencing-overview-15057455.html.

Appendix C: AgriPrep Index Information and Demultiplexing Guidance

AgriPrep utilizes a combinatorial dual indexing (CDI) strategy: all 16 Indexing Reagent Plates have the same 96 i7 indices but each plate has a different i5 index, providing 1,536 barcode combinations in total. Please refer to the AgriPrep index list in spreadsheet form on our website, for a complete list of all i7 indices (listed by row and column formats as well as in plate layout) and the i5 indices: https://seqwell.com/resource-category/index-list/

i7 Index Plate Map for AgriPrep

	1	2	3	4	5	6	7	8	9	10	11	12
A	GTCAAGTCCA	CAACTAACTC	ATAACCTGAC	CAGGTACTTC	AACCGAGCCA	CAACGTCATT	ATTGGTCAGA	ATGTTGCGGA	CACCAATAAC	TGTCCGTCTT	CGAAGGACTG	TAGTTATCGC
B	TATCTCTTCC	GTACTGGATT	TGCGGTTCCA	CAGGAATATG	TGGATTCAAG	AAGAACGATG	AAGACCTGTT	AATGCTAACC	GCGTCCACAA	CATGAGTAAC	TCTACCGTCA	TGCAGGTGAT
C	AATCTCGTGG	CATCGGAGGA	AATACTTGCC	AACGCACAAT	CAACAGATAC	CTGCAATTAC	CGCTAATGAA	GTAACACGTA	ATGTGCGCTT	CTGCGCGAAT	GGTAATATCG	TGTGAAGCTA
D	CCTACTCGGA	ATTCTGATGG	AATCGCGGAA	ATTGAGAAGG	AAGGTAACTC	CGCCGATGAT	CAGAGTGCAT	GATATACGGA	TATCTAGTGC	CTGTAGTATG	TGTGCGAGTT	CAACTCCTGA
E	TTCGTATCAC	TATCGTTACC	CAGAACGCGA	ATTGCACCTT	TGACAATACG	CGGACAAGAC	GGTAAGCTGA	ATCCGAGAGG	GCCTACAATG	GAGCCGTACA	TTATCGCTGA	TGTAGCAACG
F	TCCTCCATCC	TGGTCTGTTA	TGAACCAAGG	AAGTGGATAC	GATTGTGCAT	CGTACTCCTC	GTATTCAGTG	CAATTCACAC	AAGCTCAGTT	CATTCTTAGG	GGTTGAGTTC	CGGTAACGCA
G	ATAACATCGC	TGCCAACATG	TGTTAGTCAG	GATAAGATGC	GCACACCATT	TGCATGAGTT	GCGCTACGTT	TTGTCAGTTC	TACGCTTAGA	TGACCGACAA	ATTGGACGCC	GTAGCAGCAG
H	GATATGCGTT	TCATTACACG	CAGTAGGTAA	GACATACACG	TCGTTATTCC	AATCTGGAGC	TTGTCATAGC	ATACGCCATT	ATCCACTAGG	TGTGTAACCG	TATGTGTGTG	CTACAGCCGA

i5 Index Read

Set	Index Name	i5 Forward	i5 Reverse
Set 1000	1001	GTAACACAGA	TCTGTGTTAC
	1002	CAAGAGCGTG	CACGCTCTTG
	1003	CCGAGGTTAG	CTAACCTCGG
	1004	TGGAGCGATG	CATCGCTCCA
Set 2000	2001	ATCTCCACGG	CCGTGGAGAT
	2002	ATTCCGCTTA	TAAGCGGAAT
	2003	TTGTTCTGCG	CGCAGAACAA
	2004	CCTCTGAACA	TGTTCAGAGG
Set 3000	3001	CTGATTAGGA	TCCTAATCAG
	3002	CAATGCGGAG	CTCCGCATTG
	3003	GTATCTTAGG	CCTAAGATAC
	3004	TCGCGGACAT	ATGTCCGCGA
Set 4000	4001	TAAGTTGTGG	CCACAACTTA
	4002	CCGTAATCGA	TCGATTACGG
	4003	CTCAGTAGAC	GTCTACTGAG
	4004	CTTATCCAGG	CCTGGATAAG

***Important Notes**: Illumina sample sheets for on-board demultiplexing use the i5 index forward read sequence. The software will automatically generate the reverse complement for sequencers that rely on the i5 index reverse read sequence. Please refer to Illumina's recommendations for optimal color balancing for different sequencing chemistries.*

Optimal Demultiplexing Guidance:

In accordance with Illumina's guidelines, it is recommended to allow for 1 mismatch in the barcode reads during demultiplexing. This allows for the capture of many more reads than 0 mismatches at a reduced risk, due to sufficient Hamming distances between barcodes.

Refer to *Illumina's mismatch guidelines here:* https://knowledge.illumina.com/software/general/software-general-reference_material-list/000007484.

For the XLEAP chemistry:
For NovaSeq X, NextSeq 2000, and MiSeq i100 running XLEAP-SBS chemistry, an increased rate of 1 mismatch in the barcode reads has been observed. Hence, it is especially important to allow for 1 mismatch on these instruments to capture all expected data for each barcode.

Appendix D: Using PhiRx Indexed Control in Illumina sequencing

Each AgriPrep kit comes with a vial of PhiRx™ Indexed Control, which is a dual-indexed control library made from phiX174 genomic DNA and optimized for Illumina sequencing platforms, particularly two-color systems like XLEAP SBS chemistry on NextSeq 1000/2000, MiSeq i100 and NovaSeq X. By improving color balancing for combinatorial indexed AgriPrep libraries (where there are only 1-4 i5 indexes used), PhiRx Indexed Control ensures cleaner, more accurate sequencing results and can be used as a 1:1 substitution for Illumina's PhiX Control V3.

Before starting procedure:

Review FAQs. FAQs can be found here: https://seqwell.com/resource-category/faqs/.

Thaw and mix reagent. Thaw reagent if necessary, pulse-spin down, and keep on ice. Pipette mix 10x before using.

Procedure

1. Dilution, aliquoting and denaturation

Please refer to Illumina's sequencer-specific recommendations for dilution and loading: https://knowledge.illumina.com/instrumentation/general.

PhiRx Indexed Control is a **non-denatured**, double-stranded library that is ready to be mixed with **non-denatured** target libraries. For applications requiring a denatured format, PhiRx Indexed Control can also be denatured following standard procedures and mixed exclusively with other **denatured** libraries.

Follow the procedure below for proper dilution, aliquoting, and storage:

a. **Non-Denatured Use**

i. **Determine Concentration**: Select the appropriate concentration and aliquot volume based on your sequencing run's loading requirements.

ii. **Low-Concentration Dilutions**: For dilutions below 1 nM, prepare aliquots sized for 1–2 weeks of use. Store long-term aliquots at -20°C, keeping only the aliquot in active use at 4°C.

b. **Denatured Use**

   i. **Dilution and Denaturation**: Dilute PhiRx Indexed Control to the required concentration, then denature according to Illumina's standard protocol, mixing by vortex and briefly spinning down.

   ii. **Storage**: Store the denatured PhiRx Indexed Control at -20°C. Prepare single-use aliquots, discarding any leftover volume after loading to prevent freeze-thaw degradation.

2. Sequencing on Illumina platforms

Check Illumina recommendations specific to your sequencing platform and analysis software version to determine the appropriate PhiRx Indexed Control spike-in percentage.

Recommended Spike-In Ratios by Application:

a. **For Sequencing Quality Monitoring**: Add 1–2% of PhiRx Indexed Control to the target library.

b. **For Color Balancing of Index Reads with XLEAP-SBS**: Begin with 5–10% PhiRx Indexed Control. Per Illumina's recommendation for their PhiX Sequencing Control V3, using a higher percentage of PhiRx (up to 40%) may improve performance, especially with XLEAP-SBS 600-cycle kits.

c. **For Low-Diversity Libraries (Color Balancing of Insert Reads)**: Start with 15–20% PhiRx Indexed Control and adjust upward if needed to enhance sequencing performance.

3. Sample sheet setup and bioinformatics information

PhiRx Indexed Control is designed with randomized mixed bases at each index position, meaning it cannot be demultiplexed from the run as a distinct sample. As with PhiX Sequencing control V3, PhiRx reads will appear in the undetermined category after demultiplexing, so no additional information is required during sample sheet setup.

For additional support with read filtering or bioinformatics applications, please contact technical support at support@seqwell.com.

Technical Assistance

Please review FAQs at https://seqwell.com/resource-category/faqs/.

For additional technical assistance with the AgriPrep Library Prep Kit, contact seqWell Technical Support.

E-mail: support@seqwell.com

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