

Notices

Limitations of use

For research use only. Not for use in diagnostic procedures. Unless otherwise agreed to in writing, IDT does not intend these products to be used in clinical applications and does not warrant their fitness or suitability for any clinical diagnostic use. Purchaser is solely responsible for all decisions regarding the use of these products and any associated regulatory or legal obligations.

Safety data sheets pertaining to the products used in this protocol, please contact the original product manufacturer.

Safety Notices



Reminder symbols call attention to minor details that may be easily overlooked and compromise the procedure resulting in decreased assay performance.



Caution symbols denote critical steps in the procedure where risk of protocol failure or damage to the product itself could occur if not carefully observed.



Stop symbols indicate where this procedure may be safely suspended and resumed at a later time without risk of compromised assay performance. Make note of these steps and plan your workflow accordingly.

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Revision history

Document Number	Date	Description of change
<i>PRO036.1</i>	August 2022	<ul style="list-style-type: none"> Updated and reformatting to Archer branding. Updated estimated fragment lengths. Added list of reagents.
<i>PRO036.2</i>	March 2023	<ul style="list-style-type: none"> Updated formatting and branding.
<i>RA-DOC-054/REV01</i>	June 2023	<ul style="list-style-type: none"> Updated document number from PRO036 to RA-DOC-054. Updated formatting and branding.
<i>RA-DOC-054/REV02</i>	November 2023	<ul style="list-style-type: none"> Updated typo in step 4b of “MiSeq Loading Guidelines” section. Updated typos in step 3 of “MiSeq Loading Guidelines” and “NextSeq 500/550 Loading Guidelines” sections. Updated formatting and branding. Updated links and email addresses.
<i>RA-DOC-054/REV03</i>	June 2024	<ul style="list-style-type: none"> Added loading recommendations for NextSeq2000 platform.



Protocol

Quantify, Normalize, and Sequence for Illumina®

Technical support

Contact us directly at archer-tech@idtdna.com.

Overview

Intended use

This protocol is intended for research applications in conjunction with Archer[™] reagent kits and corresponding target-enrichment panels to produce high-complexity libraries which are sequenced using the Illumina next-generation sequencing (NGS) platforms.

Sequencing data produced by this method should be processed using Archer Analysis software—a complete bioinformatics suite that leverages Anchored Multiplex PCR (AMP[™]) chemistry to identify unique sequence fragments, thus enabling error correction, read deduplication, and ultimately high-confidence alignment and mutation calling. Archer Analysis takes demultiplexed FASTQ files straight from the sequencer as input and produces both high-level and detailed mutation reporting, as well as raw text and BAM outputs for full transparency of the pipeline.



Materials required, but not supplied have been optimized for proper application of the Archer Library preparation. Use of other materials have not been tested by IDT.

Materials Required, but not Supplied		
Description	Supplier	Part Number
1 M Tris-HCl, pH 8.0 (molecular biology grade)	Various	-
Ultrapure water (molecular biology grade)	Various	-
200 mM Tris-HCl, pH 7.0 (for sequencing)	Various	-
Concentrated NaOH solution (ACS grade)	Various	-
RNase AWAY™	Thermo Fisher Scientific	7003
KAPA Universal Library Quantification Kit	KAPA Biosystems	KK4824
MiSeq® or NextSeq® Reagent Kit (300 cycle minimum)	Illumina	-
PhiX Control v3	Illumina	FC-110-3001
Real-Time PCR thermal cycler	Various	-
qPCR tubes	Various	-
0.2 mL PCR tubes	Various	-
Microcentrifuge	Various	-
Plate centrifuge	Various	-
Pipettes (P10, P20, P200 and P1000)	Pipetman or equivalent	-
Sterile, nuclease-free aerosol barrier pipette tips	Various	-
Vortex mixer	Various	-
Gloves	Various	-

Before getting started



Important precautions

- **Read through the entire protocol before starting.**
- Use good laboratory practices to prevent cross-contamination of samples.
- Use nuclease-free PCR tubes, microcentrifuge tubes, and aerosol-barrier pipette tips.
- Wipe down workstation and pipettes with nuclease and nucleic acid cleaning products (e.g., RNase AWAY, Thermo Fisher Scientific).
- Review sequencer specific information from the manufacturer prior to starting.

Reagents to prepare before starting

- Make **20 pM** denatured PhiX according to Illumina's recommendations.
- Make at least 1.5 mL fresh 10 mM Tris-HCl, pH 8.0 from 1M Tris-HCl, pH 8.0 and ultrapure water.
 - Mix **15 µL** of 1M Tris-HCl, pH 8.0 with **1,485 µL** ultrapure water.
- Make 200 mM Tris-HCl, pH 7.0 from 1M Tris-HCl, pH 7.0 and ultrapure water.
 - Mix **200 µL** of 1M Tris-HCl, pH 7.0 with **800 µL** ultrapure water. Vortex to mix.
- Make **0.2M** NaOH from concentrated NaOH and ultrapure water.
 - If starting with 1M NaOH, mix **200 µL** of 1 M NaOH with **800 µL** of ultrapure water. Vortex to mix.
 - If starting with 5 M NaOH, mix **40 µL** of 5 M NaOH with **960 µL** of ultrapure water. Vortex to mix.



Note: 0.2M NaOH needs to be prepared freshly to help ensure successful sequencing.

Protocol

Quantify, Normalize, and Sequence

Quantify

1. Quantify the concentration of each library by qPCR following the instructions provided with the KAPA Universal Library Quantification Kit.



Note: Archer libraries are very concentrated and need to be diluted via a dilution series to 1:10,000-1:250,000 for quantification with KAPA qPCR.

2. The Illumina Platform Loading Calculator spreadsheet can be used for quantifying, normalizing, and sequencing calculations.
3. The recommended size-adjustment correction factor for calculating the concentration of a library is dependent on the input type and quality. The estimated fragment size values below account for adapter size (157 bp) plus the average insert fragment length.
 - a. For VARIANTPlex™ libraries made with DNA or total nucleic acid (TNA) extracted from:
 - i. High quality inputs (e.g., fresh tissue) should use an estimated fragment size of 407 bp.
 - ii. Low quality inputs (e.g., FFPE tissue) should use an estimated fragment size of 257 bp.
 - b. For FUSIONPlex™ libraries made with RNA/TNA inputs, use an estimated fragment size of 357 bp.
 - c. For LIQUIDPlex™ libraries made with cf/ctDNA inputs, use an estimated fragment size of 307 bp.
 - d. For IMMUNOVerser™ libraries made with RNA/TNA inputs, use an estimated fragment size of 407 bp.



Note: Quantification with a fluorometric or electrophoresis-based method is not recommended.

Normalize and Pool

4. After library quantification, dilute each library to a target concentration of 5-10 nM using 10 mM Tris-HCl, pH 8.0 so that they are equimolar.
5. To acquire the desired read depth for each sample, combine the appropriate amount of each diluted library. This is the normalized library pool.
 - a. The recommended minimum normalized library pool volume is 50 µL. This is to enable both quantification of the normalized library pool and loading of the sequencer.
6. It is recommended to perform quantification of the normalized library pool using the KAPA Universal Library Quantification Kit. To do so, dilute the normalized library pool via a dilution series to 1:10,000 and follow the instructions provided with the KAPA Universal Library Quantification Kit.
 - a. When using the Illumina Platform Loading Calculator spreadsheet, the normalized library pool concentration (nM) should be used in the “Sequencer Loading Sheet” tab to calculate the correct denature and dilution ratios.

Sequence

Loading recommendations for Illumina MiSeq and NextSeq 500/550 platforms are provided below. While example calculations are included, **the final loading concentration must be optimized by each user.** For sequencing recommendations using other Illumina platforms, please contact archer-tech@idtdna.com for more information.

7. Prepare the sequencer according to manufacturer instructions.

For reference sample sheets, index information and additional recommendations, please contact archer-tech@idtdna.com for more information.

8. Use the read lengths in the table below.

Product Line	Number of Cycles	
	VARIANTPlex, FUSIONPlex, LIQUIDPlex, IMMUNOVerser TCR	IMMUNOVerser BCR
Read 1 (R1)	151	301
Index Read 1 (R2)	8	8
Index Read 2 (R3)	If using lyophilized adapters set to 8 If using liquid adapters set to 10	
Read 2 (R4)	151	301



Note: The recommended amount of PhiX depends on the complexity and diversity of the normalized library pool. Please consult archer-tech@idtdna.com if unsure of the optimal amount of PhiX to use.

MiSeq Loading Guidelines

Loading recommendations for Miseq

Illumina MiSeq kit version	V2	V3
Loading concentration of final pool (pM)	6-10*	6-20*
% PhiX recommended	5*	

*Each lab should empirically determine their optimal loading conditions. If libraries were prepared using liquid adapters, PhiX spike-in can be reduced to 1-2%.

1. Dilute normalized library pool to 4 nM using ultrapure water or 10 mM Tris-HCl, pH 8.0.
2. Denature 4 nM normalized library pool.
 - a. Combine 10 µL of the 4 nM normalized library pool with 10 µL 0.2M NaOH in a 1.5 mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at room temperature.

4 nM normalized library pool (µL)	10
0.2M NaOH (µL)	10

3. Neutralize and dilute to 40 pM denatured library pool.
 - a. Add 10 µL 200 mM Tris-HCl, pH 7.0 and vortex briefly to mix. Add 970 µL ice-cold Illumina Hybridization Buffer (HT1) and vortex briefly to mix.

Normalized library pool + NaOH from Step 2 (µL)	20
200mM Tris-HCl, pH 7.0 (µL)	10
Hybridization Buffer (µL)	970

4. Add denatured PhiX and dilute to loading concentration.
 - a. Combine denatured PhiX with the 40 pM denatured library pool so that the desired % PhiX is achieved. Dilute to the desired loading concentration.
 - b. Example for a 10 pM library with 5% PhiX. Combine in a new 1.5 mL microcentrifuge tube and vortex briefly to mix.

Desired loading concentration (pM)	10
40 pM denatured library pool from Step 3 (µL)	237.5

20 pM denatured PhiX (μL)	25
Hybridization Buffer (μL)	737.5

5. Load final pool into cartridge.
 - a. Spin down and load the entire volume (1 mL) of this final pool into the appropriate well of the MiSeq cartridge.

Final pool (μL)	1000
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NextSeq 500/550 Loading Guidelines

Loading recommendations for NextSeq 500/550

Illumina NextSeq 500/550 kit type	Mid	High
Loading concentration of final pool (pM)	1.0 – 1.5*	1.3 – 1.8*
% PhiX recommended	20*	

*Each lab should empirically determine their optimal loading conditions. If libraries were prepared using liquid adapters, PhiX spike-in can be reduced to 1-2%.

1. Dilute normalized library pool to 4 nM using ultrapure water or 10 mM Tris-HCl, pH 8.0
2. Denature 4 nM normalized library pool.
 - a. Combine 10 μL of the 4 nM normalized library pool with 10 μL 0.2M NaOH in a 1.5 mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at room temperature.

4 nM normalized library pool (μL)	10
0.2M NaOH (μL)	10

3. Neutralize and dilute to 40 pM denatured library pool.
 - a. Add 10 μL 200 mM Tris-HCl, pH 7.0 and vortex briefly to mix. Add 970 μL ice-cold Illumina Hybridization Buffer (HT1) and vortex briefly to mix.

Normalized library pool + NaOH from Step 2 (μL)	20
200mM Tris-HCl, pH 7.0 (μL)	10
Hybridization Buffer (μL)	970

4. Add denatured PhiX and dilute to loading concentration.
 - a. Combine denatured PhiX with the 40 pM denatured library pool so that the desired % PhiX is achieved. Dilute to the desired loading concentration.

- b. Examples for a 1.2 pM (Mid output) and 1.5 pM (High output) library with 20% PhiX. Combine in a new 1.5 mL microcentrifuge tube and vortex briefly to mix.

Desired loading concentration (pM)	1.2	1.5
40 pM denatured library pool from Step 3 (µL)	31	39
20 pM denatured PhiX (µL)	16	19
Hybridization Buffer (µL)	1253	1242

5. Load final pool into cartridge.

- a. Spin down and load the entire volume (1.3 mL) of this final pool into the appropriate well of the NextSeq 500/550 cartridge.

Final pool (µL)	1300
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NextSeq 2000 Loading Guidelines

Loading recommendations for NextSeq 2000

Illumina NextSeq 2000 kit type	P1 / P2 / P3 / P4
Loading concentration of final pool (pM) – on board denaturation	650*
% PhiX recommended	20*

*Each lab should empirically determine their optimal loading conditions. If libraries were prepared using liquid adapters, PhiX spike-in can be reduced to 1-2%.

The following instructions are for use with onboard denaturation and dilution on the NextSeq 2000. If desired, the libraries and PhiX can be denatured manually according to Illumina's recommendations.

1. Dilute normalized library pool to 1.25 nM using RSB with Tween 20.
2. Dilute PhiX to 780 pM.
 - a. Combine 2 µL of the PhiX (10 nM) with 23.6 µL of RSB with Tween 20 in a 1.5 mL microcentrifuge tube. Vortex briefly to mix and spin down using microcentrifuge.

PhiX control – 10 nM (µL)	2
RSB with Tween 20 (µL)	23.6

- Combine 10 μL of RSB with Tween 20, 10 μL of 1.25 nM library pool and 4 μL of 780 pM PhiX for a total of 24 μL . Vortex briefly to mix and spin down using microcentrifuge.

RSB with Tween 20 (μL)	10
1.25 nM normalized library pool (μL)	10
PhiX control – 780 pM (μL)	4

- Load 20 μL final pool into the appropriate well of the NextSeq 2000 cartridge and start run using on board denaturation and dilution.

Data analysis

Archer AMP libraries should be demultiplexed to FASTQs prior to analysis. Analyze data with Archer Analysis using either a local software installation or Archer Unlimited. If preparing libraries with liquid adapters reference Product Insert Liquid P5 MBC P7 Adapter Kits for Illumina (RA-DOC-001) for additional information on Archer Analysis step up. Visit our website or contact archer-tech@idtdna.com for more information.

Archer assays require a one-time upload of a GTF file (a text file, in GTF format, which directs the software on how to analyze data from the panel). Additionally, if the SNV/indel pipeline is chosen, there is an option to select a targeted mutation file (a text file, in VCF format, which lists specific variants of interest). This file also requires a one-time upload. Both files can be obtained by contacting archer-tech@idtdna.com.

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