Archer® Immunoverse™-HS TCR Protocol

for Illumina®

Released November 14, 2019

Notices

Limitations of Use

For Research Use Only. Not for use in diagnostic procedures. Not intended to be used for treatment of human or animal diseases.

Safety data sheets pertaining to this product are available upon request.

Safety Notices

Caution symbols denote critical steps in the procedure where risk of assay 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.

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

In This Guide

This protocol is a guide to using Archer NGS library preparation kits, MBC adapters and gene enrichment panels for targeted sequencing of select genes and regions of interest using next-generation sequencing (NGS).

Overview – Page 3
This section contains the intended use statement, test principle and a high level overview of the workflow as well as a description of how the required reagents are supplied.

Materials Required But Not Supplied – Page 6
This section describes the materials that will be required to complete this protocol, but are not supplied in the kit.

Before Getting Started – Page 7
This section contains critical guidance for the successful implementation of the protocol and library preparation. This should be read and understood before laboratory work is initiated.

Protocol – Page 12
The section is the step-by-step protocol describing how to perform the workflow.

Additional Resources

View videos and additional resources for Archer products at:

http://archerdx.com/videos/

Technical Support

Visit http://archerdx.com/faqs for a list of helpful answers to frequently asked questions or contact us directly at tech@archerdx.com

Overview

Intended Use

The Archer Immunoverse-HS TCR Protocol is intended for use with Archer reagent kits and corresponding target enrichment panels to produce high-complexity libraries for use with Illumina® next-generation sequencing (NGS) instruments.

Immunoverse sequencing data produced with this method should be processed using **Archer Analysis** software - a complete bioinformatics suite that leverages AMP™ chemistry to detect unique sequence fragments, enabling error correction, read deduplication, and high-confidence alignment and mutation detection. Archer Analysis takes demultiplexed FASTQ files straight from the sequencer as input, and produces both high-level and detailed clonotype calling, as well as CSV and other file-type outputs for full transparency of the pipeline.

Test Principle

AMP, or Anchored Multiplex PCR, is a rapid and scalable method to generate target-enriched libraries for NGS. AMP technology can be used for applications in targeted RNA sequencing, genomic DNA sequencing and genotyping applications to generate a sequencing library in a matter of hours. Compatible with low nucleic acid input, this process delivers robust performance across a variety of sample types.

AMP utilizes unidirectional gene-specific primers (GSPs) that enrich for both known and unknown mutations. Adapters that contain both molecular barcodes and sample indices permit quantitative multiplex data analysis, read deduplication and accurate clonotype calling.

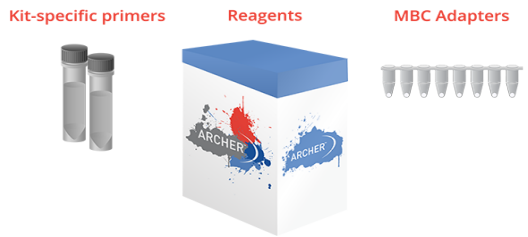
The Archer Analysis software utilizes these molecular barcodes for duplicate read binning, error correction and read deduplication to support quantitative multiplex data analysis and confident clonotype detection. Analysis reports both sequencing metrics and number of unique observations supporting called clonotypes.

Archer Library preparation reagents include:

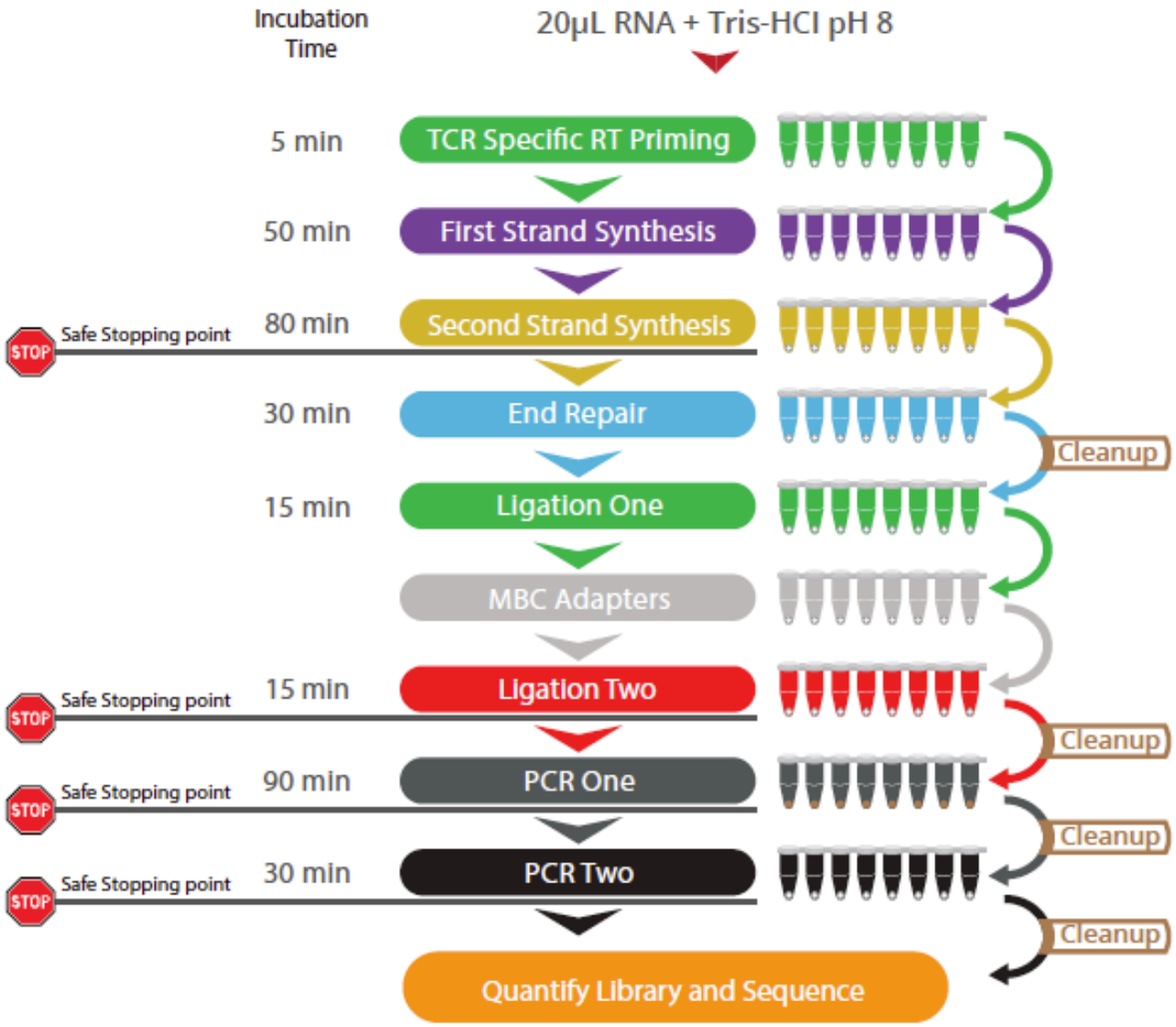
- Archer Immunoverse TCR reagents in lyophilized format.
- Gene specific primers (GSPs) that target panel specific regions of interest during PCR amplification.
- Archer Molecular Barcode Adapters (MBCs) are proprietary adapters that tag each unique molecule with a barcode and common region prior to amplification.

Modular Assay Format

Archer Kits include library preparation reagents and assay-specific liquid primers, which are used in conjunction with Archer MBC Adapters to construct sequencing-ready libraries from total nucleic acid or RNA samples. See individual kit inserts for assay targets and read depth requirements.



Protocol Overview



Supplied Reagents

Immunoverse™-HS TCR Reagents, for Illumina® (dSK0159)

Store at 2°C to 8°C

Allow pouches to reach room temperature before opening.

Description	Part Number	Quantity
TCR Specific RT Priming	SA0201	1 pouch (8 reactions/8 tube strip)
First Strand cDNA Synthesis	SA0002	
Second Strand cDNA Synthesis	SA0003	
End Repair	SA0204	
Ligation Step 1, 2.0	SA0202	
Ligation Step 2	SA0197	
First PCR for Illumina®-HS	SA0359	
Second PCR Reactions 1 thru 8 for Illumina®-HS	SA0360	
Ligation Cleanup Buffer	SA0209	1 tube (sufficient for processing 8 samples)
Ligation Cleanup Beads	SA0210	
500 mM Tris-HCl, pH 8.0	SA0020	
Ultra-Pure Water	SA0213	
Ultra-Pure Water for Ethanol Dilution	SA0022	1 bottle (sufficient for processing 8 samples)

Immunoverse™ Frozen Components (part # varies)

Store at -30°C to -10°C

Description	Part Number	Quantity
Immunoverse™ GSP	Varies	8 reactions

Materials required but not supplied

Additional Materials Required for Archer Library Preparation

Description	Supplier	Part Number
Archer MBC Adapters for Illumina®	ArcherDX	Varies
If extracting nucleic acid from FFPE samples: **Promega ReliaPrep™** **Agencourt® FormaPure®** **Promega Maxwell® RSC RNA FFPE Kit**	 Promega Beckman Coulter Promega	 Z1001 A33341 AS1440
Agencourt® AMPure® XP Beads	Beckman Coulter	A63880
100% Ethanol (ACS grade)	Various	-
RNase AWAY®	Thermo Fisher Scientific	7003
10mM NaOH (ACS grade)	Various	-
KAPA Universal Library Quantification Kit	KAPA Biosystems	KK4824
MiSeq® or NextSeq® Reagent Kit (300 cycle minimum)	Illumina	-
PhiX Control v3	Illumina	FC-110-3001
200mM Tris pH 7.0 (for sequencing)	Various	-
Standard PCR Thermal Cycler	Various	-
Real-Time PCR Thermal Cycler (PreSeq QC Assay)	Various	-
qPCR tubes (PreSeq QC Assay)	Various	-
0.2mL PCR tubes	Various	-
DynaMag™-96 Side Magnet	Thermo Fisher Scientific	12331D
Microcentrifuge	Various	-
Plate centrifuge	Various	-
Pipettes (P10, P20, P200 and P1000)	Pipetman P10, P20, P200, P1000 or equivalent	-
Sterile, nuclease-free aerosol barrier pipette tips	Various	-
Vortex Mixer	Various	-
PCR tube cooling block	Various	-
Gloves	Various	-
Qubit® 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Qubit® RNA HS Assay Kit	Thermo Fisher Scientific	Q32852

Before Getting Started

Important Precautions

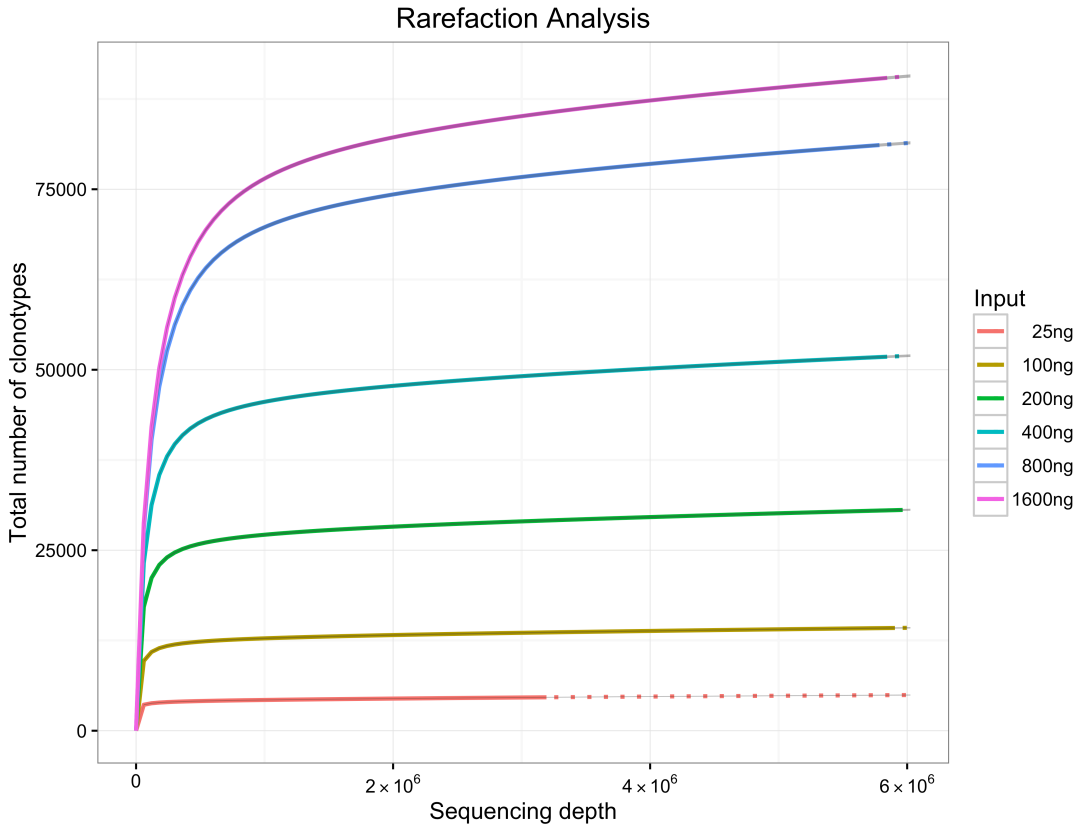
- **Read through the entire protocol before beginning library preparation.**
- Take note of stopping points throughout the protocol where samples can be safely frozen (-30°C to -10°C) to plan your workflow.
- Use good laboratory practices to prevent contamination of samples by PCR products.
- Use nuclease-free PCR tubes, microcentrifuge tubes and aerosol-barrier pipette tips.
- Wipe down workstation and pipettes with nuclease and nucleic acid cleaning products (such as RNase AWAY, Thermo Fisher Scientific).
- Verify that the thermal cycler used for library preparation is in good working order and currently calibrated according to manufacturer specifications.
- Reaction cleanup with AMPure XP beads is performed at room temperature (20°C to 25°C) and is used repeatedly throughout the Archer library prep workflow. Take care that AMPure XP beads are equilibrated to room temperature and fully resuspended by vortexing until homogenous in both color and appearance prior to drawing out material for each use.

Working with Lyophilized Reaction Pellets

- Archer reagents are provided as individually lyophilized reaction pellets in 0.2mL PCR tube strips.
- Allow pouches to reach room temperature (20°C to 25°C) before opening in order to prevent moisture condensation on tubes.
- Always centrifuge tubes briefly before opening to pull contents down.
- Detach the required number of reaction tubes using a clean razor blade and return any unused portion to the pouch with desiccant packet, reseal and store at 2°C to 8°C. It is recommended to use the remaining reactions within 4 weeks after opening.
    - For MBC Adapters and Second PCR tubes remember to label prior to returning unused portions to storage.
- Dissolve, mix and spin down:
    - **Never touch the lyosphere with the pipette tip.**
    - Add sample/reagents to pellet in tubes while on ice.
    - Allow at least 5 seconds for pellets to dissolve.
    - Pipette up and down 8 times to mix after the lyosphere has dissolved.
    - Briefly centrifuge and return to ice before proceeding.

Input Nucleic Acid

- Input nucleic acid (TNA or RNA) in *EDTA-free* buffer or Ultra-Pure Water is the optimal starting template for Archer AMP Library Preparation. Do not use EDTA-containing buffers exceeding 1mM.
- Use the maximum allowable input mass (ng) whenever possible.
- The minimum recommended input mass for Archer Immunoverse assays is dependent on experiment type, tissue type and tissue quality:
  - 20ng of RNA for screening of high abundance clones from high-quality PBMC samples
  - 400ng of RNA for deep sequencing applications from high-quality, high-complexity PBMC samples
  - 400ng of RNA from fresh-frozen tissue samples
  - >400ng of RNA for FFPE input for TIL analysis
- The rarefaction analysis below derived from varying amounts of high-quality input from a DNase-treated PBL sample illustrates the relationship between input amounts, sequencing depths and clonotype count:



- If using FFPE sample types, we recommend extracting TNA using Promega ReliaPrep™, Agencourt® FormaPure® or Promega Maxwell® RSC RNA FFPE Kit with the following modifications to the published manufacturer protocol:
  Promega ReliaPrep:
  - After step 6B Sample Lysis 5, incubate for **1** hour at **80**°C
  - At step 7 Column Washing and Elution 9, elute in a minimal elution volume of **40**µL using Ultra Pure Water (SA0213)
  - Do not use water baths

  Agencourt FormaPure:
  - After step 5, incubate for **1** hour at **80**°C
  - At step 23, elute in a minimal elution volume of **40**µL using Ultra-Pure Water (SA0213)
  - Do not use water baths

  Promega Maxwell RSC RNA FFPE Kit:
  - Skip DNase I preparation
  - Skip DNase I treatment of samples

Reagents to Prepare Before Starting

- Make fresh 10mM Tris-HCl pH 8.0 by mixing **30**µL 500mM Tris-HCl, pH 8.0 (SA0020) with **1470**µL Ultra-Pure Water (SA0213).
  - 10mM Tris-HCl pH 8.0 is appropriate for use for up to one week after mixing
- Make fresh 70% ethanol by adding **14**mL 100% ethanol to the bottle labeled Ultra-Pure Water for Ethanol Dilution (SA0022).
  - 70% ethanol is appropriate for use for up to one week after mixing
  - Tightly close the bottle cap to minimize evaporation when not in use
- Make fresh 5mM NaOH
  - If working from 1M NaOH, add 5uL of 1M NaOH to 995uL of ultra pure water to yield 5mM final NaOH
  - If working from 5M, add 10uL of 5M NaOH to 990uL of ultra pure water to yield 50mM NaOH. Mix well and briefly spin down. Take 100uL of 50mM NaOH and combine with 900uL of ultra pure water to yield 5mM NaOH. Mix well and briefly spin down.

Thermal Cycler Protocols

- Pre-program your thermal cycler with the following protocols
- Use the appropriate protocols for specific Archer Assays
- Verify programming prior to initiating runs

Specific RT Priming	Step	Temperature (ºC)	Time (min)
	1	65	5
	2	4	Hold

First Strand cDNA Synthesis	Step	Temperature (ºC)	Time (min)
	1	50	30
	2	80	20
	3	4	Hold

Second Strand cDNA Synthesis	Step	Temperature (ºC)	Time (min)
	1	16	60
	2	75	20
	3	4	Hold

End Repair	Step	Temperature (ºC)	Time (min)
	1	25	30
	2	4	Hold

Ligation Step 1	Step	Temperature (ºC)	Time (min)
	1	37	15
	2	4	Hold

Ligation Step 2	Step	Temperature (ºC)	Time (min)
	1	25	15
	2	4	Hold

First PCR Reaction	Step	Temperature	Time	Cycles
	1	95	3 min	1
	2	95	30 sec	24
	3	65	3 min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1

Second PCR Reaction	Step	Temperature (°C)	Time	Cycles
	1	95	3 min	1
	2	95	30 sec	8
	3	65	3 min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1

Molecular Barcoding, Sample Indexing & Multiplexed Sequencing

Molecule-level barcoding (or unique molecule identifier tagging) and sample-level barcoding (also known as index tagging) are both incorporated during Archer MBC ligation. Molecular barcodes are an integral component of the Archer Analysis software suite, (visit http://archerdx.com/archer-analysis for details). Sample barcodes (i.e. index tags) allow pooled libraries to be sequenced simultaneously thereby enabling maximum sequencing throughput and data demultiplexing during downstream bioinformatics analysis.

Sample Multiplexing

1) In order to efficiently utilize the throughput of the MiSeq (or other Illumina sequencing platform) as well as prevent low index diversity within your sequencing run, multiple samples should be sequenced simultaneously. Samples can be identified through a combination of two unique nucleotide sequences (see below for more details), which are subsequently read during the sequencing process. The unique nucleotide sequence is often termed an “index”.
2) Archer library preparation reagents for Illumina utilize a combination of two indices to distinguish between samples. The first index is added during Ligation Step 2 and is embedded in the Archer MBC Adapters for Illumina (p5/i5 index). The second index is added in Second PCR and is embedded in MiSeq Index 1 Primers (p7/i7) within the Second PCR reaction pellets.
3) In order to maintain appropriate coverage depth, it is recommended that users determine the maximum number of samples that can be run on a MiSeq flow cell (assuming 12 million reads per run using MiSeq reagents v2 or 22 million reads using Miseq reagents v3). In general, larger panels with more targets will require higher sequencing coverage depth and should be run with fewer samples.

Barcode Diversity

4) The Illumina MiSeq will work best when index diversity within a run is high. For example, if eight samples are included in a run, and the user chooses to use only one MBC Adapter paired with eight different MiSeq Index 1 Primers, the run may fail due to low barcode diversity. In this example it is best to use eight different Archer MBC Adapters paired with eight different MiSeq Index 1 Primers.
5) If using more than 48 MBCs, refer to http://archerdx.com/faqs/ for adapter compatibility.

Visit http://archerdx.com/faqs for a list of helpful answers to frequently asked questions or contact us directly at tech@archerdx.com

Protocol

Step 1: TCR Specific RT Priming

1) Pre-heat thermal cycler to 65°C with heated lid option on.
2) Place an appropriate number of TCR Specific RT Priming (SA0201) reaction tubes on ice.
3) Combine the appropriate amounts of Ultra-Pure Water (SA0213) and purified RNA (for input guidelines see page 8) in new PCR tubes.

Ultra-Pure Water (SA0021)	20 - XµL
Purified RNA	XµL
Total	**20µL**

4) Transfer **20**µL of RNA mixture to the TCR Specific RT Priming tubes.
   a) Dissolve, mix and spin down (See **Working with Lyophilized Reaction Pellets** in the preceding section, **Before Getting Started**).
   b) Return tubes to ice
5) After the program has reached 65°C, transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:

a) Use a heated lid (≥100°C)

TCR Specific RT Priming

Step	Temperature (°C)	Time (min)
1	65	5
2	4	Hold

b) Place samples in the thermal cycler and start program.
c) After the program has reached 4°C, place tubes on ice for at least 2 minutes.

Step 2: First Strand cDNA Synthesis

1) Place an appropriate number of First Strand cDNA Synthesis (SA0002) reaction tubes on ice.
2) Spin down the TCR Specific RT Priming mixture and transfer **20**µL to the First Strand cDNA Synthesis tubes.
   a) Dissolve, mix and spin down
   b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:

a) Use a heated lid (≥100°C)

First Strand cDNA Synthesis

Step	Temperature (°C)	Time (min)
1	50	30
2	80	20
3	4	Hold

b) Place samples in the thermal cycler and start program.
c) After the program has reached 4ºC, briefly spin down reactions and place on ice.

Step 3: Second Strand cDNA Synthesis

1) Place an appropriate number of Second Strand cDNA Synthesis (SA0003) reaction tubes on ice.
2) Add **20**µL of Ultra-Pure Water (SA0213) to each tube containing the **20**µL of First Strand cDNA Synthesis reaction.
   a) Pipette up and down to mix
3) Pipette **40**µL of each First Strand reaction into the Second Strand cDNA Synthesis tubes.
   a) Dissolve, mix and spin down
   b) Return tubes to ice
4) Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:

a) Use a heated lid (≥100C°)

Second Strand cDNA Synthesis

Step	Temperature (°C)	Time (min)
1	16	60
2	75	20
3	4	Hold

b) Place samples in the thermal cycler and start program.
c) After the program has reached 4C, briefly spin down reactions and place on ice

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Step 4: End Repair

1) Place an appropriate number of End Repair (SA0204) reaction tubes on ice.
2) Pipette **40**µL of the Second Strand cDNA Synthesis product into the End Repair tubes.
   a) Dissolve, mix and spin down
   b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:

a) Heated lid off

End Repair

Step	Temperature (°C)	Time (min)
1	25	30
2	4	Hold

   b) Place samples in the thermal cycler and start program.
   c) After the program has reached 4°C, briefly spin down reactions and place on ice.

Reaction cleanup after End Repair

See Important Precautions section above for guidance on working with AMPure XP beads.

1) Completely resuspend AMPure Beads by vortexing.
2) Add **2.5X** volume (**100**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a homogenous mixture.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes and **ensure beads are fully pelleted** against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipet tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.
9) Move tube strip to next adjacent row on magnetic block.
10) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tubes.
11) Remove and discard the supernatant.
12) Repeat steps 8-11 for a total of **two washes** in 70% ethanol.
13) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **5** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
14) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
15) Place tubes back on the magnet for **2** minutes.

Step 5: Ligation step 1, 2.0

1) Place an appropriate number of Ligation Step 1, 2.0 (SA0202) reaction tubes on ice.

2) Transfer **20**µL of purified DNA from **Reaction cleanup after End Repair** Step 15 into Ligation step 1, 2.0 tubes. Be sure to avoid transferring beads to Ligation step 1.
   a) Dissolve, mix and spin down
   b) Return tubes to ice
3) Transfer reactions to a pre-heated thermal cycler and initiate an incubation using the following program and guidelines:

a) Use a heated lid (≥100°C)

Ligation Step 1, 2.0 Incubation Conditions:

Step	Temperature (°C)	Time (min)
1	37	15
2	4	Hold

   b) Place samples in the thermal cycler and start program.
   c) After the program has reached 4ºC, briefly spin down reactions and place on ice.
4) Add 20µL of 10mM Tris-HCL, pH 8.0, mix well by pipetting up and down and briefly spin down.

Step 6: MBC Adapter Incorporation

1) Label MBC Adapter tubes with:
   a) The sample index tag letter (A, B, or C) and number (1-48) from the MBC Adapters pouch label.
   b) Use a permanent laboratory marker taking care to orient lid hinges to the back as illustrated:

***Important* This step incorporates your P5 index tag for sample-level tracking, take care to record this number for future reference and record which MBC adapter is being used for each sample. Unused tubes must be labeled before returning to the pouch.**

2) Place an appropriate number of MBC Adapter reaction tubes on ice.
3) Add **40**µL of the cDNA sample from **Ligation Step 1, 2.0**, Step 5 .
   a) Dissolve, mix and spin down.
4) Immediately proceed to **Step 7: Ligation Step 2**.

Step 7: Ligation Step 2

1) Place an appropriate number of Ligation Step 2 (SA0197) reaction tubes on ice.
2) Transfer the entire volume of each purified DNA sample from **MBC Adapter Incorporation** Step 4 to Ligation Step 2 tubes.
   a) Dissolve, mix and spin down
   b) Return tubes to ice

3) Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:

a) Heated lid off

Ligation Step 2 Incubation Conditions:

Step	Temperature (°C)	Time (min)
1	25	15
2	4	Hold

b) Place samples in the thermal cycler and start program

c) After the program has reached 4°C, briefly spin down reactions and place on ice.

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Reaction Cleanup after Ligation Step 2

Prepare Ligation Cleanup Beads:

1) Completely resuspend Ligation Cleanup Beads by vortexing.
2) For each reaction, pipette **50**µL of Ligation Cleanup Beads (SA0210) into a new 0.2mL PCR tube for each library.
3) Place tube(s) on the magnet for **1** minute or until the beads are pelleted.
4) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipet tip, return contents to the tube and repeat magnetic pelleting step).
5) Pipette **50**µL of Ligation Cleanup Buffer (SA0209) into each tube to resuspend beads.

Ligation cleanup procedure:

Caution: Vortex PCR tubes with fingers firmly placed on all lids as the detergent may compromise sealing of lids.

1) Transfer the entire volume of Ligation Step 2 reaction into the tubes with Ligation Cleanup Beads and Buffer.
2) Mix samples by vortexing
3) Incubate reactions at room temperature for **5** minutes.
4) Mix samples by vortexing.
5) Incubate reactions at room temperature for **5** minutes.
6) Briefly spin down tubes.
7) Place tubes on the magnet for **1** minute and ensure beads are fully pelleted against the tube wall.
8) Carefully pipette off and discard supernatant (**100**µL) without disturbing the beads.
9) Wash beads **two times** with Ligation Cleanup Buffer.

a) Resuspend beads in **200**µL Ligation Cleanup Buffer (SA0209) by vortexing, briefly spin down, and place back on magnet for **1** minute.
b) Once slurry has cleared, discard supernatant.

10) Wash beads once with Ultra-Pure Water (SA0213):
a) Resuspend beads in 200µL of Ultra-Pure Water by vortexing, briefly spin down and place back on magnet.
b) Once slurry has cleared discard supernatant.
c) Take care to ensure that all supernatant has been removed from beads

11) Elute DNA from ligation cleanup beads:
a) Resuspend ligation cleanup beads in 36µL of 5mM NaOH.
b) Transfer beads to thermal cycler and incubate at 75°C for 10 minutes then cool to 4°C.
i) Use a heated lid
c) After sample has reached 4°C, briefly spin down, and transfer to the magnet.
d) Wait for beads to clear and leave it there while you prepare **Step 8: First PCR.**

Step 8: First PCR

Note: The Immunoverse kits do not contain gene-specific primers in the reaction pellet. Please use the panel-specific GSPs provided.

1) Place an appropriate number of First PCR HS (SA0359) reaction tubes on ice.
a) Label tubes by sample number
2) Pipette 4µL of panel-specific GSP mix into each First PCR HS tube.
3) Transfer 36µL supernatant from Step 11 above into appropriately labeled PCR1 tube.
a) Dissolve, mix, and spin down.
b) Return tubes to ice
4) Transfer reactions to a thermal cycler and immediately initiate a run using the following program and guidelines:

a) Use a heated lid (≥100°C)

First PCR Cycling Conditions:

Step	Temperature °C	Time	Cycles
1	95	3 min	1
2	95	30 sec	24
3	65	3 min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

b) Place samples in the thermal cycler and start program.
c) After the program has reached 4°C, briefly spin down reactions and place on ice. It is also acceptable to leave tubes in the thermal cycler at 4°C overnight.

Reaction cleanup after First PCR

See Important Precautions section above for guidance on working with AMPure XP beads

1) Completely resuspend AMPure Beads by vortexing.
2) Add **1.2**X volume (**48**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a homogenous mixture.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes and ensure beads are fully pelleted against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipet tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.
9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tube(s).
10) Remove and discard supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **3** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **44**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.
15) Transfer **42**µL of purified solution to a new 0.2ml PCR tube and proceed directly to **Step 9: Second PCR**
    a) Be sure to avoid transferring beads to the fresh tube.

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Step 9: Second PCR

1) Place an appropriate number of Second PCR HS (SA0360) reaction tubes on ice.
    a) Use a permanent marker to label the tubes 1 to 8 from left to right as shown below. (See **Molecular Barcoding, Sample Indexing & Multiplexed Sequencing** in the **Before Getting Started section**).

***Important* This step incorporates your P7 index tag for sample-level tracking, take care to record this number for future reference and record which MBC adapter is being used for each sample. Unused tubes must be labeled before returning to the pouch.**

Index 1 (P7) sequence table

Sample Number	Illumina Index 1 P7/i7 Sequence
1	TAAGGCGA
2	CGTACTAG
3	AGGCAGAA
4	TCCTGAGC
5	GGACTCCT
6	TAGGCATG
7	CTCTCTAC
8	CAGAGAGG

2) On ice, add **40**µL of the purified library DNA from **First PCR cleanup** (step 15).
   a) Dissolve, mix and spin down
   b) Return tubes to ice
3) Transfer reactions to a thermal cycler and initiate a run using the following program and guidelines:

a) Use a heated lid (≥100°C)

Second PCR Reaction Cycling Conditions

Step	Temperature (°C)	Time	Cycles
1	95	3 min	1
2	95	30 sec	8*
3	65	3 min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

*Note: If >200nM yield is consistently generated, consider decreasing the number of PCR2 cycles for improved assay performance

b) Place samples in the thermal cycler and start program.

c) After the program has reached 4°C, briefly spin down reactions and place on ice.

Cleanup after Second PCR

See "Important Precautions" section above for guidance on working with AMPure XP beads

1) Completely resuspend AMPure Beads by vortexing.
2) Add **1.2**X volume (**48**µL) of AMPure to each reaction.
3) Vortex well or pipette 10 times to mix and visually inspect the color of the sample to ensure a homogenous mixture.
4) Incubate for **5** minutes at room temperature (20°C to 25°C).

5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes and ensure beads are fully pelleted against the tube wall.
7) Without disturbing the bead pellet, use a pipette to remove and discard the supernatant. (If the pellet becomes dislodged from the magnet and a portion is drawn into the pipet tip, return contents to the tube and repeat magnet incubation step).
8) With tubes still on the magnet, add **200**µL of 70% ethanol.
9) Incubate for **30** seconds to allow the bead pellet to fully pellet against the side of the tube(s).
10) Remove and discard supernatant.
11) Repeat steps 8-10 for a total of **two washes** in 70% ethanol.
12) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **3** minutes at room temperature with open lids. Take care not to over-dry beads as this will significantly decrease overall recovery (yield) of nucleic acid.
13) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes
15) Transfer the **18**µL of purified solution to a new 0.2ml PCR tube and proceed directly to **Quantify, Normalize and Sequence**.
    a) Be sure to avoid transferring beads to the fresh tube.

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C.

Quantify, Normalize and Sequence

Quantify

1) Quantify the concentration of each library using the KAPA Universal Library Quantification Kit
    a) It is recommended customers use 250 bps as the size correction factor.
    b) Archer libraries are very concentrated. You will need to dilute libraries 1:10,000-1:250,000 for quantification with KAPA qPCR.

Normalize

2) After quantification, pool libraries at equimolar concentrations and load the sequencer according to manufacturer instructions. For reference sample sheets and additional recommendations, visit our website at http://archerdx.com/mbc-adapters and http://archerdx.com/faqs.

Sequence

3) Loading recommendations:
    *Note: The loading concentration should be optimized by the user per library type.
    a) For MiSeq, use the read level sequence in the tables below.
        i) If using the 300bp kit

Level	Read Length
(R1) Read 1	151
(R2) Index Read 1	8

Level	Read Length
(R3) Index Read 2	8
(R4) Read 2	151

ii) If using the 600bp kit

Level	Read Length
(R1) Read 1	301
(R2) Index Read 2	8
(R3) Index Read 2	8
(R4) Read 2	301

iii) In addition, a reference sample sheet is available for download at: http://www.archerdx.com/mbc-adapters/. Fill out the sample sheet according to the MiSeq protocol.

b) Load sequencing libraries with PhiX, prepared as follows:

i) Dilute and denature PhiX to 10pM or 20pM (depending on desired final PhiX concentration) according to the Illumina protocol.

***Important* The amount of PhiX depends on the complexity of the final library pool. A higher concentration of PhiX is recommended for samples that contain one dominant clone at high frequency resulting in low complexity libraries. A recommended starting concentration is 10% PhiX.**

MiSeq Loading guidelines when starting from low quality input (such as FFPE)

ii) Begin with a 4nM pool of your barcoded libraries:

(1) Combine 10µL of the 4nM library pool with 10µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at room temperature.

(2) Add 10µL 200mM Tris pH 7.0 and vortex briefly to mix.

(3) Add 970µL ice-cold Illumina Hyb buffer and vortex briefly to mix. This makes 40pM library.

(4) Refer to the table below for amounts of pooled library, PhiX and Hyb Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.

(5) Load the entire volume (1.0mL) of the final pool into the appropriate well of the MiSeq cartridges

MiSeq Loading Guidelines

1	**Denature pooled libraries:**	**Volume (µL)**				
	Pooled 4nM libraries	10	10	10	10	10
	0.2N NaOH	10	10	10	10	10
	Incubate 5 minutes					
2	**Neutralize and Dilute to 40pM**					
	Pooled libraries + NaOH (from Step 1)	20	20	20	20	20
	200mM Tris pH 7.0	10	10	10	10	10
	Hyb Buffer	970	970	970	970	970

3	**Dilute library to pM concentration:**					
4	**v2 chemistry, 2x151bp final loading concentration at 20pM by %PhiX**	**5%**	**10%**	**15%**	**20%**	**25%**
	40pM Libraries from step 2	500	500	500	500	500
	10pM Denatured PhiX	105	222	353	-	-
	20pM Denatured PhiX	-	-	-	250	333
	Hyb Buffer	395	278	147	250	167
4	**v3 chemistry, 2x300bp final loading concentration at 20pM by %PhiX**	**5%**	**10%**	**15%**	**20%**	**25%**
	40pM Libraries from step 2	500	500	500	500	500
	10pM Denatured PhiX	105	222	353	-	-
	20pM Denatured PhiX	-	-	-	250	333
	Hyb Buffer	395	278	147	250	167
5	**Load Final Pool into Cartridge**	**1000**	**1000**	**1000**	**1000**	**1000**

MiSeq Loading guidelines when starting from high quality input

iii) Begin with a 6nM pool of your barcoded libraries:

(1) Combine 10µL of the 6nM library pool with 10µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at room temperature.

(2) Add 10µL 200mM Tris pH 7.0 and vortex briefly to mix.

(3) Add 970µL ice-cold Illumina Hyb buffer and vortex briefly to mix. This makes 60pM library.

(4) Refer to the table below for amounts of pooled library, PhiX and Hyb Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.

(5) Load the entire volume (1.0mL) of the final pool into the appropriate well of the MiSeq cartridges

1	**Denature pooled libraries:**	**Volume (µL)**	
	Pooled 6nM libraries	10	10
	0.2N NaOH	10	10
	Incubate 5 minutes		
2	**Neutralize and Dilute to 60pM**		
	Pooled libraries + NaOH (from Step 1)	20	20
	200mM Tris pH 7.0	10	10
	Hyb Buffer	970	970
3	**Dilute library to pM concentration:**		
4	**v2 chemistry, 2x151bp final loading concentration at 25pM by %PhiX**	**5%**	**10%**
	60pM Libraries from step 2	416.7	416.7
	10pM Denatured PhiX	131.6	277.8
	Hyb Buffer	451.8	305.6
4	**v3 chemistry, 2x300bp final loading concentration at 25pM by %PhiX**	**5%**	**10%**
	60pM Libraries from step 2	416.7	416.7
	10pM Denatured PhiX	131.6	277.8
	Hyb Buffer	451.8	305.6
5	**Load Final Pool into Cartridge**	**1000**	**1000**

c) For NextSeq, load sequencing libraries with 20% PhiX, prepared as follows:
   i) Dilute and denature PhiX to 20pM according to the Illumina protocol
      (1) Combine 10µL 4nM library pool with 10µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at ambient room temperature.
      (2) Add 970µL ice-cold HT1 buffer and vortex briefly to mix. This makes 40pM library.
      (3) Refer to the table below for amounts of pooled library, PhiX and HT1 Buffer to combine in a new 1.5mL microcentrifuge tube and vortex briefly to mix.
      (4) Spin down and load the entire volume (1.3mL) of this final pool in 20% PhiX into the appropriate well of the NextSeq cartridge.

NextSeq Loading Guidelines

1	**Denature pooled libraries:**	**Volume (µL)**		
	Pooled 4nM libraries	10	10	10
	0.2N NaOH	10	10	10
	Incubate 5 minutes			
2	**Neutralize and Dilute to 40pM**			
	Pooled libraries + NaOH (from Step 1)	20	20	20
	200mM Tris pH 7.0	10	10	10
	Hyb Buffer	970	970	970
3	**Dilute library to desired pM concentration:**			
4	**Final loading concentration (pM) →**	**1.4**	**1.6**	**1.8**
	40pM Libraries from step 2	46	52	59
	20pM Denatured PhiX	22	26	29
	Hyb Buffer	1232	1222	1212
5	**Load Final Pool into Cartridge**	**1300**	**1300**	**1300**

Data Analysis

Analyze data with Archer Analysis using either a local software installation, Archer Vault or Archer Unlimited. Visit our website at http://archerdx.com/software/analysis for more information.

Immunoverse assays may also require a one-time upload of a Target Region file (a text file, in GTF format, which directs the software on how to analyze data from the panel). This file can be obtained by contacting tech@archerdx.com.

Demultiplex NextSeq libraries according to recommendations in FAQs: http://archerdx.com/faqs/

Limitations of Use

For Research Use Only. Not for use in diagnostic procedures. Not intended to be used in treatment of animal or human diseases.

Safety data sheets pertaining to this product are available upon request.

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ArcherDX, Inc.
2477 55th Street, Suite 202
Boulder, CO 80301
303-357-9001
www.archerdx.com