Archer® VariantPlex® HS/HGC Protocol *for Illumina®*

Released November 12, 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 5
This section describes the materials that will be required to complete this protocol, but are not supplied in the kit.

Before Getting Started – page 6
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 10
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 https://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 VariantPlex® 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.

Sequencing data produced by this method should be processed using **Archer Analysis** software - a complete bioinformatics suite that leverages 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 file 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. 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.

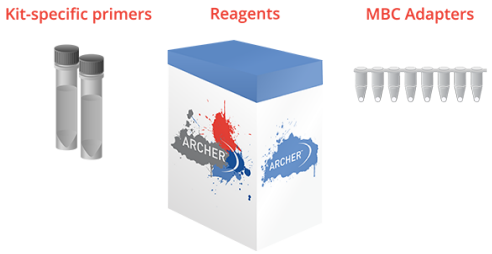
The Archer Analysis software utilizes these molecular barcodes for duplicate read binning, error correction and read deduplication to support quantitative multiplex data analysis and confident mutation detection. Analysis reports both sequencing metrics and number of unique observations supporting called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

Archer Library preparation reagents include:

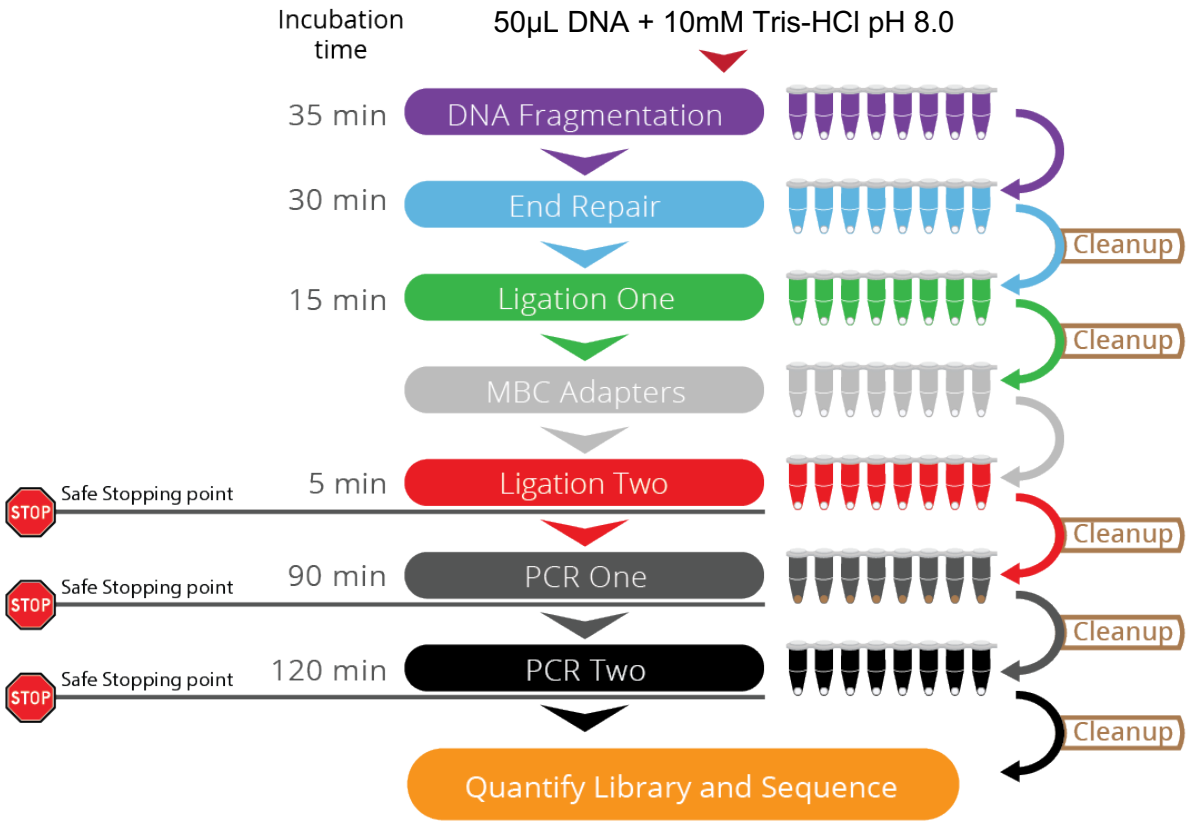
- *Archer VariantPlex reagents in lyophilized format for each step of library preparation.*
- *Gene specific primers (GSPs) that target panel specific regions of interest during PCR amplification.*
- *Archer Molecular Barcode Adapters (MBCs) are proprietary adapters that tag each unique molecule with 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 DNA samples. See individual product inserts for assay targets and read depth requirements.



Protocol Overview



Supplied Reagents

VariantPlex®-HS/HGC Reagents, for Illumina® (HGC, SK0115) (HS, SK0117)

Store at 2°C to 8°C - Allow pouches to reach room temperature before opening.

Description	Part Number (SK0115)	Part Number (SK0117)	Quantity
DNA Fragmentation	SA0370	SA0370	1 pouch (8 reactions/ 8 tube strip)
End Repair	SA0204	SA0204	
Ligation Step 1	SA0196	SA0196	
Ligation Step 2	SA0197	SA0197	
First PCR for Illumina®-HGC	SA0361	-	
Second PCR Reactions 1 thru 8 for Illumina®-HGC	SA0362	-	
First PCR for Illumina®-HS	-	SA0359	
Second PCR Reactions 1 thru 8 for Illumina®-HS	-	SA0360	
500 mM Tris-HCl, pH 8.0	SA0020	SA0020	1 tube/bottle (sufficient for processing 8 samples)
Ultra-Pure Water	SA0213	SA0213	
Ultra-Pure Water for Ethanol Dilution	SA0022	SA0022	
Ligation Cleanup Buffer	SA0209	SA0209	
Ligation Cleanup Beads	SA0210	SA0210	

VariantPlex Frozen Components (part number varies)

Store at -30°C to -10°C

Description	Part Number	Quantity
VariantPlex GSP1	Refer to product insert	Varies (Refer to product Insert)
VariantPlex GSP2	Refer to product insert	
PreSeq® DNA QC Assay Standard	SA0597	
PreSeq® DNA QC Assay 10x Primer Mix	SA0598	

Required Materials Not Supplied

Additional Materials Required for Archer Library Preparation

Description	Supplier	Part Number
Archer MBC Adapters for Illumina®	ArcherDX	Varies
Agencourt® AMPure® XP Beads	Beckman Coulter	A63880
100% Ethanol (ACS grade)	Various	-
RNase AWAY™	Thermo Fisher Scientific	7003
Concentrated NaOH Solution (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	Various	-
qPCR tubes/plates	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® DNA HS Assay Kit	Thermo Fisher Scientific	Q32851

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.

Before Getting Started

Input Nucleic Acid

- Input DNA in *EDTA-free* Tris buffer (pH 7-8) or Ultra-Pure Water is the optimal starting template for VariantPlex Library Preparation.
- Commercial purification kits recommended by Archer can be found on our website at: http://archerdx.com/faqs.

Important: Archer PreSeq DNA QC kit is recommended for use prior to library preparation to indicate the quality of your sample and number of amplifiable genomes present. The PreSeq DNA QC score is tied to Archer Analysis QC and can indicate sample failure or estimated sensitivity for CNV and variant calling prior to beginning library preparation. If you are using PreSeq prior to library preparation, please refer to the PreSeq DNA QC kit protocol at http://archerdx.com/preseq-calculator/.

Reagents to Prepare Before Starting

- Make fresh 10mM Tris-HCl pH 8.0 by mixing 30µL 500mM Tris-HCl, pH 8.0 (SA0020) with 1470µL 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 14mL 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 5µL of 1M NaOH to 995µL of Ultra Pure Water to yield 5mM final NaOH
  - If working from 5M, add 10µL of 5M NaOH to 990µL of Ultra Pure Water to yield 50mM NaOH. Mix well and briefly spin down. Take 100µL of 50mM NaOH and combine with 900µL 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)
Fragmentation	1	4	1
	2	37	12
	3	72	20
	4	4	Hold

	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 (ºC)	Time (min)
Ligation Elution	1	75	10
	2	4	Hold

	Step	Temperature (°C)	Time	Cycles
First PCR Reaction	1	95	3 min	1
	2	95	30 sec	See **Product Insert**
	3	Varies - See **Product Insert**		
	4	72	3 min	1
	5	4	Hold	1

	Step	Temperature (°C)	Time	Cycles
Second PCR Reaction	1	95	3 min	1
	2	95	30 sec	See **Product Insert**
	3	Varies - See **Product Insert**		
	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) The Archer Library Preparation Reagents for Illumina utilize a combination of two indices to distinguish between samples. The first index is added during Adapter Ligation 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 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. Refer to product insert for specific panel read depth recommendations

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

DNA Fragmentation

End Repair

Protocol

Step 1: DNA Fragmentation

1) Place an appropriate number of DNA Fragmentation (SA0370) reaction tubes **on ice**.
2) Adjust purified DNA samples, diluted in 10mM Tris-HCL ph 8.0 to a final volume of **50**µL, combine with DNA Fragmentation lyosphere on ice.
   a) Dissolve, mix and spin down (See **Working with Lyophilized Reaction Pellets** in the preceding section, **Before Getting Started**).
   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) Using a heated lid (≥100°C), start the following thermal cycler program and transfer reactions to the block when it reaches 4°C. Pause the program if necessary.

Incubation Conditions:

Step	Temperature (°C)	Time (minutes)
1	4	1
2	37	12
3	72	20
4	4	Hold

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

Step 2: End Repair

1) Place an appropriate number of End Repair (SA0204) reaction tubes on ice.
2) Transfer the entire volume of **50**µL of DNA Fragmentation reaction to the End Repair lyosphere
   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) Heated lid off

Incubation Conditions:

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

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

Cleanup after End Repair

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

1) Completely resuspend AMPure XP beads by vortexing.
2) Add **2.5**X volume (**125**µL) of AMPure XP beads to each End Repair reaction.

End Repair

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) Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
   a) Add **200**µL 70% ethanol
   b) Incubate for 30 seconds at room temperature (20°C to 25°C)
   c) Carefully remove ethanol and discard
9) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **3-5** 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.
10) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
11) Place tubes back on the magnet for **2** minutes.

Ligation Step 1

Step 3: 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 11 into Ligation Step 1 tubes. (It is acceptable for a small amount of AMPure beads to be carried over to this step.)
   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 Incubation Conditions:

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

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

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 **2.5X** volume (**50**µL) of AMPure to each Ligation Step 1 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 **3-5** 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 4: 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 below:

***Important* This step incorporates the 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) Combine **40**µL of each DNA sample from **Reaction cleanup after Ligation Step 1** step above with a unique Archer MBC Adapter reaction tube. **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 5: Ligation Step 2**.

Step 5: 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 above to Ligation Step 2 tubes.
   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) Heated lid off

Ligation Step 2 Incubation Conditions:

Step	Temperature (°C)	Time (minutes)
1	22	5
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.

Cleanup after Ligation Step 2

Prepare Ligation Cleanup Beads:

1) Completely resuspend Ligation Cleanup Beads by vortexing.
2) For each library, pipette **50**µL of Ligation Cleanup Beads (SA0210) into a new 0.2mL PCR tube.
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: When vortexing PCR tubes in the subsequent steps, maintain firm pressure on all lids as the detergent may allow lids to open.

1) Transfer **50**µL of Ligation Step 2 reaction into the tubes with Ligation Cleanup Beads and Buffer. Your total volume will be **100**µL
   a) Mix samples by vortexing.
   b) Incubate reactions at room temperature for **5** minutes.
   c) Mix samples by vortexing.
   d) Incubate reactions at room temperature for **5** minutes.
   e) Briefly spin down tubes.
2) Place tubes on the magnet for **1** minute **or until beads are fully pelleted** against the tube wall.
3) Carefully pipette off and discard supernatant (**100**µL) without disturbing the beads.
4) Wash beads **two times** with Ligation Cleanup Buffer (SA0209). For each wash:
   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.

5) 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
6) Resuspend ligation cleanup beads in the volume of 5mM NaOH specified in the **Product Insert (A)** corresponding to your target enrichment panel.
7) 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 Elution Incubation Conditions:

Step	Temperature (°C)	Time (minutes)
1	75	10
2	4	Hold

b) After sample has reached 4°C, briefly spin down, and transfer to the magnet.
c) Allow beads to remain on the magnet and fully pellet against the tube wall while you prepare **Step 6: First PCR.**

Step 6: First PCR

1) Place an appropriate number of First PCR HS/HGC (SA0359/SA0361) reaction tubes on ice.
   a) Label tubes by sample number
2) Pipette the volume of GSP1 mix specified in the **Product Insert (B)** into each First PCR tube.
   a) Spin down and return tubes to ice
3) Transfer the entire volume of eluate from **Step 5: Ligation Step 2, 7(c)** above into appropriately labeled PCR1 tube(s).
   a) Mix and spin down.
   b) Return tubes to ice
4) Transfer reactions to a thermal cycler and initiate the program specified in the **Product Insert (First PCR Reaction)**.
5) When the run has completed, briefly spin down reactions and place on ice.

Cleanup after First PCR

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

1) Completely resuspend AMPure XP beads by vortexing.
2) Add **0.8**X volume (**32**µL) of AMPure XP beads to each PCR1 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) Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
   a) Add **200**µL 70% ethanol
   b) Incubate for 30 seconds at room temperature (20°C to 25°C)
   c) Carefully remove ethanol and discard
9) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **3-5** 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.
10) Elute DNA by resuspending beads in the volume of 10mM Tris-HCl pH 8.0 specified in the **Product Insert (C)**.
11) Place tubes back on the magnet for **2** minutes.
12) Carefully transfer the volume of purified eluate specified in the **Product Insert (D)** to a new 0.2mL PCR tube or proceed directly to **Step 7 Second PCR.**

Safe Stopping Point: It is OK to stop and store the reactions at -30°C to -10°C (or leave in thermal cycler on 4°C hold for no more than 24 hours).

Step 7: Second PCR

1) Place an appropriate number of Second PCR HS/HGC (SA0360/SA0362) 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 the P7 index tag for sample-level tracking, take care to record this number for future reference and record which index 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, transfer the entire volume of purified First PCR eluate into each Second PCR tube.

Be sure to record which P7 index sequence is used with which sample.

3) Pipette the volume of GSP2 mix specified in the **Product Insert (E)** 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 the program specified in the **Product Insert (Second PCR Reaction)**.
5) After the program has reached 4°C, briefly spin down reactions and place on ice or immediately proceed to **Cleanup after Second PCR**. It is also acceptable to leave tubes in the thermal cycler overnight at 4°C.

Cleanup after Second PCR

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

1) Completely resuspend AMPure XP beads by vortexing.
2) Add **0.8**X volume (**32**µL) of AMPure XP beads to each PCR2 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) Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
   a) Add **200**µL 70% ethanol
   b) Incubate for 30 seconds at room temperature (20°C to 25°C)
   c) Carefully remove ethanol and discard
9) After the final wash, use a pipette (≤20µL capacity) to completely remove visible supernatant residue and allow tubes to dry for **3-5** 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.
10) Elute DNA by resuspending beads in **20**µL 10mM Tris-HCl pH 8.0.
11) Place tubes back on the magnet for **2** minutes.
12) Carefully transfer **18**µL of the purified solution to a fresh 0.2mL PCR tube. Stop or proceed directly to **Quantify, Normalize and Sequence**. Be sure to avoid transferring beads to the new 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 VariantPlex libraries for the size-adjustment calculation is 250bp.
   b) Archer libraries are very concentrated. You will need to dilute libraries 1:20,000-1:250,000 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/documents/ and http://archerdx.com/faqs.

Sequence

Loading recommendations are provided below. **The final loading concentration must be optimized by each user.**

3) For MiSeq, use the read level sequence depth in the table below:

Recommended MiSeq read lengths:

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

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

Note: The amount of PhiX depends on the complexity of the final library pool. A higher concentration of PhiX is recommended for 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

1	Denature pooled libraries:	Volume (µL)					
	Pooled 4nM libraries	10	10	10	10	10	10
	0.2N NaOH	10	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	20
	200mM Tris pH 7.0	10	10	10	10	10	10
	Hyb Buffer	970	970	970	970	970	970
3	Dilute library to desired pM concentration:						
Final loading concentration (pM) →		**13**	**14**	**15**	**16**	**17**	**18**
	40pM Libraries from step 2	325	350	375	400	425	450
	10pM Denatured PhiX	68	74	79	84	89	95
	Hyb Buffer	607	576	546	516	486	455
5	Load Final Pool into Cartridge	1000	1000	1000	1000	1000	1000

4) For NextSeq, load sequencing libraries with 20% PhiX as a starting point, prepared as follows:
   a) Dilute and denature PhiX to 20pM according to the Illumina protocol.
   b) Begin with a 4nM pool of your barcoded libraries:
      i) 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.
      ii) Add **10**µL 200mM Tris pH 7.0 and vortex briefly to mix.
      iii) Add **970**µL ice-cold HT1 buffer and vortex briefly to mix. This makes 40pM library.
      iv) 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.
      v) 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:			
Final loading concentration (pM) →		**1.4**	**1.6**	**1.8**
	40pM Libraries from step 2	46	52	58
	20pM Denatured PhiX	23	26	29
	Hyb Buffer	1231	1222	1213
5	Load Final Pool into Cartridge	1300	1300	1300

Analysis

Data Analysis

Upon completion of the run, the data should be analyzed using Archer Analysis at http://analysis.archerdx.com/. Archer Analysis may be run via either a local software installation or Archer Unlimited.

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

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