

# AmpliSeq for Illumina Custom and Community Panels

## Reference Guide



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## Revision History

Document	Date	Description of Change
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 Ligase 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.

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# Chapter 1 Overview

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## Introduction

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

The workflow requires the following components:

- ▶ AmpliSeq Library PLUS for Illumina
- ▶ AmpliSeq Custom and Community Panels for Illumina
- ▶ AmpliSeq CD Indexes for Illumina

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.

## AmpliSeq for Illumina Panels

AmpliSeq Custom and Community 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 these panels from the Illumina website or use them as a starting point for a custom design.

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

## DNA Input Recommendations

The AmpliSeq for Illumina 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) gDNA 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 gDNA 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 gDNA preparations.

## Limited Samples

Degraded samples with average fragment sizes that are shorter than amplicon sizes can still yield AmpliSeq Custom and Community Panels libraries. Only primer pairs designed for FFPE samples are recommended for degraded samples.

## RNA Input Recommendations

The AmpliSeq for Illumina 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

Visit the AmpliSeq for Illumina Custom and Community Panels support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

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

Resource	Description
Custom Protocol Selector	<a href="https://support.illumina.com/custom-protocol-selector.html">support.illumina.com/custom-protocol-selector.html</a> A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
<i>AmpliSeq for Illumina Custom and Community Panels DNA Checklist (document # 1000000039391)</i>	Provides a checklist of the DNA protocol steps. The checklist is intended for experienced users.
<i>AmpliSeq for Illumina Custom and Community Panels RNA Checklist (document # 1000000047981)</i>	Provides a checklist of the RNA protocol steps. The checklist is intended for experienced users.
<i>AmpliSeq for Illumina Custom and Community Panels Consumables &amp; Equipment List (document # 1000000039393)</i>	Provides an interactive checklist of user-provided consumables and equipment.
<i>Index Adapters Pooling Guide (document # 1000000041074)</i>	Provides pooling guidelines and dual indexing strategies for AmpliSeq for Illumina libraries.

# Chapter 2 Protocol for DNA Panels

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## Introduction

This chapter describes the AmpliSeq for Illumina protocol for DNA.

- ▶ Review best practices before proceeding. See *Additional Resources on page 3* for information on accessing Best Practices on the Illumina website.
- ▶ Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information on page 39*.
- ▶ 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<sup>®</sup> 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.

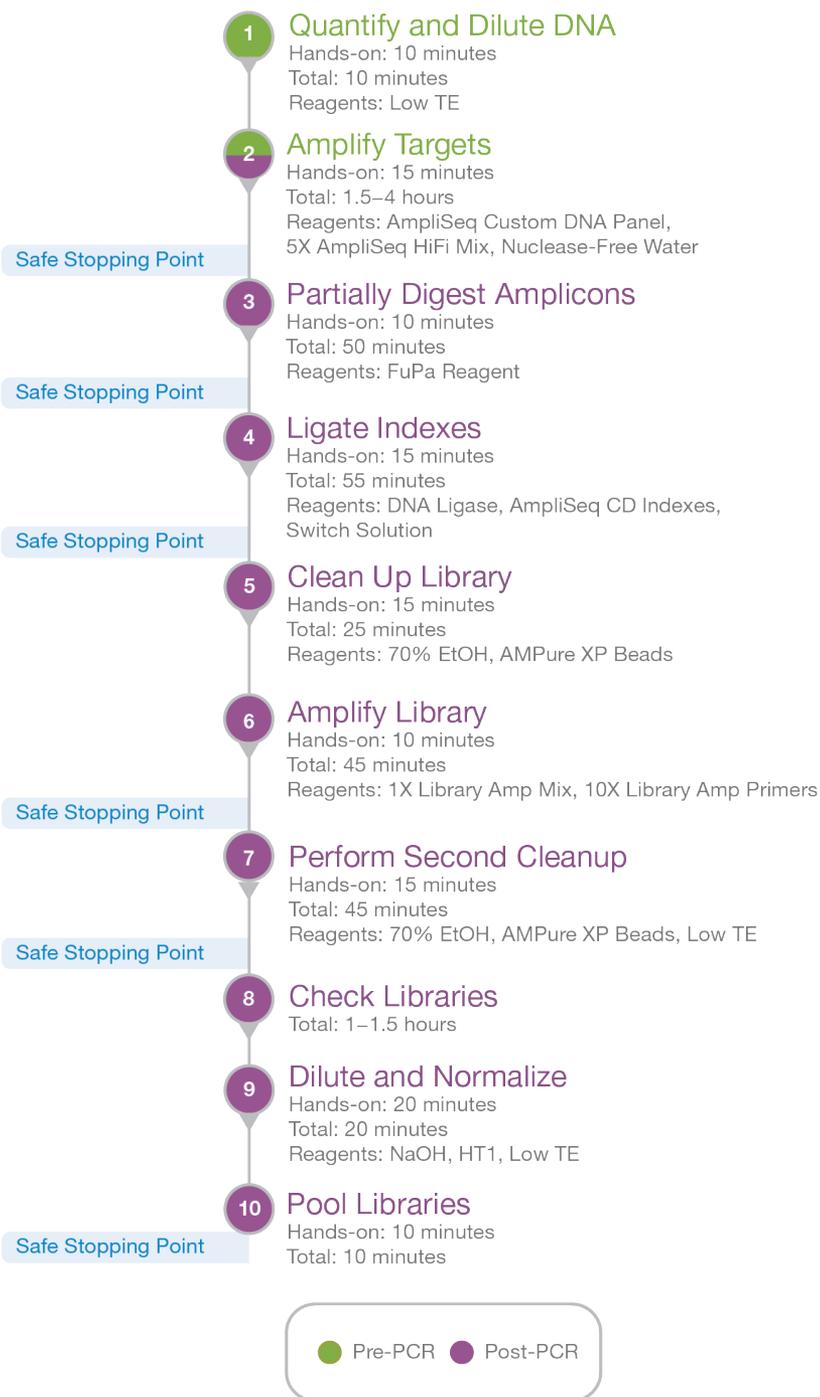
## Handling Beads

- ▶ Pipette bead suspensions slowly.
- ▶ Before use, allow the beads to come to 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 DNA workflow. Safe stopping points are marked between steps.

**Figure 1** AmpliSeq for Illumina 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.

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).

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)

- ▶ 5X AmpliSeq HiFi Mix (red cap)
- ▶ 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)	-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 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 *X Cycles and X Minutes* without adjustments.
- ▶ If you are using a different input amount or low-quality DNA, adjust X number of cycles and X minutes per the *Adjustments to Thermal Cycler Program* table.

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

**Table 3 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

**Table 4 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 Add the following volumes to one well of a new 96-well PCR plate. For multiple samples, combine all reagents except DNA in a 1.5 ml tube to prepare a master mix. Add 10% extra volume of each reagent to account for pipetting errors. Pipette or vortex briefly, and then centrifuge briefly. Pipette master mix into each well of the plate, and then add DNA.

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	4
2X AmpliSeq Custom DNA Panel	10
DNA (1–100 ng)	≤ 6
Nuclease-free water	To reach total required volume
<b>Total Volume</b>	<b>20</b>

- 2 Seal the plate, and then centrifuge briefly.
- 3 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP\_DNA program.
- 4 Proceed to *Partially Digest Amplicons* on page 29.

### 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

- Combine the following volumes per sample in a 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
<b>Total Volume</b>	<b>12.5</b>

Extra volume is prepared to account for small pipetting errors.

- Pipette to mix, and then centrifuge briefly.
- Transfer each sample from the tube to a new PCR plate as follows.
  - Transfer 5 µl master mix to one well.
  - Transfer 5 µl of the same master mix to a second well.
  - Add 5 µl 2X AmpliSeq Custom DNA Pool 1 to the first well.
  - Add 5 µl 2X AmpliSeq Custom DNA Pool 2 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.

- Seal the plate, and then centrifuge briefly.
- Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP\_DNA program.
- Proceed to *Partially Digest Amplicons* on page 29.

### 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

- Combine the following volumes per sample in a 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
<b>Total Volume</b>	<b>17.5</b>

Extra volume is prepared to account for small pipetting errors.

- Pipette to mix, and then centrifuge briefly.

- 3 Transfer each sample from the tube to a new PCR plate as follows.
  - a Transfer 5  $\mu$ l master mix to one well.
  - b Transfer 5  $\mu$ l of the same master mix to a second well, and then transfer another 5  $\mu$ l to a third well.
  - c Add 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 1 to the first well.
  - d Add 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 2 to the second well, and 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 3 to the third well.

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

- 4 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.
- 6 Proceed to *Partially Digest Amplicons* on page 29.

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

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

Extra volume is prepared to account for small pipetting errors.

- 2 Pipette to mix, and then centrifuge briefly.
- 3 Transfer each sample from the tube to a new PCR plate as follows.
  - a Transfer 5  $\mu$ l master mix to four wells of the plate so that each of the four wells contains 5  $\mu$ l of the same master mix.
  - b Add 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 1 to the first well, and 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 2 to the second.
  - c Add 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 3 to the third well, and 5  $\mu$ l 2X AmpliSeq Custom DNA Pool 4 to the fourth.

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

- 4 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.

## 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 for one or two primer pools, 33 µl for three primer pools, or 44 µl for four primer pools
  - ▶ 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 Centrifuge briefly to collect contents, and then unseal.
- 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

Number of Primer Pools	FuPa Volume ( $\mu$ l)
3	3
4	4

The total volume per sample is 22  $\mu$ l, 33  $\mu$ l, or 44  $\mu$ l.

- 4 Seal the plate.
- 5 Vortex briefly, and then centrifuge briefly.
- 6 Place on the thermal cycler, cover with a compression pad (if applicable) and run the FUPA program.

### SAFE STOPPING POINT

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

## Ligate Indexes

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

- 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. When adding AmpliSeq CD Indexes, 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 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	2	3	4
3	DNA Ligase (blue cap)	2	3	4
	<b>Total Volume</b> (including 22 µl digested amplicons)	<b>30</b>	<b>45</b>	<b>60</b>



### CAUTION

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

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

### SAFE STOPPING POINT

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

## Clean Up Library

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

### Consumables

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

### About Reagents

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

## Preparation

- 1 Prepare the following consumables:

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

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

## Procedure

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

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

Number of Primer Pools	Bead Volume ( $\mu$ l)
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 two times as follows.
- Add 150  $\mu$ l freshly prepared 70% EtOH to each well.
  - Incubate at room temperature until the solution is clear (~30 seconds).
  - 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.
- Use a 20  $\mu$ l pipette to remove residual EtOH from each well.
  - Air-dry on the magnetic stand for 10 minutes. Leave uncovered.
  - Inspect each well to make sure that the EtOH has completely evaporated.
  - 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.

## 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
<b>Total Volume per reaction</b>	<b>50</b>

- 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 gDNA 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 10 ml fresh 70% EtOH from absolute ethanol.  
This volume is sufficient to clean up 24 reactions.

### Procedure

- 1 Briefly centrifuge the plate to collect contents, and then unseal.
- 2 Add 25  $\mu$ l AMPure XP beads to each well containing  $\sim$ 50  $\mu$ l 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 ( $\sim$ 5 minutes).
- 6 Unseal the plate.
- 7 Transfer the *entire* supernatant ( $\sim$ 75  $\mu$ l), *which contains the desired amplicon library*, to a new plate.  
Small amounts of bead carryover do not affect performance.

- 8 Add 60  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ .

## 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  $\mu$ l undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
  - ▶ Analyze 1  $\mu$ l library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.

- 3 [Optional] Determine quantity using smear analysis for Fragment Analyzer or region analysis for Bioanalyzer.

## Quantify Library

- 1 Quantify the library using one of the following methods:
  - ▶ Analyze 2  $\mu$ l undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
  - ▶ Analyze 1  $\mu$ l library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
  - ▶ Analyze 2  $\mu$ l 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  $\mu$ l library using the AccuClear Ultra High Sensitivity dsDNA Quantitation Kit.
  - ▶ Analyze 2  $\mu$ l library using the Quant-iT PicoGreen dsDNA Assay Kit.

## Dilute and Normalize Libraries

### Prepare Reagents

#### Prepare a Fresh Dilution of NaOH

- 1 Combine the following volumes in a microcentrifuge tube:
  - ▶ Laboratory-grade water (800  $\mu$ l)
  - ▶ Stock 1.0 N NaOH (200  $\mu$ l)
 The result is 1 ml of 0.2 N NaOH.
- 2 Invert the tube several times to mix.



#### NOTE

Use the fresh dilution within **12 hours**.

#### Prepare HT1

- 1 Remove HT1 from -25°C to -15°C storage and thaw at room temperature.
- 2 Store at 2°C to 8°C until you are ready to dilute denatured libraries.

#### Prepare Low TE

- 1 If frozen, remove Low TE from -25°C to -15°C storage and thaw at room temperature.
- 2 Store thawed Low TE at room temperature until you are ready to dilute libraries.

## Dilute Libraries

- 1 In a new 96-well LoBind PCR plate, dilute each library to 2 nM using Low TE. Libraries that contain shorter amplicons cluster more efficiently. When a range of loading concentrations is possible, start on the lower end of the range to optimize cluster density.

## Pool Libraries

- 1 Transfer equal volumes of each 2 nM library from the plate to a 1.5 mL LoBind tube.

- 2 Vortex each tube to mix.
- 3 Centrifuge each tube briefly.

## Denature Libraries

- 1 Combine the following volumes of library and freshly diluted 0.2 N NaOH in a microcentrifuge tube.

Reagent	Volume (µl)
Pooled libraries	10
0.2 N NaOH	10

- 2 Vortex briefly and then centrifuge briefly.
- 3 Incubate at room temperature for 5 minutes.
- 4 Add 10 µl 200 mM Tris-HCl, pH 7.0 to the tube containing 2 nM pooled libraries.
- 5 Vortex briefly and then centrifuge briefly.

## Dilute Denatured Libraries to 20 pM

- 1 Add 970 µl prechilled HT1 to the tube of 2 nM denatured library pool. The result is a 20 pM denatured library.
- 2 Vortex briefly and then centrifuge briefly.
- 3 Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

## Dilute Libraries to Final Loading Concentration

Final loading concentration and volume vary depending on library preparation, quantification methods, and sequencing instrument. For information about the number of libraries supported per sequencing run, refer to the AmpliSeq for Illumina Custom and Community Panels support page on the Illumina website.

- 1 Use prechilled HT1 to dilute the denatured 20 pM library solution to the desired final loading concentration.

**Table 5 Suggested Final Loading Concentrations**

Quantification system	Suggested Final Loading Concentration (pM)		
	MiSeq	MiniSeq	NextSeq
Bioanalyzer	~7	~1.1	~1.1
Fragment Analyzer	~7	~1.1	~1.1
Accuclear	~7	~1.1	~1.1
PicoGreen	~8.4	~1.3	~1.3
Qubit HS	~8.4	~1.3	~1.3
qPCR (size adjusted)	~18	~3.0	~3.0

- 2 Invert to mix and then centrifuge briefly.

### SAFE STOPPING POINT

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

# Chapter 3 Protocol for RNA Panels

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## Introduction

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

- ▶ Review best practices before proceeding. See [Additional Resources on page 3](#) for information on accessing Best Practices on the Illumina website.
- ▶ Confirm kit contents and make sure that you have the required equipment and consumables. See [Supporting Information on page 39](#).
- ▶ 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<sup>®</sup> 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.

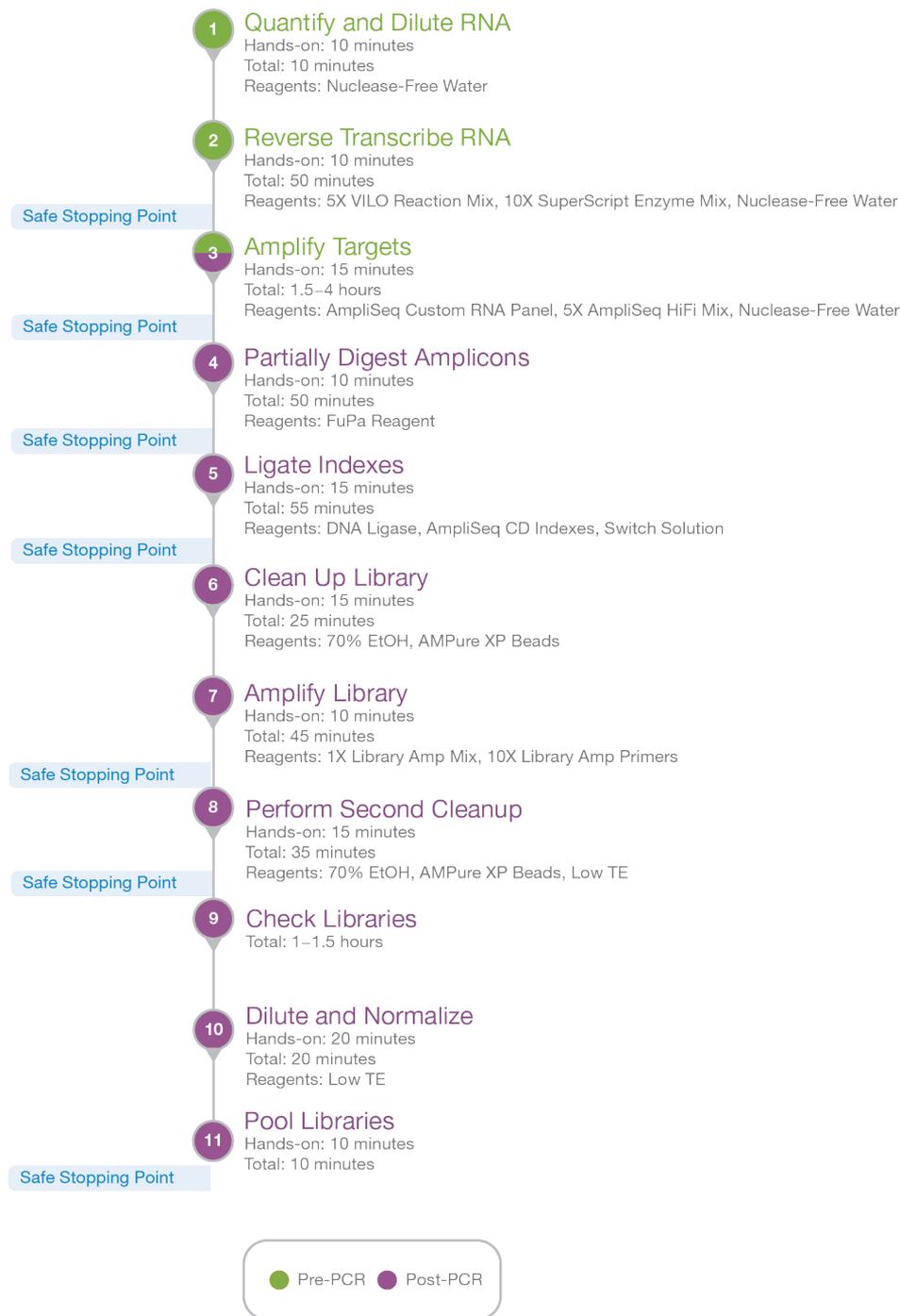
## Handling Beads

- ▶ Pipette bead suspensions slowly.
- ▶ Before use, allow the beads to come to 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 Custom and Community Panels RNA workflow. Safe stopping points are marked between steps.

**Figure 2** AmpliSeq 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 2](#).  
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 SuperScript VILO cDNA Synthesis Kit to reverse transcribe RNA to cDNA.

### Consumables

- ▶ Total RNA (1–100 ng per pool)
- ▶ SuperScript VILO cDNA Synthesis Kit
- ▶ 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:

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

## 3 Save the following RT program a the 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, prepare a master mix without RNA in a 1.5 ml tube.

- ▶ 5X VILO Reaction Mix (2 µl)
- ▶ 10X SuperScript Enzyme Mix (1 µl)
- ▶ Total RNA (1–100 ng per pool) (≤ 7 µl)
- ▶ Nuclease-free water (to 10 µl)

These volumes result in 10 µl sample master mix 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.

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 HiFi Mix (red cap)
- ▶ 5X AmpliSeq Custom RNA Panel (1–2 pools) (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 Custom RNA Panel (red cap)	-25°C to -15°C	Thaw at room temperature. Vortex briefly, then centrifuge briefly.

- 2 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 *X Cycles and X Minutes* without adjustments.
- ▶ If you are using a different input amount or low-quality RNA, adjust X number of cycles and X minutes per the *Adjustments to Thermal Cycler Program* table.

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

**Table 6 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 7 Adjustments to Thermal Cycler Program**

Condition	Adjustment
The panel has two primer pools in different cycle categories.	Use the greater number of cycles.

Condition	Adjustment
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.

## Procedure for One Primer Pool

- Briefly centrifuge the plate to collect contents, and then unseal.
- Add the following volumes per sample to each well containing 10  $\mu$ l cDNA. For multiple samples, prepare a master mix in a 1.5 ml tube.
  - ▶ 5X AmpliSeq HiFi Mix (4  $\mu$ l) (red cap)
  - ▶ 5X AmpliSeq Custom RNA Panel (4  $\mu$ l) (red cap)
  - ▶ Nuclease-free water (2  $\mu$ l)
 Each well contains 20  $\mu$ l cDNA and master mix.
- Pipette to mix.
- Seal the plate, and then centrifuge briefly.
- Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP\_RNA program.
- Proceed to *Partially Digest Amplicons* on page 29.

### 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

- Briefly centrifuge the plate to collect contents, and then unseal.
- Add the following volumes per sample to each well containing 10  $\mu$ l cDNA. For multiple samples, prepare a master mix in a 1.5 ml tube.
  - ▶ 5X AmpliSeq HiFi Mix (4.5  $\mu$ l) (red cap)
  - ▶ Nuclease-free water (3.5  $\mu$ l)
 Each well contains 18  $\mu$ l cDNA and master mix.
- Pipette to mix.
- Seal the plate, and then centrifuge briefly.
- Transfer each sample from the tube to a new PCR plate as follows.
  - a Transfer 8  $\mu$ l master mix to one well.
  - b Transfer 8  $\mu$ l of the same master mix to a second well.
  - c Add 2  $\mu$ l 5X AmpliSeq Custom RNA Pool 1 to the first well.
  - d Add 2  $\mu$ l 5X AmpliSeq Custom RNA Pool 2 to the second well.
 Each of the two wells contains 8  $\mu$ l sample master mix and 2  $\mu$ l primer pool for a total of 10  $\mu$ l per well.
- Seal the plate.
- 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.

**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 Centrifuge briefly to collect contents, and then unseal.
- 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 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
- ▶ 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	-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.
Agencourt AMPure XP beads	2°C to 8°C	Bring to room temperature in preparation for a later procedure. Vortex thoroughly to resuspend.

- 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, 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	2
3	DNA Ligase (blue cap)	2
<b>Total Volume</b> (including 22 µl digested amplicons)		<b>30</b>



### CAUTION

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

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

## Clean Up Library

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

### Consumables

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

### About Reagents

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

## Preparation

- 1 Prepare the following consumables:

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

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

## Procedure

- 1 Briefly centrifuge the plate to collect contents, and then unseal.
- 2 Add 30  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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.

## 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  $\mu$ l
- ▶ 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
<b>Total Volume per reaction</b>	<b>50</b>

- 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 gDNA 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 10 ml fresh 70% EtOH from absolute ethanol.  
This volume is sufficient to clean up 24 reactions.

## Procedure

- 1 Briefly centrifuge the plate to collect contents, and then unseal.
- 2 Add 25  $\mu$ l AMPure XP beads to each well containing  $\sim$ 50  $\mu$ l 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 ( $\sim$ 5 minutes).
- 6 Unseal the plate.
- 7 Transfer the *entire* supernatant ( $\sim$ 75  $\mu$ l), *which contains the desired amplicon library*, to a new plate.  
Small amounts of bead carryover do not affect performance.
- 8 Add 60  $\mu$ l 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 ( $\sim$ 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  $\mu$ l freshly prepared 70% EtOH to each well.
  - b Incubate at room temperature until the solution is clear ( $\sim$ 30 seconds)
  - c Without disturbing the pellet, remove and discard supernatant.
- 15 Use a 20  $\mu$ l 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  $\mu$ l 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 ( $\sim$ 5 minutes).

- 21 Unseal the plate.
- 22 Transfer 27  $\mu$ l 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  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ .

## 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  $\mu$ l undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
  - ▶ Analyze 1  $\mu$ l library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
- 3 [Optional] Determine library quantity using smear analysis for Fragment Analyzer or region analysis for Bioanalyzer.

### Quantify Library

- 1 Quantify the library using one of the following methods:
  - ▶ Analyze 2  $\mu$ l undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
  - ▶ Analyze 1  $\mu$ l library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
  - ▶ Analyze 2  $\mu$ l 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  $\mu$ l library using the AccuClear Ultra High Sensitivity dsDNA Quantitation Kit.
  - ▶ Analyze 2  $\mu$ l library using the Quant-iT PicoGreen dsDNA Assay Kit.

## Dilute and Normalize Libraries

### Prepare Reagents

#### Prepare a Fresh Dilution of NaOH

- 1 Combine the following volumes in a microcentrifuge tube:
  - ▶ Laboratory-grade water (800  $\mu$ l)
  - ▶ Stock 1.0 N NaOH (200  $\mu$ l)

The result is 1 ml of 0.2 N NaOH.

- 2 Invert the tube several times to mix.



#### NOTE

Use the fresh dilution within **12 hours**.

## Prepare HT1

- 1 Remove HT1 from -25°C to -15°C storage and thaw at room temperature.
- 2 Store at 2°C to 8°C until you are ready to dilute denatured libraries.

## Prepare Low TE

- 1 If frozen, remove Low TE from -25°C to -15°C storage and thaw at room temperature.
- 2 Store thawed Low TE at room temperature until you are ready to dilute libraries.

## Dilute Libraries

- 1 In a new 96-well LoBind PCR plate, dilute each library to 2 nM using Low TE.  
Libraries that contain shorter amplicons cluster more efficiently. When a range of loading concentrations is possible, start on the lower end of the range to optimize cluster density.

## Pool Libraries

- 1 Transfer equal volumes of each 2 nM library from the plate to a 1.5 mL LoBind tube.
- 2 Vortex each tube to mix.
- 3 Centrifuge each tube briefly.

## Denature Libraries

- 1 Combine the following volumes of library and freshly diluted 0.2 N NaOH in a microcentrifuge tube.

Reagent	Volume (µl)
Pooled libraries	10
0.2 N NaOH	10

- 2 Vortex briefly and then centrifuge briefly.
- 3 Incubate at room temperature for 5 minutes.
- 4 Add 10 µl 200 mM Tris-HCl, pH 7.0 to the tube containing 2 nM pooled libraries.
- 5 Vortex briefly and then centrifuge briefly.

## Dilute Denatured Libraries to 20 pM

- 1 Add 970 µl prechilled HT1 to the tube of 2 nM denatured library pool.  
The result is a 20 pM denatured library.
- 2 Vortex briefly and then centrifuge briefly.
- 3 Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

## Dilute Libraries to Final Loading Concentration

Final loading concentration and volume vary depending on library preparation, quantification methods, and sequencing instrument. For information about the number of libraries supported per sequencing run, refer to the AmpliSeq for Illumina Custom and Community Panels support page on the Illumina website.

- 1 Use prechilled HT1 to dilute the denatured 20 pM library solution to the desired final loading concentration.

**Table 8 Suggested Final Loading Concentrations**

Quantification system	Suggested Final Loading Concentration (pM)		
	MiSeq	MiniSeq	NextSeq
Bioanalyzer	~7	~1.1	~1.1
Fragment Analyzer	~7	~1.1	~1.1
Accuclear	~7	~1.1	~1.1
PicoGreen	~8.4	~1.3	~1.3
Qubit HS	~8.4	~1.3	~1.3
qPCR (size adjusted)	~18	~3.0	~3.0

- 2 Invert to mix and then centrifuge briefly.

### SAFE STOPPING POINT

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

# Supporting Information

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## Kit Contents

The AmpliSeq for Illumina protocol requires the AmpliSeq Library PLUS kit, AmpliSeq for Illumina Custom and Community Panels, and AmpliSeq CD Indexes.

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
Indexes	AmpliSeq CD Indexes Set A for Illumina (96 Indexes, 96 Samples)	20019105

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

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 CD Indexes for Illumina Contents, Store at -25°C to -15°C

Quantity	Description
1	AmpliSeq CD Indexes Set A plate (96 indexes, 96 samples)

## Consumables and Equipment

In addition to the AmpliSeq Library PLUS, AmpliSeq CD Indexes, and AmpliSeq for Illumina Custom and Community Panels, 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
[RNA] SuperScript VILO cDNA Synthesis Kit	Thermo Fisher Scientific, catalog # 11754050
Eppendorf DNA LoBind Microcentrifuge Tubes, 1.5 ml	Fisher Scientific, catalog # 13-698-791
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific, catalog # 4306311
One of the following 96-well PCR plates: 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

Item	Supplier
One of the following kits, depending on quantification method: <ul style="list-style-type: none"> <li>• [Bioanalyzer] Agilent DNA 1000 Kit</li> <li>• [Fluorometer] [DNA] Qubit dsDNA HS Assay Kit</li> <li>• [Fluorometer] [RNA] Qubit RNA HS Assay Kit</li> <li>• [Fluorometer] [RNA] QuantiT RiboGreen RNA Assay Kit</li> <li>• [Fragment Analyzer] Standard Sensitivity NGS Fragment Analyzer Kit (1 bp – 6,000 bp)</li> <li>• [qPCR] KAPA Library Quantification Kit (Universal)</li> <li>• AccuClear Ultra High Sensitivity dsDNA Quantitation Kit</li> <li>• Quant-iT PicoGreen dsDNA Assay Kit</li> </ul>	One of the following suppliers, depending on kit: <ul style="list-style-type: none"> <li>• Agilent, catalog # 5067-1504</li> <li>• Thermo Fisher Scientific, catalog # Q32851 or Q32854</li> <li>• Thermo Fisher Scientific, catalog # Q32852 or Q32855</li> <li>• Thermo Fisher Scientific, catalog # R11490</li> <li>• Advanced Analytical Technologies, Inc., part # DNF-473</li> <li>• Kapa Biosystems, catalog # 4468802</li> <li>• Biotium, catalog # 31028</li> <li>• Thermo Fisher catalog # P11496</li> </ul>
[RNA] One of the following FFPE isolation kits: <ul style="list-style-type: none"> <li>• RecoverAll Nucleic Acid Isolation Kit for FFPE</li> <li>• AllPrep DNA/RNA FFPE Kit</li> </ul>	One of the following suppliers, depending on kit: <ul style="list-style-type: none"> <li>• Thermo Fisher Scientific, catalog # AM1975</li> <li>• QIAGEN, catalog # 80234</li> </ul>
[Optional] One of the following positive sample controls: <ul style="list-style-type: none"> <li>• [DNA] Quantitative Multiplex Reference Standard</li> <li>• [DNA] Tru-Q 2 (5% Tier)</li> <li>• [DNA] Acrometrix Oncology Hotspot Control</li> <li>• [DNA] NA 12878 Coriell DNA</li> <li>• [RNA] Universal Reference RNA</li> <li>• [RNA] Human Brain Total RNA</li> </ul>	One of the following suppliers, depending on sample control: <ul style="list-style-type: none"> <li>• Horizon, catalog # HD701</li> <li>• Horizon, catalog # HD729</li> <li>• Thermo Fisher Scientific, catalog # 969056</li> <li>• Coriell, catalog # NA12878</li> <li>• Agilent, catalog # 740000</li> <li>• Ambion, catalog # AM7962</li> </ul>
HT1 (Hybridization Buffer)	Illumina
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: <ul style="list-style-type: none"> <li>• DynaMag-96 Side Magnet</li> </ul> For use with Hard-Shell 96-Well Skirted PCR Plates: <ul style="list-style-type: none"> <li>• DynaMag-96 Side Skirted Magnet</li> </ul>	One of the following suppliers, depending on magnetic stand type: <ul style="list-style-type: none"> <li>• Thermo Fisher Scientific, catalog # 12331D</li> <li>• Thermo Fisher Scientific, catalog # 12027</li> </ul>
Fisher Scientific Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	Fisher Scientific, catalog # 14-100-143
Fragment Analyzer Automated CE System	Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10
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: <ul style="list-style-type: none"> <li>• SimpliAmp Thermal Cycler</li> <li>• Applied Biosystems 2720 Thermal Cycler</li> <li>• Veriti 96-Well Thermal Cycler</li> <li>• ProFlex 96-well PCR System</li> <li>• GeneAmp PCR System 9700<sup>2</sup> or Dual 96-well Thermal Cycler</li> </ul> Bio-Rad thermal cyclers: <ul style="list-style-type: none"> <li>• C1000 Touch Thermal Cycler</li> <li>• S1000 Thermal Cycler</li> </ul>	Thermo Fisher Scientific, see web product pages for catalog numbers  Bio-Rad: <ul style="list-style-type: none"> <li>• Part # 1851196</li> <li>• Part # 1852196</li> </ul>

Item	Supplier
<p>One of the following instruments, depending on quantification method:</p> <ul style="list-style-type: none"> <li>• [Bioanalyzer] Agilent 2100 Bioanalyzer</li> <li>• [Fluorometer] Qubit 3.0 Fluorometer or Qubit 2.0 Fluorometer<sup>2</sup></li> <li>• [Fragment Analyzer] Fragment Analyzer Automated CE System</li> <li>• [qPCR] Real-time PCR instrument<sup>1</sup></li> </ul>	<p>One of the following suppliers, depending on instrument:</p> <ul style="list-style-type: none"> <li>• Agilent, catalog # G2939AA</li> <li>• Thermo Fisher Scientific, catalog # Q33216</li> <li>• Advanced Analytical Technologies, Inc., part # FSv2-CE2 or FSv2-CE10</li> <li>• General lab supplier</li> </ul>

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

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

# Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

## Illumina Customer Support Telephone Numbers

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

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



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