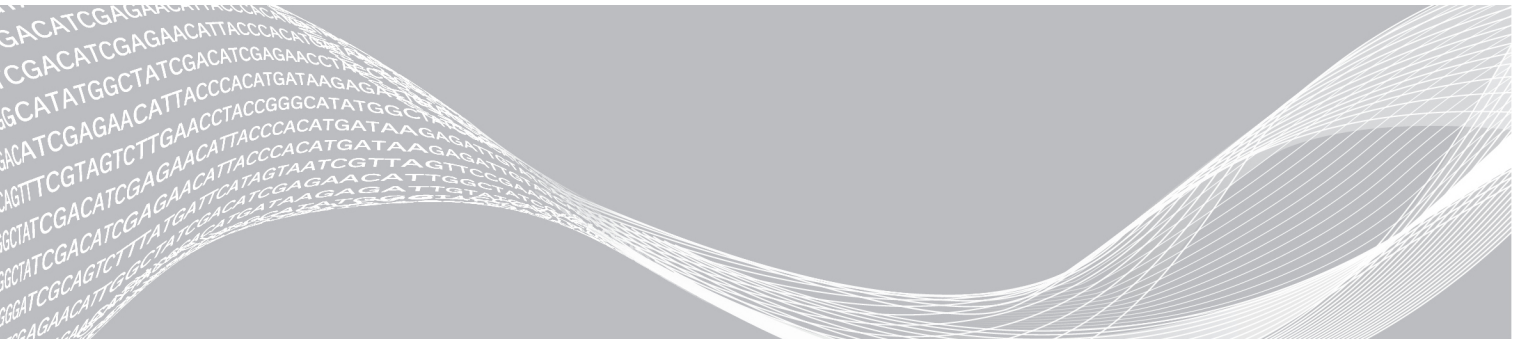
illumina®

AmpliSeq for Illumina On-Demand, Custom, and Community Panels

Reference Guide



Document # 1000000036408 v09

ILLUMINA PROPRIETARY

May 2020

Revision History

Document	Date	Description of Change
Document # 1000000036408 v09	May 2020	Added dilution information for the NextSeq 2000 Sequencing System.
Document # 1000000036408 v08	November 2019	Clarified the number of days a plate can be stored. Removed requirements for 50X AmpliSeq Custom DNA Panel spike-in.
Document # 1000000036408 v07	May 2019	Added support for AmpliSeq Custom RNA Fusion panel. Clarified one primer pool volume when partially digesting amplicons.
Document # 1000000036408 v06	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. Added optional steps to spike in AmpliSeq ERCC RNA Spike–In Mix for Illumina and AmpliSeq ERCC RNA Companion Panel 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 # 1000000036408 v05	October 2018	Added optional steps to spike in a custom DNA panel to an AmpliSeq On-Demand Panel. Added optional instructions for using the AmpliSeq Library Equalizer for Illumina.
Document # 1000000036408 v04	July 2018	Changed reference guide name to AmpliSeq for Illumina On-Demand, Custom and Community Panels Reference Guide. Added instructions on using AmpliSeq On-Demand Panel for Illumina. Added optional steps to spike in AmpliSeq Sample ID Panel for Illumina. Referenced the *AmpliSeq Direct FFPE DNA Kit for Illumina Reference Guide (document # 1000000056164)* for information on FFPE tissue sample preparation. Added step for transferring to the post-PCR area. Expanded available primer pairs per pool in the X Cycles and X Minutes table. Added information on using DNA prepared with the AmpliSeq Direct FFPE DNA Kit for Illumina. Clarified difference between shipping and storage temperature for AmpliSeq CD Indexes for Illumina, AmpliSeq Custom Panel for Illumina, and AmpliSeq On-Demand Panel for Illumina. Removed optional step for determining library quantity. Added formula for calculating molarity when using fluorometric methods. Corrected digested amplicons volume in the Ligate Indexes section.

Document	Date	Description of Change
Document # 1000000036408 v03	April 2018	Added option to use AmpliSeq CD Indexes Large Volume for Illumina in automated workflows. Changed gDNA to DNA throughout. Added several entries to the Additional Resources table. Moved Dilute and Normalize Libraries procedure into the appropriate denature and dilute libraries guides. Changed SuperScript VILO cDNA Synthesis Kit to AmpliSeq cDNA Synthesis for Illumina Kit.
Document # 1000000036408 v02	January 2018	Changed DNA and RNA input volumes to 100 ng per pool. Changed Quantify and Dilute RNA procedure to use nuclease-free water. Removed 3- and 4-pool configuration volumes from RNA Ligate Indexes procedure. Added tube quantities for 96- and 384-reaction kits.
Document # 1000000036408 v01	January 2018	Clarified wording of genome copy equivalents. Corrected panel name. Removed duplicate Amplify cDNA procedure.
Document # 1000000036408 v00	January 2018	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

This guide explains how to prepare up to 384 uniquely indexed libraries of genomic DNA or total RNA using the AmpliSeq™ for Illumina® workflow.

NOTE

If preparing the maximum number of libraries per kit, more than one kit may be required to accommodate 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.
- Scalable library prep with 12–24, 576 primer pairs per pool from 1–100 ng DNA per pool or 1–100 ng RNA per pool.
- Compatibility with FFPE samples.
- Generation of sequence-ready libraries from DNA or RNA 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.

AmpliSeq for Illumina Panels

AmpliSeq Library PLUS panels provide primer pools for amplification of target regions. Proprietary primer modifications allow the removal of primer sequences during library preparation for efficient target assessment during sequencing.

Multiple primer pools (up to four) can create overlapping amplicons for complete coverage of large targets. Nucleic acid from various sources, including FFPE tissue, can be used as starting material.

Community and Custom Panels

Community panels are available in DNA and RNA configurations. Order community panels through the Illumina website or use them as a starting point for a custom design. Order custom panels through DesignStudio™.

Each community and custom panel includes at least one primer pool of primer pairs. DNA panels include up to four pools at 2X concentration, and RNA panels include one or two pools at 5X concentration. Each tube of primer pool contains 1500 µl.

Table 1 DNA Panel (2X Concentration)

Number of Amplicons per Pool	One Primer Pool	Two Primer Pools	Three Primer Pools	Four Primer Pools
≤ 96	5 tubes	2 × 5 tubes	3 × 5 tubes	4 × 5 tubes
> 96	20 tubes	2 × 20 tubes	3 × 20 tubes	4 × 20 tubes

Table 2 RNA Panel (5X Concentration)

Number of Amplicons per Pool	One Primer Pool	Two Primer Pools
12–1200	3 tubes	2 × 3 tubes

On-Demand Panels

On-Demand panels are available in DNA configurations only. Order On-Demand panels through DesignStudio™. On-Demand panels are available in 24 or 96 reaction sizes and include two pools at 2X concentration. On-Demand panels can be used with or without a 50X AmpliSeq Custom DNA Panel spike-in.

Table 3 DNA Panel (2X Concentration)

Reaction Size	Number of Amplicons per Pool	Two Primer Pools
24	Varies	1 × 2 tubes
96	Varies	4 × 2 tubes

Table 4 Custom DNA Spike-In Panel (50X Concentration)

Reaction Size	Number of Amplicons per Pool	Two Primer Pools
14,400	up to 123	1 × 2 tubes

DNA Input Recommendations

The AmpliSeq for Illumina On-Demand, Custom, and Community Panels are provided as single or multiple DNA primer pools. The protocol supports 1–100 ng per pool (where 1 ng is equivalent to ~300 genome copies) 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 On-Demand, Custom, and Community Panels libraries. Only primer pairs designed for FFPE samples are recommended for degraded samples.

RNA Input Recommendations

The AmpliSeq for Illumina On-Demand, Custom, and Community Panels RNA protocol reverse-transcribes RNA into cDNA. Each reverse transcription reaction requires 1–100 ng per pool of DNase-treated total RNA. The recommended input is 10 ng RNA per pool. 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.
  - Use 1 ng total RNA per pool only with high-quality, well-quantified samples.
- 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.
- Library yield can be lower for degraded library samples such as FFPE RNA.

Additional Resources

The AmpliSeq for Illumina On-Demand, Custom, and Community Panels support pages on the Illumina website provide software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Resource	Description
Custom Protocol Selector	A tool for generating end-to-end instructions tailored to your library prep method, run parameters, and analysis method, with options to refine the level of detail.
AmpliSeq for Illumina On-Demand, Custom and Community Panels DNA Checklist (document # 1000000039391)	Provides a checklist of the DNA protocol steps. The checklist is intended for experienced users.
AmpliSeq for Illumina On-Demand, Custom and Community Panels RNA Checklist (document # 1000000047981)	Provides a checklist of the RNA and RNA Fusion protocol steps. The checklist is intended for experienced users.
AmpliSeq for Illumina On-Demand, Custom and Community Panels Consumables & Equipment List (document # 1000000039393)	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.
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.
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.

Resource	Description
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 for DNA Panels

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 41.
- 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.
- Prepare aliquots as needed to minimize freeze-thaw cycles of AmpliSeq panels. Panels can be stored at 2°C to 8°C for up to one year.
- 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 for DNA

The following diagram illustrates the AmpliSeq for Illumina On-Demand, Custom, and Community Panels DNA workflow. Safe stopping points are marked between steps.

Figure 1 AmpliSeq On-Demand, Custom, and Community Panels Workflow

Quantify and Dilute DNA

This step quantifies and dilutes input DNA to the appropriate concentration for subsequent steps. The volume of diluted DNA required depends on the number of primer pools.

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.
The following table lists example dilutions to result in standard DNA input per pool.

Number of Pools	DNA Concentration (ng/µl)	Diluted DNA Volume (µl)	Total DNA Input (ng)
1	2	5	10
2	4	5	20
3	6	5	30
4	8	5	40

Amplify DNA Targets

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

The target amplification procedure depends on the number of primer pools in the DNA panel. Make sure that you follow the appropriate procedure for the number of pools in your panel (one, two, three, or four). Use the procedure for two primer pools for all AmpliSeq for Illumina On-Demand panels.

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

Consumables

- 2X AmpliSeq Custom DNA Panel (1–4 pools) (cap color varies) or 2X AmpliSeq On-Demand DNA Panel (two pools) (clear cap)
- 5X AmpliSeq HiFi Mix (red cap)
- [Optional] 20X AmpliSeq Sample ID Panel for Illumina
- [Optional] 50X AmpliSeq Custom DNA Panel (spike-in to On-Demand panels)
- DNA (1–100 ng per pool)
- 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.
2X AmpliSeq Custom DNA Panel (cap color varies) or 2X AmpliSeq On-Demand DNA Panel (clear cap)	-25°C to -15°C	Thaw at room temperature. Vortex briefly, then centrifuge briefly.
[Optional] 20X AmpliSeq Sample ID Panel for Illumina	-25°C to -15°C	Thaw at room temperature. Vortex briefly, then centrifuge briefly.
[Optional] 50X AmpliSeq Custom DNA Panel	-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 [Optional] If using 50X AmpliSeq Custom DNA Panel spike-in, do as follows.

   a Add 5 µl AmpliSeq Custom DNA Panel spike-in pool 1 to AmpliSeq On-Demand DNA Panel pool 1 tube.
   b Add 5 µl AmpliSeq Custom DNA Panel spike-in pool 2 to AmpliSeq On-Demand DNA Panel pool 2 tube.

3 Transfer to the post-PCR area.

4 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 20 µl for one pool or 10 µl for two, three, or four pools
   - 99°C for 2 minutes
   - X cycles of:
     - 99°C for 15 seconds
     - 60°C for X minutes
   - Hold at 10°C for up to 24 hours

   Use the following two tables to determine X number of cycles and X minutes.
   - If you are using 10 ng high-quality input per pool (10–40 ng total) use the values in Table 5 without adjustments.
   - If you are using a different input amount or low-quality DNA, adjust X number of cycles and X minutes per Table 6.
   - If you are using AmpliSeq Spike-In Panel for Illumina, add the primer pairs from both the AmpliSeq for Illumina On-Demand panel and the spike-in when referencing Table 5.

   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 5 X Cycles and X Minutes

Primer Pairs per Pool	X Cycles for 10 ng High-Quality DNA (3000 Copies)	X Minutes
12–24	21	4
25–48	20	4
49–96	19	4
97–192	18	4
193–384	17	4
385–768	16	4
769–1,536	15	8
1,537–3,072	14	8
3,073–6,144	13	16
6,145–24,576	12	16

Table 6 Adjustments to Thermal Cycler Program

Condition	Adjustment
The panel has two primer pools in different cycle categories.	Use the greater number of cycles.
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.
The panel uses a 375 bp amplicon design.	Add four minutes.

Procedure for One Primer Pool

1 [One sample] Add the following volumes to one well of a new 96-well PCR plate.

2 [Multiple samples] Prepare master mix as follows.

   a Combine all the following reagents except DNA in a 1.5 ml tube. Add 10% extra volume of each reagent to account for pipetting errors.

b Pipette or vortex briefly, and then centrifuge briefly.
c Dispense master mix into each well of the plate, and then add DNA.

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 (µl)
5X AmpliSeq HiFi Mix (red cap)	4
2X AmpliSeq Custom DNA Panel (cap color varies)	10
[Optional] 20X AmpliSeq Sample ID Panel for Illumina	1
DNA (1–100 ng)	≤ 6 (≤ 5 if using AmpliSeq Sample ID Panel for Illumina)
Nuclease-free water	To reach total required volume
Total Volume	**20**

3 Pipette to mix, seal the plate, and then centrifuge briefly.
4 Place on the preprogrammed thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA program.
5 Proceed to *Partially Digest Amplicons* on page 14

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.

Procedure for Two Primer Pools

1 [Optional] If using AmpliSeq Sample ID Panel for Illumina, do as follows.

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 DNA Panel Pool 1 (cap color varies) (5 µl)

b Pipette to mix, and then centrifuge briefly.

2 For each sample, combine the following volumes 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 (µl)
5X AmpliSeq HiFi Mix (red cap)	5
DNA (2–200 ng)	≤ 7.5
Nuclease-free water	To reach total required volume
Total Volume	**12.5**

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 µl master mix to two wells so each of the two wells contains 5 µl of the same master mix.
b Add 5 µl 2X AmpliSeq DNA Panel Pool 1 (cap color varies) to the first well.
c Add 5 µl 2X AmpliSeq DNA Panel Pool 2 (cap color varies) to the second well.

Each of the two wells contains 5 µl sample master mix and 5 µl primer pool for a total of 10 µl 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 µl master mix to two wells so each of the two wells contains 5 µl of the same master mix.
b Add 5.5 µl 2X AmpliSeq DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
c Add 5 µl 2X AmpliSeq DNA Panel Pool 2 (cap color varies) to the second well.

The result is a total volume of 10.5 µl for the first well and 10 µl for the second well.

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

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

8 Proceed to *Partially Digest Amplicons* on page 14

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.

Procedure for Three Primer Pools

1 [Optional] If using AmpliSeq Sample ID Panel for Illumina, do as follows.

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 Custom DNA Panel Pool 1 (cap color varies) (5 µl)

b Pipette to mix, and then centrifuge briefly.

2 For each sample, combine the following volumes 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 (µl)
5X AmpliSeq HiFi Mix (red cap)	7
DNA (3–300 ng)	≤ 10.5
Nuclease-free water	To reach total required volume
Total Volume	**17.5**

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 µl master mix to three wells of the plate so each of the three wells contains 5 µl of the same master mix.

b Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) to the first well.
c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.

Each of the three wells contains 5 µl sample master mix and 5 µl primer pool for a total of 10 µl 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 µl master mix to three wells of the plate so each of the three wells contains 5 µl of the same master mix.
b Add 5.5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.

The result is a total volume of 10.5 µl for the first well and 10 µl for the second and third wells.

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

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

8 Proceed to *Partially Digest Amplicons* on page 14

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.

Procedure for Four Primer Pools

1 [Optional] If using AmpliSeq Sample ID Panel for Illumina, do as follows.

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 Custom DNA Panel Pool 1 (cap color varies) (5 µl)

b Pipette to mix, and then centrifuge briefly.

2 For each sample, combine the following volumes 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 (µl)
5X AmpliSeq HiFi Mix (red cap)	9
DNA (4–400 ng)	≤ 13.5
Nuclease-free water	To reach total required volume
Total Volume	**22.5**

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 µl master mix to four wells of the plate so each of the four wells contains 5 µl of the same master mix.
b Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) to the first well.
c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.
e Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 4 (cap color varies) to the fourth well.

Each of the three wells contains 5 µl sample master mix and 5 µl primer pool for a total of 10 µl 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 µl master mix to four wells of the plate so each of the four wells contains 5 µl of the same master mix.
b Add 5.5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.
e Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 4 (cap color varies) to the fourth well.

The result is a total volume of 10.5 µl for the first well and 10 µl for the remaining wells.

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

7 Place on the preprogrammed 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.

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:

Reagent	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	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.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

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:

Number of Primer Pools	Reaction Volume (µl)
1 or 2	22
3	33
4	44

- 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.
[One primer pool] The total volume is 20 µl.

2 [Multiple primer pools] For each sample, use a multichannel pipette to combine the two, three, or four 10 µl target amplification reactions into the well containing pool 1, without changing tips.
The total volume per sample is 20 µl, 30 µl, or 40 µl.

3 Add the appropriate volume of 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 volumes.

Number of Primer Pools	FuPa Volume (µl)
1 or 2	2
3	3
4	4

The total volume per sample is 22 µl, 33 µl, or 44 µl.

4 Seal the plate.

5 Vortex briefly, and then centrifuge briefly.

6 Place on the preprogrammed 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 (document # 1000000041074)*.

Consumables

- AmpliSeq CD Indexes or UD Indexes for Illumina
- DNA Ligase (blue cap)
- Switch Solution (yellow 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
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.
Switch Solution (yellow cap)	-25°C to -15°C	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.
Agencourt AMPure XP beads*	2°C to 8°C	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

2 Save the following LIGATE program on the thermal cycler:
- Choose the preheated lid option and set to 105°C
- Set the reaction volume:

Number of Primer Pools	Reaction Volume (µl)
1 or 2	30
3	45
4	60 (or highest available volume)

- 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.
- Use a multichannel pipette when adding AmpliSeq CD Indexes or UD Indexes for Illumina 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.

Order of Addition	Reagent	Volume for One or Two Primer Pools (µl)	Volume for Three Primer Pools (µl)	Volume for Four Primer Pools (µl)
1	Switch Solution (yellow cap)	4	6	8
2	AmpliSeq CD Indexes or UD Indexes for Illumina	2	3	4
3	DNA Ligase (blue cap)	2	3	4
	Total Volume (including 22 µl, 33 µl, or 44 µl digested amplicons)	**30**	**45**	**60**

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 preprogrammed 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	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	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

2 Prepare 10 ml fresh 70% EtOH from absolute EtOH.
This volume is sufficient to clean up 24 reactions.

Procedure

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

2 Add the appropriate volume of AMPure XP beads to each library, and then seal the plate.

Number of Primer Pools	Bead Volume (µl)
1 or 2	30
3	45
4	60

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 beads two times as follows.

   a Add 150 µl freshly prepared 70% ethanol to each well.

b Incubate at room temperate until the solution is clear (~30 seconds).
c Without disturbing the pellet, remove and discard supernatant from each well.

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 46. Otherwise, continue to *Amplify Library* on page 36.

NOTE

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

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:

Reagent	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	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

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 preprogrammed 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	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 10 ml fresh 70% EtOH from absolute EtOH.
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 beads two times as follows.

a Add 150 µl freshly prepared 70% ethanol to each well.
b Incubate at room temperate until the solution is clear (~30 seconds).
c Without disturbing the pellet, remove and discard supernatant from each well.

15 Use a 20 µl pipette to remove residual EtOH from each well.

16 Discard unused 70% EtOH.

17 Air-dry on the magnetic stand for 5 minutes.

18 Remove from the magnetic stand.

19 Add 30 µl Low TE to each well, and then seal the plate.

20 Vortex briefly to disperse the beads, and then centrifuge briefly.

21 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

22 Unseal the plate.

23 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 for up to 30 days.

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.

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
NextSeq 2000	2	750

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.

Chapter 3 Protocol for RNA Panels

Introduction

This chapter describes the AmpliSeq for Illumina protocol for total RNA.

- Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information* on page 41.
- 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.
- Prepare aliquots as needed to minimize freeze-thaw cycles of AmpliSeq panels. Panels can be stored at 2°C to 8°C for up to one year.
- 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 for RNA

The following diagram illustrates the AmpliSeq for Illumina On-Demand, Custom, and Community Panels RNA workflow. Safe stopping points are marked between steps.

Figure 2 AmpliSeq On-Demand, Custom, and Community Panels 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 per pool. For more information, see *RNA Input Recommendations* on page 3.
Example: If your final RNA concentration is 2 ng/µl, add 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

- AmpliSeq cDNA Synthesis for Illumina Kit
- [Optional] AmpliSeq ERCC RNA Spike-In Mix for Illumina
- Total RNA (1–100 ng per pool)
- Nuclease-free water
- MicroAmp Clear Adhesive Film
- 96-well PCR plate compatible with your thermal cycler

Preparation

1 If RNA was prepared from FFPE tissue and never heat-treated, heat at 80°C for 10 minutes and then cool to room temperature.

2 Prepare the following consumables:

Reagent	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.
[Optional] AmpliSeq ERCC RNA Spike-In Mix for Illumina	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly. Keep on ice during the procedure.

3 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 10 µl
- 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, combine the following reagents except RNA in a 1.5 ml tube to prepare master mix.
- 5X AmpliSeq cDNA Reaction Mix (2 µl)
- 10X AmpliSeq RT Enzyme Mix (1 µl)
- [Optional] AmpliSeq ERCC RNA Spike-In Mix for Illumina as follows.

Total RNA Input (ng)	Volume (µl)
10	1 (1:5000 dilution)
20	2 (1:5000 dilution)
50	1 (1:1000 dilution)
100	2 (1:1000 dilution)

- Total RNA (1–100 ng per pool) (≤ 7 µl)
- Nuclease-free water (to 10 µl)

Results in 10 µl reaction volume per sample.

2 Seal the plate.

3 Vortex thoroughly, and then centrifuge briefly.

4 Place on the preprogrammed 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.

The procedure depends on the number of primer pools in your AmpliSeq RNA panel. Make sure that you follow the appropriate procedure for the number of pools in your panel (one or two).

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

Consumables

- 5X AmpliSeq Custom RNA Panel or AmpliSeq Custom RNA Fusion Panel (1–2 pools) (cap color varies)
- 5X AmpliSeq HiFi Mix (red cap)
- cDNA
- [Optional] AmpliSeq ERCC RNA Companion Panel for Illumina
- 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:

Reagent	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 Custom RNA Panel (cap color varies)	-25°C to -15°C	Thaw at room temperature. Vortex briefly, then centrifuge briefly.
[Optional] AmpliSeq ERCC RNA Companion Panel for Illumina	-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:

Number of Primer Pools	Reaction Volume (µl)
1	20
2	10

- 99°C for 2 minutes
- X cycles of
  - 99°C for 15 seconds
  - 60°C for X minutes
- Hold at 10°C

Use the following two tables to determine X number of cycles and X minutes.
- If you are using 10 ng high-quality input per pool (10–20 ng total) use the values in Table 7 without adjustments.
- If you are using a different input amount or low-quality RNA, adjust X number of cycles and X minutes per Table 8.
- If you are using the AmpliSeq Custom RNA Fusion panel, adjust X number of cycles and X minutes per Table 8. Refer to the DesignStudio design for the number of gene expression targets.

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 7 X Cycles and X Minutes

Primer Pairs per Pool	X Cycles for 10 ng High-Quality RNA per Pool	X Minutes
12–24	21	4
25–48	20	4
49–96	19	4
97–192	18	4
193–384	17	4
385–768	16	4
769–1200	15	8

Table 8 Adjustments to Thermal Cycler Program

Condition	Adjustment
The panel has two primer pools in different cycle categories.	Use the greater number of cycles.
Input is 1 ng RNA per pool.	Add three cycles.
Input is 100 ng RNA per pool.	Subtract three cycles.
RNA is low-quality (FFPE).	Add three cycles.
Using the Custom RNA Fusion Panel with ≤ 48 gene expression targets.	Use 27 cycles for 4 minutes.
Using the Custom RNA Fusion Panel with > 48 gene expression targets.	Base the number of cycles on the number of gene expression targets per pool. Use Table 7 to determine X minutes.

Procedure for One Primer Pool

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

2 Add the following volumes per sample to each well containing 10 µl cDNA. For multiple samples, combine the following volumes in a 1.5 ml tube to prepare a master mix.
- 5X AmpliSeq HiFi Mix (4 µl) (red cap)
- 5X AmpliSeq Custom RNA Panel or AmpliSeq Custom RNA Fusion Panel (4 µl) (cap color varies)
- [Optional] AmpliSeq ERCC RNA Companion Panel for Illumina (1 µl)
- Nuclease-free water (2 µl)

3 Pipette to mix.

4 Seal the plate, and then centrifuge briefly.

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

6 Proceed to *Partially Digest Amplicons* on page 31.

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.

Procedure for Two Primer Pools

1 [Optional] If using AmpliSeq ERCC RNA Companion Panel for Illumina, do as follows.

a For each sample, combine the following volumes in a 1.5 ml tube:
   - 20X AmpliSeq ERCC RNA Companion Panel for Illumina (0.5 µl)
   - 2X AmpliSeq RNA Panel Pool 1 (cap color varies) (5 µl)

b Pipette to mix, and then centrifuge briefly.

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

3 Add the following volumes per sample to each well containing 10 µl cDNA. For multiple samples, combine the following volumes in a 1.5 ml tube to prepare master mix.
   - 5X AmpliSeq HiFi Mix (4.5 µl) (red cap)
   - Nuclease-free water (3.5 µl)

   Each well contains 18 µl cDNA and master mix.

4 Pipette to mix.

5 Seal the plate, and then centrifuge briefly.

6 If not using AmpliSeq ERCC RNA Companion Panel for Illumina, transfer each sample from the tube to a new PCR plate as follows.

   a Transfer 8 µl master mix to two wells so each of the two wells contains 8 µl of the same master mix.
   b Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 1 (cap color varies) to the first well.
   c Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 2 (cap color varies) 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.

7 If using AmpliSeq ERCC RNA Companion Panel for Illumina, transfer each sample from the tube to a new PCR plate as follows.

   a Transfer 8 µl master mix to two wells so each of the two wells contains 8 µl of the same master mix.
   b Add 2.5 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 1 (cap color varies) plus AmpliSeq ERCC RNA Companion Panel for Illumina mix to the first well.
   c Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 2 (cap color varies) to the second well.

   The result is a total volume of 10.5 µl for the first well and 10 µl for the second well.

8 Seal the plate.

9 Place on the preprogrammed 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.

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	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.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

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.
[One primer pool] The total volume per sample is 20 µl.

2 [Two primer pools] For each sample, use a multichannel pipette to combine the 10 µl target amplification reaction from the sample well containing pool 2 into the sample well containing pool 1.
The total volume per sample is 20 µl.

3 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.

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 preprogrammed 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 (document # 1000000041074)*.

Consumables

- AmpliSeq CD Indexes or UD Indexes for Illumina
- DNA Ligase (blue cap)
- Switch Solution (yellow 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:

Reagent	Storage	Instructions
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 (blue 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	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.
Agencourt AMPure XP beads*	2°C to 8°C	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

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 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.

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.

4 Seal the library plate.

5 Vortex briefly, and then centrifuge briefly.

6 Place on the preprogrammed 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.

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	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	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

2 Prepare 10 ml fresh 70% EtOH from absolute EtOH.
This volume is sufficient to clean up 24 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 beads two times as follows.

   a Add 150 µl freshly prepared 70% ethanol to each well.
   b Incubate at room temperate until the solution is clear (~30 seconds).
   c Without disturbing the pellet, remove and discard supernatant from each well.

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 46. Otherwise, continue to *Amplify Library* on page 36.

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:

Reagent	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	Bring to room temperature. Vortex thoroughly to resuspend.

* If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

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 preprogrammed 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	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 10 ml fresh 70% EtOH from absolute EtOH.
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 beads two times as follows.

a Add 150 µl freshly prepared 70% ethanol to each well.
b Incubate at room temperate until the solution is clear (~30 seconds).
c Without disturbing the pellet, remove and discard supernatant from each well.

15 Use a 20 µl pipette to remove residual EtOH from each well.

16 Discard unused 70% EtOH.

17 Air-dry on the magnetic stand for 5 minutes.

18 Remove from the magnetic stand.

19 Add 30 µl Low TE to each well, and then seal the plate.

20 Vortex briefly to disperse the beads, and then centrifuge briefly.

21 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

22 Unseal the plate.

23 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 for up to 30 days.

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.

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.

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

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
NextSeq 2000	2	750

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.

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 On-Demand, Custom, and Community Panels 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 Custom DNA Large Panel for Illumina	20020497
	AmpliSeq Custom DNA Panel for Illumina	20020495
	AmpliSeq Custom RNA Panel for Illumina	20020496
	AmpliSeq Custom RNA Fusion Panel for Illumina	20032798
	AmpliSeq On-Demand Panel for Illumina (24 reactions, 1-50 genes)	20023977
	AmpliSeq On-Demand Panel for Illumina (24 reactions, 51-300 genes)	20023983
	AmpliSeq On-Demand Panel for Illumina (24 reactions, 301-500 genes)	20023978
	AmpliSeq On-Demand Panel for Illumina (96 reactions, 1-50 genes)	20023979
	AmpliSeq On-Demand Panel for Illumina (96 reactions, 51-300 genes)	20023980
	AmpliSeq On-Demand Panel for Illumina (96 reactions, 301-500 genes)	20023981
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
	AmpliSeq CD Indexes Large Volume for Illumina (96 Indexes, 96 Samples)	20019108
Reverse Transcriptase	[RNA] AmpliSeq cDNA Synthesis for Illumina Kit	20022654

Panel configurations with three or four primer pools require more DNA and reagents than panels with one or two primer pools.

AmpliSeq Library PLUS Configuration	Total Number of Libraries Supported (DNA and RNA)		
	One or Two Pools	Three Pools	Four Pools
24 reactions	24	16	12
96 reactions	96	64	48
384 reactions	384	256	192

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 Custom 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.

The quantity and concentration of primer pools depends on the panel. Panels with more than 96 amplicons are packaged in four duplicate boxes.

Quantity	Reagent
1–4	AmpliSeq Custom RNA or DNA Panel Pools

AmpliSeq Custom RNA Fusion 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
1–2	AmpliSeq Custom RNA Fusion Panel Pools

AmpliSeq On-Demand 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	AmpliSeq On-Demand DNA Panel Pool 1
1	AmpliSeq On-Demand DNA Panel Pool 2

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), AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples), or AmpliSeq CD Indexes Large Volume (96 indexes, 96 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 On-Demand, Custom, and Community Panels 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 Sample ID Panel for Illumina	Illumina, catalog # 20019162
[Optional]AmpliSeq ERCC RNA Spike-In Mix for Illumina	Illumina, catalog # 20030696
[Optional] AmpliSeq ERCC RNA Companion Panel for Illumina	Illumina, catalog # 20030696
[Optional] 50X AmpliSeq Custom DNA Panel spike-in	Illumina, catalog # 20020495
[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
[RNA] One of the following FFPE isolation kits: • RecoverAll Nucleic Acid Isolation Kit for FFPE • AllPrep DNA/RNA FFPE Kit	One of the following suppliers, depending on kit: • Thermo Fisher Scientific, catalog # AM1975 • QIAGEN, catalog # 80234
[Optional] One of the following positive sample controls: • **[DNA]** Quantitative Multiplex Reference Standard • **[DNA]** Tru-Q 2 (5% Tier) • **[DNA]** Acrometrix Oncology Hotspot Control • **[DNA]** NA12878 • **[RNA]** Universal Reference RNA • **[RNA]** Human Brain Total RNA	One of the following suppliers, depending on sample control: • Horizon, catalog # HD701 • Horizon, catalog # HD729 • Thermo Fisher Scientific, catalog # 969056 • Coriell, catalog # NA12878 • Agilent, catalog # 740000 • Ambion, catalog # AM7962
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)

   Reagent overage is included 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 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
Germany	+49 8001014940	+49 8938035677
Hong Kong, China	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	
South Korea	+82 80 234 5300	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan, China	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 from support.illumina.com.

Document # 1000000036408 v09

Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

For Research Use Only. Not for use in diagnostic procedures.

illumina®