Notices

Limitations of use

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

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

Safety Notices

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

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

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

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Revision history

Document Number	Date	Description of change
RA-DOC-049/REV01	June 2023	• Initial release.
RA-DOC-049/REV02	August 2023	• Updated coloring of figure under “Workflow” section.
RA-DOC-049/REV03	September 2023	• Under section “Working with liquid reagents”, added enzyme and buffer component freeze thaw information
RA-DOC-049/REV04	November 2023	• Updated First and Second PCR cycling conditions to include separate anneal and extended steps. • Edited reaction mix table under “Step 4: PreSeq RNA QC Assay” for clarity. • Updated branding. • Updated links and email addresses.
RA-DOC-049/REV05	January 2025	• Updated ethanol volume in cleanup steps. • Updated reagent overage percentages. • Added an automation note to increase elution volume where necessary. • Updated PCR1 and PCR2 with automation note. • Made note to scale volumes in “Reagents to Prepare Before Starting” section. • Updated wording for unsealing liquid adapters for clarity.

Technical support

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

Overview

Intended use

The Archer FUSION*Plex*-HT protocol is intended for research applications in conjunction with Archer reagent kits and corresponding target-enrichment panels to produce high-complexity libraries for use with Illumina next- generation sequencing (NGS) platforms.

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

Test principle

Anchored Multiplex PCR (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 (MBCs) 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 fusions.

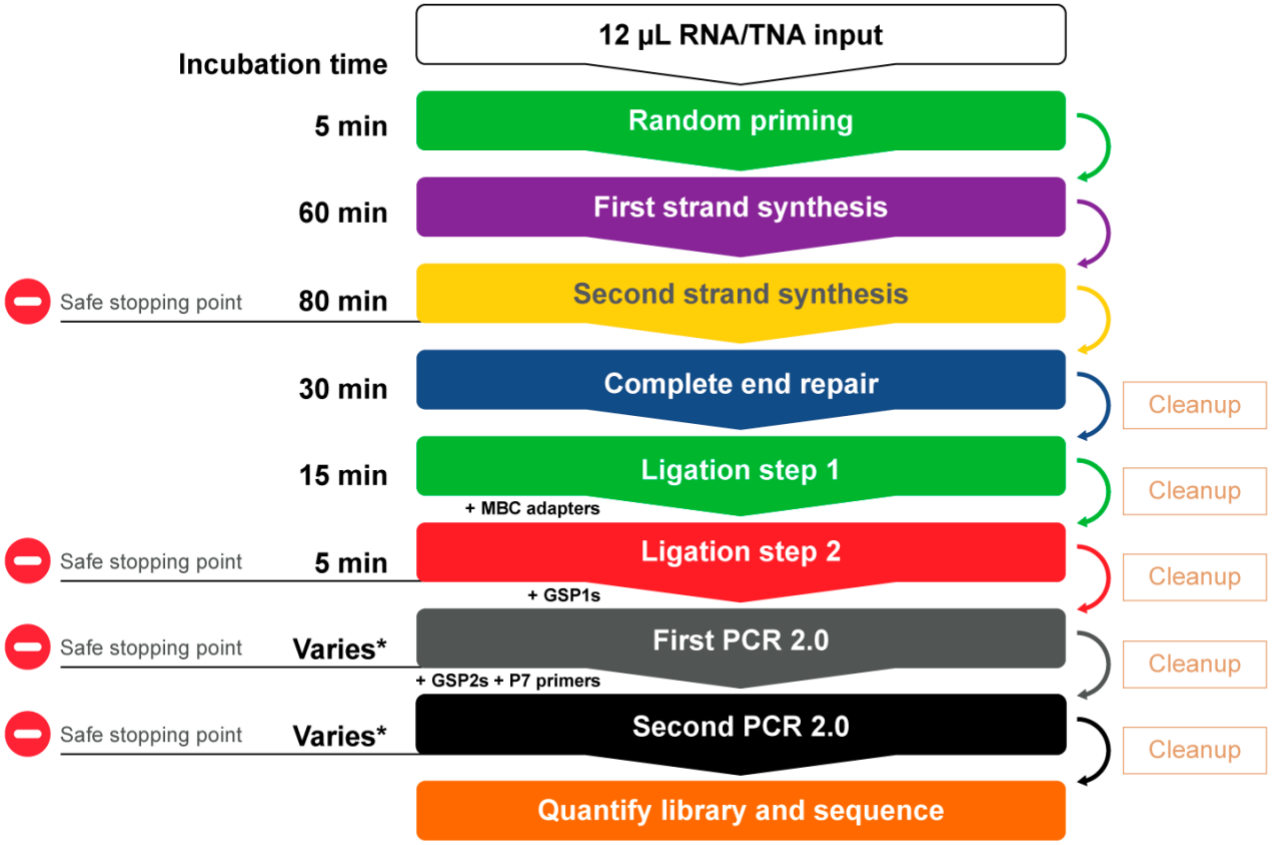
Modular assay format Archer library preparation reagents include:

- Archer FUSION*Plex*-HT reagents in liquid format for each step of library preparation.
- GSPs that target panel-specific regions of interest during PCR amplification.
- Archer MBC adapters are proprietary adapters that tag each unique molecule with a barcode and common region prior to amplification.
- Archer PreSeq™ RNA QC assay to determine the quality of starting material.

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 (TNA) or RNA samples. See individual product inserts for panel targets and read depth requirements.

Workflow



** First and Second PCR times vary based on the specific panel as well as individual lab cycling conditions. See Product Insert for more information.*

FUSION*Plex*-HT Liquid Reagents for Illumina (SK0197, SK0191)

Store at -30°C to -10°C

Materials Supplied		
Description	**Part Number – 24 reaction kit (SK0197)**	**Part Number – 96 reaction kit (SK0191)**
Random Priming Buffer Mix	*SA0826*	*SA0821*
First Strand cDNA Synthesis Buffer Mix	*SA0827*	*SA0818*
First Strand Synthesis Enzyme A	*SA0828*	*SA0819*
First Strand Synthesis Enzyme B	*SA0829*	*SA0820*
Second Strand Synthesis Buffer Mix	*SA0830*	*SA0823*
Second Strand Synthesis Enzyme A	*SA0831*	*SA0824*
Second Strand Synthesis Enzyme B	*SA0832*	*SA0825*
Complete End Repair Buffer Mix	*SA0804*	*SA0686*
Complete End Repair Enzyme A	*SA0805*	*SA0680*
Complete End Repair Enzyme B	*SA0806*	*SA0681*
Ligation Step 1 Buffer Mix	*SA0807*	*SA0685*
Ligation Step 1 Enzyme	*SA0808*	*SA0682*
Ligation Step 2 Buffer Mix 2.0	*SA0810*	*SA0816*
Ligation Step 2 Enzyme	*SA0809*	*SA0683*
First PCR Buffer Mix 2.0	*SA0834*	*SA0817*
Second PCR Buffer Mix 2.0	*SA0835*	*SA0822*
PCR Enzyme	*SA0837*	*SA0836*

Library Prep Cleanup Reagents (SK0190, SK0179)

Store at 2°C to 8°C

Materials Supplied		
Description	**Part Number – 24 reaction kit (SK0190)**	**Part Number – 96 reaction kit (SK0179)**
Ligation Cleanup Beads	*SA0655*	*SA0689*
Ligation Cleanup Buffer	*SA0656*	*SA0690*

Additional components

Store at -30°C to -10°C

Materials Supplied		
Description	**Part Number**	**Quantity**
FUSION*Plex-HT Panel GSP1*	*Refer to product insert*	*24 or 96 reactions*
FUSION*Plex-HT Panel GSP2*	*Refer to product insert*	
10X VCP Primer Mix	*Varies*	

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

Materials Required, But Not Supplied		
Description	**Supplier**	**Part Number**
Liquid P5 MBC + P7 Adapter Kit, 2304 reactions	*Archer*	*Set A – SK0180* *Set B – SK0181*
Liquid P5 MBC + P7 Adapter Kit, 96 reactions	*Archer*	*Set A – SK0186* *Set B – SK0187*
AMPure® XP beads	*Beckman Coulter*	*A63880*
iTaq™ Universal SYBR® Green Supermix	*Bio-Rad Laboratories*	*172-5120*
1 M Tris-HCl, pH 8.0 (molecular biology grade)	*Various*	-
Ultrapure water (molecular biology grade)	*Various*	-
200 mM Tris-HCl, pH 7.0 (for sequencing)	*Various*	-
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*
Standard PCR thermal cycler	*Various*	-
Real-Time PCR thermal cycler	*Various*	-
qPCR tubes	*Various*	-
0.2 mL PCR tubes or 96-well plates	*Various*	-

DynaMag™-96 Side Magnet	*Thermo Fisher Scientific*	*12331D*
Microcentrifuge	*Various*	-
Plate centrifuge	*Various*	-
Pipettes (P10, P20, P200 and P1000)	*Pipetman or equivalent*	-
Sterile, nuclease-free aerosol barrier pipette tips	*Various*	-
Vortex mixer	*Various*	-
PCR tube cooling block	*Various*	-
Gloves	*Various*	-
Qubit® Fluorometer (3.0 or higher)	*Thermo Fisher Scientific*	*Q33216*
Qubit RNA HS Assay Kit	*Thermo Fisher Scientific*	*Q32852*
-20℃ 1.5mL Tube Benchtop Cooler Box	*Various*	-

Before getting started

Important precautions

- **Read through the entire protocol before starting your library preparation.**
- Take note of safe 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 (e.g., 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. When using a thermal cycler with a 100% ramp rate >6°C/sec, set the ramp rate to go no higher than 6°C/sec for First PCR and Second PCR.
- Reaction cleanup with AMPure XP beads (Beckman Coulter) is performed at room temperature (20°C to 25°C) and is used repeatedly throughout the 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.
- If utilizing 96-well plates, make sure they are nuclease free, wells are of sufficient volume and plate seals are sufficient to prevent moisture loss.

Working with liquid reagents

- FUSION*Plex*®-HT enzymes are sensitive to frequent temperature changes and potential freeze-thaw events.
- Enzymes will not freeze when stored at recommended storage temperature range but may freeze during shipment.
- For best results, it is recommended that you store the enzymes provided in a -20°C benchtop cooler box and use this to transport enzymes between workspaces and freezers.
- Enzyme components can undergo up to 5 freeze-thaw cycles without significant effect on functionality.
- Buffer mixes will freeze at recommended storage temperature and should be thawed on ice prior to use. For best results, buffer mixes should be aliquoted to appropriate batch sizes for single use with adequate overage, such that aliquots are not re-frozen after thawing.

- Buffer components can undergo up to 15 freeze-thaw cycles without significant effect on functionality.
- If using adapter plates which contain 24 reactions per well, it is recommended to aliquot into batch sizes for single use with adequate overage to avoid excessive freeze thaw cycles.
- Please reference Product Insert Liquid P5 MBC + P7 Adapter Kits for Illumina (RA-DOC-001) for more information on working with liquid adapters.
- If preparing reagents for a liquid handler protocol, we recommend a batch size that does not exceed 3 reagent withdrawals from the stock tubes to ensure enough overage is always present (e.g., 8 samples per run using a 24-reaction kit).

Input nucleic acid

- Input nucleic acid (TNA, RNA) in EDTA-free buffer (pH 7-8) or ultrapure water is the optimal starting template for AMP library preparation. Do NOT use EDTA-containing buffers.
    - Note: Some FUSION*Plex* 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.
- Contact technical support (archer-tech@idtdna.com) for commercially available extraction kit recommendations.
- Use the maximum allowable input mass (ng) whenever possible. Higher input quantities enable more sensitive fusion detection:
    - 10 – 200 ng of RNA/TNA for FUSION*Plex*-HT
- If using 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.

Reagents to prepare before starting

- The volumes shown below need to be increased if you are preparing more than 24 libraries.
- Make at least **10 mL** fresh 10 mM Tris-HCl, pH 8.0 from 1M Tris-HCl, pH 8.0 and ultrapure water.
    - Mix **100 µL** 1M Tris-HCl, pH 8.0 with **9,900 µL** ultrapure water.
    - 10 mM Tris-HCl, pH 8.0 can be used for up to one week after mixing.
- Make at least **50 mL** fresh 70% ethanol from 100% ethanol and ultrapure water.
    - Add **35 mL** 100% ethanol to **15 mL** ultrapure water.
    - Tightly close the cap to minimize evaporation when not in use.
    - 70% ethanol is appropriate for use for up to one week after mixing.

- Make at least **1 mL** fresh 5 mM NaOH working stock from concentrated NaOH and ultrapure water.
    - If working from 1 M NaOH, add **5 µL** of 1 M NaOH to **995 µL** of ultrapure water to yield 5 mM final NaOH.
    - If working from 5 M, add **10 µL** of 5 M NaOH to **990 µL** of ultrapure water to yield 50 mM NaOH. Mix well and briefly spin down. Take **100 µL** of 50 mM NaOH and combine with **900 µL** of ultrapure water to yield 5 mM NaOH. Mix well and briefly spin down.

Thermal cycler programs

- Preprogram your thermal cycler with these specific programs.
- Use the appropriate programs for the specific Archer assay.
- Verify programming before initiating runs.

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

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

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

	Step	Temperature (°C)	Time (min)	Data Acquisition	Cycles
Preseq RNA QC Assay *(on Real-Time PCR Instrument)*	*Activation*	*95*	*20 [20*]*	*Off*	*1*
	Denaturation	*95*	*15 [3*]*	*Off*	*35*
	Primer annealing & extension	*60*	*60 [30*]*	*On*	
	Melt-curve gradient	*60-95*	*0.5°C/sec increment*	*On*	*1*

*Times in [ ] are for fast-mode cycling. Only use fast-mode conditions if specifically supported by the qPCR instrument.

	Step	Temperature (°C)	Time (min)
Complete End Repair *(Heated lid off)*	*1*	*25*	*30*
	2	*4*	*Hold*

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

	Step	Temperature (°C)	Time (min)
Ligation Step 2 *(Heated lid off)*	*1*	*22*	*5*
	2	*4*	*Hold*

	Step	Temperature (°C)	Time (min)
Ligation Elution	*1*	*75*	*10*
	2	*4*	*Hold*

	Step	Temperature (°C)	Time	Cycles
First PCR	*1*	*95*	*3 min*	*1*
	2	*95*	*30 sec*	*Varies–See panel specific product insert)*
	3	*Varies–See panel specific product insert)*	*10 sec*	
	4		*Varies–See panel specific product insert) 100% ramp rate**	
	5	*72*	*3 min*	*1*
	6	*4*	*Hold*	*1*

*See ramp rate guidelines in the “Before getting started” section.

	Step	Temperature (°C)	Time	Cycles
Second PCR	*1*	*95*	*3 min*	*1*
	2	*95*	*30 sec*	*Varies–See panel specific product insert)*
	3	*Varies–See panel specific product insert)*	*10 sec*	
	4		*Varies–See panel specific product insert) 100% ramp rate**	
	5	*72*	*3 min*	*1*
	6	*4*	*Hold*	*1*

*See ramp rate guidelines in the “Before getting started” section.

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. 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

- 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”.
- The Archer Library Preparation Reagents for Illumina use 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) within the Liquid Adapter Plate. Index 1 is added during the Second PCR and is embedded in Illumina Index 1 Primers (p7/i7) within the Liquid P7 Index Plate.
- To maintain appropriate coverage depth, it is recommended that users determine the maximum number of samples that can be run on a flow cell. 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 panel specific read depth recommendations.

Barcode diversity

- Illumina sequencers 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 Index 1 Primers, the run may fail due to low barcode diversity. In this example it is recommended to use eight different Archer MBC adapters paired with eight different Index 1 Primers.
- Each Liquid P5 MBC + P7 Adapter Kit contains a Liquid P5 MBC Adapter Plate and a Liquid P7 Index Plate each containing 96 unique indexes. When used together, these two plates can generate up to 96 unique dual indexed samples. For best results, use at least 6 sequential adapters per sequencing run. If less than 6 sequential adapters are used, the percent of phiX should be increased.

Protocol

Before beginning, review precautions in "Working with Liquid Reagents" section for additional information regarding best practices for enzymes and buffers.

Step 1: Random Priming

1. Thaw the **Random Priming Mix** on ice. Briefly vortex and spin down buffer mix.
2. Adjust purified RNA/TNA samples to a final volume of **12 µL** and transfer to a new 0.2 mL 8-strip tube or plate wells. Keep input samples on ice.

Component	Reaction Mix
Ultrapure water	12 - Xµl
Purified nucleic acid or RNA	Xµl
Total volume	*12µl*

3. On ice, aliquot enough **Random Priming Buffer Mix** based on the desired number of reactions (including but not exceeding 50% overage) using the table below.

Random Priming Master Mix	1X (µL)	___X (µL)
Random Priming Buffer Mix (SA0826 or SA0821)	3	

   a. Mix by briefly vortexing and spin down.
   b. Keep tubes on ice.
4. To each **12 µL** input sample, add **3 µL** Random Priming Master Mix.
   a. Mix by briefly vortexing and spin down.
   b. Return tubes to ice.
5. Start the following thermal cycler program, and only transfer reactions to the block once temperature reaches 65°C. Pause the program if necessary.
   a. Use a heated lid (≥100°C).

Random Priming incubation conditions

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

b. After the program has reached 4°C, briefly spin down reactions and place on ice for **at least 2 minutes.**

Step 2: First Strand cDNA Synthesis

1. Thaw the **First Strand cDNA Synthesis Buffer Mix on ice.** Briefly vortex buffer mix and either vortex or pipette mix enzymes, then spin down.
2. On ice, make a **First Strand cDNA Synthesis Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

First Strand cDNA Synthesis Master Mix	1X (µL)	___X (µL)
First Strand cDNA Synthesis Buffer Mix (SA0827 or SA0818)	3	
First Strand cDNA Synthesis Enzyme A (SA0828 or SA0819)	1	
First Strand cDNA Synthesis Enzyme B (SA0829 or SA0820)	1	
Total volume	*5*	

   a. Mix by briefly vortexing and spin down.

   b. Keep master mix on ice.

3. To each **15 µL** Random Priming reaction from Step 1: Random Priming, add **5 µL** First Strand cDNA Synthesis Master Mix.

   a. Mix by briefly vortexing and spin down.

   b. Return tubes to ice.

4. Transfer reactions to a preheated thermal cycler and initiate an incubation using the following program and guidelines:

   a. Use a heated lid (≥100°C).

First Strand cDNA Synthesis incubation conditions

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

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

5. Make diluted cDNA samples for the PreSeq RNA QC Assay.
    a. Place a new, empty 8-strip of PCR tubes on ice.
    b. For each sample, pipette **9 µL** of ultrapure water into the new PCR strip.
    c. Pipette **1 µL** of each First Strand cDNA synthesis reaction into the water, mix briefly by vortexing and spin down.
    d. Keep on ice for use in Step 4: PreSeq RNA QC Assay.

Step 3: Second Strand cDNA Synthesis

1. Thaw the **Second Strand Synthesis Buffer Mix** on ice. Briefly vortex buffer mix and either vortex or pipette mix enzymes, then spin down.
2. On ice, make a **Second Strand Synthesis Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

Second Strand Synthesis Master Mix	1X (µL)	___X (µL)
Second Strand Synthesis Buffer Mix (SA0830 or SA0823)	15	
Second Strand Synthesis Enzyme A (SA0831 or SA0824)	4	
Second Strand Synthesis Enzyme B (SA0832 or SA0825)	1	
Nuclease-free water *(omit if Step 4: PreSeq QC is not needed)*	1	
Total volume	*21*	

    a. Mix by briefly vortexing and spin down.
    b. Keep master mix on ice.

3. To each **19 µL** First Strand Synthesis sample from Step 2: First Strand cDNA Synthesis, add **21 µL** of Second Strand cDNA Synthesis Master Mix. If Step 4: PreSeq QC is not needed, to each **20 µL** First Strand Synthesis sample, add **20 µL** of Second Strand cDNA Synthesis Master Mix.
    a. Mix by briefly vortexing and spin down.
    b. Return tubes to ice.
4. Transfer reactions to a preheated thermal cycler and initiate an incubation using the following program and guidelines:
    a. Use a heated lid (≥100°C).

Second Strand cDNA Synthesis incubation conditions

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

    b. Place samples in the thermal cycler and start the program.
    c. While the samples are incubating, proceed to Step 4: PreSeq RNA QC Assay.
    d. When the run has completed, briefly spin down reactions and place on ice.

Safe stopping point: It is okay 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 the **10X VCP Primer Mix** at room temperature. Once thawed, store 10X VCP Primer Mix on ice.
2. Place a fresh qPCR plate or tubes appropriate to your Real-Time PCR instrument on ice.
3. Prepare sufficient qPCR reaction mix for:
    a. **Duplicate reactions** of each diluted cDNA sample from Step 2: First Strand cDNA Synthesis.
    b. **One No Template Control (NTC)** made using ultrapure water.

Component	Part Number	Reaction Mix (n = 1)
iTaq SYBR Green Supermix	Not Supplied	5 µL
10X VCP Primer Mix	Varies	1 µL
Total reaction mix volume	-	*6 µL*

4. Pipette **6 µL** of the reaction mix into each assigned well of a qPCR plate/tube.
5. Pipette **4 µL** of the diluted cDNA samples or NTC (ultrapure water) into the assigned wells/tubes containing reaction mix for a total of **10 µL.**
    a. Mix slowly to avoid introducing bubbles, cap or seal the reactions and spin down.
6. Transfer reactions to a Real Time-PCR instrument and initiate a run using the following program:

PreSeq RNA QC Assay Real-Time incubation conditions

Step	Temperature (°C)	Time (sec)	Data Acquisition	Cycles
Activation	95	20 [20*]	Off	1
Denaturation	95	15 [3*]	Off	35
Primer annealing & extension	60	60 [30*]	On	
Melt-curve gradient	60-95	0.5°C/sec increment	On	1

*Times in [ ] are for fast-mode cycling. Only use fast-mode conditions if specifically supported by the qPCR instrument.

7. After completion of the program, review the Ct values of each sample.
    a. A low Ct value generally correlates with a high probability for good QC values when analyzing the samples in Archer Analysis.
    b. A high Ct value or N/A result indicates issues with the sample quality or quantity.
    c. A lab should establish their own Ct-value threshold for when the prospects of success for individual samples are too low to process further in the library prep.

Step 5: Complete End Repair

1. Thaw the **Complete End Repair Buffer Mix** on ice. Briefly vortex buffer mix and either vortex or pipette mix enzymes, then spin down.
2. On ice, make a **Complete End Repair Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

Complete End Repair Master Mix	1X (µL)	___X (µL)
Complete End Repair Buffer Mix (SA0804 or SA0686)	8.86	
Complete End Repair Enzyme A (SA0805 or SA0680)	1	
Complete End Repair Enzyme B (SA0806 or SA0681)	0.14	
Total Volume	*10*	

   a. Mix by briefly vortexing and spin down.

   b. Keep master mix on ice.

3. To each **40** µL cDNA sample from Step 3: Second Strand cDNA Synthesis, add **10** µL of Complete End Repair Master Mix.

   c. Mix by briefly vortexing and spin down.

   d. Return tubes to ice.

4. Transfer reactions to a preheated thermal cycler and initiate an incubation using the following program and guidelines:

   e. Heated lid off. If the lid is still hot, consider using a different thermal cycler or leaving the lid open.

Complete End Repair incubation conditions

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

   f. Place samples in the thermal cycler and start the program.

   g. When the run has completed, briefly spin down reactions and place on ice.

Reaction Cleanup after Complete End Repair

Refer to Important Precautions section for guidance on working with AMPure XP beads.

1. Completely resuspend AMPure XP beads by vortexing.
2. Remove tubes from ice and add **2.5X** volume (**125 µL**) of AMPure XP beads to each Complete End Repair 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 **180 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 (≤20 µL 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 **16 µL** 10mM Tris-HCl, pH 8.0.

    ***Note that for automated workflows it is acceptable to add 1 µL 10mM Tris-HCl, pH 8.0 to the bead resuspension volume shown above. This extra 1 µL should be left behind when removing the purified DNA in upcoming steps.**

11. Place tubes back on the magnet for **2 minutes**.

Step 6: Ligation Step 1

1. Thaw the **Ligation Step 1 Buffer Mix** on ice. Briefly vortex buffer mix and either vortex or pipette mix enzymes, then spin down.

2. On ice, make a **Ligation Step 1 Master Mix** working solution based on the desired number of reactions (including but not exceeding 50% overage) using the table below.

Ligation Step 1 Master Mix	1X (µL)	___X (µL)
Ligation Step 1 Buffer Mix (SA0807 or SA0685)	3.7	
Ligation Step 1 Enzyme (SA0808 or SA0682)	0.3	
Total Volume	*4*	

   a. Mix by briefly vortexing and spin down.

   b. Keep master mix on ice.

3. Transfer **16 µL** of eluted DNA from Step 5: Complete End Repair into new 0.2mL 8-strip tubes or plate wells. It is acceptable for a small amount of AMPure XP beads to be transferred.

4. Add **4 µL** of Ligation Step 1 Master Mix to each sample.

   a. Mix by briefly vortexing and spin down.

   b. Return tubes to ice.

5. Transfer reactions to a preheated 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 the program.

   c. After the program has reached 4°C, remove tubes from the temperature block, briefly spin down reactions and place on ice.

Reaction Cleanup after Ligation Step 1

Refer to Important Precautions section for guidance on working with AMPure XP beads.

1. Completely resuspend AMPure XP beads by vortexing.

2. Remove tubes from ice and add **2.5X** volume (**50 µL**) of AMPure XP beads 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 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. Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
    a. Add **180 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 (≤20 µL 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 **25 µL** 10 mM Tris-HCl, pH 8.0.
11. Place tubes back on the magnet for **2 minutes**.

Step 7: MBC Adapter Incorporation

1. Thaw **Liquid P5 MBC Adapter Plate(s)** (SA0691, SA0693, SA0800, or SA0802). Spin down before unsealing plate. It is acceptable to pierce the foil seal with a pipette tip to access the liquid adapters.

    ***Important* As this step incorporates the P5 index tag for sample-level tracking, be sure to record which MBC adapter is being used for each sample.**

2. Transfer **23 µL** of eluted DNA from the cleanup after Step 6: Ligation Step 1 above to new 0.2mL 8-strip tubes or plate wells. **Avoid pipetting AMPure XP beads into this reaction.**
3. Add **2 µL** of respective unique **Liquid P5 MBC Adapter** for a total of **25 µL**. Re-seal adapter plate using a new seal after all P5 MBC adapters have been added to respective samples.
    a. Mix by vortexing and spin down.

b. Return tubes to ice.

4. Immediately proceed to Step 8: Ligation Step 2.

Step 8: Ligation Step 2

1. Thaw the **Ligation Step 2 Buffer Mix 2.0** on ice. Vortex buffer mix well and spin down. Either vortex or pipette mix enzymes then spin down.
2. On ice, make a **Ligation Step 2 Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

Due to the viscosity of the Ligation Step 2 Buffer Mix 2.0 components, it is essential to slowly pipette mix the stock solution at least 10 times then vortex for 10 seconds BEFORE aliquoting the required volume for the working solution and again AFTER the addition of the Ligation Step 2 Enzyme.

Ligation Step 2 Master Mix	1X (µL)	___X (µL)
Ligation Step 2 Buffer Mix 2.0 (SA0810 or SA0816)	24.6	
Ligation Step 2 Enzyme (SA0809 or SA0683)	0.4	
Total volume	*25*	

a. Mix by briefly vortexing and spin down.

b. Keep master mix on ice.

3. Transfer **25 µL** of Ligation Step 2 Master Mix into each tube or well containing the **25 µL** of each sample (DNA + Liquid MBC Adapter) from Step 7: MBC Adapter Incorporation above.

a. Mix well and spin down. **Due to the viscosity of these components, carefully pipette mixing 10 times in addition to vortexing is required to ensure complete mixing.**

b. Return tubes to ice.

4. Transfer reactions to a preheated 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. After the program has reached 4ºC, briefly spin down reactions and place on ice.

Safe stopping point: It is okay to stop and store the reactions at -30°C to -10°C.

Reaction Cleanup after Ligation Step 2

Caution: This step uses Ligation Cleanup Beads (SA0655 or SA0689) and Ligation Cleanup Buffer (SA0656 or SA0690) instead of AMPure XP beads and 70% ethanol. Make sure to use Ligation Cleanup Beads and Ligation Cleanup Buffer that come with the kit for this step.

Prepare Ligation Cleanup Beads:

1. Completely resuspend **Ligation Cleanup Beads** by vortexing.
2. For each reaction, pipette **50 µL** of Ligation Cleanup Beads into new 0.2 mL 8-strip 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** 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 contained detergent may allow lids to open.

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 **180 µL** Ligation Cleanup Buffer by vortexing, briefly spinning down, and placing back on magnet for **1 minute**.
    b. Once slurry has cleared, discard supernatant.
10. Wash beads **once** with **ultrapure water**:
    a. Resuspend beads in **180 µL** of ultrapure water by vortexing, briefly spinning down and placing back on magnet.
    b. Once slurry has cleared, discard supernatant.
    c. Take care to ensure that all supernatant has been removed from tubes.
11. Elute DNA from Ligation Cleanup Beads:
    a. Resuspend ligation cleanup beads in **24 µL** of 5 mM NaOH.

    ***Note that for automated workflows it is acceptable to add an additional 1 µL 5 mM NaOH to the ligation bead resuspension volume shown above. This extra 1 µL should be left behind when removing the purified DNA in upcoming steps.**

    b. Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:
    c. Use a heated lid (≥100°C).

Ligation Elution incubation conditions

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

    d. After sample has reached 4°C, briefly spin down and transfer to the magnet.

Step 9: First PCR

1. Thaw the **First PCR Buffer Mix 2.0** and **GSP1** on ice. Briefly vortex and spin down buffer mix and primers. Either vortex or pipette mix enzymes then spin down.
2. On ice, make a **First PCR Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

   ***Note that the PCR Enzyme will also be utilized in Second PCR so do not discard unused enzyme after making First PCR Master Mix.**

First PCR Master Mix	1X (µL)	___X (µL)
First PCR Buffer Mix 2.0 (SA0834 or SA0817)	11.2	
PCR Enzyme (SA0837 or SA0836)	0.8	
Total volume	*12*	

   a. Mix by briefly vortexing and spin down.

   b. Keep master mix on ice.

3. To new 0.2mL 8-strip tubes or plate wells add:

   a. **12 µL** First PCR Master Mix

   b. **4 µL** GSP1

   ***Note that for automated workflows, the GSPs for the panel(s) in use should be added to their own master mix instead of directly to the sample.**

   c. **24 µL** of purified DNA from Step 8: Ligation 2 Cleanup.

4. Mix by briefly vortexing and spin down.
5. Return tubes to ice.
6. Transfer reactions to a preheated thermal cycler and immediately initiate the program specified in the panel specific **Product Insert (First PCR Reaction).**

   a. Use a heated lid (≥100°C).

   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

Refer to Important Precautions section for guidance on working with AMPure XP beads.

1. Completely resuspend AMPure XP beads by vortexing.
2. Add **1.2X** volume (**48 µL**) of AMPure XP beads 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. Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
    a. Add **180 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 (≤20 µL 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 **22 µL** of 10 mM Tris-HCl, pH 8.0.
11. Place tubes back on the magnet for **2 minutes**.
12. Transfer **20 µL** of purified eluate 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 okay to stop and store the reactions at -30°C to -10°C.

Step 10: Second PCR

1. Thaw the **Second PCR Buffer Mix 2.0**, **GSP2**, and **Liquid P7 Index Plate(s)** (SA0692, SA0694, SA0801, or SA0803) on ice. Vortex buffer and GSP2 then spin down. Spin down P7 plate before unsealing. It is acceptable to pierce the foil seal with a pipette tip to access the liquid adapters.
2. On ice, make a **Second PCR Master Mix** working solution based on the desired number of reactions (including but not exceeding 20% overage) using the table below.

Second PCR Master Mix	1X (µL)	___X (µL)
Second PCR Buffer Mix 2.0 (SA0835 or SA0822)	11.2	
PCR Enzyme (SA0837 or SA0836)	0.8	
Total volume	*12*	

   a. Mix by briefly vortexing and spin down.

   b. Keep master mix on ice.

3. To new 0.2mL 8-strip tubes or plate wells add:

   a. **12 µL** Second PCR Master Mix

   b. **4 µL** respective Liquid P7 Index according to sample index planning. Re-seal P7 plate using a new seal after adding P7 index to respective wells.

   c. **4 µL** GSP2

   ***Note that for automated workflows, the GSPs for the panel(s) in use should be added to their own master mix instead of directly to the sample.**

   d. Transfer **20 µL** of purified DNA from Step 9: First PCR.

   ***Important* As this step incorporates the P7 index tag for sample-level tracking, be sure to record which Liquid P7 Index is being used for each sample.**

4. Mix by briefly vortexing and spin down.
5. Return tubes to ice.
6. Transfer reactions to a preheated thermal cycler and immediately initiate the program specified in the panel specific **Product Insert (Second PCR Reaction).**

   a. Use a heated lid (≥100°C).

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

Refer to Important Precautions section for guidance on working with AMPure XP beads.

1. Completely resuspend AMPure XP beads by vortexing.
2. Add **1.2X** volume (**48 µL**) of AMPure XP beads to each Second PCR 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. Wash beads **two times** with 70% ethanol while still on the magnet. For each wash:
    a. Add **180 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 (≤20 µL 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** 10 mM Tris-HCl, pH 8.0.
11. Place tubes back on the magnet for **2 minutes**.
12. Transfer **18 µL** of the purified solution to a new 0.2mL PCR tube. Be sure to avoid transferring beads to the fresh tube.
13. Stop or proceed directly to Quantify, Normalize and Sequence.

Safe stopping point: It is okay to stop and store the reactions at -30°C to -10°C.

Quantify, Normalize, and Sequence

Refer to the Quantify, Normalize, and sequence Protocol for Illumina (RA-DOC-054) for information on quantifying, normalizing and sequencing Archer AMP libraries.

Data analysis

Archer AMP libraries should be demultiplexed to FASTQs prior to analysis. Analyze data with Archer Analysis v7 or higher using either a local software installation or Archer Unlimited. Reference Product Insert Liquid P5 MBC P7 Adapter Kits for Illumina (RA-DOC-001) for additional information on Archer Analysis step up. Visit our website or contact archer-tech@idtdna.com for more information.

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