Archer® FusionPlex® Protocol
for Illumina®

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.

Revision History

Version	Revisions
LA135.1 Release July 23, 2020	• Updated table formatting. • Removal of recommendation to run fragment analysis on final libraries. • Updated PCR cycling tables to refer to the product insert for panel-specific cycling recommendations. • Updated vendor part numbers for materials required but not supplied.

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).

Additional Resources

View videos and additional resources for Archer products at http://archerdx.com/videos/

Technical Support

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

Overview

Intended Use

The Archer FusionPlex 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) platforms.

FusionPlex 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 detect 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.

Test Principle

AMP 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. Designed for 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 mutation calling.

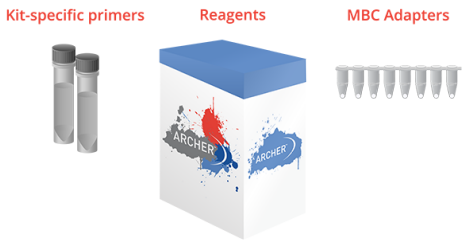
The Archer Analysis software utilizes these molecular barcodes for duplicate read binning, error correction and read deduplication to support quantitative multiplex data analysis and confident mutation detection. Analysis reports both sequencing metrics and number of unique observations supporting called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

Archer Library preparation reagents include:

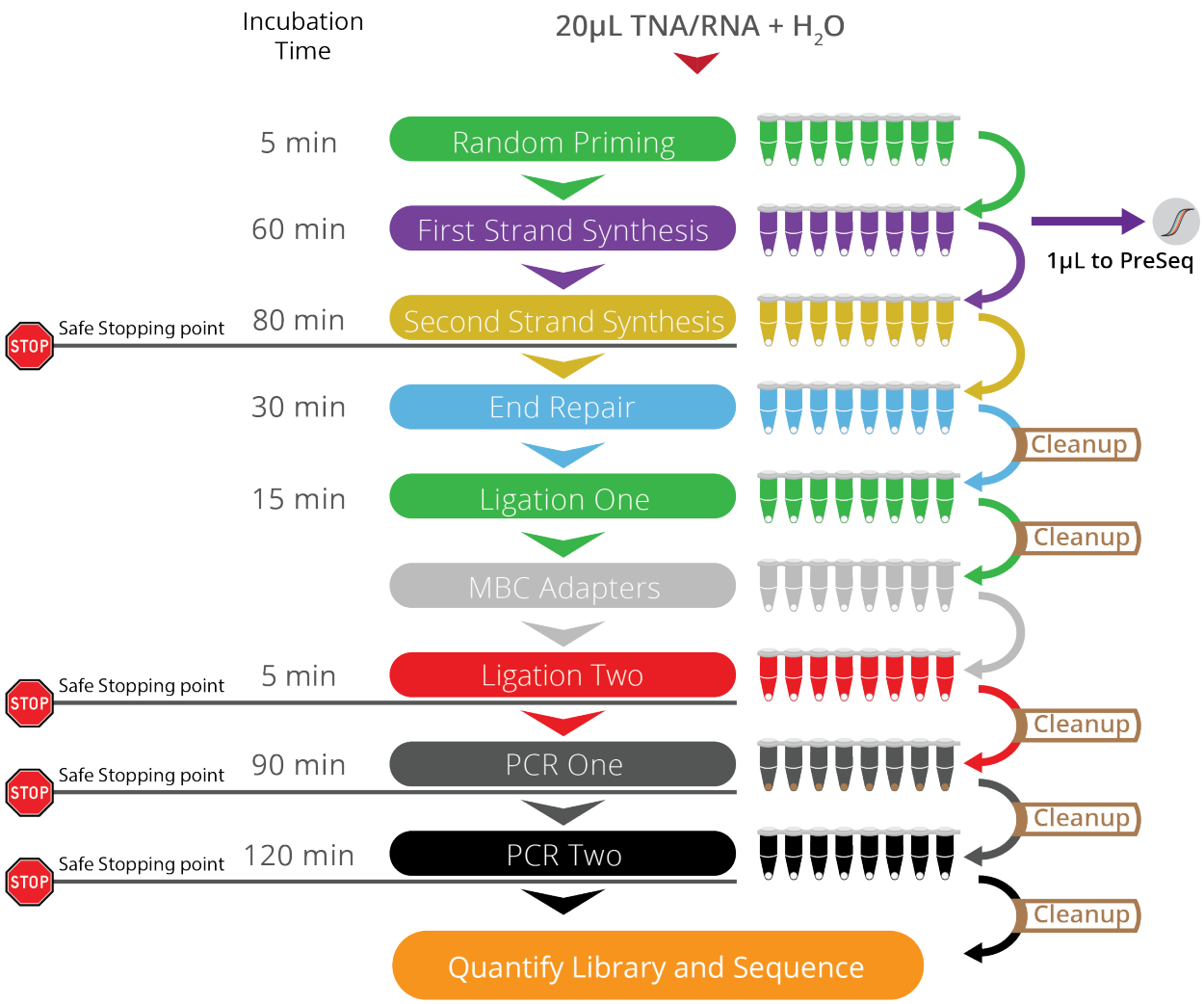
- Archer FusionPlex reagents in lyophilized format for each step of library preparation.
- Gene specific primers (GSPs) that target panel specific regions of interest during PCR amplification.
- Archer PreSeq® RNA QC assay to determine the quality of starting material.
- Archer Molecular Barcode (MBC) Adapters 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 product inserts for assay targets and read depth requirements.



Protocol Overview



Supplied Reagents

FusionPlex Reagents, for Illumina (SK0093)

Store at 2°C to 8°C

Allow pouches to reach room temperature before opening.

Description	Part Number	Quantity
Random Priming 2.0	SA0194	2 pouches (16 reactions/two 8-tube strips)
First Strand cDNA Synthesis	SA0002	
Second Strand cDNA Synthesis	SA0003	
End Repair	SA0204	1 pouch (8 reactions/one 8-tube strip)
Ligation Step 1	SA0196	
Ligation Step 2	SA0197	
First PCR (Illumina-P)	SA0109	
Second PCR Reactions 1 thru 8 (Illumina-P)	SA0110	
Ligation Cleanup Beads	SA0210	1 tube (sufficient for processing 8 samples)
Ligation Cleanup Buffer	SA0209	
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)

FusionPlex Frozen Components (part number varies)

Store at -30°C to -10°C

Description	Part Number	Quantity
FusionPlex panel GSP1	Refer to product insert	8 reactions
FusionPlex panel GSP2	Refer to product insert	
10X VCP Primer Mix	SA0126	16 reactions*

*Supplied volume is sufficient to run duplicate qPCR reactions for 16 samples and 4 No Template Controls (NTC).

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	-
Agencourt® AMPure® XP Beads	Beckman Coulter	A63880
iTaq™ Universal SYBR® Green Supermix	Bio-Rad Laboratories	172-5120
100% Ethanol (ACS grade)	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
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 FusionPlex Reagents workflow. Ensure 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.
    - Note: Some FusionPlex panels contain gene-specific primers designed for sample tracking that target non-expressed DNA sequence (intronic or intergenic). When

using SEX_ID and SNP_ID-based sample tracking, TNA should be used as the starting template.

- Use the maximum allowable input mass (ng) whenever possible. Higher input quantities enable more sensitive variant and fusion detection:
  - 20 – 250ng of RNA for FusionPlex (somatic mutation detection)
- If using total nucleic acid (TNA), DO NOT pretreat with DNase. DNA found in total nucleic acid can act as an internal control, verifying assay performance in the absence of RNA.
- 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 (Optional)
  - Skip DNase I treatment of samples (Optional)

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 **5**µL of 1M NaOH to **995**µL of Ultra Pure Water to yield 5mM final NaOH
  - If working from 5M, add **10**µL of 5M NaOH to **990**µL of Ultra Pure Water to yield 50mM NaOH. Mix well and briefly spin down. Take **100**µL of 50mM NaOH and combine with **900**µL 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

	Step	Temperature (ºC)	Time (min)
Random Priming 2.0	**1**	65	5
	2	4	Hold

	Step	Temperature (ºC)	Time (min)
First Strand cDNA Synthesis	**1**	25	10
	2	42	30
	3	80	20
	4	4	Hold

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

	Step	Temperature (°C)	Time (Sec)	Cycles
PreSeq RNA QC Assay	**Activation**	95	20 [20*]	1
	Denaturation	95	3 [15*]	35
	Primer Annealing & Extension	60	30 [60*]	
	Melt-curve gradient	60-95	0.5°C/sec increment	1

*Times in [ ] are for standard cycling.

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

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

	Step	Temperature (ºC)	Time (min)
Ligation Step 2	**1**	22	5
	2	4	Hold

	Step	Temperature (°C)	Time	Cycles
First PCR Reaction	1	95	3 min	1
	2	95	30 sec	15*
	3	65*	5* min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1

*Refer to product insert for panel-specific parameters.

	Step	Temperature (°C)	Time	Cycles
Second PCR Reaction	1	95	3 min	1
	2	95	30 sec	20**
	3	65*	5* min (100% ramp rate)	
	4	72	3 min	1
	5	4	Hold	1

*Refer to product insert for panel-specific parameters.
** If you regularly experience library yields higher than 200nM you can decrease cycle number

Molecular Barcoding, Sample Indexing, and 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 https://archerdx.com/technology-platform/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) The FusionPlex reagents for Illumina utilize a combination of two indices to distinguish between samples. Index 2 is added during Ligation Step 2 and is embedded in the Archer MBC Adapters for Illumina (p5/i5 index). Index 1 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 and 25 million reads per run 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

1) The Illumina sequencers 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.
2) If using more than 48 MBCs, refer to http://archerdx.com/mbc-adapters for adapter compatibility.

Step 1: Random Priming 2.0

1) Pre-heat the thermal cycler to 65°C with heated lid option on.
2) Place an appropriate number of Random Priming 2.0 (SA0194) reaction tubes on ice.
3) Combine the appropriate amounts of Ultra-Pure Water (SA0213) and purified total nucleic acid or RNA (20-250ng) in new PCR tubes.

Component	Reaction Mix
Ultra-Pure Water (SA0213)	20 – X µL
Purified Total Nucleic Acid or RNA	X µL
(Total)	(20 µL)

4) Transfer **20**µL TNA/Water mix to the Random Priming 2.0 reaction tubes.
   a) Dissolve, mix and spin down (see **Working with Lyophilized Reaction Pellets** section above)
   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)
   b) Place samples in the thermal cycler, close the lid and start program
   c) After the program has reached 4°C, place tubes on ice for at least 2 minutes

Random Priming 2.0 Thermal Cycler Protocol

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

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 Random Priming 2.0 mixture and transfer **20**µL to the First Strand cDNA Synthesis tube(s).
   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 Thermal Cycler Protocol

Step	Temperature (°C)	Time (min)
1	25	10
2	42	30
3	80	20
4	4	Hold

   b) After the program has reached 4ºC, briefly spin down reactions and place on ice.
4) Make diluted cDNA samples for PreSeq RNA QC assay.
   a) For each sample, pipette **9**µL of Ultra-Pure Water (SA0213) into a new PCR tube.
   b) Pipette **1**µL of each First Strand cDNA Synthesis reaction into the water and pipette up and down to mix.
   c) Keep on ice for use in Step 4 (PreSeq RNA QC assay).

Step 3: Second Strand cDNA Synthesis

1) Place an appropriate number of Second Strand cDNA Synthesis (SA0003) reaction tubes on ice.
2) Add **21**µL of Ultra-Pure Water (SA0213) to each tube containing the **19**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 (≥100°C)

Second Strand cDNA Synthesis

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

b) Start the program and pause once block has reached 16ºC
c) Place samples in the thermal cycler; close the lid and resume program
d) While sample(s) are incubating, proceed to RNA PreSeq QC assay (step 4)
e) After the program has reached 4ºC, 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. It is recommended to review the qPCR results from the PreSeq RNA QC assay at this time to determine predicted sample success.

Step 4: PreSeq RNA QC Assay

1) Thaw 10X VCP Primer Mix (SA0126) at room temperature.
2) Prepare sufficient qPCR reaction mix for
   a) Duplicate reactions of each diluted cDNA sample
   b) One No Template Control (NTC) made from **10**µL Ultra-Pure Water (SA0213)

Component	Part Number	Reaction Mix (n=1)	Reaction Master Mix (n=20)
iTaq SYBR Green Supermix	Not Supplied	5µL	100µL
10X VCP Primer Mix	SA0126	1µL	20µL
Diluted cDNA sample or NTC	-	4µL	-
(Total)		(10µL)	(120µL)

3) Pipette 6µL of the reaction mix into each assigned well of a qPCR plate/tube.
4) Pipette 4µL of the diluted cDNA samples or NTC into assigned wells/tubes containing reaction mix.
   a) Mix slowly to avoid introducing bubbles, cap or seal the reactions and spin down
5) Transfer reactions to a thermal cycler and initiate a run using the following program:

PreSeq RNA QC Assay qPCR Instrument Protocol

Step	Temperature (°C)	Time (Sec)	Cycles
Activation	95	20 (20*)	1
Denaturation	95	3 (15*)	35
Primer Annealing & Extension	60	30 (60*)	
Melt-curve gradient	60-95	0.5°C/sec increment	1

*Times in ( ) are for standard cycling.

Step 5: 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 Thermal Cycler Protocol

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

   b) Place samples in the thermal cycler; close the lid and start program
   c) When the run has completed, 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 **2.5**X 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 **or until 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 pipette 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 tubes.
10) Remove and discard the 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 **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.
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.

Step 6: Ligation Step 1

1) Place an appropriate number of Ligation Step 1 (SA0196) reaction tubes on ice.
2) Transfer **20**µL of purified DNA from Reaction Cleanup after End Repair step 14 into Ligation Step 1 tubes. (It is acceptable for a small amount of AMPure beads to be transferred).
   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)

Ligation Step 1 Thermal Cycler Protocol

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

   b) After the program has reached 4ºC, briefly spin down reactions and place on ice.

Reaction Cleanup after Ligation Step 1

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

1) Completely resuspend AMPure Beads by vortexing.
2) Add **2.5**X volume (**50**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 even mixing.
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 **or until 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 pipette 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 tubes.
10) Remove and discard the 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 **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.
13) Elute DNA by resuspending beads in **42**µL 10mM Tris-HCl pH 8.0.
14) Place tubes back on the magnet for **2** minutes.

Step 7: MBC Adapter Incorporation

1) Label MBC Adapter tubes with the sample index tag letter (A, B, or C) and number (1-48) from the MBC Adapters pouch label

    a) Use a permanent laboratory marker and orient lid hinges to the back as illustrated:

***Important* As this step incorporates your index tag for sample-level tracking, be sure to 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 purified cDNA sample from Reaction Cleanup after Ligation Step 1, Step 6. **Avoid pipetting AMPure beads into this reaction**. If minute amounts of AMPure beads were carried over, simply place MBC Adapter tubes on magnet for one minute and transfer all liquid to the next tubes while MBC Adapter tubes remain on the magnet.
    a) Dissolve, mix and spin down.
4) Immediately proceed to Step 8 Ligation Step 2.

Step 8: 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 Step 7 MBC Adapters 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 Thermal Cycler Protocol

Step	Temperature (°C)	Time (min)
1	22	5
2	4	Hold

   b) After the program has reached 4ºC, 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 (SA0210) by vortexing.
2) For each reaction, pipette **50**µL of Ligation Cleanup Beads into new 0.2mL PCR tubes.
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 pipette 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) Pipette 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 **or until 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 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 18µL 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.

Step 9: First PCR

1) Place an appropriate number of First PCR (SA0109) reaction tubes on ice.
   a) Label tubes by sample number
2) Pipette **2**µL of **GSP1** into each First PCR tube.
   a) Spin down and return tubes to ice
3) Pipette **18**µL of supernatant from Step 11 above into appropriately labeled First PCR 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 Reaction Thermal Cycler Protocol

Step	Temperature (°C)	Time	Cycles
1	95	3 min	1
2	95	30 sec	15*
3	65*	5* min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

**Refer to product insert for panel-specific parameters.*

   b) After the program has reached 4ºC, 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 (**24**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 **or until 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 pipette 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 **20**µL of purified solution to a new 0.2mL PCR tube and store reactions as indicated below or proceed directly to Step 10: Second PCR

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

Step 10: Second PCR

1) Place an appropriate number of Second PCR (SA0110) reaction tubes on ice.
Important: The Index 1 (P7) index tag is incorporated during this step.
   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).

   b) 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) Pipette **2**µL of GSP2 into each Second PCR tube.

a) Record which P7 index sequence is used with which sample.

3) Pipette **18**µL of First PCR cleanup elution into each Second PCR tube.
   a) Dissolve, mix and spin down
   b) Return tubes to ice
4) 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 Thermal Cycler Protocol

Step	Temperature (°C)	Time	Cycles
1	95	3 min	1
2	95	30 sec	20**
3	65*	5* min (100% ramp rate)	
4	72	3 min	1
5	4	Hold	1

**Refer to product insert for panel-specific parameters.*
*** If you regularly experience library yields higher than 200nM you can decrease cycle number*

   b) After the program has reached 4ºC, 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 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 (**24**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 (20ºC to 25°C).
5) Briefly spin down tubes.
6) Place tubes on the magnet for **4** minutes **or until 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 pipette 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 the 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 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.
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 **18**µL of the purified solution to a new 0.2mL PCR tube. Stop or 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) The recommended average fragment length of FusionPlex libraries for the size-adjustment calculation is 200bp.
   b) Archer libraries are very concentrated. You will need to dilute libraries 1:10,000-1:250,000 for quantification with KAPA qPCR.

Normalize

1) 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/support/faqs.

Sequence

1) Loading recommendations are provided below. **The final loading concentration must be optimized by each user.**
   a) For MiSeq, use the read level sequence in the table below.

Level	Read Length
(R1) Read 1	151
(R2) Index Read 1	8
(R3) Index Read 2	8
(R4) Read 2	151

      i) In addition, a reference sample sheet is available for download at: http://archerdx.com/mbc-adapters. Fill out the sample sheet according to the MiSeq protocol.
   b) Load sequencing libraries with **5%** PhiX, prepared as follows:
      i) Dilute and denature PhiX to 10pM according to the Illumina protocol.

Note: The amount of PhiX depends on the complexity and diversity of the final library pool. A higher concentration of PhiX is recommended for libraries prepared from low input masses resulting in low complexity libraries.

      ii) Begin with a **4nM** pool of your barcoded libraries:
         (1) Combine **10**µL of the 4nM library pool with **10**µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for **5** minutes at room temperature.
         (2) Add **10**µL 200mM Tris pH 7.0 and vortex briefly to mix.
         (3) Add **970**µL 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 cartridge.

MiSeq Loading Guidelines

Desired concentration of final pool (pM)	13	14	15	16	17	18
1) Denature pooled libraries:						
Pooled 4nM libraries (µL)	10	10	10	10	10	10
0.2N NaOH (µL)	10	10	10	10	10	10
2) Incubate for 5 minutes						
3) Neutralize and Dilute to 40pM						
Pooled libraries + NaOH from Step 1 (µL)	20	20	20	20	20	20
200mM Tris pH 7.0 (µL)	10	10	10	10	10	10
Hyb Buffer (µL)	970	970	970	970	970	970
4) Dilute library to desired loading concentration (pM)	**13**	**14**	**15**	**16**	**17**	**18**
40pM Libraries from step 2 (µL)	325	350	375	400	425	450
10pM Denatured PhiX (µL)	68	74	79	84	89	95
Hyb Buffer (µL)	607	576	546	516	486	455
5) Load Final Pool into Cartridge						
Final pool (µL)	1000	1000	1000	1000	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
   ii) Begin with a 4nM pool of your barcoded libraries:
      (1) Combine **10**µL 4nM library pool with **10**µL 0.2N NaOH in a 1.5mL microcentrifuge tube. Vortex briefly to mix and incubate for 5 minutes at ambient room temperature.
      (2) Add **10**µL 200mM Tris pH 7.0 and vortex briefly to mix.
      (3) Add **970**µL ice-cold HT1 buffer and vortex briefly to mix. This makes 40pM library.
      (4) 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.
      (5) 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

Desired concentration of final pool (pM)		1.4	1.6	1.8
1) Denature pooled libraries:				
	Pooled 4nM libraries (µL)	10	10	10
	0.2N NaOH (µL)	10	10	10
2) Incubate for 5 minutes				
3) Neutralize and Dilute to 40pM				
	Pooled libraries + NaOH from Step 1 (µL)	20	20	20
	200mM Tris pH 7.0 (µL)	10	10	10
	Hyb Buffer (µL)	970	970	970
4) Dilute library to desired loading concentration (pM)		**1.4**	**1.6**	**1.8**
	40pM Libraries from step 2 (µL)	46	52	58
	20pM Denatured PhiX (µL)	23	26	29
	Hyb Buffer (µL)	1231	1222	1213
5) Load Final Pool into Cartridge				
	Final pool (µL)	1300	1300	1300

Analysis

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

FusionPlex assays may also 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 RNA SNP/InDel pipeline is chosen, there is also an option to select a target mutation file (a text file, in VCF format, which lists specific variants of interest). This file also requires a one-time upload. Both of these files can be obtained by contacting tech@archerdx.com.

Demultiplex NextSeq libraries according to recommendations in FAQs:
https://support.archerdx.com/

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