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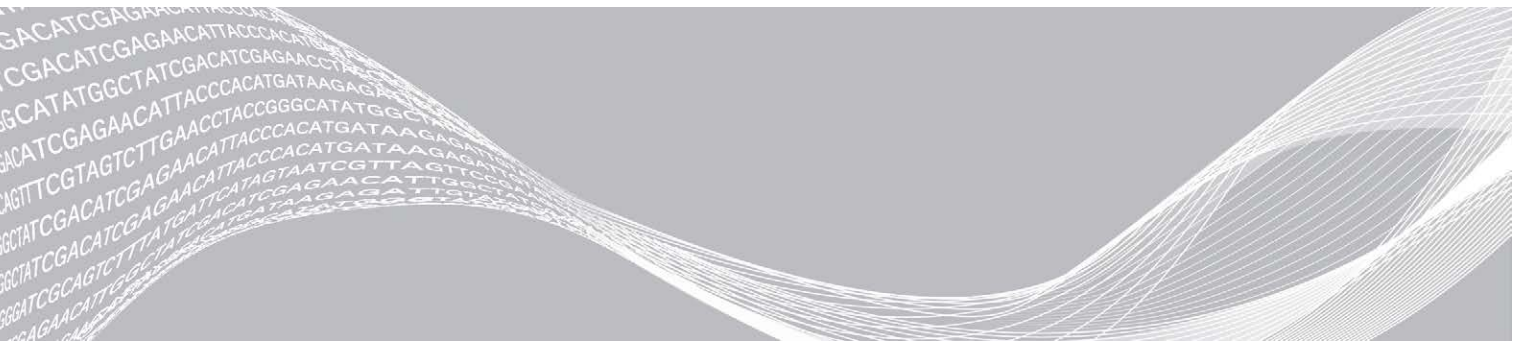
AmpliSeq for Illumina Exome Panel

Reference Guide



Document # 1000000036409 v06

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February 2019

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Revision History

Document	Date	Description of Change
Document # 1000000036409 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 instructions to vortex briefly after adding master mix in the Amplify Libraries step. 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 # 1000000036409 v05	October 2018	Added optional instructions for using the AmpliSeq Library Equalizer for Illumina.
Document # 1000000036409 v04	July 2018	Added table outlining required number of kits. Removed the following documents from the Additional Resources table: • *MiSeq System Denature and Dilute Libraries Guide* • *MiniSeq System Denature and Dilute Libraries Guide* • *iSeq 100 Sequencing System Guide Guide* Added step for transferring to the post-PCR area. Clarified difference between shipping and storage temperature for AmpliSeq CD Indexes Large Volume for Illumina and AmpliSeq Exome Panel for Illumina. Removed optional step for determining library quantity. Removed expected library yield range. Added formula for calculating molarity when using fluorometric methods.
Document # 1000000036409 v03	April 2018	Changed required kits to include AmpliSeq CD Indexes Large Volume for Illumina. Removed steps from the Ligate Indexes procedure to accommodate the AmpliSeq CD Indexes Large Volume. Changed gDNA to DNA. Added several entries to the Additional Resources table. Moved Dilute and Normalize Libraries procedure into the appropriate denature and dilute libraries guides.
Document # 1000000036409 v02	January 2018	Corrected total volume per sample in Ligate Indexes procedure. Added tube quantities for 96- and 384-reaction kits.
Document # 1000000036409 v01	January 2018	Added panel specification table. Corrected mean library yield example total input amount.
Document # 1000000036409 v00	January 2018	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

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

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

Kit	Quantity		
	8 samples	32 samples	128 samples
	8 DNA libraries	32 DNA libraries	128 DNA libraries
AmpliSeq Exome Panel for Illumina	1	4	16
AmpliSeq Library PLUS for Illumina	1 × 24-reaction kit (20019101)	1 × 96-reaction kit (20019102)	1 × 384-reaction kit (20019103)
AmpliSeq CD Indexes Large Volume for Illumina*	1	1	2
AmpliSeq UD Indexes for Illumina (24 Indexes, 24 Samples)*	1	2	6

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

NOTE

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

NOTE

The AmpliSeq Exome Panel for Illumina uses 3X the reagents for standard library prep. A 24–reaction kit is needed for eight reactions.

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

Library PLUS offers:

- Preparation of dual-index libraries for high-throughput sample multiplexing.
  - Prepare up to eight libraries from 50–100 ng of DNA with 12 pools, 24,492 amplicons per pool
- Faster and more efficient library normalization using the optional AmpliSeq Library Equalizer™ for Illumina.

Panel Specifications

Panel Name	Number of Pools	Number of Amplicons	Average Amplicon Length (bp)	Average Library Length (bp)
AmpliSeq Exome Panel for Illumina	12	293,903	205	345

DNA Input Recommendations

The AmpliSeq Exome Panel for Illumina protocol supports 50–100 ng of input DNA diluted in up to 56 µl of nuclease-free water.

- Use 100 ng DNA where DNA is not limiting.
- The AmpliSeq Exome Panel for Illumina does not support FFPE DNA samples.

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.

Additional Resources

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

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

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
AmpliSeq for Illumina Exome Panel Checklist (document # 1000000039400)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
AmpliSeq for Illumina Exome Panel Consumables & Equipment List (document # 1000000039401)	Provides an interactive checklist of user-provided consumables and equipment.
Index Adapters Pooling Guide (document # 1000000041074)	Provides pooling guidelines and dual indexing strategies for Library PLUS library prep.
NextSeq System Denature and Dilute Libraries Guide (document # 15048776)	Provides instructions on how to denature and dilute prepared libraries for sequencing on the Illumina NextSeq™ Sequencing System.

Chapter 2 Protocol

Introduction

This chapter describes the AmpliSeq for Illumina protocol.

- Confirm kit contents and make sure that you have the required equipment and consumables. See *Supporting Information* on page 18.
- The thermal cyclers recommended for this protocol require different plates, seals, and magnetic stands. Make sure that you use the appropriate compatible supplies for your thermal cycler.
- Make sure that reagents are not expired. Using expired reagents might negatively affect performance.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Do not allow more than six freeze-thaw cycles of reagents.

Tips and Techniques

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

Avoiding Cross-Contamination

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

Sealing the Plate

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

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

Plate Transfers

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

Covering the Plate

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

Vortexing and Centrifugation

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

Handling Beads

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

Library Prep Workflow

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

Figure 1 AmpliSeq Exome Panel Workflow

Quantify and Dilute DNA

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

Consumables

- Low TE
- DNA
- 1.5 ml tube

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instruction
DNA	-25°C to -15°C (long-term) 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 20 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 Dilute DNA to desired final concentration using Low TE.
Standard input is 50–100 ng high-quality DNA per sample. For more information, see *DNA Input Recommendations* on page 2.

Amplify Targets

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

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

Consumables

- 5X AmpliSeq HiFi Mix (red cap)
- DNA (50–100 ng)
- Nuclease-free water
- AmpliSeq Exome Panel plate
- 1.5 ml tube
- 8-tube strips
- MicroAmp Clear Adhesive Film

About Reagents

- HiFi Mix is viscous. Pipette slowly.
- The primers are dried and include a blue dye. Each row contains 12 primer pools for one sample.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
5X AmpliSeq HiFi Mix	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge briefly. Keep on ice during the procedure.
DNA	-25°C to -15°C	If frozen, thaw on ice. Invert or flick the thawed tubes to mix, and then centrifuge briefly.

2 Transfer to the post-PCR area.

3 Save the following AMP_10 program on a thermal cycler with a heated lid.
- Choose the preheated lid option and set to 105°C
- Set the reaction volume to 5 µl (or lowest available volume)
- 99°C for 2 minutes
- 10 cycles of:
  - 99°C for 15 seconds
  - 60°C for 16 minutes
- Hold at 10°C for up to 24 hours

Procedure

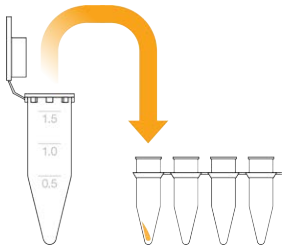
1 Combine the following volumes per sample in a 1.5 ml tube to prepare amplification master mix.
- 5X AmpliSeq HiFi Mix (14 µl)
- 50–100 ng DNA (≤ 56 µl)
- Nuclease-free water (to 70 µl)

These volumes result in 70 µl master mix per sample.

2 Pipette or vortex briefly to mix, and then centrifuge briefly.

3 For each sample, transfer 70 µl master mix into a new well of an 8-tube strip.

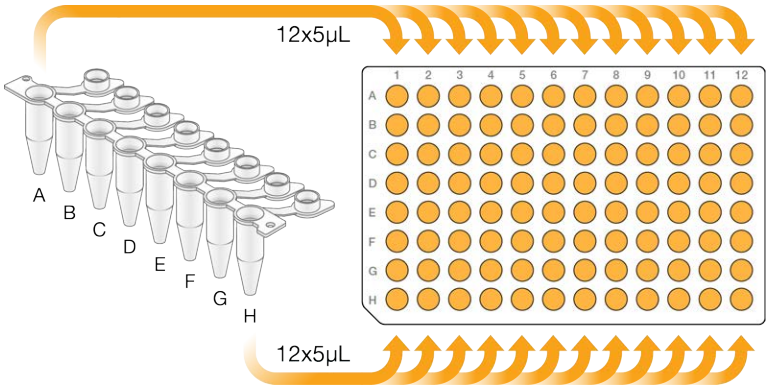
Figure 2 Transfer to 8-Tube Strip



4 Unseal the AmpliSeq Exome Panel plate.

5 For each sample, add 5 µl master mix to each well in one row of the AmpliSeq Exome Panel plate (12 wells) without changing tips.

Figure 3 Transfer to Plate



6 Seal the plate, vortex briefly, and then centrifuge briefly.

7 Place on the thermal cycler and run the AMP_10 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
- 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	-25°C to -15°C	Thaw on ice. Centrifuge briefly. Keep on ice during the procedure.
Switch Solution	-25°C to -15°C	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

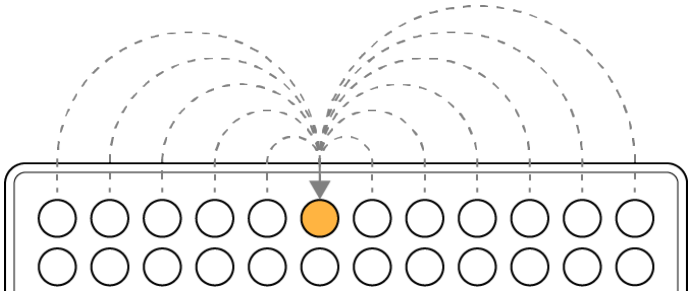
- Set the reaction volume to 60 µl
- 50°C for 20 minutes
- 55°C for 20 minutes
- 62°C for 20 minutes
- Hold at 10°C for up to one hour

Procedure

1 Briefly centrifuge to collect contents, and then unseal.

2 For each row, use a multichannel pipette to combine the 12 wells into the column 6 well, without changing tips.

Figure 4 Combine Samples in Column 6



3 Add 6 µl FuPa Reagent to each well in column 6, and then seal the plate.

4 Vortex briefly, and then centrifuge briefly.

5 Place on the thermal cycler, cover with a compression pad, and run the FUPA program.

SAFE STOPPING POINT

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

Ligate Indexes

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

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

Consumables

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

About Reagents

- DNA Ligase is viscous. Pipette slowly.
- Switch Solution is viscous. Pipette slowly.
- Beads take approximately 30 minutes to reach room temperature.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
AmpliSeq CD Indexes Large Volume or UD Indexes for Illumina	-25°C to -15°C*	Thaw at room temperature. Vortex briefly to mix, and then centrifuge.
DNA Ligase	-25°C to -15°C	Thaw on ice. Centrifuge briefly. Keep on ice during the procedure.
Agencourt AMPure XP beads	2°C to 8°C	If you are not stopping after this procedure is complete, bring to room temperature in preparation for a later procedure. Vortex thoroughly to resuspend.

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

2 Save the following LIGATE program on a thermal cycler with a heated lid:
- Choose the preheated lid option and set to 105°C
- Set the reaction volume to 80 µl
- 22°C for 30 minutes
- 72°C for 10 minutes
- Hold at 10°C for up to one hour

Procedure

1 Add the following volumes ***in the order listed*** to each well containing digested amplicons. Make sure to add DNA Ligase to the wells last. When adding AmpliSeq CD Indexes Large Volume 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.

Reagent	Volume (µl)
Switch Solution	12
AmpliSeq CD Indexes Large Volume or UD Indexes for Illumina	6
DNA Ligase	6
Total Volume per sample	**80**

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

2 Seal the plate.

3 Vortex briefly, and then centrifuge briefly.

4 Place on the thermal cycler, cover with a compression pad, and run the LIGATE 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.

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)
- Nuclease-free water
- 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.
Low TE	-25°C to -15°C	If frozen, thaw at room temperature for 20 minutes. Vortex to mix. This reagent can be stored at room temperature.

2 Prepare 5 ml fresh 70% EtOH from absolute ethanol.

Procedure

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

2 Add 80 µl AMPure XP beads to each library, and then seal the plate.

3 Vortex briefly.

4 Inspect each well to make sure that the mixture is homogeneous.

5 Centrifuge briefly.

6 Incubate at room temperature for 5 minutes.

7 Place on a magnetic stand and wait until the mixture is clear (~2 minutes). Keep on the magnetic stand until step 11.

8 Unseal the plate.

9 Remove and discard entire supernatant from each well.

10 Wash two times as follows.

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

11 Immediately seal the plate and centrifuge briefly.

12 Place on the magnetic stand, and then unseal. Make sure that the plate is returned to the same orientation on the magnet.

NOTE

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

13 Immediately remove all residual EtOH as follows.

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

CAUTION

Residual EtOH causes library prep to fail by inhibiting amplification.

14 If you are using the AmpliSeq Library Equalizer for Illumina, proceed to *Equalize Libraries* on page 21. Otherwise, continue to *Amplify Library* on page 1.

NOTE

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

Amplify Library

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

Consumables

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

About Reagents

- Beads take approximately 30 minutes to reach room temperature.

Preparation

1 Prepare the following consumables:

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

2 Save the following AMP_7 program on a thermal cycler with a heated lid:
- Choose the preheated lid option and set to 105°C
- Set the reaction volume to 50 µl
- Hold at 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 one hour

Procedure

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

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

2 Vortex briefly, and then centrifuge briefly.

3 Remove the plate from the magnetic stand.

4 Add 50 µl amplification master mix to each library well, and then seal the plate.

5 Vortex briefly, and then centrifuge briefly.

6 Place on the thermal cycler, cover with a compression pad, 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 cleanup.
- 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% ethanol (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
Low TE	15°C to 30°C	If frozen, thaw at room temperature for 20 minutes. Vortex to mix.

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

Procedure

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

2 Add 25 µl AMPure XP beads to each well containing ~50 µl library, and then seal the plate.
This step adds beads to the beads already in the reaction.

3 Vortex briefly, and then centrifuge briefly. The beads already in the reaction do not need to be fully resuspended.

4 Incubate at room temperature for 5 minutes.

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

6 Unseal the plate.

7 Transfer the *entire* supernatant (~75 µl), *which contains the desired amplicon library*, to a new plate.
Small amounts of bead carryover do not affect performance.

8 Add 60 µl AMPure XP beads to each well containing the transferred supernatant, and then seal the plate.

9 Vortex briefly, and then centrifuge briefly.

10 Incubate at room temperature for 5 minutes.

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

12 Unseal the plate.

13 Without disturbing the beads, remove and discard all supernatant from each well.
The amplicon library is captured by the beads, which remain in the wells.

14 Wash two times as follows.

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

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

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

17 Remove from the magnetic stand.

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

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

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

21 Unseal the plate.

22 Transfer 27 µl supernatant to a new LoBind PCR plate.
The supernatant contains the amplicon library.

SAFE STOPPING POINT

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

Check Libraries

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

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

Assess Library Quality

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

CAUTION

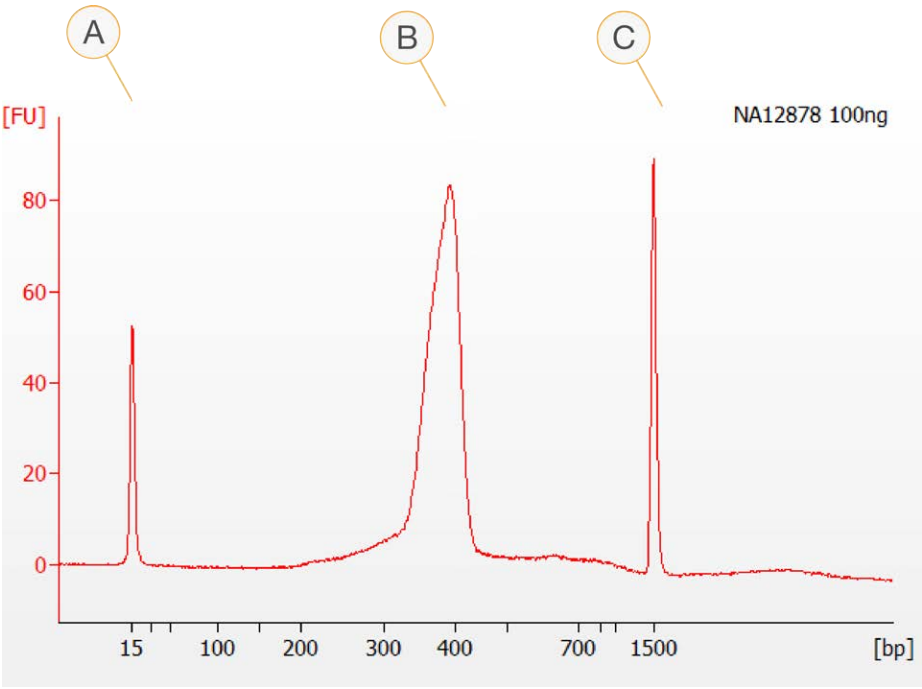
Bead carryover can affect cluster density.

2 Assess library quality using one of the following methods:

- Analyze 2 µl undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
- Analyze 1 µl library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.

Figure 5 provides an example Bioanalyzer trace of a successfully sequenced library. Typical libraries show a size distribution from 292–376 bp. The suggested size distribution for quantification is 250–450 bp.

Figure 5 Example Bioanalyzer Trace



A Lower marker
B Expected libraries
C Upper marker

Quantify Library

1 Quantify the library using one of the following methods:
- Analyze 2 µl undiluted library using the Fragment Analyzer with the Standard Sensitivity NGS Fragment Analysis Kit and *PROSize* Data Analysis Software.
- Analyze 1 µl library using the Agilent 2100 Bioanalyzer with the Agilent DNA 1000 Kit.
- Analyze 2 µl library using the Qubit 2.0 or 3.0 Fluorometer with the Qubit DNA HS Assay Kit.
- Analyze 1:10,000 diluted library using the KAPA Library Quantification Kit (Universal). For qPCR instructions, see the *Obsolete*.
- 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)
NextSeq 550 and NextSeq 500	2	1.1–1.9

3 Dilute libraries using Low TE:
- **Libraries quantified as a pool**—Dilute the pool to the starting concentration for your system.
- **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a pool.

4 Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
- For the iSeq 100 System, see the system guide for dilution instructions (libraries are automatically denatured).
- For all other systems, see the denature and dilute libraries guide.

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

Supporting Information

Kit Contents

The AmpliSeq for Illumina protocol requires the AmpliSeq Library PLUS kit for Illumina, AmpliSeq Exome Panel for Illumina, and AmpliSeq CD Indexes Large Volume or UD Indexes for Illumina.

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

Component	Kit	Catalog #
Library PLUS Kit	AmpliSeq Library PLUS for Illumina (24 reactions)	20019101
	AmpliSeq Library PLUS for Illumina (96 reactions)	20019102
	AmpliSeq Library PLUS for Illumina (384 reactions)	20019103
Panel	AmpliSeq Exome Panel for Illumina	20019166
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) AmpliSeq CD Indexes Large Volume for Illumina (96 Indexes, 96 Samples)	20019105 20019106 20019107 20019167 20019104 20019108

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 Exome Panel for Illumina Contents, Store at Room Temperature

Quantity	Component
1	AmpliSeq Exome Panel plate

AmpliSeq CD Indexes Large Volume 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 Large Volume (96 indexes, 96 samples)

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

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

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

Consumables and Equipment

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

Consumables

Item	Source
Absolute ethanol, molecular biology grade	General lab supplier
Agencourt AMPure XP	Thermo Fisher Scientific, catalog # NC9959336 or NC9933872
Eppendorf DNA LoBind Microcentrifuge Tubes, 1.5 ml	Thermo Fisher Scientific, catalog # 13-698-791
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific, catalog # 4306311
MicroAmp EnduraPlate Optical 96-Well Clear Reaction Plates with Barcode	Thermo Fisher Scientific, catalog # 4483352 or 4483354
Eppendorf twin.tec 96 Well LoBind PCR Plates, Semi-skirted	Thermo Fisher Scientific, catalog # E0030129504
MicroAmp Optical Film Compression Pad	Thermo Fisher Scientific, catalog # 4312639
Nuclease-free water	Thermo Fisher Scientific, catalog # AM9932
Pipettes, 2–200 µl, and low-retention filtered pipette tips	Thermo Fisher Scientific
8-tube strips	General lab supplier
One of the following kits, depending on quantification method: • [Bioanalyzer] Agilent High Sensitivity DNA Kit • [Fluorometer] Qubit dsDNA HS 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	One of the following suppliers, depending on instrument: • Agilent, catalog # 5067-1504 • Thermo Fisher Scientific, catalog # Q32851 or Q32854 • Advanced Analytical Technologies, Inc., catalog # DNF-473 • Kapa Biosystems, catalog # KK4824 • Biotium, catalog # 31028
[Optional] MagMax DNA Multi-Sample Kit	Thermo Fisher Scientific, catalog # 4413020
[Optional] PureLink Genomic DNA Mini Kit	Thermo Fisher Scientific, catalog # K182000

Item	Source
[Optional] NA 12878 Coriell DNA (for positive control sample)	Coriell, catalog # NA12878
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 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 • Promega, catalog #Z5342
MicroAmp Adhesive Film Applicator	Thermo Fisher Scientific, catalog # 4333183
Vortexer with 96-well plate attachment	General lab supplier
One of the following 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	Thermo Fisher Scientific, see web product pages for catalog numbers
One of the following instruments, depending on quantification method: • [Fluorometer] Qubit 3.0 Fluorometer or Qubit 2.0 Fluorometer[1] • [Bioanalyzer] Agilent 2100 Bioanalyzer • [Fragment Analyzer] Fragment Analyzer Automated CE System • [qPCR] Real-time PCR instrument[2]	One of the following suppliers, depending on instrument: • Thermo Fisher Scientific, catalog # Q32866 • Agilent G2939AA • Advanced Analytical Technologies, Inc., catalog # FSv2-CE2 or FSv2-CE10 • General lab supplier

[1] No longer available for purchase.

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

[Optional] AmpliSeq Library Equalizer for Illumina

Equalize Libraries

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

Consumables

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

Preparation

1 Prepare the following consumables:

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

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

Amplify Library

1 Remove the plate with purified libraries from the magnetic stand.

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

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

3 Vortex briefly, and then centrifuge briefly.

4 Add 50 µl amplification master mix to each library well, and then seal the plate.

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

Wash Equalizer Beads

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

   Extra volume is included here to account for small pipetting errors.

2 Pipette to mix.

3 Place on the magnetic stand and wait until the liquid is clear (~3 minutes).

4 Without disturbing the pellet, remove and discard all supernatant from the test tube.

5 Remove from the magnetic stand.

6 For each reaction, add 7 µl Equalizer Wash Buffer. Pipette to resuspend.
   These steps result in washed Equalizer Beads ready for use later in the protocol.

NOTE

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

Add Equalizer Capture

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

2 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

3 Transfer 45 µl of supernatant from each well of the library plate to the corresponding well of a new plate.

4 Add 10 µl Equalizer Capture to each well.

5 Seal the plate, vortex to mix, and then briefly centrifuge to collect contents.

6 Incubate at room temperature for 5 minutes.

Perform Second Cleanup

1 Unseal the plate.

2 Vortex or pipette washed Equalizer Beads to mix.

3 Add 6 µl Equalizer Beads to each well.

4 Seal the plate, vortex thoroughly, and then centrifuge briefly to collect contents.

5 Incubate at room temperature for 5 minutes.

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

7 Unseal the plate.

8 Without disturbing the pellet, remove and discard all supernatant from each well.

9 Wash two times as follows.

   a Add 150 µl Equalizer Wash Buffer to each well.
   b Incubate at room temperature until the solution is clear (~30 seconds).
   c Without disturbing the pellet, remove and discard supernatant.

Elute Library

1 Remove the plate from the magnetic stand.

2 Add 30 µl Equalizer Elution Buffer to each well.

3 Seal the plate, vortex thoroughly, and then centrifuge briefly to collect contents.

4 Elute the library by incubating on a thermal cycler at 45°C for 5 minutes.

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

6 Unseal the plate.

7 Transfer 27 µl supernatant to a new LoBind PCR plate.
The supernatant contains the amplicon library.

SAFE STOPPING POINT

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

Denature and Dilute Libraries

1 Denature and dilute libraries for loading on the sequencing instrument you are using.
For detailed instructions, refer to the system guide or denature and dilute libraries guide for your sequencing instrument.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
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Illumina Customer Support Telephone Numbers

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

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

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