

# AmpliSeq for Illumina Comprehensive Cancer Panel

Reference Guide



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# Revision History

| Document                        | Date             | Description of Change   |
|---------------------------------|------------------|---|
| Document #<br>1000000039408 v06 | February<br>2019 | <p>Added support for AmpliSeq UD Indexes for Illumina, AmpliSeq CD Indexes Set B for Illumina, AmpliSeq CD Indexes Set C for Illumina, and AmpliSeq CD Indexes Set D for Illumina.</p> <p>Corrected run format from 2 x 101 to 2 x 151.</p> <p>Clarified that MiSeq starting and final loading concentrations are for the v3 reagent kit.</p> <p>Fixed Amplify Library step in Appendix B to include adding master mix.</p>   |
| Document #<br>1000000039408 v05 | October<br>2018  | <p>Added optional instructions for using the AmpliSeq Library Equalizer for Illumina.</p>   |
| Document #<br>1000000039408 v04 | July<br>2018     | <p>Added table outlining required number of kits.</p> <p>Added optional steps to spike in AmpliSeq Sample ID Panel for Illumina.</p> <p>Referenced the <i>AmpliSeq Direct FFPE DNA Kit for Illumina Reference Guide</i> (document # 100000056164) for information on FFPE tissue sample preparation.</p> <p>Added step for transferring to the post-PCR area.</p> <p>Added information on using DNA prepared with the AmpliSeq Direct FFPE DNA Kit for Illumina.</p> <p>Clarified difference between shipping and storage temperature for AmpliSeq CD Indexes for Illumina and AmpliSeq Comprehensive Cancer Panel for Illumina.</p> <p>Removed optional step for determining library quantity.</p> <p>Removed expected library yield range.</p> <p>Added formula for calculating molarity when using fluorometric methods.</p> <p>Corrected the volume of digested amplicons to 44 µl.</p> |
| Document #<br>1000000039408 v03 | April<br>2018    | <p>Added option to use AmpliSeq CD Indexes Large Volume for Illumina in automated workflows.</p> <p>Changed gDNA to DNA.</p> <p>Added several entries to the Additional Resources table.</p> <p>Moved Dilute and Normalize Libraries procedure into the appropriate denature and dilute libraries guides.</p>   |
| Document #<br>1000000039408 v02 | January<br>2018  | <p>Changed DNA input volume to 100 ng per pool.</p> <p>Added tube quantities for 96- and 384-reaction kits.</p>   |
| Document #<br>1000000039408 v01 | January<br>2018  | <p>Clarified wording of genome copy equivalents.</p> <p>Corrected specification table heading.</p>  |
| Document #<br>1000000039408 v00 | January<br>2018  | Initial release.  |

# Table of Contents

|  |           |
|--|-----------|
| <b>Chapter 1 Overview .....</b>  | <b>1</b>  |
| Introduction .....   | 1         |
| Panel Specifications .....   | 2         |
| DNA Input Recommendations .....  | 2         |
| Additional Resources .....   | 2         |
| <br>   |           |
| <b>Chapter 2 Protocol .....</b>  | <b>4</b>  |
| Introduction .....   | 4         |
| Tips and Techniques .....  | 4         |
| Library Prep Workflow .....  | 6         |
| Quantify and Dilute DNA .....  | 7         |
| Amplify Targets .....  | 7         |
| Partially Digest Amplicons .....   | 9         |
| Ligate Indexes .....   | 10        |
| Clean Up Library .....   | 12        |
| Amplify Library .....  | 14        |
| Perform Second Cleanup .....   | 15        |
| Check Libraries .....  | 17        |
| Dilute Libraries to the Starting Concentration .....                       | 18        |
| <br>   |           |
| <b>Appendix A Supporting Information .....</b>                             | <b>20</b> |
| Kit Contents .....   | 20        |
| Consumables and Equipment .....  | 21        |
| <br>   |           |
| <b>Appendix B [Optional] AmpliSeq Library Equalizer for Illumina .....</b> | <b>24</b> |
| Equalize Libraries .....   | 24        |
| Preparation .....  | 24        |
| Amplify Library .....  | 25        |
| Wash Equalizer Beads .....   | 25        |
| Add Equalizer Capture .....  | 25        |
| Perform Second Cleanup .....   | 26        |
| Elute Library .....  | 26        |
| Denature and Dilute Libraries .....  | 26        |
| <br>   |           |
| <b>Technical Assistance .....</b>  | <b>27</b> |

# Chapter 1 Overview

|                                 |   |
|---------------------------------|---|
| Introduction .....              | 1 |
| Panel Specifications .....      | 2 |
| DNA Input Recommendations ..... | 2 |
| Additional Resources .....      | 2 |

## Introduction

This guide explains how to prepare up to 96 uniquely indexed libraries of genomic DNA using the AmpliSeq™ for Illumina® workflow.

The following kits are required to prepare the appropriate number of DNA samples:

| Kit  | Quantity                           |                                    |                                     |
|--|------------------------------------|------------------------------------|-------------------------------------|
|  | 24 samples                         | 96 samples                         | 384 samples                         |
|  | 24 DNA libraries                   | 96 DNA libraries                   | 384 DNA libraries                   |
| AmpliSeq Comprehensive Cancer Panel for Illumina           | 1                                  | 4                                  | 16                                  |
| AmpliSeq Library PLUS for Illumina                         | 2 × 24-reaction kits<br>(20019101) | 2 × 96-reaction kits<br>(20019102) | 2 × 384-reaction kits<br>(20019103) |
| AmpliSeq CD Indexes for Illumina*                          | 1                                  | 1                                  | 4                                   |
| AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples)* | 1                                  | 4                                  | 16                                  |

\* Either AmpliSeq CD Indexes or UD Indexes for Illumina can be used to complete the protocol.

AmpliSeq Comprehensive Cancer Panel is provided as four primer pools and requires more DNA and reagents than panels with one or two primer pools. For more information on kit requirements, see *Kit Contents* on page 20.



### NOTE

If preparing the maximum number of libraries per kit, more than one kit may be required to accommodate for higher dead volume requirements associated with automated platforms and any variation in overfill volumes by original reagent manufacturer.

Reagents provided in these kits are used to amplify target regions from DNA and add adapter sequences to the amplicons. The result is targeted libraries from DNA for sequencing on Illumina systems.

AmpliSeq for Illumina offers:

- ▶ Preparation of dual-index libraries for high-throughput sample multiplexing.
- ▶ Sample input from 4–400 ng DNA.
- ▶ Compatibility with FFPE samples.
- ▶ High sensitivity for somatic variant calling down to ~5%.
- ▶ Generation of sequence-ready libraries from DNA in less than eight hours.
- ▶ Sample signatures using the optional AmpliSeq Sample ID Panel for Illumina.
- ▶ Faster and more efficient library normalization using the optional AmpliSeq Library Equalizer™ for Illumina.

## Panel Specifications

| Panel Name                                       | Number of Pools | Concentration | Number of Amplicons | Average Amplicon Length (bp) | Average Library Length (bp) |
|--|-----------------|---------------|---------------------|------------------------------|-----------------------------|
| AmpliSeq Comprehensive Cancer Panel for Illumina | 4               | 2X            | 15,992              | 109                          | 249                         |

## DNA Input Recommendations

The AmpliSeq for Illumina Comprehensive Cancer Panel protocol supports 1–100 ng per pool (where 1 ng is equivalent to ~300 genome copies) of human DNA from high-quality sample or FFPE tissue. Recommended input is 10 ng high-quality DNA per pool. Before starting the protocol, quantify and dilute input DNA to the desired concentration.

- ▶ Increasing the amount of input DNA within this range typically results in higher library quality, especially when DNA quality is unknown.
  - ▶ Do not exceed the maximum supported amount of input DNA.
  - ▶ Use 1 ng DNA per pool only with high-quality, well-quantified samples.
- ▶ Library yield can be lower for degraded library samples such as FFPE DNA. Inhibitors such as high melanin content can reduce the efficiency of target amplification.

## Input DNA Quantification

- ▶ Quantify the starting DNA using a fluorescence-based quantification method, such as a Qubit dsDNA HS Assay Kit or PicoGreen. Do not use a UV spectrometer method.
  - ▶ Fluorescence-based methods employ a dye specific to double-stranded DNA (dsDNA) and specifically and accurately quantify dsDNA, even when many common contaminants are present.
  - ▶ In contrast, UV spectrometer methods based on 260 OD readings can overestimate DNA concentrations. The overestimation is due to the presence of RNA and other contaminants common to DNA preparations.

## Limited Samples

Degraded samples with average fragment sizes that are shorter than amplicon sizes can still yield AmpliSeq Comprehensive Cancer Panel libraries.

## Additional Resources

Visit the AmpliSeq for Illumina Comprehensive Cancer Panel support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

The following documentation is available for download from the Illumina website.

| Resource  | Description   |
|---|---|
| Custom Protocol Selector  | <a href="http://support.illumina.com/custom-protocol-selector.html">support.illumina.com/custom-protocol-selector.html</a><br>A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run. |
| <i>AmpliSeq for Illumina Comprehensive Cancer Panel Checklist (document # 1000000039410)</i>                        | Provides a checklist of the protocol steps. The checklist is intended for experienced users.  |
| <i>AmpliSeq for Illumina Comprehensive Cancer Panel Consumables &amp; Equipment List (document # 1000000039411)</i> | Provides an interactive checklist of user-provided consumables and equipment.   |
| <i>Index Adapters Pooling Guide (document # 1000000041074)</i>  | Provides pooling guidelines and dual indexing strategies for AmpliSeq for Illumina libraries.   |
| AmpliSeq Direct FFPE DNA Kit for Illumina Reference Guide (document # 1000000056164)                                | Provides instructions on preparing DNA from unstained, slide-mounted, formalin-fixed, paraffin-embedded (FFPE) tissue samples.  |
| NextSeq System Denature and Dilute Libraries Guide (document # 15048776)  | Provides instructions on how to denature and dilute prepared libraries for sequencing on the Illumina NextSeq™ Sequencing System.   |

# Chapter 2 Protocol

|  |    |
|--|----|
| Introduction .....                                   | 4  |
| Tips and Techniques .....                            | 4  |
| Library Prep Workflow .....                          | 6  |
| Quantify and Dilute DNA .....                        | 7  |
| Amplify Targets .....                                | 7  |
| Partially Digest Amplicons .....                     | 9  |
| Ligate Indexes .....                                 | 10 |
| Clean Up Library .....                               | 12 |
| Amplify Library .....                                | 14 |
| Perform Second Cleanup .....                         | 15 |
| Check Libraries .....                                | 17 |
| Dilute Libraries to the Starting Concentration ..... | 18 |

## Introduction

This chapter describes the AmpliSeq for Illumina protocol.

- ▶ Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information* on page 20.
- ▶ The thermal cyclers recommended for this protocol require different plates, seals, and magnetic stands. Make sure that you use the appropriate compatible supplies for your thermal cycler.
- ▶ Make sure that reagents are not expired. Using expired reagents might negatively affect performance.
- ▶ Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- ▶ Do not allow more than six freeze-thaw cycles of reagents.

## Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

### Avoiding Cross-Contamination

- ▶ When adding or transferring samples, change tips between *each sample* unless instructed otherwise.
- ▶ Set up PCR in an area or room that is free of amplicon contamination.

### Sealing the Plate

- ▶ Always seal the 96-well plate with MicroAmp™ Clear Adhesive Film before the following steps in the protocol:
  - ▶ Shaking steps
  - ▶ Vortexing steps
  - ▶ Centrifugation steps
  - ▶ Thermal cycling steps
- ▶ Apply the MicroAmp Clear Adhesive Film to cover the plate, and seal with the MicroAmp Adhesive Film Applicator.
- ▶ MicroAmp Clear Adhesive Film is effective for shaking, vortexing, centrifuging, thermal cycling, and storage.

- ▶ Remove MicroAmp Clear Adhesive Film carefully. If the seal on a cooled plate is difficult to remove, warm the plate in a nonheated thermal cycler with the heated lid set to 105°C for 10 seconds, and then remove the seal.

## Plate Transfers

- ▶ When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

## Covering the Plate

- ▶ When using MicroAmp EnduraPlates, always place a compression pad on the sealed plate before thermal cycling.

## Vortexing and Centrifugation

- ▶ When vortexing briefly, vortex three times for three seconds on the maximum setting.
- ▶ When centrifuging briefly, centrifuge at 280 × g for ten seconds.

## Handling Beads

- ▶ Pipette bead suspensions slowly.
- ▶ Before use, allow the beads to reach room temperature.
- ▶ Immediately before use, vortex the beads thoroughly until they are well resuspended. The color of the liquid must appear homogeneous.
- ▶ If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- ▶ When washing beads:
  - ▶ Use the appropriate magnetic stand for the plate.
  - ▶ Keep the plate on the magnetic stand until the instructions specify to remove it.
  - ▶ Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.

## Library Prep Workflow

The following diagram illustrates the AmpliSeq for Illumina Comprehensive Cancer Panel workflow. Safe stopping points are marked between steps.

**Figure 1** AmpliSeq Comprehensive Cancer Panel Workflow



## Quantify and Dilute DNA

This step quantifies and dilutes input DNA to the appropriate concentration for subsequent steps.

### Consumables

- ▶ Low TE
- ▶ DNA
- ▶ 1.5 ml tube

### Preparation

- 1 Prepare the following consumables:

| Reagent | Storage   | Instruction   |
|---------|---|---|
| DNA     | -25°C to -15°C (long-term)<br>2°C to 8°C (short-term) | Thaw at room temperature.<br>Invert or flick to mix, and then centrifuge briefly.                                     |
| Low TE  | -25°C to -15°C  | If frozen, thaw at room temperature for 45 minutes. Vortex to mix.<br>This reagent can be stored at room temperature. |

### Procedure

- 1 Quantify DNA using a fluorometric method, such as Qubit or PicoGreen.
- 2 If enough DNA is available, dilute to an intermediate concentration as follows.
  - a Dilute to a concentration of ~20–50 ng/µl using Low TE.
  - b Requantify the diluted DNA using the same fluorometric quantification method.
- 3 Dilute DNA to desired final concentration using Low TE.  
 Standard input is 10 ng high-quality DNA per pool. For more information, see *DNA Input Recommendations on page 2*.  
 Example: If your final DNA concentration is 8 ng/µl, add 5 µl diluted DNA to result in 40 ng total input.

## Amplify Targets

This step uses PCR to amplify target regions of the DNA sample. Optionally, add an available spike-in panel before target amplification.

For information on pooling and plate layout, see the *Index Adapters Pooling Guide*.

### Consumables

- ▶ 2X AmpliSeq Comprehensive Cancer Panel (four pools) (blue cap)
- ▶ 5X AmpliSeq HiFi Mix (red cap)
- ▶ [Optional] 20X AmpliSeq Sample ID Panel for Illumina
- ▶ DNA (4–400 ng)
- ▶ Nuclease-free water
- ▶ MicroAmp Clear Adhesive Film
- ▶ 1.5 ml tube

- ▶ 96-well PCR plate compatible with your thermal cycler

## About Reagents

- ▶ HiFi Mix is viscous. Pipette slowly and mix thoroughly.

## Preparation

- 1 Prepare the following consumables:

| Item  | Storage        | Instructions  |
|---|----------------|---|
| 5X AmpliSeq HiFi Mix (red cap)                              | -25°C to -15°C | Thaw on ice. Invert to mix, then centrifuge briefly.<br>Keep on ice during the procedure.     |
| 2X AmpliSeq Comprehensive Cancer Panel (blue cap)           | -25°C to -15°C | Thaw at room temperature.<br>Vortex briefly, then centrifuge briefly.                         |
| <b>[Optional]</b> 20X AmpliSeq Sample ID Panel for Illumina | -25°C to -15°C | Thaw at room temperature.<br>Vortex briefly, then centrifuge briefly.                         |
| DNA   | -25°C to -15°C | If frozen, thaw on ice. Invert or flick the thawed tubes to mix, and then centrifuge briefly. |

- 2 Transfer to the post-PCR area.
- 3 Save the following AMP program on a thermal cycler with a heated lid:
  - ▶ Choose the preheated lid option and set to 105°C
  - ▶ Set the reaction volume to 10 µl
  - ▶ 99°C for 2 minutes
  - ▶ 13 cycles of:
    - ▶ 99°C for 15 seconds
    - ▶ 60°C for 8 minutes
  - ▶ Hold at 10°C for up to 24 hours

The thermal cycler program is designed for 10 ng high-quality input per pool (40 ng total). If you are using a different input amount or low-quality DNA, adjust the program per [Table 1](#).

When multiple samples are amplified in one plate, make sure that the input for each sample is the same. Similar input optimizes cycle numbers for all samples.

**Table 1** Adjustments to Thermal Cycler Program

| Condition                     | Adjustment             |
|-------------------------------|------------------------|
| Input is 1 ng DNA per pool.   | Add three cycles.      |
| Input is 100 ng DNA per pool. | Subtract three cycles. |
| DNA is low quality (FFPE).    | Add three cycles.      |

## Procedure

- 1 **[Optional]** If using AmpliSeq Sample ID Panel for Illumina:
  - a For each sample, combine the following volumes in a 1.5 ml tube:
    - ▶ 20X AmpliSeq Sample ID Panel for Illumina (0.5 µl)
    - ▶ 2X AmpliSeq Comprehensive Cancer Panel Pool 1 (blue cap) (5 µl)
  - b Pipette to mix, and then centrifuge briefly.
- 2 Combine the following volumes per sample in a 1.5 ml tube.  
If using the AmpliSeq Direct FFPE DNA Kit for Illumina, remove up to the maximum volume indicated

below from the aqueous phase of the well and add to the 1.5 ml tube.

| Reagent                        | Volume ( $\mu$ l)              |
|--------------------------------|--------------------------------|
| 5X AmpliSeq HiFi Mix (red cap) | 9                              |
| DNA (4–400 ng)                 | $\leq 13.5$                    |
| Nuclease-free water            | To reach total required volume |
| <b>Total Volume</b>            | <b>22.5</b>                    |

Extra volume is prepared to account for small pipetting errors.

- 3 Pipette to mix, and then centrifuge briefly.
- 4 If not using AmpliSeq Sample ID Panel for Illumina, transfer each sample from the tube to a new PCR plate as follows.
  - a Transfer 5  $\mu$ l master mix to four wells of the plate so that each of the four wells contains 5  $\mu$ l of the same master mix.
  - b Add 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 1 (blue cap) to the first well, and 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 2 (blue cap) to the second.
  - c Add 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 3 (blue cap) to the third well, and 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 4 (blue cap) to the fourth.

Each of the three wells contains 5  $\mu$ l sample master mix and 5  $\mu$ l primer pool for a total of 10  $\mu$ l per well.

- 5 If using AmpliSeq Sample ID Panel for Illumina, transfer each sample from the tube to a new PCR plate as follows.
  - a Transfer 5  $\mu$ l master mix to four wells of the plate so that each of the four wells contains 5  $\mu$ l of the same master mix.
  - b Add 5.5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 1 (blue cap) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
  - c Add 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 2 (blue cap) to the second well, 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 3 (blue cap) to the third well, and 5  $\mu$ l 2X AmpliSeq Comprehensive Cancer Panel Pool 4 (blue cap) to the fourth well.

The result is a total volume of 10.5  $\mu$ l for the first well and 10  $\mu$ l for the remaining wells.

- 6 Pipette to mix, seal the plate, and then centrifuge briefly.
- 7 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP program.

## SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 24 hours. For longer durations, store at -25°C to -15°C.

## Partially Digest Amplicons

This step uses FuPa Reagent to digest primer dimers and partially digest amplicons.

### Consumables

- ▶ FuPa Reagent (brown cap)
- ▶ MicroAmp Clear Adhesive Film
- ▶ 8-tube strip

- ▶ Prepare for a later procedure:
  - ▶ Switch Solution (yellow cap)

## About Reagents

- ▶ FuPa Reagent is viscous. Pipette slowly.

## Preparation

- 1 Prepare the following consumables:

| Item                            | Storage        | Instructions   |
|---------------------------------|----------------|--|
| FuPa Reagent<br>(brown cap)     | -25°C to -15°C | Thaw on ice. Centrifuge briefly.<br>Keep on ice during the procedure.  |
| Switch Solution<br>(yellow cap) | -25°C to -15°C | If you are not stopping after this procedure is complete, thaw at room temperature in preparation for a later procedure. Vortex to mix, and then centrifuge briefly.<br>If precipitate is observed in the solution or cap, vortex or pipette to resuspend. |

- 2 Save the following FUPA program on a thermal cycler with a heated lid:

- ▶ Choose the preheated lid option and set to 105°C
- ▶ Set the reaction volume to 44 µl
- ▶ 50°C for 10 minutes
- ▶ 55°C for 10 minutes
- ▶ 62°C for 20 minutes
- ▶ Hold at 10°C for up to one hour

## Procedure

- 1 Briefly centrifuge to collect contents, and then unseal.
- 2 For each sample, use a multichannel pipette to combine the 10 µl target amplification reactions from sample wells containing pools 2–4 into the well containing pool 1, without changing tips.  
The total volume per sample is 40 µl.
- 3 Add 4 µl FuPa Reagent (brown cap) to each target amplification reaction. If you are using a multichannel pipette, pre aliquot FuPa Reagent into an 8-tube strip, and then transfer the appropriate volume.  
The total volume per sample is 44 µl.
- 4 Seal the plate.
- 5 Vortex briefly, and then centrifuge briefly.
- 6 Place on the thermal cycler, cover with a compression pad (if applicable), and run the FUPA program.

### SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 1 hour. For longer periods, store at -25°C to -15°C.

## Ligate Indexes

This step ligates Index 1 (i7) and Index 2 (i5) adapters to each sample. The indexes are premixed in a single-use plate to ensure unique combinations. Each library must have a unique index combination for dual-index sequencing.

For more information, see the *Index Adapter Pooling Guide*.

## Consumables

- ▶ Switch Solution (yellow cap)
- ▶ AmpliSeq CD Indexes or UD Indexes for Illumina
- ▶ DNA Ligase (blue cap)
- ▶ MicroAmp Clear Adhesive Film
- ▶ Prepare for a later procedure:
  - ▶ Agencourt AMPure XP beads

## About Reagents

- ▶ DNA Ligase is viscous. Pipette slowly.
- ▶ Switch Solution is viscous. Pipette slowly.
- ▶ The index plate wells cannot be reused.
- ▶ Beads take approximately 30 minutes to reach room temperature.

## Preparation

- 1 Prepare the following consumables:

| Item  | Storage         | Instructions  |
|---|-----------------|---|
| Switch Solution<br>(yellow cap)                         | -25°C to -15°C  | If you are resuming the protocol after a safe stopping point, thaw at room temperature. Vortex to mix, and then centrifuge briefly.<br>If precipitate is observed in the solution or cap, vortex or pipette to resuspend. |
| AmpliSeq CD<br>Indexes or<br>UD Indexes for<br>Illumina | -25°C to -15°C* | Thaw at room temperature. Vortex briefly to mix, and then centrifuge.   |
| DNA Ligase  | -25°C to -15°C  | Thaw on ice. Centrifuge briefly.<br>Keep on ice during the procedure.   |
| Agencourt<br>AMPure XP beads                            | 2°C to 8°C      | If you are not stopping after this procedure is complete, bring to room temperature in preparation for a later procedure. Vortex thoroughly to resuspend.   |

\*Ships at room temperature, but must be stored at -25°C to -15°C.

- 2 Save the following LIGATE program on the thermal cycler:

- ▶ Choose the preheated lid option and set to 105°C
- ▶ Set the reaction volume to 60 µl
- ▶ 22°C for 30 minutes
- ▶ 68°C for 5 minutes
- ▶ 72°C for 5 minutes
- ▶ Hold at 10°C for up to 24 hours

## Procedure

- 1 Briefly centrifuge the library plate to collect contents, and then unseal.
- 2 Remove the seal from the index plate.

- 3 Add the following volumes ***in the order listed*** to each well containing digested amplicons. Make sure to add DNA Ligase last. When adding AmpliSeq CD Indexes or UD Indexes for Illumina, use a multichannel pipette to transfer the appropriate volume from the wells of the index plate to the corresponding wells of the PCR plate.

For automated library preparation solutions that have higher reagent fill volume requirements, you may choose to use AmpliSeq CD Indexes Large Volume for Illumina (96 Indexes, 96 Samples, p/n 20019108).

| Order of Addition   | Reagent  | Volume ( $\mu$ l) |
|---|--|-------------------|
| 1   | Switch Solution (yellow cap)                   | 8                 |
| 2   | AmpliSeq CD Indexes or UD Indexes for Illumina | 4                 |
| 3   | DNA Ligase (blue cap)                          | 4                 |
| <b>Total Volume</b> (including 44 $\mu$ l digested amplicons) |  | <b>60</b>         |



### CAUTION

To avoid library prep failure, do not combine these components outside the wells containing digested amplicons.

- 4 Seal the library plate.
- 5 Vortex briefly, and then centrifuge briefly.
- 6 Place on the thermal cycler, cover with a compression pad (if applicable), and run the LIGATE program.
- 7 If the index plate contains unused indexes, seal the plate and return to storage.

### SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 24 hours. For longer durations, store at -25°C to -15°C.

## Clean Up Library

This step uses Agencourt AMPure XP beads to clean up the library. The beads are carried over for the next procedure.

### Consumables

- ▶ Agencourt AMPure XP beads
- ▶ Freshly prepared 70% ethanol (EtOH)
- ▶ Prepare for a later procedure:
  - ▶ 1X Lib Amp Mix (black cap)
  - ▶ 10X Library Amp Primers (pink cap)

### About Reagents

- ▶ Pipette beads slowly and mix thoroughly.
- ▶ Beads take approximately 30 minutes to reach room temperature.

## Preparation

- 1 Prepare the following consumables:

| Item                               | Storage        | Instructions  |
|------------------------------------|----------------|---|
| 1X Lib Amp Mix (black cap)         | -25°C to -15°C | If you are not stopping after this procedure is complete, thaw on ice in preparation for a later procedure. Invert or flick to mix, and then centrifuge briefly.      |
| 10X Library Amp Primers (pink cap) | -25°C to -15°C | If you are not stopping after this procedure is complete, thaw at room temperature in preparation for a later procedure. Vortex briefly, and then centrifuge briefly. |
| Agencourt AMPure XP beads          | 2°C to 8°C     | Bring to room temperature. Vortex thoroughly to resuspend.  |

- 2 Prepare 10 ml fresh 70% EtOH from absolute ethanol.

This volume is sufficient to clean up 24 reactions.

## Procedure

- 1 Briefly centrifuge the plate to collect contents, and then unseal.
- 2 Add 60 µl AMPure XP beads to each library, and then seal the plate.
- 3 Vortex briefly.
- 4 Inspect each well to make sure that the mixture is homogeneous.
- 5 Centrifuge briefly.
- 6 Incubate at room temperature for 5 minutes.
- 7 Place on a magnetic stand and wait until the mixture is clear (~2 minutes). Keep on the magnetic stand until step 11.
- 8 Unseal the plate.
- 9 Remove and discard entire supernatant from each well.
- 10 Wash two times as follows.
  - a Add 150 µl freshly prepared 70% EtOH to each well.
  - b Incubate at room temperature until the solution is clear (~30 seconds).
  - c Without disturbing the pellet, remove and discard supernatant.
- 11 Immediately seal the plate and centrifuge briefly.
- 12 Place on the magnetic stand, and then unseal. Make sure that the plate is returned to the same orientation on the magnet.



### NOTE

Using the original orientation on the magnet keeps the beads on the same side of the well.

13 Immediately remove all residual EtOH as follows.

- a Use a 20 µl pipette to remove residual EtOH from each well.
- b Air-dry on the magnetic stand for 10 minutes. Leave uncovered.
- c Inspect each well to make sure that the EtOH has completely evaporated.
- d If EtOH remains in the wells, continue to air-dry until EtOH is no longer visible. Overdried or cracked beads do not affect performance.



#### CAUTION

Residual EtOH causes library prep to fail by inhibiting amplification.

14 If you are using the AmpliSeq Library Equalizer for Illumina, proceed to [Equalize Libraries on page 24](#). Otherwise, continue to [Amplify Library on page 14](#).



#### NOTE

Make sure to follow the appropriate instructions for your normalization method, either the standard workflow or using the AmpliSeq Library Equalizer for Illumina.

## Amplify Library

This second amplification step amplifies libraries to ensure sufficient quantity for sequencing on Illumina systems. The amplification reaction contains the beads, which are carried over from the previous step.

### Consumables

- ▶ 1X Lib Amp Mix (black cap)
- ▶ 10X Library Amp Primers (pink cap)
- ▶ MicroAmp Clear Adhesive Film
- ▶ Prepare for a later procedure:
  - ▶ Agencourt AMPure XP beads

### About Reagents

- ▶ Beads take approximately 30 minutes to reach room temperature.

## Preparation

- 1 Prepare the following consumables:

| Item                               | Storage        | Instructions  |
|------------------------------------|----------------|---|
| 1X Lib Amp Mix (black cap)         | -25°C to -15°C | Thaw on ice. Invert or flick to mix, and then centrifuge briefly.   |
| 10X Library Amp Primers (pink cap) | -25°C to -15°C | Thaw at room temperature. Vortex briefly, and then centrifuge briefly.  |
| Agencourt AMPure XP beads          | 2°C to 8°C     | If you are not stopping after this procedure is complete, bring to room temperature in preparation for a later procedure. Vortex thoroughly to resuspend. |

- 2 Save the following AMP\_7 program on a thermal cycler with a heated lid:
- ▶ Choose the preheated lid option and set to 105°C
  - ▶ Set the reaction volume to 50 µl
  - ▶ 98°C for 2 minutes
  - ▶ 7 cycles of:
    - ▶ 98°C for 15 seconds
    - ▶ 64°C for 1 minute
  - ▶ Hold at 10°C for up to 24 hours

## Procedure

- 1 For each reaction, combine the following volumes to prepare amplification master mix.

| Reagent                            | Volume (µl) |
|------------------------------------|-------------|
| 1X Lib Amp Mix (black cap)         | 45          |
| 10X Library Amp Primers (pink cap) | 5           |
| Total Volume per reaction          | 50          |

- 2 Vortex briefly, and then centrifuge briefly.
- 3 Remove the plate from the magnetic stand.
- 4 Add 50 µl amplification master mix to each library well, and then seal the plate.
- 5 Vortex briefly, and then centrifuge briefly.
- 6 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP\_7 program.

### SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 24 hours. For longer durations, store at -25°C to -15°C.

## Perform Second Cleanup

This second cleanup step uses Agencourt AMPure XP beads to perform two rounds of purification.

- ▶ **First round**—High molecular-weight DNA is captured by the beads and discarded. The library and primers are retained in the supernatant and transferred to a fresh plate for the second round of purification.

- ▶ **Second round**—Libraries in the saved supernatant are captured by the beads while primers remain in the supernatant. The bead pellet is saved, and libraries are eluted from the beads.

## Consumables

- ▶ Agencourt AMPure XP beads
- ▶ Freshly prepared 70% EtOH
- ▶ Low TE
- ▶ 96-well LoBind PCR plates
- ▶ MicroAmp Clear Adhesive Film

## About Reagents

- ▶ Pipette beads slowly and mix thoroughly.
- ▶ Beads take approximately 30 minutes to reach room temperature.

## Preparation

- 1 Prepare the following consumables:

| Item                      | Storage        | Instructions   |
|---------------------------|----------------|--|
| Agencourt AMPure XP beads | 2°C to 8°C     | If you are resuming the protocol after a safe stopping point, bring to room temperature. Vortex thoroughly to resuspend. |
| Low TE                    | -25°C to -15°C | If frozen, thaw at room temperature for 45 minutes. Vortex to mix.<br>This reagent can be stored at room temperature.    |

- 2 Prepare 10 ml fresh 70% EtOH from absolute ethanol.

This volume is sufficient to clean up 24 reactions.

## Procedure

- 1 Briefly centrifuge the plate to collect contents, and then unseal.
- 2 Add 25 µl AMPure XP beads to each well containing ~50 µl library, and then seal the plate.  
This step adds beads to the beads already in the reaction.
- 3 Vortex briefly, and then centrifuge briefly. The beads already in the reaction do not need to be fully resuspended.
- 4 Incubate at room temperature for 5 minutes.
- 5 Place the plate on a magnetic stand and wait until the liquid is clear (~5 minutes).
- 6 Unseal the plate.
- 7 Transfer the **entire** supernatant (~75 µl), **which contains the desired amplicon library**, to a new plate.  
Small amounts of bead carryover do not affect performance.
- 8 Add 60 µl AMPure XP beads to each well containing the transferred supernatant, and then seal the plate.
- 9 Vortex briefly, and then centrifuge briefly.
- 10 Incubate at room temperature for 5 minutes.
- 11 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 12 Unseal the plate.

- 13 Without disturbing the beads, remove and discard all supernatant from each well.  
The amplicon library is captured by the beads, which remain in the wells.
- 14 Wash two times as follows.
  - a Add 150 µl freshly prepared 70% EtOH to each well.
  - b Incubate at room temperature until the solution is clear (~30 seconds).
  - c Without disturbing the pellet, remove and discard supernatant.
- 15 Use a 20 µl pipette to remove and discard residual EtOH from each well.
- 16 Air-dry on the magnetic stand for 5 minutes.
- 17 Remove from the magnetic stand.
- 18 Add 30 µl Low TE to each well, and then seal the plate.
- 19 Vortex briefly to disperse the beads, and then centrifuge briefly.
- 20 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 21 Unseal the plate.
- 22 Transfer 27 µl supernatant to a new LoBind PCR plate.  
The supernatant contains the amplicon library.

#### SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C.

### Check Libraries

Perform the following procedures for quality control analysis and to ensure optimum cluster densities on the flow cell.

The Fragment Analyzer and Bioanalyzer methods can be used to quantify and qualify libraries.

### Assess Library Quality

- 1 Place the plate on the magnetic stand. Keep the plate on the stand while performing normalization and pooling.

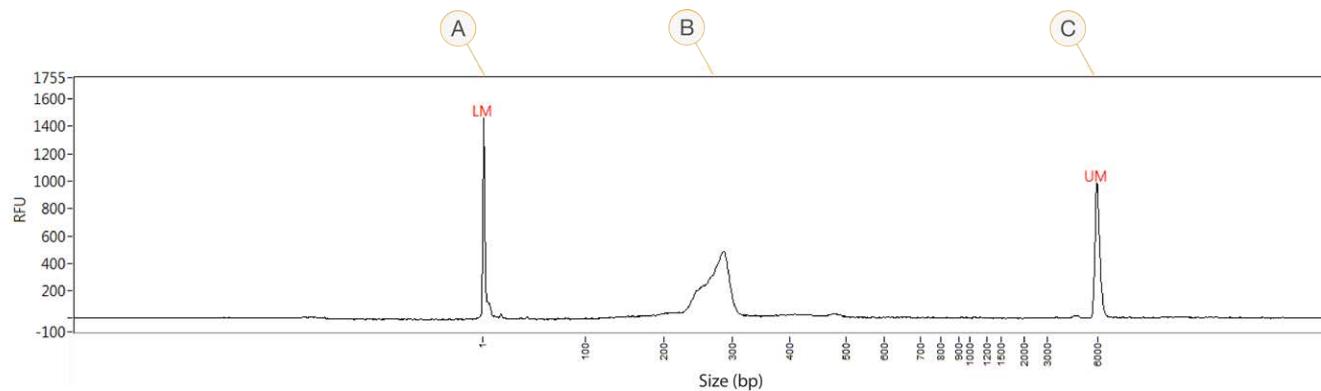


#### CAUTION

Bead carryover can affect cluster density.

- 2 Assess library quality using one of the following methods:
  - ▶ Analyze 2 µl undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and PROSize Data Analysis Software.
  - ▶ Analyze 1 µl library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.

Figure 2 provides an example Fragment Analyzer trace of a successfully sequenced library. Typical libraries show a size distribution from 186–323 bp. The suggested size distribution for quantification is 200–320 bp.

**Figure 2** Example Fragment Analyzer Trace

- A Lower marker
- B Expected libraries
- C Upper marker

## Quantify Library

- 1 Quantify the library using one of the following methods:
  - ▶ Analyze 2 µl undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and PROSize Data Analysis Software.
  - ▶ Analyze 1 µl library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
  - ▶ Analyze 2 µl library using the Qubit 2.0 or 3.0 Fluorometer with the Qubit DNA HS Assay Kit.
  - ▶ Analyze 1:10,000 diluted library using the KAPA Library Quantification Kit (Universal). For qPCR instructions, see the *Sequencing Library qPCR Quantification Guide* (document # 11322363).
  - ▶ Analyze 2 µl library using the AccuClear Ultra High Sensitivity dsDNA Quantitation Kit.
  - ▶ Analyze 2 µl library using the Quant-iT PicoGreen dsDNA Assay Kit.
- 2 For fluorometric methods, calculate the molarity of the library using the following formula:

$$\frac{\text{ng} / \mu\text{l} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times \text{average library size (bp)}} = \text{Molarity (nM)}$$

## Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read (2 × 151 run format).

- 1 Calculate the molarity value of the library or pooled libraries using the following formula.
  - ▶ For libraries qualified on a Bioanalyzer or Fragment Analyzer, use the average size obtained for the library.
  - ▶ For all other qualification methods, use 350 bp as the average library size.
- 2 Using the molarity value, calculate the volumes of Low TE and library needed to dilute libraries to the starting concentration for your system.

| Sequencing System           | Starting Concentration<br>(nM) | Final Loading Concentration<br>(pM) |
|-----------------------------|--------------------------------|-------------------------------------|
| NextSeq 550 and NextSeq 500 | 2                              | 1.1–1.9                             |

3 Dilute libraries using Low TE:

- ▶ **Libraries quantified as a pool**—Dilute the pool to the starting concentration for your system.
- ▶ **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a pool.

4 Follow the denature and dilute instructions for your system to dilute to the final loading concentration.

- ▶ For the iSeq 100 System, see the system guide for dilution instructions (libraries are automatically denatured).
- ▶ For all other systems, see the denature and dilute libraries guide.

The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

# Supporting Information

|                                 |    |
|---------------------------------|----|
| Kit Contents .....              | 20 |
| Consumables and Equipment ..... | 21 |

## Kit Contents

The AmpliSeq for Illumina protocol requires the AmpliSeq Library PLUS kit for Illumina, AmpliSeq Comprehensive Cancer Panel for Illumina, and AmpliSeq CD Indexes or UD Indexes for Illumina.

The following products are available to order through Illumina to support the AmpliSeq for Illumina workflow.

| Component        | Kit  | Catalog # |
|------------------|--|-----------|
| Library PLUS Kit | AmpliSeq Library PLUS for Illumina (24 reactions)                      | 20019101  |
|                  | AmpliSeq Library PLUS for Illumina (96 reactions)                      | 20019102  |
|                  | AmpliSeq Library PLUS for Illumina (384 reactions)                     | 20019103  |
| Panel            | AmpliSeq Comprehensive Cancer Panel for Illumina                       | 20019160  |
| Indexes          | AmpliSeq CD Indexes Set A for Illumina (96 Indexes, 96 Samples)        | 20019105  |
|                  | AmpliSeq CD Indexes Set B for Illumina (96 Indexes, 96 Samples)        | 20019106  |
|                  | AmpliSeq CD Indexes Set C for Illumina (96 Indexes, 96 Samples)        | 20019107  |
|                  | AmpliSeq CD Indexes Set D for Illumina (96 Indexes, 96 Samples)        | 20019167  |
|                  | AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples)              | 20019104  |
|                  | AmpliSeq CD Indexes Large Volume for Illumina (96 Indexes, 96 Samples) | 20019108  |

AmpliSeq Comprehensive Cancer Panel is provided as four primer pools and requires more DNA and reagents than panels with one or two primer pools.

- ▶ Use the 24-reaction configuration to prepare 12 libraries.
- ▶ Use the 96-reaction configuration to prepare 48 libraries.
- ▶ Use the 384-reaction configuration to prepare 192 libraries.

## AmpliSeq Library PLUS for Illumina Contents, Store at -25°C to -15°C

| 24-reaction | 96-reaction | 384-reaction | Quantity                | Reagent |
|-------------|-------------|--------------|-------------------------|---------|
|             |             |              |                         |         |
| 1           | 4           | 16           | 1X Lib Amp Mix          |         |
| 1           | 1           | 4            | 10X Library Amp Primers |         |
| 1           | 1           | 4            | DNA Ligase              |         |
| 1           | 1           | 4            | 5X AmpliSeq HiFi Mix    |         |
| 1           | 1           | 4            | FuPa Reagent            |         |
| 1           | 2           | 8            | Low TE*                 |         |
| 1           | 1           | 4            | Switch Solution         |         |

\* Low TE can be stored at room temperature.

## AmpliSeq Comprehensive Cancer Panel for Illumina Contents, Store at -25°C to -15°C

These reagents are shipped at room temperature. Promptly store reagents at the indicated temperature to ensure proper performance.

| Quantity | Reagent                                       |
|----------|---|
| 1        | 2X AmpliSeq Comprehensive Cancer Panel Pool 1 |
| 1        | 2X AmpliSeq Comprehensive Cancer Panel Pool 2 |
| 1        | 2X AmpliSeq Comprehensive Cancer Panel Pool 3 |
| 1        | 2X AmpliSeq Comprehensive Cancer Panel Pool 4 |

## AmpliSeq CD Indexes or UD Indexes for Illumina Contents, Store at -25°C to -15°C

These reagents are shipped at room temperature. Promptly store reagents at the indicated temperature to ensure proper performance.

| Quantity | Description   |
|----------|---|
| 1        | AmpliSeq CD Indexes Set A, B, C, or D plate (96 indexes, 96 samples) or AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples), or AmpliSeq CD Indexes Large Volume (96 indexes, 96 samples) |

## AmpliSeq Library Equalizer for Illumina, Store at 2°C to 8°C

The AmpliSeq Library Equalizer Kit provides an optional method for normalizing library concentration without quantification. Use this kit when library yields are consistently above the minimum expected concentration.

| Quantity | Reagent                  |
|----------|--------------------------|
| 1        | Equalizer Beads          |
| 1        | Equalizer Capture        |
| 1        | Equalizer Elution Buffer |
| 1        | Equalizer Wash Buffer    |

## Consumables and Equipment

In addition to the AmpliSeq Library PLUS kit for Illumina, AmpliSeq Comprehensive Cancer Panel for Illumina, and AmpliSeq CD Indexes or UD Indexes for Illumina, make sure that you have the required consumables and equipment before starting the protocol.

### Consumables

| Item  | Supplier  |
|---|---|
| Absolute ethanol, molecular biology grade                 | General lab supplier                                |
| Agencourt AMPure XP                                       | Fisher Scientific, catalog # NC9959336 or NC9933872 |
| <b>[Optional]</b> AmpliSeq Sample ID Panel for Illumina   | Illumina, catalog # 20019162                        |
| <b>[Optional]</b> AmpliSeq Library Equalizer for Illumina | Illumina, catalog # 20019171                        |

| Item  | Supplier   |
|---|--|
| Eppendorf DNA LoBind Microcentrifuge Tubes, 1.5 ml  | Fisher Scientific, catalog # 13-698-791                      |
| MicroAmp Clear Adhesive Film  | Thermo Fisher Scientific, catalog # 4306311                  |
| One of the following 96-well PCR plates:<br>For use with Thermo Fisher thermal cyclers:     | One of the following suppliers, depending on plate type:     |
| • MicroAmp EnduraPlate Optical 96-Well Clear Reaction Plates with Barcode                   | • Thermo Fisher Scientific, catalog # 4483352 or 4483354     |
| For use with Bio-Rad thermal cyclers:   |  |
| • Hard-Shell 96-Well Skirted PCR Plates, low-profile, skirted                               | • Bio-Rad, catalog # HSP-9601                                |
| Eppendorf twin.tec 96 Well LoBind PCR Plates, Semi-skirted                                  | Fisher Scientific, catalog # E0030129504                     |
| MicroAmp Optical Film Compression Pad (required for use with Thermo Fisher thermal cyclers) | Thermo Fisher Scientific, catalog # 4312639                  |
| Nuclease-free water   | Thermo Fisher Scientific, catalog # AM9932                   |
| Pipettes, 2–200 µl, and low-retention filtered pipette tips                                 | Fisher Scientific  |
| 8-tube strips   | General lab supplier   |
| One of the following kits, depending on quantification method:                              | One of the following suppliers, depending on kit:            |
| • [Bioanalyzer] Agilent DNA 1000 Kit  | • Agilent, catalog # 5067-1504                               |
| • [Fluorometer] Qubit dsDNA HS Assay Kit  | • Thermo Fisher Scientific, catalog # Q32851 or Q32854       |
| • [Fragment Analyzer] Standard Sensitivity NGS Fragment Analyzer Kit (1 bp – 6,000 bp)      | • Advanced Analytical Technologies, Inc., part # DNF-473     |
| • [qPCR] KAPA Library Quantification Kit (Universal)  | • Kapa Biosystems, catalog # KK4824                          |
| • AccuClear Ultra High Sensitivity dsDNA Quantitation Kit                                   | • Biotium, catalog # 31028                                   |
| • Quant-iT PicoGreen dsDNA Assay Kit  | • Thermo Fisher catalog # P11496                             |
| [Optional] One of the following positive sample controls:                                   | One of the following suppliers, depending on sample control: |
| • Quantitative Multiplex Reference Standard   | • Horizon, catalog # HD701                                   |
| • Tru-Q 2 (5% Tier)   | • Horizon, catalog # HD729                                   |
| • Acrometrix Oncology Hotspot Control   | • Thermo Fisher Scientific, catalog # 969056                 |
| NaOH, molecular biology-grade   | General lab supplier   |
| Tris-HCl, pH 7.0  | General lab supplier   |

## Equipment

| Item  | Supplier   |
|---|--|
| One of the following magnetic stands:<br>For use with MicroAmp EnduraPlates:<br>• DynaMag-96 Side Magnet  | One of the following suppliers, depending on magnetic stand type:<br>• Thermo Fisher Scientific, catalog # 12331D  |
| For use with Hard-Shell 96-Well Skirted PCR Plates:<br>• DynaMag-96 Side Skirted Magnet   | • Thermo Fisher Scientific, catalog # 12027  |
| For use with 1.5 ml tubes:<br>• MagneSphere® Technology Magnetic Separation Stands (12 position, 1.5 ml)  | • Promega, catalog #Z5342  |
| Fisher Scientific Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge   | Fisher Scientific, catalog # 14-100-143  |
| MicroAmp Adhesive Film Applicator   | Thermo Fisher Scientific, catalog # 4333183  |
| Vortexer with 96-well plate attachment  | General lab supplier   |
| One of the following thermal cyclers.<br>Thermo Fisher thermal cyclers:<br>• SimpliAmp Thermal Cycler<br>• Applied Biosystems 2720 Thermal Cycler<br>• Veriti 96-Well Thermal Cycler<br>• ProFlex 96-well PCR System<br>• GeneAmp PCR System 9700 <sup>2</sup> or Dual 96-well Thermal Cycler                     | Thermo Fisher Scientific, see web product pages for catalog numbers  |
| Bio-Rad thermal cyclers:<br>• C1000 Touch Thermal Cycler<br>• S1000 Thermal Cycler  | Bio-Rad:<br>• Part # 1851196<br>• Part # 1852196   |
| One of the following instruments, depending on quantification method:<br>• [Bioanalyzer] Agilent 2100 Bioanalyzer<br>• [Fluorometer] Qubit 3.0 Fluorometer or Qubit 2.0 Fluorometer <sup>2</sup><br>• [Fragment Analyzer] Fragment Analyzer Automated CE System<br>• [qPCR] Real-time PCR instrument <sup>1</sup> | One of the following suppliers, depending on instrument:<br>• Agilent, catalog # G2939AA<br>• Thermo Fisher Scientific, catalog # Q33216<br><br>• Advanced Analytical Technologies, Inc., part # FSv2-CE2 or FSv2-CE10<br>• General lab supplier |

<sup>1</sup> For example: Applied Biosystems 7900HT, 7500, StepOne, StepOnePlus, ViiA 7 Systems, or QuantStudio 12K Flex Real-Time PCR System.

<sup>2</sup> No longer available for purchase.

# [Optional] AmpliSeq Library Equalizer for Illumina

|                                     |     |
|-------------------------------------|-----|
| Equalize Libraries .....            | .24 |
| Preparation .....                   | .24 |
| Amplify Library .....               | .25 |
| Wash Equalizer Beads .....          | .25 |
| Add Equalizer Capture .....         | .25 |
| Perform Second Cleanup .....        | .26 |
| Elute Library .....                 | .26 |
| Denature and Dilute Libraries ..... | .26 |

## Equalize Libraries

Use the AmpliSeq Library Equalizer for Illumina to normalize library concentration without quantification.

### Consumables

- ▶ AmpliSeq Library Equalizer for Illumina
- ▶ 1X Lib Amp Mix (black cap)
- ▶ 10X Library Amp Primers (pink cap)
- ▶ MicroAmp Clear Adhesive Film
- ▶ 1.5 ml tube

## Preparation

- 1 Prepare the following consumables:

| Item                               | Storage                        | Instructions   |
|------------------------------------|--------------------------------|--|
| Equalizer Beads                    | 2°C to 8°C                     | Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly.             |
| Equalizer Capture                  | 2°C to 8°C                     | Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly.             |
| Equalizer Elution Buffer           | 2°C to 8°C                     | Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly.             |
| Equalizer Wash Buffer              | 2°C to 8°C or room temperature | If chilled, let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly. |
| 1X Lib Amp Mix (black cap)         | -25°C to -15°C                 | Thaw on ice. Invert or flick to mix, and then centrifuge briefly.  |
| 10X Library Amp Primers (pink cap) | -25°C to -15°C                 | Thaw at room temperature. Vortex briefly, and then centrifuge briefly.   |

- 2 Save the following EQUAL program on a thermal cycler with a heated lid:
  - ▶ Choose the preheated lid option and set to 105°C
  - ▶ Set the reaction volume to 50 µl
  - ▶ 98°C for 2 minutes
  - ▶ 9 cycles of:
    - ▶ 98°C for 15 seconds
    - ▶ 64°C for 1 minute
  - ▶ Hold at 10°C for up to 1 hour

## Amplify Library

- 1 Remove the plate with purified libraries from the magnetic stand.
- 2 For each reaction, combine the following volumes to prepare amplification master mix.

| Reagent                            | Volume (µl) |
|------------------------------------|-------------|
| 1X Lib Amp Mix (black cap)         | 45          |
| 10X Library Amp Primers (pink cap) | 5           |
| Total Volume per reaction          | 50          |

- 3 Vortex briefly, and then centrifuge briefly.
- 4 Add 50 µl amplification master mix to each library well, and then seal the plate.
- 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the EQUAL program.

## Wash Equalizer Beads

- 1 For each reaction, combine the following volumes in a 1.5 ml tube:
  - ▶ Equalizer Beads (7 µl)
  - ▶ Equalizer Wash Buffer (14 µl)

Extra volume is included here to account for small pipetting errors.
- 2 Pipette to mix.
- 3 Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
- 4 Without disturbing the pellet, remove and discard all supernatant from the test tube.
- 5 Remove from the magnetic stand.
- 6 For each reaction, add 7 µl Equalizer Wash Buffer. Pipette to resuspend.  
These steps result in washed Equalizer Beads ready for use later in the protocol.



### NOTE

Equalizer Beads can be prepared in bulk and stored at 4°C for at least six months.

## Add Equalizer Capture

- 1 Briefly centrifuge the library plate to collect contents, and then unseal.
- 2 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
- 3 Transfer 45 µl of supernatant from each well of the library plate to the corresponding well of a new plate.
- 4 Add 10 µl Equalizer Capture to each well.

- 5 Seal the plate, vortex to mix, and then briefly centrifuge to collect contents.
- 6 Incubate at room temperature for 5 minutes.

## Perform Second Cleanup

- 1 Unseal the plate.
- 2 Vortex or pipette washed Equalizer Beads to mix.
- 3 Add 6 µl Equalizer Beads to each well.
- 4 Seal the plate, vortex thoroughly, and then centrifuge briefly to collect contents.
- 5 Incubate at room temperature for 5 minutes.
- 6 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 7 Unseal the plate.
- 8 Without disturbing the pellet, remove and discard all supernatant from each well.
- 9 Wash two times as follows.
  - a Add 150 µl Equalizer Wash Buffer to each well.
  - b Incubate at room temperature until the solution is clear (~30 seconds).
  - c Without disturbing the pellet, remove and discard supernatant.

## Elute Library

- 1 Remove the plate from the magnetic stand.
- 2 Add 30 µl Equalizer Elution Buffer to each well.
- 3 Seal the plate, vortex thoroughly, and then centrifuge briefly to collect contents.
- 4 Elute the library by incubating on a thermal cycler at 45°C for 5 minutes.
- 5 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 6 Unseal the plate.
- 7 Transfer 27 µl supernatant to a new LoBind PCR plate.  
The supernatant contains the amplicon library.

### SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C.

## Denature and Dilute Libraries

- 1 Denature and dilute libraries for loading on the sequencing instrument you are using.  
For detailed instructions, refer to the system guide or denature and dilute libraries guide for your sequencing instrument. See *Additional Resources* on page 2.

# Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: [www.illumina.com](http://www.illumina.com)  
Email: [techsupport@illumina.com](mailto:techsupport@illumina.com)

## Illumina Customer Support Telephone Numbers

| Region          | Toll Free       | Regional       |
|-----------------|-----------------|----------------|
| North America   | +1.800.809.4566 |                |
| Australia       | +1.800.775.688  |                |
| Austria         | +43 800006249   | +43 19286540   |
| Belgium         | +32 80077160    | +32 34002973   |
| China           | 400.066.5835    |                |
| Denmark         | +45 80820183    | +45 89871156   |
| Finland         | +358 800918363  | +358 974790110 |
| France          | +33 805102193   | +33 170770446  |
| Germany         | +49 8001014940  | +49 8938035677 |
| Hong Kong       | 800960230       |                |
| Ireland         | +353 1800936608 | +353 016950506 |
| Italy           | +39 800985513   | +39 236003759  |
| Japan           | 0800.111.5011   |                |
| Netherlands     | +31 8000222493  | +31 207132960  |
| New Zealand     | 0800.451.650    |                |
| Norway          | +47 800 16836   | +47 21939693   |
| Singapore       | +1.800.579.2745 |                |
| Spain           | +34 911899417   | +34 800300143  |
| Sweden          | +46 850619671   | +46 200883979  |
| Switzerland     | +41 565800000   | +41 800200442  |
| Taiwan          | 00806651752     |                |
| United Kingdom  | +44 8000126019  | +44 2073057197 |
| Other countries | +44.1799.534000 |                |

Safety data sheets (SDSs)—Available on the Illumina website at [support.illumina.com/sds.html](http://support.illumina.com/sds.html).

Product documentation—Available for download in PDF from the Illumina website. Go to [support.illumina.com](http://support.illumina.com), select a product, then select Documentation & Literature.



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