

## 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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### 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 [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com).

## Overview

### Intended use

The Archer FusionPlex 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 Ion Torrent<sup>™</sup> next-generation sequencing (NGS) platforms.

FusionPlex sequencing data produced by this method should be processed using Archer<sup>™</sup> Analysis software—a complete bioinformatics suite that leverages Anchored Multiplex PCR (AMP<sup>™</sup>) 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 variants.

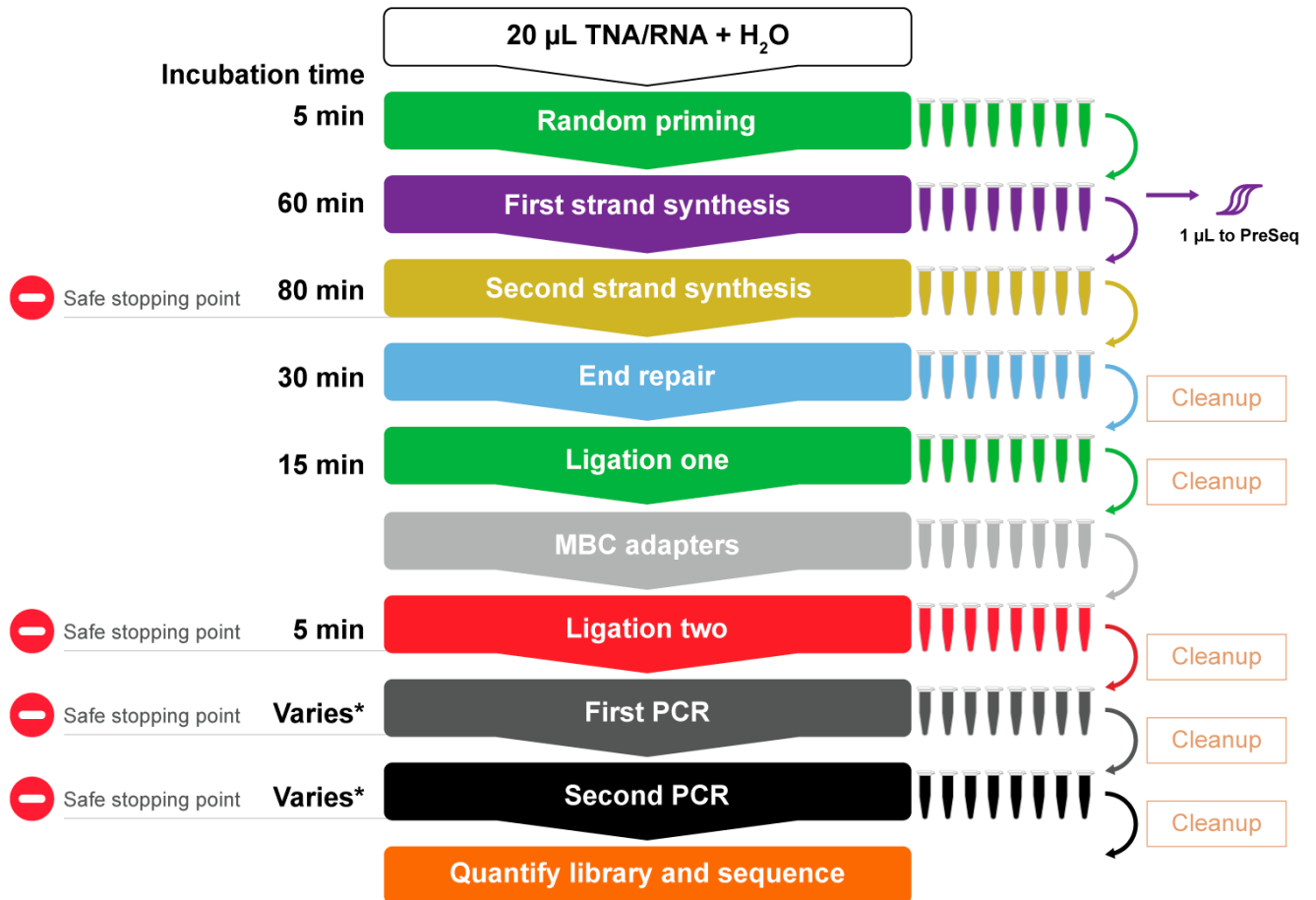
### Modular assay format Archer library preparation reagents include:

- Archer FusionPlex reagents in lyophilized 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<sup>™</sup> 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.

### FusionPlex Reagents for Ion Torrent (SK0094)

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

Materials Supplied		
Description	Part Number	Quantity
<i>Random Priming 2.0</i>	SA0194	1 pouch (8 reactions/one 8-tube strip)
<i>First Strand cDNA Synthesis</i>	SA0002	
<i>Second Strand cDNA Synthesis</i>	SA0003	
<i>End Repair</i>	SA0204	
<i>Ligation Step 1</i>	SA0196	
<i>Ligation Step 2</i>	SA0197	
<i>First PCR (Ion Torrent-P)</i>	SA0111	
<i>Second PCR (Ion Torrent-P)</i>	SA0112	
<i>Ligation Cleanup Beads</i>	SA0210	1 tube
<i>Ligation Cleanup Buffer</i>	SA0209	(sufficient for processing 8 samples)

### Additional components

Store at -30°C to -10°C

Materials Supplied		
Description	Part Number	Quantity
<i>FusionPlex Panel GSP1</i>	<i>Refer to product insert</i>	8 reactions
<i>FusionPlex Panel GSP2</i>	<i>Refer to product insert</i>	
<i>10X VCP Primer Mix</i>	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 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
<i>Archer MBC adapters for Ion Torrent</i>	<i>Archer</i>	<i>Varies</i>
<i>AMPure<sup>®</sup> XP beads</i>	<i>Beckman Coulter</i>	<i>A63880</i>
<i>iTaq<sup>™</sup> Universal SYBR<sup>®</sup> Green Supermix</i>	<i>Bio-Rad Laboratories</i>	<i>172-5120</i>
<i>1 M Tris-HCl, pH 8.0 (molecular biology grade)</i>	<i>Various</i>	<i>-</i>
<i>Ultrapure water (molecular biology grade)</i>	<i>Various</i>	<i>-</i>
<i>200 mM Tris-HCl, pH 7.0 (for sequencing)</i>	<i>Various</i>	<i>-</i>
<i>100% ethanol (ACS grade)</i>	<i>Various</i>	<i>-</i>
<i>Concentrated NaOH solution (ACS grade)</i>	<i>Various</i>	<i>-</i>
<i>RNase AWAY<sup>™</sup></i>	<i>Thermo Fisher Scientific</i>	<i>7003</i>
<i>Ion Library TaqMan<sup>™</sup> Quantification Kit</i>	<i>Thermo Fisher Scientific</i>	<i>4468802</i>
<i>Genexus<sup>™</sup> Sequencing Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A40271</i>
<i>Ion 510<sup>™</sup> Chip Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A34292</i>
<i>Ion 520<sup>™</sup> Chip Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A27762</i>
<i>Ion 530<sup>™</sup> Chip Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A27764</i>
<i>Ion 540<sup>™</sup> Chip Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A27765 or A27766</i>
<i>Ion 550<sup>™</sup> Chip Kit</i>	<i>Thermo Fisher Scientific</i>	<i>A34537 or A34538</i>
<i>Ion S5<sup>™</sup> Controls Kit Plus</i>	<i>Thermo Fisher Scientific</i>	<i>A30729</i>
<i>Standard PCR thermal cycler</i>	<i>Various</i>	<i>-</i>

<i>Real-Time PCR thermal cycler</i>	<i>Various</i>	-
<i>qPCR tubes</i>	<i>Various</i>	-
<i>0.2 mL PCR tubes</i>	<i>Various</i>	-
<i>DynaMag<sup>™</sup>-96 Side Magnet</i>	<i>Thermo Fisher Scientific</i>	<i>12331D</i>
<i>Microcentrifuge</i>	<i>Various</i>	-
<i>Plate centrifuge</i>	<i>Various</i>	-
<i>Pipettes (P10, P20, P200 and P1000)</i>	<i>Pipetman or equivalent</i>	-
<i>Sterile, nuclease-free aerosol barrier pipette tips</i>	<i>Various</i>	-
<i>Vortex mixer</i>	<i>Various</i>	-
<i>PCR tube cooling block</i>	<i>Various</i>	-
<i>Gloves</i>	<i>Various</i>	-
<i>Qubit<sup>®</sup> Fluorometer (3.0 or higher)</i>	<i>Thermo Fisher Scientific</i>	<i>Q33216</i>
<i>Qubit RNA HS Assay Kit</i>	<i>Thermo Fisher Scientific</i>	<i>Q32852</i>

## 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.

### Working with lyophilized reagents

- Archer reagents are provided as individually lyophilized reaction pellets in 0.2 mL PCR tube strips.
- Allow pouches to reach room temperature (20°C to 25°C) before opening to prevent moisture condensation on tubes.
- Always centrifuge tubes briefly before opening to pull contents down.
- Detach the required number of reaction tubes 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 adapter tubes remember to label prior to returning unused portions to storage.
- Dissolve, mix and spin down:
  - **Never touch the lysosphere with the pipette tip.**



- Add sample/reagents to pellets in tubes while on ice.
- Allow at least 5 seconds for pellets to dissolve.
- Pipette up and down 8 times to mix after the lysosphere has dissolved.
- Briefly centrifuge and return to ice before proceeding.

### Input nucleic acid

- Input nucleic acid (TNA or 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.
- Use the maximum allowable input mass (ng) whenever possible. Higher input quantities enable more sensitive variant and fusion detection:
  - 20 - 250 ng of RNA for FusionPlex
- 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.
- Contact technical support ([archer-tech@idtdna.com](mailto:archer-tech@idtdna.com)) for commercially available extraction kit recommendations.

### Reagents to prepare before starting

- 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 from concentrated NaOH and ultrapure water on the day of use.
  - If working from 1M NaOH, add **5 µL** of 1M NaOH to **995 µL** of ultrapure water to yield 5 mM final NaOH.
  - If working from 5M NaOH, add **10 µL** of 5M 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)
<b>Random Priming 2.0</b>	1	65	5
	2	4	Hold

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

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

	Step	Temperature (°C)	Time (sec)	Data Acquisition	Cycles
<b>PreSeq RNA QC Assay</b> (on Real-Time PCR instrument)	<i>Activation</i>	95	20 [20*]	Off	1
	<i>Denaturation</i>	95	15 [3*]	Off	35
	<i>Primer annealing &amp; extension</i>	60	60 [30*]	On	
	<i>Melt-curve gradient</i>	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)
<b>End Repair</b> (Heated lid off)	1	25	30
	2	4	Hold

	Step	Temperature (°C)	Time (min)
<b>Ligation Step 1</b>	1	37	15
	2	4	Hold

	Step	Temperature (°C)	Time (min)
<b>Ligation Step 2</b> (Heated lid off)	1	22	5
	2	4	Hold

	Step	Temperature (°C)	Time (min)
<b>Ligation Elution</b>	1	75	10
	2	4	Hold

	Step	Temperature (°C)	Time	Cycles
<b>First PCR</b>	1	95	3 min	1
	2	95	30 sec	<i>Varies—See panel specific product insert)</i>
	3	<i>Varies—See panel specific product insert)</i>	<i>Varies—See panel specific product insert) 100% ramp rate*</i>	
	4	72	3 min	1
	5	4	Hold	1

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

	Step	Temperature (°C)	Time	Cycles
<b>Second PCR</b>	1	95	3 min	1
	2	95	30 sec	<i>Varies—See panel specific product insert)</i>
	3	<i>Varies—See panel specific product insert)</i>	<i>Varies—See panel specific product insert) 100% ramp rate*</i>	
	4	72	3 min	1
	5	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 (visit [archerdx.com/technology-platform/analysis](http://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

- For Ion Torrent kits, the protocol uses a single index to distinguish between samples.
- To efficiently utilize the throughput of the Ion Torrent sequencer, multiple samples should be sequenced simultaneously. Samples can be identified through a unique nucleotide sequence that is part of the molecular barcode adapter ligated to the nucleic acid sequence of interest during library construction, and which is subsequently read during the sequencing process. The unique nucleotide sequence is often termed an “index”. The index is added during Ligation Step 2 and is embedded in the molecular barcode adapter.
- To maintain appropriate coverage depth, it is recommended that users determine the maximum number of samples that can be run on the chip being used. In general, larger panels with more targets will require higher sequencing coverage depth and should be run with fewer samples per chip.
- Contact [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com) to receive tools that can help defining the appropriate number of samples per chip.

## Protocol

Before beginning, review precautions in “Working with Lyophilized Reagents” section for additional information regarding best practices using lyophilized reagents.

### Step 1: Random Priming 2.0

- Place an appropriate number of **Random Priming 2.0** (SA0194) reaction tubes on ice.
- Adjust purified RNA/TNA (20 - 250 ng) samples to a final volume of 20  $\mu\text{L}$  and transfer to new PCR tubes. Keep input samples on ice.

Component	Reaction Mix
Ultrapure water	20 - X $\mu\text{L}$
Purified nucleic acid or RNA	X $\mu\text{L}$
<i>Total volume</i>	<i>20<math>\mu\text{L}</math></i>

- Transfer **20  $\mu\text{L}$**  reaction mix to the Random Priming 2.0 reaction tubes.
  - Dissolve, mix and spin down.
  - Return tubes to ice.
- Start the following thermal cycler program, and only transfer reactions to the block once temperature reaches 65°C. Pause the program if necessary.
  - Use a heated lid ( $\geq 100^\circ\text{C}$ ).

#### *Random Priming 2.0 Incubation conditions*

Step	Temperature ( $^\circ\text{C}$ )	Time (minutes)
1	65	5
2	4	Hold

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

### Step 2: First Strand cDNA Synthesis

- 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 tubes.
  - a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
3. Transfer reactions to a preheated thermal cycler and initiate an incubation using the following program and guidelines:
  - a. Use a heated lid ( $\geq 100^{\circ}\text{C}$ ).

***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  $4^{\circ}\text{C}$ , briefly spin down reactions and place on ice.
4. 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 and pipette up and down to mix.
  - d. 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 ultrapure water 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 diluted First Strand reaction into the new Second Strand cDNA Synthesis tubes.

- a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
4. Start the following thermal cycler program, and only transfer reactions to the block once temperature reaches 16°C. Pause the program if necessary.
- a. Use a heated lid ( $\geq 100^{\circ}\text{C}$ ).

***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 sample(s) are incubating, proceed to Step 4: PreSeq RNA QC Assay.
- d. After 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  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$ . 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** (SA0126) 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	SA0126	1 µL
Diluted cDNA sample or NTC	-	4 µL
<i>Total volume</i>	-	<i>10 µL</i>

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.
  - 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. It is possible to purchase a set of the first 3 steps of the FusionPlex library prep individually (SK0107 PreSeq RNA QC Assay Reagents - 16 reactions), to allow for affordable repetition of difficult samples, without wasting a whole reaction.

## 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 preheated thermal cycler and initiate an incubation using the following program and guidelines:
  - a. Heated lid off. If the lid is still hot, consider using a different thermal cycler or leaving the lid open.

### *End Repair Incubation conditions*

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

- b. Place samples in the thermal cycler and start the program.
- c. 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 (**100 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 **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 (≤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** 10mM Tris-HCl, pH 8.0.
11. 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 cDNA from Reaction Cleanup after End Repair, step 11 into Ligation Step 1 tubes. It is acceptable for a small amount of AMPure XP beads to be transferred.
  - a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
3. 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 **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 (≤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 **42 µL** 10 mM Tris-HCl, pH 8.0.
11. Place tubes back on the magnet for **2 minutes**.

## Step 7: MBC Adapter Incorporation

1. Label MBC adapter tubes with the sample index 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 the 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 11 to a unique MBC adapter reaction tube. **Avoid pipetting AMPure XP beads into this reaction.**
  - a. If AMPure XP beads are carried over, place the MBC adapter tubes on the magnet for one minute and transfer all liquid to the Ligation Step 2 tubes while the MBC adapter tubes remain on the magnet.
  - b. 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 cDNA sample from Step 7: MBC Adapter Incorporation to Ligation Step 2 tubes.
  - a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
3. Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:
  - a. Heated lid off.

### *Ligation Step 2 Incubation conditions*

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

b. After the program has reached 4°C, 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 (SA0210) and Ligation Cleanup Buffer (SA0209) 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 **200 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 **200 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 **18 µL** of 5 mM NaOH.
  - b. Transfer reactions to a thermal cycler and initiate an incubation using the following program and guidelines:
  - c. Use a heated lid ( $\geq 100^{\circ}\text{C}$ ).

***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. Place an appropriate number of **First PCR** (SA0111) reaction tubes on ice.
  - a. Label tubes by sample number.
2. Pipette **2 µL** of **GSP1 (red cap)** into each First PCR tube.
  - a. Spin down and return tubes to ice.

3. Pipette **18 µL** of supernatant from Reaction Cleanup after Ligation Step 2, step 11 into appropriately labeled First PCR tubes.
  - a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
4. 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 ( $\geq 100^{\circ}\text{C}$ ).
  - b. After the program has reached  $4^{\circ}\text{C}$ , briefly spin down reactions and place on ice. It is also acceptable to leave tubes in the thermal cycler at  $4^{\circ}\text{C}$  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 (**24 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 ( $20^{\circ}\text{C}$  to  $25^{\circ}\text{C}$ ).
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 ( $20^{\circ}\text{C}$  to  $25^{\circ}\text{C}$ )
  - c. Carefully remove ethanol and discard
9. After the final wash, use a pipette ( $\leq 20$  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** 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 (or leave in thermal cycler on 4°C hold for no more than 24 hours).

## Step 10: Second PCR

1. Place an appropriate number of **Second PCR** (SA0112) reaction tubes on ice.
2. Pipette **2 µL** of **GSP2 (black cap)** into each Second PCR tube.
  - a. Record which P7 index sequence is used with which sample.
3. Pipette **18 µL** of purified First PCR reaction into each Second PCR tube.
  - a. Dissolve, mix and spin down.
  - b. Return tubes to ice.
4. 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 ( $\geq 100^{\circ}\text{C}$ ).
  - b. After the program has reached 4°C, briefly spin down reactions and place on ice. It is also acceptable to leave tubes in the thermal cycler at 4°C overnight.



### Reaction Cleanup after 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 (**24 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 (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 (≤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

### Quantification

1. Quantify the concentration of each library using the Ion Library TaqMan® Quantitation Kit following the instructions provided by the manufacturer under consideration of the following notes:
  - a. FusionPlex libraries are very concentrated and dilutions of **1:10,000-1:250,000** are usually required for accurate quantification with the Ion Library TaqMan Quantitation Kit.
  - b. When creating the dilutions, use a dilution series and avoid pipetting volumes of less than 2 µL to avoid inconsistent quantifications due to pipetting errors.

- c. Libraries and standards should be measured in triplicate.
- 2. Refer to the Real Time-PCR cycling conditions listed in Table 4 of the Ion Library TaqMan Quantitation Kit User Guide (version C.0) for use with FusionPlex libraries.
- 3. Please contact [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com) for further assistance for the conversion of qPCR results to concentrations.

### Normalization

- 1. After quantification, dilute all samples to an equal concentration. Usual concentrations for this step range from 4 - 10 nM.
- 2. After this normalization step, pool libraries proportional to the desired, panel-specific read depth. This information can be found in the product insert.
  - a. Please contact [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com) for further assistance regarding appropriate pooling.
- 3. To ensure accurate loading, it is strongly recommended to quantify the final pool following the instructions of the Ion Library TaqMan Quantitation Kit.
- 4. Dilute the re-quantified pool to the appropriate loading concentration for the selected chip. Recommendations can be found in the next section.

### Sequencing

- 1. Please contact [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com) to obtain the required barcode information for sample demultiplexing as well as further assistance for the run setup.
- 2. Loading recommendations for template preparation:

Sequencer	Pooled cDNA Libraries (pM)
Ion PGM Template OT2 200 kit (or equivalent)	13
Ion Chef PGM	25
Ion Chef S5 (Ion 520 or 530 chips)	40
Genexus (GX5 chip)	200

Note: The loading concentrations are only starting points and will need to be optimized per library type.

- 3. Perform template preparation according to the manufacturer's protocol.
- 4. Once the template preparation is complete, load the sequencer according to manufacturer instructions and initiate the sequencing run.

## Data analysis

Analyze data with Archer Analysis using either a local software installation or Archer Unlimited. Visit our website or contact [archer-tech@idtdna.com](mailto:archer-tech@idtdna.com) for more information.

FusionPlex 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](mailto:archer-tech@idtdna.com).

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