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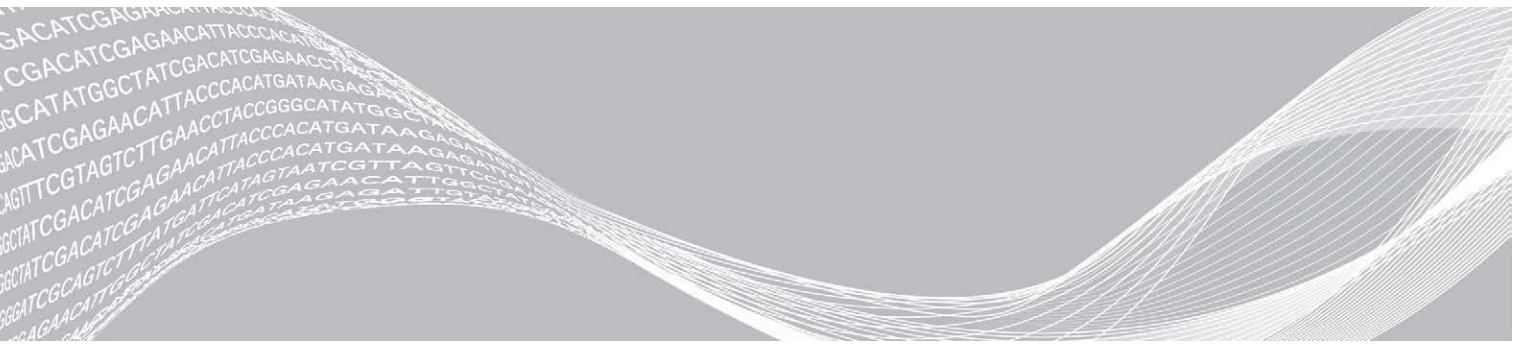
AmpliSeq for Illumina Myeloid Panel

Reference Guide



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Revision History

Document	Date	Description of Change
Document # 1000000056148 v02	February 2019	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. Corrected run format from 2 x 101 to 2 x 151. Clarified that MiSeq starting and final loading concentrations are for the v3 reagent kit. Fixed Amplify Library step in Appendix B to include adding master mix.
Document # 1000000056148 v01	October 2018	Added optional instructions for using the AmpliSeq Library Equalizer for Illumina.
Document # 1000000056148 v00	July 2018	Initial release.

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Chapter 1 Overview

Introduction

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

The following table shows required kit quantities and assumes that DNA and RNA libraries come from the same sample:

Kit	Quantity		
	24 samples[1]	96 samples[1]	384 samples[1]
	48 libraries (24 DNA + 24 RNA)[2]	192 libraries (96 DNA + 96 RNA)[2]	768 libraries (384 DNA + 384 RNA)[2]
AmpliSeq Myeloid Panel for Illumina	1	4	16
AmpliSeq Library PLUS for Illumina	2 × 24-reaction kits (20019101)	2 × 96-reaction kits (20019102)	2 × 384-reaction kits (20019103)
AmpliSeq CD Indexes for Illumina[3]	1	2	8
AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples)[3]	2	8	32
AmpliSeq cDNA Synthesis for Illumina	1	1	4

[1] A sample is a specimen of nucleic acid from an individual source. Samples can be DNA, RNA, or Paired.

[2] A library is nucleic acids from a sample after preparation and contains the required ends for sequencing. Because DNA and RNA are prepared separately, each preparation is considered a unique library. Therefore, a single sample can give rise to 2 libraries (1 for DNA and 1 for RNA).

[3] Either AmpliSeq CD Indexes or UD Indexes for Illumina can be used to complete the protocol.

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 or cDNA and add adapter sequences to the amplicons. The result is targeted libraries from DNA or RNA for sequencing on Illumina systems.

AmpliSeq for Illumina offers:

- Preparation of dual-index libraries for high-throughput sample multiplexing.
- Sample input from 20–200 ng DNA and 10–100 ng RNA.
- Generation of sequence-ready libraries from DNA or RNA in less than eight hours.
- Sample signatures using the built-in AmpliSeq Sample ID Panel for Illumina.
- Faster and more efficient library normalization using the optional AmpliSeq Library Equalizer™ for Illumina.

Panel Specifications

The AmpliSeq Myeloid Panel includes a DNA component and an RNA component with different specifications. Complete the DNA procedures using the DNA pools and the RNA procedures using the RNA pool.

Panel Name	Component	Number of Pools	Concentration	Number of Amplicons	Average Amplicon Length (bp)	Average Library Length (bp)
AmpliSeq Myeloid Panel for Illumina	DNA	2	5X	526	230	370
	RNA	1	5X	700	100	240

Combined Workflow

The AmpliSeq Myeloid Panel combined DNA and RNA workflow supports up to 48 samples (24 DNA samples and 24 RNA samples) in a single plate. After the libraries are amplified, pools 1 and 2 of the DNA panel are transferred and combined in empty wells on the RNA plate.

For more information, see *DNA Input Recommendations* on page 2.

DNA Input Recommendations

The AmpliSeq for Illumina Myeloid Panel DNA protocol supports 10–100 ng per pool (where 1 ng is equivalent to ~300 genome copies) of human DNA from high-quality sample. 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.

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.

RNA Input Recommendations

The AmpliSeq for Illumina Myeloid Panel RNA protocol reverse-transcribes RNA into cDNA. Each reverse transcription reaction requires 10–100 ng of DNase-treated total RNA. The recommended input is 10 ng RNA. Before starting the protocol, quantify and dilute input RNA to the desired concentration.

- Increasing the amount of input RNA within this range typically results in higher-quality libraries, especially when RNA quality is unknown.
  - Do not exceed the maximum supported amount of input RNA.
- Isolate total RNA using a standard nucleic acid purification kit.
- Quantify the starting RNA using a fluorescence-based quantification method, such as the Qubit RNA HS Assay Kit or QuantiT RiboGreen RNA Assay Kit. Do not use a UV-spectrometer-based method.

Additional Resources

Visit the AmpliSeq for Illumina Myeloid 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	support.illumina.com/custom-protocol-selector.html 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.
AmpliSeq for Illumina Myeloid Panel Checklist (document # 1000000056159)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
AmpliSeq for Illumina Myeloid Panel Consumables & Equipment List (document # 1000000056161)	Provides an interactive checklist of user-provided consumables and equipment.
Index Adapters Pooling Guide (document # 1000000041074)	Provides pooling guidelines and dual indexing strategies for AmpliSeq for Illumina libraries.
MiSeq System Denature and Dilute Libraries Guide (document # 15039740)	Provides instructions on how to denature and dilute prepared libraries for sequencing on the Illumina MiSeq™ Sequencing System.
MiniSeq System Denature and Dilute Libraries Guide (document # 1000000002697)	Provides instructions on how to denature and dilute prepared libraries for sequencing on the Illumina MiniSeq™ Sequencing System.
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.
iSeq 100 Sequencing System Guide (document # 1000000036024)	Provides instructions on how to denature and dilute prepared libraries for sequencing on the Illumina iSeq™ 100 Sequencing System.

Chapter 2 Protocol

Introduction

This chapter describes the AmpliSeq for Illumina protocol for DNA.

- Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information* on page 24.
- 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 Myeloid Panel workflow. Safe stopping points are marked between steps.

Figure 1 AmpliSeq Myeloid Panel Workflow

Quantify and Dilute RNA

This step quantifies and dilutes input RNA to the appropriate concentration in the required diluent for subsequent steps.

Consumables

- Total RNA
- Nuclease-free water
- 1.5 ml tube

Preparation

1 Prepare the following consumable:

Reagent	Storage	Instruction
RNA	-80°C	Thaw on ice immediately before use. Invert or flick to mix, and then centrifuge briefly. Keep on ice during the procedure.

Procedure

1 Quantify RNA using a fluorometric method, such as Qubit or RiboGreen.

2 If enough RNA is available, dilute to an intermediate concentration as follows.

   a Dilute to a concentration of ~20–50 ng/µl using nuclease-free water.
   b Requantify the diluted RNA using the same fluorometric quantification method.

3 Dilute RNA to desired final concentration.
Standard input is 10 ng high-quality RNA. For more information, see *RNA Input Recommendations* on page 2.
Example: If your final RNA concentration is 4 ng/µl, add 2.5 µl diluted RNA to result in 10 ng total input.

Reverse Transcribe RNA

This step uses the AmpliSeq cDNA Synthesis for Illumina Kit to reverse transcribe RNA to cDNA.

Consumables

- Total RNA (10–100 ng)
- AmpliSeq cDNA Synthesis for Illumina Kit
- Nuclease-free water
- MicroAmp Clear Adhesive Film
- 96-well PCR plate compatible with your thermal cycler

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
5X AmpliSeq cDNA Reaction Mix	-25°C to -15°C	Thaw at room temperature. Vortex briefly or pipette to mix.
10X AmpliSeq RT Enzyme Mix	-25°C to -15°C	Thaw on ice. Vortex briefly or pipette to mix. Keep on ice during the procedure.

2 Save the following RT program on a thermal cycler with a heated lid:
- Choose the preheated lid option and set to 105°C
- Set the reaction volume to 5 µl (or lowest available volume)
- 42°C for 30 minutes
- 85°C for 5 minutes
- Hold at 10°C

Procedure

1 For one sample, combine the following volumes in one well of a 96-well PCR plate. For multiple samples, prepare a master mix without RNA in a 1.5 ml tube.
- 5X AmpliSeq cDNA Reaction Mix (1 µl)
- 10X AmpliSeq RT Enzyme Mix (0.5 µl)
- Total RNA (10–100 ng) (≤ 3.5 µl)
- Nuclease-free water (to 5 µl)

Results in 5 µl reaction volume per sample.

2 Seal the plate.

3 Vortex thoroughly, and then centrifuge briefly.

4 Place on the thermal cycler, cover with a compression pad (if applicable), and run the RT program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 16 hours. For longer durations, store at -25°C to -15°C.

Amplify cDNA Targets

This step uses PCR to amplify target regions of the cDNA sample.

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

Consumables

- 5X AmpliSeq HiFi Mix (red cap)
- 5X AmpliSeq Myeloid RNA Panel (red cap)
- cDNA
- Nuclease-free water
- 1.5 ml tube
- 96-well PCR plate compatible with your thermal cycler

- MicroAmp Clear Adhesive Film

About Reagents

- HiFi Mix is viscous. Pipette slowly.

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. Keep on ice during the procedure.
5X AmpliSeq Myeloid RNA Panel (red cap)	-25°C to -15°C	Thaw at room temperature. Vortex briefly, then centrifuge briefly.

2 Transfer to the post-PCR area.

3 Save the following AMP_RNA program on a thermal cycler with a heated lid.
- Choose the preheated lid option and set to 105°C
- Set the reaction volume to 20 µl
- 99°C for 2 minutes
- 25 cycles of
  - 99°C for 15 seconds
  - 60°C for 4 minutes
- Hold at 10°C

The thermal cycler program is designed for 10 ng high-quality input per pool. If you are using a different input amount, 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 100 ng RNA.	Subtract three cycles.

Procedure

1 Briefly centrifuge the plate to collect contents, and then unseal.

2 Add the following volumes per sample to each well containing 5 µl cDNA. For multiple samples, prepare a master mix in a 1.5 ml tube.
- 5X AmpliSeq HiFi Mix (4 µl) (red cap)
- 5X AmpliSeq Myeloid RNA Panel (4 µl) (red cap)
- Nuclease-free water (7 µl)

3 Pipette to mix, seal the plate, and then centrifuge briefly.

4 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_RNA program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C overnight or store at -25°C to -15°C.

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) 2°C to 8°C (short-term)	Thaw at room temperature. 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. 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 4 ng/µl, add 5 µl diluted DNA to result in 20 ng total input.

Amplify DNA Targets

This step uses PCR to amplify target regions of the DNA sample.

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

Consumables

- 5X AmpliSeq Myeloid DNA Panel (two pools) (blue cap)
- 5X AmpliSeq HiFi Mix (red cap)
- DNA (20–200 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. Keep on ice during the procedure.
5X AmpliSeq Myeloid DNA Panel (blue cap)	-25°C to -15°C	Thaw at room temperature. 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_DNA 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
- 17 cycles of:
  - 99°C for 15 seconds
  - 60°C for 4 minutes
- Hold at 10°C for up to 24 hours

The thermal cycler program is designed for 10 ng high-quality input per pool (20 ng total). If you are using a different input amount or low-quality DNA, adjust the program per Table 2.

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 2 Adjustments to Thermal Cycler Program

Condition	Adjustment
Input is 1 ng DNA.	Add three cycles.
Input is 100 ng DNA per pool.	Subtract three cycles.

Procedure

1 Combine the following volumes per sample in a 1.5 ml tube.

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	4.5
DNA (20–200 ng)	≤ 13.5
Nuclease-free water	To reach total required volume
Total Volume	**18**

Extra volume is prepared to account for small pipetting errors.

2 Pipette to mix, and then centrifuge briefly.

3 Transfer each sample from the tube to a new PCR plate as follows.

   a Transfer 8 µl master mix to one well.
   b Transfer 8 µl of the same master mix to a second well.
   c Add 2 µl 5X AmpliSeq Myeloid DNA Panel Pool 1 (blue cap) to the first well.
   d Add 2 µl 5X AmpliSeq Myeloid DNA Panel Pool 2 (blue cap) to the second well.

   Each of the two wells contains 8 µl sample master mix and 2 µl primer pool for a total of 10 µl per well.

4 Pipette to mix, seal the plate, and then centrifuge briefly.

5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA 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.

Transfer Amplicons

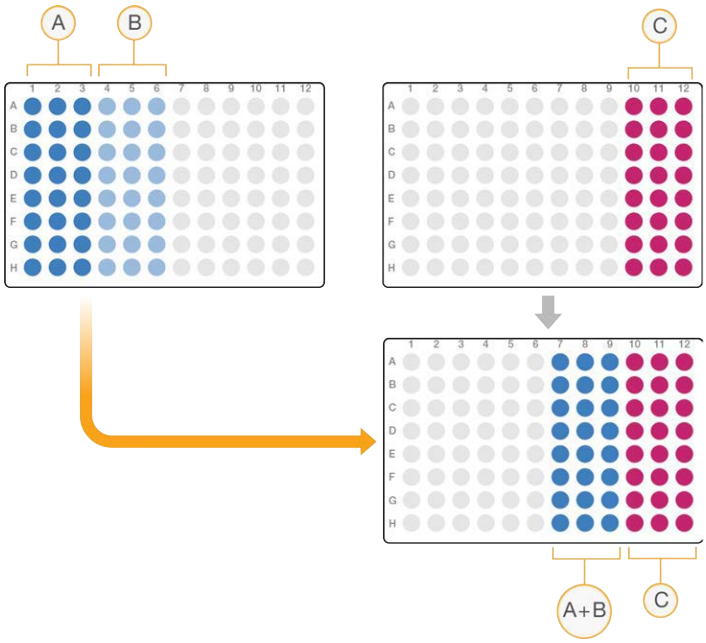
This step transfers DNA amplicons to the RNA plate.

Procedure

1 Briefly centrifuge the DNA and RNA library plates to collect contents, and then unseal.

2 For each DNA sample, use a multichannel pipette to transfer and combine the 10 µl target amplification reaction from the corresponding sample wells containing pool 1 and pool 2 to empty wells of the RNA sample plate.
   Total volume per sample is 20 µl.

Figure 2 Transfer DNA Amplicons



A DNA amplicons pool 1
B DNA amplicons pool 2
C RNA amplicons

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 (brown cap)	-25°C to -15°C	Thaw on ice. Centrifuge briefly. Keep on ice during the procedure.
Switch Solution (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. 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 22 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 Add 2 µl FuPa Reagent (brown cap) to each target amplification reaction. If you are using a multichannel pipette, prealiquot FuPa Reagent into an 8-tube strip, and then transfer the appropriate volume. The total volume per sample is 22 µl.

3 Seal the plate.

4 Vortex briefly, and then centrifuge briefly.

5 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, including DNA and RNA libraries from the same sample.

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 (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. If precipitate is observed in the solution or cap, vortex or pipette to resuspend.
AmpliSeq CD Indexes or UD Indexes for 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. Keep on ice during the procedure.
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.

*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 30 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 Remove the seal from the index plate.

2 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.

Order of Addition	Reagent	Volume (µl)
1	Switch Solution (yellow cap)	4
2	AmpliSeq CD Indexes or UD Indexes for Illumina	2
3	DNA Ligase (blue cap)	2
	Total Volume (including 22 µl digested amplicons)	**30**

CAUTION

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

3 Seal the library plate.

4 Vortex briefly, and then centrifuge briefly.

5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the LIGATE program.

6 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 20 ml fresh 70% EtOH from absolute ethanol.
This volume is sufficient to clean up 24 DNA and 24 RNA reactions (48 total reactions).

Procedure

1 Briefly centrifuge the plate to collect contents, and then unseal.

2 Add 30 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 28. Otherwise, continue to *Amplify Library* on page 17.

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. This reagent can be stored at room temperature.

2 Prepare 20 ml fresh 70% EtOH from absolute ethanol.
This volume is sufficient to clean up 24 DNA and 24 RNA reactions (48 total 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.

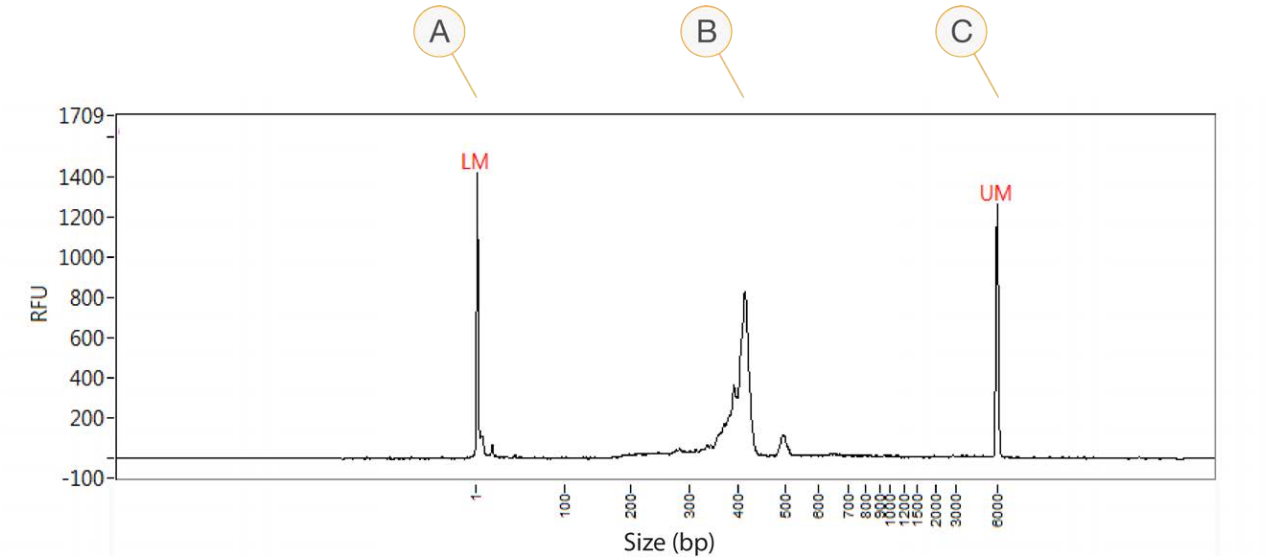
CAUTION
Bead carryover can affect cluster density.

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 3 provides an example Fragment Analyzer trace of a successfully sequenced DNA library. Typical libraries show a size distribution from 200–550 bp. The suggested size distribution for quantification is 200–450 bp.

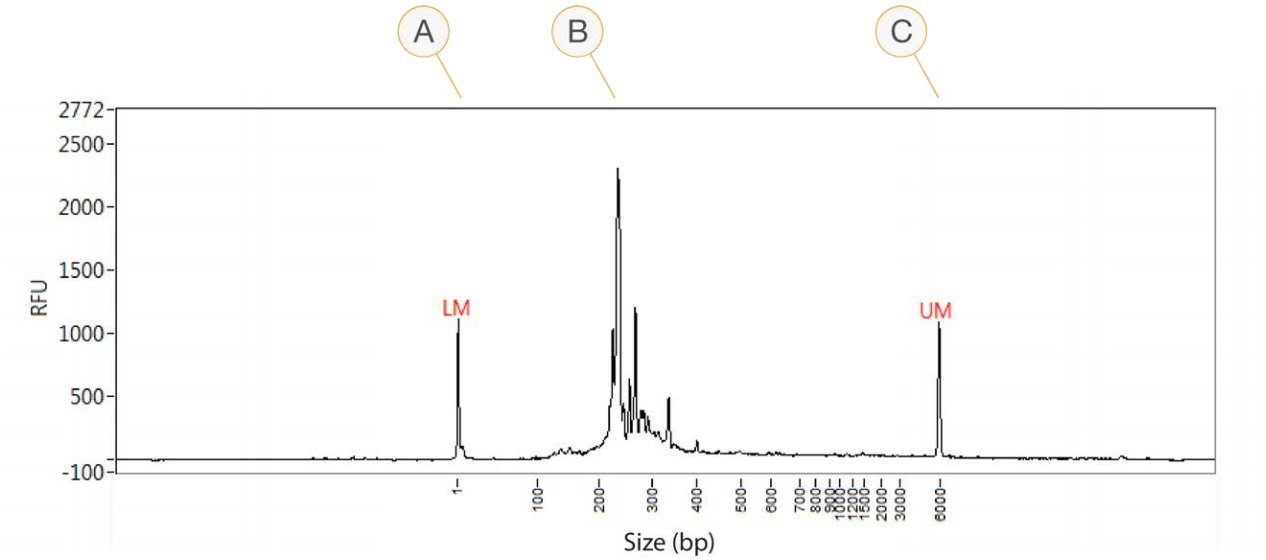
Figure 3 Example DNA Library Fragment Analyzer Trace



A Lower marker
B Expected DNA libraries
C Upper marker

Figure 4 provides an example Fragment Analyzer trace of a successfully sequenced RNA library. Typical libraries show a size distribution from 181–373 bp. The suggested size distribution for quantification is 160–500 bp.

Figure 4 Example RNA Library Fragment Analyzer Trace



A Lower marker
B Expected RNA 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:

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

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.

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

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 (nM)	Final Loading Concentration (pM)
iSeq 100 System	2	50
MiniSeq System	2	1.1–1.9
MiSeq System (v3 reagents)	2	7–9
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

The AmpliSeq for Illumina protocol requires the AmpliSeq Library PLUS kit for Illumina, AmpliSeq Myeloid Panel for Illumina, AmpliSeq CD Indexes or UD Indexes for Illumina, and [RNA] AmpliSeq cDNA Synthesis for Illumina Kit.

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 Myeloid Panel for Illumina	20024478
Indexes	AmpliSeq CD Indexes Set A for Illumina (96 Indexes, 96 Samples) AmpliSeq CD Indexes Set B for Illumina (96 Indexes, 96 Samples) AmpliSeq CD Indexes Set C for Illumina (96 Indexes, 96 Samples) AmpliSeq CD Indexes Set D for Illumina (96 Indexes, 96 Samples) AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples)	20019105 20019106 20019107 20019167 20019104
Reverse Transcriptase	[RNA] AmpliSeq cDNA Synthesis for Illumina Kit	20022654

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

Quantity			Reagent
24-reaction	96-reaction	384-reaction	
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 Myeloid 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	5X AmpliSeq Myeloid DNA Panel Pool 1
1	5X AmpliSeq Myeloid DNA Panel Pool 2
1	5X AmpliSeq Myeloid RNA Panel Pool 1

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)

AmpliSeq cDNA Synthesis for Illumina Contents, Store at -25°C to -15°C

Quantity	Description
1	5X AmpliSeq cDNA Reaction Mix
1	10X AmpliSeq RT Enzyme Mix

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 Myeloid Panel for Illumina, AmpliSeq CD Indexes or UD Indexes for Illumina, and [RNA] AmpliSeq cDNA Synthesis for Illumina Kit, 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
[Optional] AmpliSeq Library Equalizer for Illumina	Illumina, catalog # 20019171
Eppendorf DNA LoBind Microcentrifuge Tubes, 1.5 ml	Fisher Scientific, catalog # 13-698-791
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific, catalog # 4306311

Item	Supplier
One of the following 96-well PCR plates: For use with Thermo Fisher thermal cyclers: • MicroAmp EnduraPlate Optical 96-Well Clear Reaction Plates with Barcode For use with Bio-Rad thermal cyclers: • Hard-Shell 96-Well Skirted PCR Plates, low-profile, skirted	One of the following suppliers, depending on plate type: • Thermo Fisher Scientific, catalog # 4483352 or 4483354 • 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: • [Bioanalyzer] Agilent DNA 1000 Kit • [Fluorometer] [DNA] Qubit dsDNA HS Assay Kit • [Fluorometer] [RNA] Qubit RNA HS Assay Kit • [Fluorometer] [RNA] QuantiT RiboGreen RNA Assay Kit • [Fragment Analyzer] Standard Sensitivity NGS Fragment Analyzer Kit (1 bp – 6,000 bp) • [qPCR] KAPA Library Quantification Kit (Universal) • AccuClear Ultra High Sensitivity dsDNA Quantitation Kit • Quant-iT PicoGreen dsDNA Assay Kit	One of the following suppliers, depending on kit: • Agilent, catalog # 5067-1504 • Thermo Fisher Scientific, catalog # Q32851 or Q32854 • Thermo Fisher Scientific, catalog # Q32852 or Q32855 • Thermo Fisher Scientific, catalog # R11490 • Advanced Analytical Technologies, Inc., part # DNF-473 • Kapa Biosystems, catalog # KK4824 • Biotium, catalog # 31028 • Thermo Fisher catalog # P11496
[Optional] One of the following positive sample controls: • [DNA] Quantitative Multiplex Reference Standard • [DNA] Tru-Q 2 (5% Tier) • [DNA] Acrometrix Oncology Hotspot Control • [RNA] SeraSeq Myeloid Mutation DNA Mix • [RNA] SeraSeq Myeloid Fusion RNA Mix	One of the following suppliers, depending on sample control: • Horizon, catalog # HD701 • Horizon, catalog # HD729 • Thermo Fisher Scientific, catalog # 969056 • SeraCare, catalog # 0710-0408 • SeraCare, catalog # 0710-0407
NaOH, molecular biology-grade	General lab supplier
Tris-HCl, pH 7.0	General lab supplier

Equipment

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

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

[2] No longer available for purchase.

[Optional] AmpliSeq Library Equalizer for Illumina

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 3.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: 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
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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.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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