AccuFusion™ RNA Lung Cancer Panel

User Guide

This user guide is for the following products for sequencing on Illumina® and Ion Torrent™ NGS platforms:

- AccuFusion™ RNA Lung Cancer Panel

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UG3002-04
November 2024

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AccuFusion™ RNA Lung Cancer Panel User Guide

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Revision History

Document	Date	Description of Change
UG3002-01	September 2020	• Initial version
UG3002-02	February 2022	• Included section for data analysis recommendations for Ion Torrent
UG3002-03	February 2022	• Corrected major typos in procedure titles and input recommendations. • References to RNA fragmentation was incorrectly retained, and has been removed as it is not necessary for AccuFusion workflows. • Included relevant information for Ion Torrent compatibility (index information, volumes and temperatures for 2nd PCR, etc.)
UG3002-04	November 2024	• Updated data analysis for Illumina step to process STAR output files to make fusion calls.

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Overview

Product Information

AccuFusion™ RNA technology is a fast, robust, and versatile solution for target enrichment and library preparation for next-generation sequencing (NGS) on Illumina® and Ion Torrent™ sequencing platforms. AccuFusion RNA Panels generate highly accurate data from as little as 10 ng of RNA using a fast and simple workflow. AccuFusion RNA Panels are expertly optimized with predesigned primers to generate valuable insights in key cancer and disease research areas.

AccuFusion RNA Panels are powered by Paragon Genomics' proprietary CleanPlex Technology, which combines an advanced primer design algorithm and an innovative background cleaning chemistry to generate best-in-class target enrichment performance and efficient use of sequencing reads. The patented CleanPlex background cleaning chemistry effectively removes non-specific PCR products to enable ultra-high multiplexing of amplicons. More than 20,000 amplicons can be multiplexed per primer pool to build large NGS panels that can interrogate megabase-size genomic regions to unlock new applications using a streamlined workflow.

The AccuFusion product line is one of two technologies tailored specifically to your RNA-seq needs. AccuFusion panels offer robust targeted detection of fusions with known fusion partners. By contrast, the OmniFusion panels utilize single ended amplification to also discover fusions with unknown fusion partners.

AccuFusion RNA Panels feature a fast and simple workflow that can be completed in about 6 hours from input RNA to indexed NGS libraries ready for sequencing. The workflow involves just 4 steps, each consisting of a thermal-cycling or incubation reaction followed by a library purification using magnetic beads. AccuFusion RNA Panels have minimal tube-to-tube transfer, offering many benefits of a streamlined workflow such as preserving nucleotide material and preventing handling errors and sample mix-up.

The first step of the AccuFusion RNA workflow is cDNA synthesis and purification from minimal input of RNA. The second step is a multiplex PCR reaction that uses target-specific primers to amplify targets of interest. The third step is a digestion reaction that performs background cleaning by removing non-specific PCR products. The last step is a PCR reaction that uses CleanPlex Indexed PCR Primers to amplify and add sample-level indexes to the fusion libraries. CleanMag® Magnetic Beads are recommended for library purification. See the workflow section for a detailed depiction of the CleanPlex workflow.

AccuFusion Target Enrichment and Library Preparation Workflow

6 hours of total assay time, 70 minutes of hands-on time

Applications

The AccuFusion RNA Panels can be used to detect gene fusions, splice variants and the typical SNV and indels within the designed region. With its superior efficiency, the Fusion RNA technology enables gene expression profiling with high correlation, and fusion detection of known and unknown fusion partners.

AccuFusion panels are tailored towards highly specific panels for known fusions, whereas OmniFusion panels utilize single-sided targeted amplification to identify both known and unknown gene fusions. With these two methods, comprehensive RNA screening is made faster and simpler than ever.

Leveraging the CleanPlex Technology, fusion panels can be highly multiplexed to include 7 to 20,000 amplicons per primer pool for high quality libraries and high-quality sequencing.

The table below shows some of the organisms, sample types, and applications that users have studied using CleanPlex NGS Panels. This is not meant to be an exhaustive or restrictive list.

Organisms	Sample Types	Applications
• Human	• Genomic RNA from whole blood, tissues, buccal swab, and cultured cells • Degraded RNA from FFPE tissues • RNA samples from bulk or single cell	• Tumor profiling • Fusion detection and discovery • Gene expression profiling • Oncogenesis and disease progression

Compatible Sequencing Instruments

CleanPlex Indexed PCR Primers for Illumina are used to generate CleanPlex target-enriched NGS libraries that are compatible with Illumina sequencing platforms, including NovaSeq® 5000/6000 Systems, HiSeq 3000/4000 Systems, HiSeq 2500 System, NextSeq® Series, MiSeq® System, MiniSeq® System, and iSeq® System.

CleanPlex Indexed PCR Primers v2 for Ion Torrent are used to generate CleanPlex target-enriched NGS libraries that are compatible with all Ion Torrent sequencing platforms, including Ion PGM System, Ion Proton System, and Ion GeneStudio S5 Series.

Kit Contents

The protocol outlined in the AccuFusion RNA Panel User Guide requires the following components, which need to be ordered separately:

- AccuFusion RNA Lung Cancer Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)

Define Your Content

AccuFusion RNA Lung Cancer Panel

Includes:
- AccuFusion RNA Primer Pools
- AccuFusion RNA Library Kit

+

Pick Indexed PCR Primers for Your Sequencing Platform of Choice

CleanPlex Indexed PCR Primers for Illumina

Or

CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent

+

Complete Workflow with Validated Magnetic Beads

CleanMag Magnetic Beads

Panel Specifications

Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Average Amplicon Length	Average Library Length
AccuFusion RNA Lung Cancer Panel	2	10X	97	129 bp	265 bp

AccuFusion RNA Panels — Kit Contents, Store at −20°C

Panel	SKU	Size (Reactions)	Components		
			Primer Pool 1	Primer Pool 2	CleanPlex Targeted RNA Library Kit
AccuFusion RNA Lung Cancer Panel	917103	8	8 µl	8 µl	2-pool, 8 rxns
	917104	96	96 µl	96 µl	2-pool, 96 rxns
	917105	384	384 µl	384 µl	2-pool, 384 rxns

An AccuFusion RNA Library Kit is included in every AccuFusion panel kit. Please note that the AccuFusion RNA Library Kit is **not** sold separately.

AccuFusion RNA Library Kit Contents (1-Pool Panels) — Store at −20°C (not sold separately)

		Configuration	1-Pool		
		Size	**8 Rxns**	**96 Rxns**	**384 Rxns**
Component	**Cap Color**	**SKU**	**917013**	**917014**	**917015**
RT Primer Mix DP	Purple Striped		24 µl	288 µl	1152 µl
RT Enzyme	Blue Striped		8 µl	96 µl	384 µl
RT Buffer DP	Green Striped		40 µl	480 µl	1920 µl
5X mPCR Mix	Green		16 µl	192 µl	768 µl
CP Digestion Buffer	White		16 µl	192 µl	768 µl
CP Digestion Reagent	Yellow		8 µl	96 µl	384 µl
Stop Buffer	Red		200 µl	1.65 ml	2 x 1.65 ml
5X 2nd PCR Mix	Blue		64 µl	768 µl	3072 µl
TE Buffer	Clear		1.7 ml	3 x 1.7 ml	20 ml
DEPC Treated Water (Nuclease-free)	Clear		1.7 ml	3 x 1.7 ml	20 ml

AccuFusion RNA Library Kit Contents (2- Pool Panels) — Store at −20°C (not sold separately)

		Configuration	2-Pool		
		Size	**8 Rxns**	**96 Rxns**	**384 Rxns**
Component	**Cap Color**	**SKU**	**917016**	**917017**	**917018**
RT Primer Mix DP	Purple Striped		24 µl	288 µl	1152 µl
RT Enzyme	Blue Striped		8 µl	96 µl	384 µl
RT Buffer DP	Green Striped		40 µl	480 µl	1920 µl
5X mPCR Mix	Green		32 µl	384 µl	1536 µl
CP Reagent Buffer	White		16 µl	192 µl	768 µl
CP Digestion Reagent	Yellow		8 µl	96 µl	384 µl
Stop Buffer	Red		200 µl	1.65 ml	2 x 1.65 ml
5X 2nd PCR Mix	Blue		64 µl	768 µl	3072 µl
TE Buffer	Clear		1.7 ml	3 x 1.7 ml	20 ml
DEPC Treated Water (Nuclease-free)	Clear		1.7 ml	3 x 1.7 ml	20 ml

Required Materials and Equipment Not Included

- CleanPlex Indexed PCR Primers (visit www.paragongenomics.com/store for more indexing options)

CleanPlex Dual-Indexed PCR Primers for Illumina — Store at −20°C

	SKU	Size (Reactions)	Format
CleanPlex Dual-Indexed PCR Primers for Illumina Set T	716021	9	Random 3 i7 indexes + 3 i5 indexes (9 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set A	716006 716017	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Dual-Indexed PCR Primers for Illumina Set B	716018 716019	96 384	12 i7 indexes + 8 i5 indexes (20 tubes)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set A	716033 716045	96 384	Pre-pooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set B	716034 716046	96 384	Pre-pooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set C	716035 716047	96 384	Pre-pooled i5 and i7 indexes (96-well plate)
CleanPlex Plated Dual-Indexed PCR Primers for Illumina Set D	716036 716048	96 384	Pre-pooled i5 and i7 indexes (96-well plate)

CleanPlex Unique Dual-Indexed PCR Primers for Illumina — Store at −20°C

	SKU	Size (Reactions)	Format
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set C	716037 716049	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set D	716038 716050	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set E	716039 716051	96 384	96 i7 indexes + i5 indexes (96-well plate)
CleanPlex Plated Unique Dual-Indexed PCR Primers for Illumina Set F	716040 716052	96 384	96 i7 indexes + i5 indexes (96-well plate)

CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent — Store at –20°C	SKU	Size (Reactions)	Format
CleanPlex® Single-Indexed PCR Primers v2 for Ion Torrent Set T	716016	8	8 indexes, (8 tubes)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set C	716053	192	96 indexes, (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set D	716054	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set E	716055	192	96 indexes (96-well plate)
CleanPlex® Plated Single-Indexed PCR Primers v2 for Ion Torrent Set F	716056	192	96 indexes (96-well plate)

- CleanMag Magnetic Beads, or equivalent — eg. Agencourt™ AMPure™ XP Kit (Beckman Coulter, A63880, A63881, or A63882)

CleanMag Magnetic Beads — Store at 2–8°C	SKU	Size (Volume)	Reactions 1-pool panels	Reactions 2-Pool panels
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~6	~5
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~33	~25
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~130	~100
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~400	~300
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,000	~2,250

- For PCR tubes or strips, CleanMag Magnetic Rack (SKU 719001) or equivalent magnetic racks designed for PCR strip workflows
- For 96-well PCR plates, CleanMag Magnetic Plate (SKU 719002) or equivalent magnetic plates designed for PCR plate workflows

CleanMag Magnetic Rack & Plate

	SKU	Compatibility
CleanMag Magnetic Rack	719001	2 rows of 12 tubes each for PCR strip tubes
CleanMag Magnetic Plate	719002	96 well PCR plates, full or semi- skirted compatible

- 70% ethanol (freshly prepared)
- Nuclease-free water
- Nuclease-free, low bind, thin-wall PCR strip tubes with attached caps, or 96-well PCR plate with adhesive film
- Pipettors and low-retention filtered pipette tips
- Thermal cycler
- Mini-centrifuge, 96-well plate centrifuge
- Qubit® Fluorometer and dsDNA HS (high sensitivity) Assay Kit, or equivalent
- Agilent® 2100 Bioanalyzer® Instrument and Agilent High Sensitivity DNA Kit, or equivalent

Storage, Handling, and Usage

AccuFusion Panels and CleanPlex Indexed PCR Primers are shipped on blue ice (ice packs). Upon receipt, immediately store AccuFusion Panels and CleanPlex Indexed PCR Primers at −20°C in a constant-temperature freezer. Do not store in a freezer with auto-defrost or frost-free features. Do not store at −80°C.

CleanMag Magnetic Bead solution is shipped at room temperature or on blue ice (ice packs). **Magnetic beads may be frozen during transit.** This single freeze-thaw will not affect the performance of the beads. Upon receipt, immediately store CleanMag Magnetic Beads at 2°C to 8°C in a constant-temperature refrigerator. Do not freeze. Do not store at −20°C with other reagents.

Always ensure that all frozen components are fully thawed and have been vortexed and spun down to bring all liquids to the bottom of the tubes prior to use.

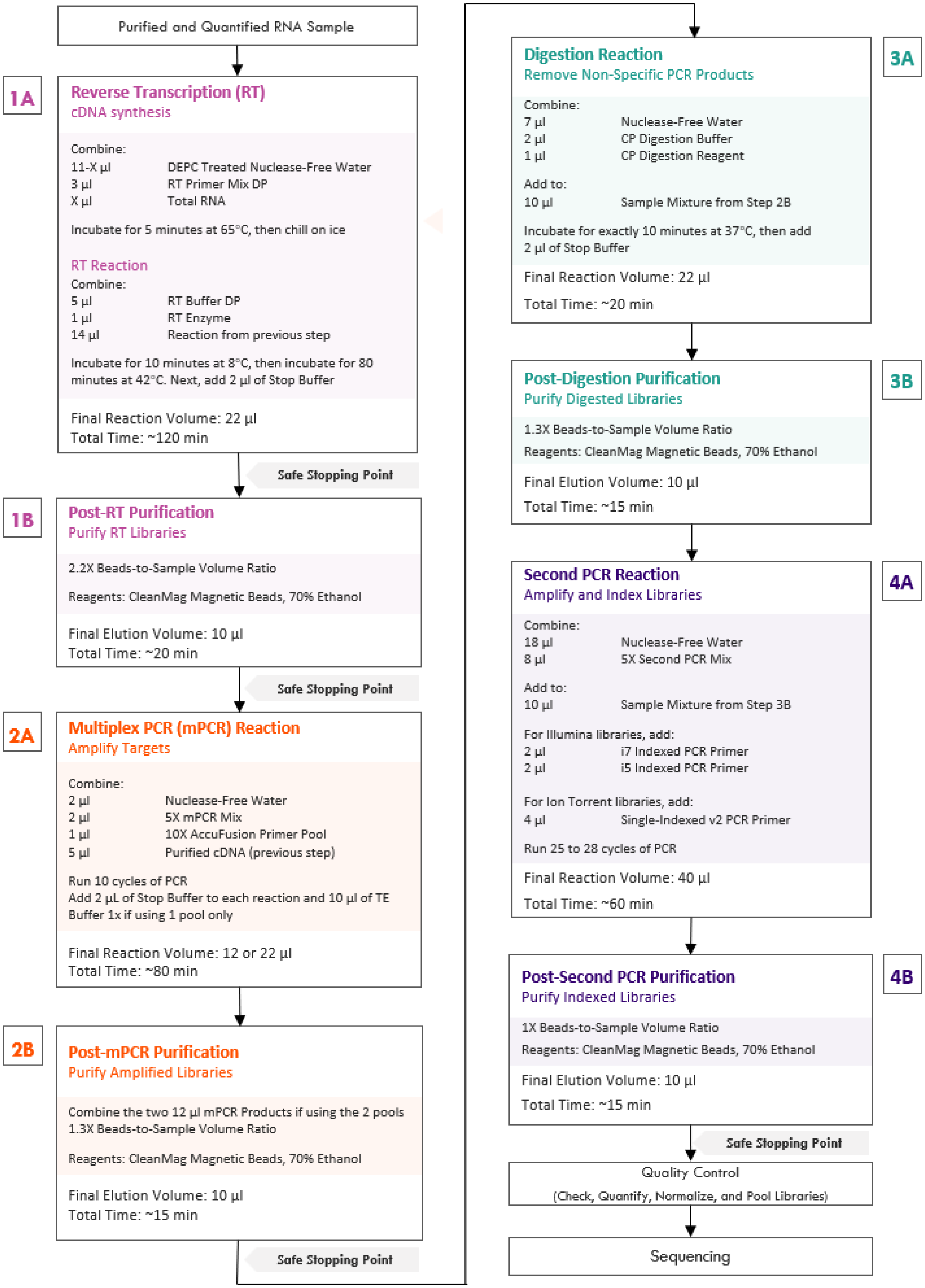
The components containing enzymes are viscous (10X AccuFusion Primer pools, RT Enzyme, 5X mPCR Mix, CP Digestion Reagent, 5X 2nd PCR Mix) and are specially formulated for storage at −20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components below −23°C.

AccuFusion Panels are developed, designed and sold exclusively for **Research Use Only**. None of the products or their individual components have been tested for use in diagnostic procedures.

For hazard information, please refer to the Safety Data Sheet (SDS), which is available upon request.

Workflow

The following diagram illustrates AccuFusion RNA Lung Cancer Panel's targeted NGS library preparation workflow.



Purified and Quantified RNA Sample

1A

Reverse Transcription (RT)
cDNA synthesis

Combine:

11-X µl	DEPC Treated Nuclease-Free Water
3 µl	RT Primer Mix DP
X µl	Total RNA

Incubate for 5 minutes at 65°C, then chill on ice

RT Reaction

Combine:

5 µl	RT Buffer DP
1 µl	RT Enzyme
14 µl	Reaction from previous step

Incubate for 10 minutes at 8°C, then incubate for 80 minutes at 42°C. Next, add 2 µl of Stop Buffer

Final Reaction Volume: 22 µl
Total Time: ~120 min

Safe Stopping Point

1B

Post-RT Purification
Purify RT Libraries

2.2X Beads-to-Sample Volume Ratio

Reagents: CleanMag Magnetic Beads, 70% Ethanol

Final Elution Volume: 10 µl
Total Time: ~20 min

Safe Stopping Point

2A

Multiplex PCR (mPCR) Reaction
Amplify Targets

Combine:

2 µl	Nuclease-Free Water
2 µl	5X mPCR Mix
1 µl	10X AccuFusion Primer Pool
5 µl	Purified cDNA (previous step)

Run 10 cycles of PCR
Add 2 µL of Stop Buffer to each reaction and 10 µl of TE Buffer 1x if using 1 pool only

Final Reaction Volume: 12 or 22 µl
Total Time: ~80 min

2B

Post-mPCR Purification
Purify Amplified Libraries

Combine the two 12 µl mPCR Products if using the 2 pools
1.3X Beads-to-Sample Volume Ratio

Reagents: CleanMag Magnetic Beads, 70% Ethanol

Final Elution Volume: 10 µl
Total Time: ~15 min

Safe Stopping Point

3A

Digestion Reaction
Remove Non-Specific PCR Products

Combine:

7 µl	Nuclease-Free Water
2 µl	CP Digestion Buffer
1 µl	CP Digestion Reagent

Add to:

10 µl	Sample Mixture from Step 2B

Incubate for exactly 10 minutes at 37°C, then add 2 µl of Stop Buffer

Final Reaction Volume: 22 µl

Total Time: ~20 min

3B

Post-Digestion Purification
Purify Digested Libraries

1.3X Beads-to-Sample Volume Ratio

Reagents: CleanMag Magnetic Beads, 70% Ethanol

Final Elution Volume: 10 µl

Total Time: ~15 min

4A

Second PCR Reaction
Amplify and Index Libraries

Combine:

18 µl	Nuclease-Free Water
8 µl	5X Second PCR Mix

Add to:

10 µl	Sample Mixture from Step 3B

For Illumina libraries, add:

2 µl	i7 Indexed PCR Primer
2 µl	i5 Indexed PCR Primer

For Ion Torrent libraries, add:

4 µl	Single-Indexed v2 PCR Primer

Run 25 to 28 cycles of PCR

Final Reaction Volume: 40 µl

Total Time: ~60 min

4B

Post-Second PCR Purification
Purify Indexed Libraries

1X Beads-to-Sample Volume Ratio

Reagents: CleanMag Magnetic Beads, 70% Ethanol

Final Elution Volume: 10 µl

Total Time: ~15 min

Safe Stopping Point

Quality Control
(Check, Quantify, Normalize, and Pool Libraries)

Sequencing

Protocol

General Best Practices

- When using the kit for the first time, briefly vortex and spin the tubes in the kit to bring the liquid to the bottom of the tubes. Store the tubes containing enzymes (RT Enzyme, 5X mPCR Mix, CP Digestion Reagent, and 5X 2nd PCR Mix) on ice during their respective procedures. All other components, including primer pools, may be thawed at room temperature, mixed thoroughly by vortexing and spun-down before use.

- Use good laboratory practices to minimize cross-contamination. If possible, perform PCR setup in an isolated area or room to minimize cross-contamination between samples, multiplex PCR (mPCR) primers, or indexed PCR primers. Always change pipette tips between samples and change gloves frequently. Clean all workstations and tools with 10% bleach followed by water, then alcohol at the end of each workday.

- Use a calibrated PCR thermal cycler as specified by the manufacturer's user guide. Validated thermal cyclers include Bio-Rad C1000, Eppendorf Mastercycler series, and Applied Biosystems GeneAmp PCR System 9700. Generally, a thermal cycler set with the highest ramp speed, such as 5°C/second or higher, is not recommended. For thermal cyclers with adjustable ramp speed, we recommend 3°C/second up and 2°C/second down speed, or use the default setting (no ramp adjustment).

- To ensure accurate assembly of reactions, withdraw viscous solutions (such as 10X AccuFusion Primer pools, RT Enzyme, 5X mPCR Mix, CP Digestion Reagent, and 5X 2nd PCR Mix) slowly from containers and dispense it slowly into the reaction mixtures. A good practice is to remove excess from outside of tip and rinse the tip by pipetting up and down several times after dispensing viscous solutions into aqueous mixture. Thoroughly mix each assembly to ensure solutions are homogeneous prior to PCR and incubations. Remember to briefly spin the PCR tubes or 96-well PCR plate after mixing.

- Always keep working solutions and PCR products on ice until needed. Combine PCR mixes immediately prior to use and do not prolong storage of combined PCR mixes and PCR products.

- The protocol is designed to minimize the number of tube-to-tube transfers in order to avoid or reduce sample loss.

- When working with 96-well PCR plates, take extra care to ensure thorough mixing of all samples and proper sealing to avoid cross contamination between samples.

- Magnetic bead purification steps should be performed carefully to minimize residual supernatant and ethanol washes, and to minimize bead loss. Using a strong magnetic rack or plate specifically designed for manual handling of PCR tubes or 96-well PCR plates is critical for successful bead purification

- Always pre-warm thermal cyclers, pre-warm a water bath or heat block to 65°C or 37°C as described in the protocol.

- Assign sample indexes to specific samples before starting the protocol.

- Always prepare a master mix of reagents when working with multiplex reactions. Prepare ~5% excess of each master mix to allow for pipetting losses.

RNA Best Practices

- RNA is a single-stranded polynucleotide that is incredibly susceptible to degradation by base- or enzyme-catalyzed hydrolysis. This means that working with RNA can be more demanding than its double-stranded counterpart, DNA, due to the chemical instability of RNA and because of the pervasive presence of RNases. Furthermore, unlike DNases, RNases have no requirement for metal ion co-factors and can remain active even after prolonged boiling or autoclaving.

- When working with RNA, wear gloves at all times. After putting on gloves, avoid touching contaminated surfaces and equipment with the gloved hands. Even if all the reagents have been decontaminated, RNases can be reintroduced by contact with ungloved hands or with unfiltered air.

- Use sterile, disposable plasticware whenever possible. These require no treatment and are considered to be RNase-free.
  Note: *Autoclaving without DEPC/DMPC treatment is insufficient for inactivating RNases.*

- If possible, designate a special area for RNA work only. Treat surfaces of benches, glassware, and pipettes with commercially available RNase inactivating agents such as RNase Zap® or equivalent agent.

- Also, wipe benches with a three-wash method, using 10% bleach, followed by water, and finish with 90-100% ethanol **each time** prior to use, in order to rid the area of microorganisms.

- Whenever possible, purchase reagents that are free of RNases. Be sure to separate reagents used for RNA work from "general use reagents" in the laboratory and use only for RNA processing.

- Prior to processing, store RNA at −70°C to −80°C, as aliquots in ethanol, isopropanol or lysis buffer as indicated by extraction method. Most RNA is relatively stable at this temperature. Centrifuge the RNA and resuspend in an appropriate RNase-free buffer before use. Use autoclaved DEPC water to elute and resuspend RNA or alternatively use molecular grade water certified as RNAse and DNAse free.

- Although DNA is relatively stable at elevated temperatures (+100°C), most RNA is not. Therefore, avoid high temperatures (above +65°C) since these affect the integrity of the RNA. This means it is critical to handle solutions of RNA on ice when using, this includes thawing and processing RNA samples and related reactions on ice.

Input RNA Requirements

Refer to the following chart for the amounts of input RNA.

Panel	RNA Input Range (per pool)	Recommended RNA Input (per pool)	Recommended FFPE RNA Input (per pool)
AccuFusion RNA Lung Cancer Panel (2-pool)	10–100 ng	10 ng	50 ng

- The maximum volume of RNA input per Reverse Transcription (RT) Reaction is 11 µl.
- Qubit RNA HS Assay Kit (Thermo Fisher, Cat. No. Q32851 or Q32854) or an equivalent fluorometric method is recommended for measuring RNA concentration. UV spectrophotometry methods (e.g. NanoDrop™ spectrophotometer) are not recommended because it can significantly overestimate the RNA concentration.
- After RNA extraction, avoid diluting RNA samples to < 10 ng/µl when possible for prolonged storage, since RNA is less stable in solution at lower concentrations.
- Avoid freeze-thawing RNA samples when possible, and measure sample concentrations immediately prior to use to avoid inaccurate sample input.
- 50 ng or higher of RNA from normal or FFPE tissue is recommended for each Reverse Transcription Reaction.
- Lower RNA input may result in biased detection of certain targets.
- When RNA quality is low or unknown (such as RNA from FFPE tissues), higher RNA input can be used to produce better library quality and reach lower limits of detection.
- Compatible RNA buffering systems are Tris HCl or TE.

Set up and Preparations

- When working with multiple samples, prepare a master mix of RT Primer Annealing Reaction Mixture for the Reverse Transcription (RT) Reaction. Mix well, aliquot into individual tubes, then add the recommended RNA input to each reaction.
- Bring magnetic bead solution to room temperature for at least 30 minutes before use. Replace in 2-8 °C storage at the end of the day.
- Freshly prepare 12mL (per 8 reactions) of 70% Ethanol by combining 8.4mL of 100% ethanol and 3.6mL nuclease-free water at volume ratios of 7 to 3 respectively. Do not top off one liquid with the other in a volumetric container because the volumetric ratio will not be accurate. When water and ethanol are mixed, the final volume will be less than the sum of individual volumes. Lower concentration of ethanol will affect final library yield.
- For all samples to be sequenced together, assign a specific index (CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent) or index combination (CleanPlex Dual-Indexed PCR Primers for Illumina) to each sample.
- Note the safe stopping points after the Reverse Transcription (RT) Reaction, Post-RT Purification, Post-mPCR Purification and Post-2nd PCR purification. After starting Digestion Reaction step, samples must be carried through to 2nd PCR purification without stopping. Plan accordingly.

1A. Reverse Transcription (RT) Reaction

1A.1. Thaw RT Primer Mix, RT Enzyme, and RT Buffer DP tubes and keep all Reverse Transcription reagents on ice. Before use, vortex and quick spin all reagents. Using thin-wall PCR strip tubes (or a 96-well PCR plate), prepare the RT Primer Annealing Reaction Mixture by adding components in the following order on ice or on a cold block.

Note: *When working with multiple samples prepare a master mix of RT Primer Annealing Reaction Mixture . Mix well, aliquot into individual tubes, then add RNA samples to each reaction.*

Note: *Pre-warm a thermal cycler or heating unit to 65°C before beginning the Reverse Transcription Reaction.*

Note: *Thaw and keep Stop Buffer and Magnetic Beads at room temperature for use at the end of step 1A and beginning of step 1B.*

⚠

Important! Use RNA Best Practices by performing all reactions on a cold block or on ice and diligently avoid contamination and introduction of RNases until RT step (1A) and RT cleanup (1B) are complete.

RT Primer Annealing Reaction Mixture

Reagent	Cap Color	Volume per reaction
DEPC Treated Nuclease-Free Water	Clear	11 – X µl
RT Primer Mix DP	Purple Striped	3 µl
Total RNA	—	X µl
Total Volume per reaction		**14 µl**

1A.2. Close the caps of the PCR tubes or seal the PCR plate with adhesive film, spin briefly to bring down the liquid, mix thoroughly by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds to collect the liquid.

Note: *It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can cause decreased yield and increase nonspecific product formation.*

Note: *If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during thermal cycling.*

1A.3. Load the tubes/plate in the thermal cycler and run the following thermal cycling protocol to anneal RT Primers.

RT Primer Annealing Reaction Incubation Protocol

Step	Temperature	Time	Preheat
Incubation	65 °C	5 min	65 °C
Hold	4 °C	∞	-

⚠

Important! Do not stop and store samples. Immediately after the RT Primer Annealing Reaction, continue to Reverse Transcription (RT) Reaction.

1A.4. Place and keep the tubes/plate on ice. Open the tubes or carefully remove the adhesive film from the PCR plate. Prepare the Reverse Transcription (RT) Reaction Mixture by adding components in the following order on ice or a cold block.

Note*: When working with multiple samples, prepare a master mix of Reverse Transcription (RT) Reaction Mixture. Mix well, aliquot into individual tubes.*

RT Reaction Mixture

Reagent	Cap Color	Volume per reaction
RT Buffer DP	Green Striped	5 µl
RT Enzyme	Blue Striped	1 µl
Previous Reaction	—	14 µl
Total Volume per reaction		**20 µl**

1A.5. Close the caps of the PCR tubes or seal the PCR plate with adhesive film. Spin briefly to bring down the liquid, mix thoroughly by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds to collect the liquid.

Note: *It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can cause decreased yield and increase nonspecific product formation.*

Note: *Avoid over vortexing. RNA is single stranded and susceptible to manual shearing and excessive fragmentation.*

1A.6. Set up the thermal cycler temperatures as shown below. Allow the cycler to reach 8°C prior to loading the tubes/plate in the thermal cycler. Run the following thermal cycling protocol.

Step	Temperature	Time	Preheat
Equilibration	8 °C	10 min	8 °C
Incubation	42 °C	80 min	
Hold	4 °C	∞	

1A.7. When thermocycling is complete, immediately add 2 µl of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid. The volume of each sample is now approximately 22 µl.

Safe Stopping Point. RT Reaction products can be stored at 10°C for up to 1 hour or at −20°C for up to 24 hours.

1B. Post-RT Cleanup Purification

⚠

Important! Ensure the Magnetic Bead Solution has come to room temperature before use.

⚠

Important! Use freshly prepared 70% ethanol. Lower concentration ethanol may result in lower yields.

1B.1. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a **2.2X** bead-based purification by adding 48 µl of magnetic beads to each sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-Reverse Transcription Purification — 2.2X Beads-to-Sample Volume Ratio

Reagent	Volume per reaction
Reverse Transcription Product	22 µl
Magnetic Beads	48 µl

⚠

Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

1B.2. Incubate the mixture for **10 minutes** at room temperature.

1B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/well. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.

Note: *If the magnetic beads are aspirated into the pipette tip at any point during the purification process, dispense the solution back into tube, remove the tip, allow the beads to migrate toward the magnet again, then repeat.*

1B.4. Cap/seal the tubes/plate, and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

Important! Removing all residual supernatant from the Post-RT Purification *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin-and-remove step ensures complete removal of supernatant.

Note: *A strong compatible magnetic rack is essential. If your magnetic rack or plate cannot collect the magnetic beads effectively, please look into a replacement.*

1B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

1B.6. Repeat step 1B.5.

1B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnet rack to gather the beads. Carefully remove the residual ethanol in each tube/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: *Over-dried beads can dislodge from the side of the tube and lead to cross contamination. They are also more difficult to resuspend during elution. Residual ethanol inhibits PCR and will result in reduced yield. Especially when working with 96-well PCR plates, make sure all residual ethanol is dried (may take longer than 5 minutes) before going to the next step.*

1B.8. Add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

1B.9. Proceed to Step 2A. Multiplex PCR Reaction.

Safe Stopping Point. Purified RT products can be stored at 10°C for up to 1 hour or at −20°C for up to 24 hours.

2A. Multiplex PCR (mPCR) Reaction

Important: Each primer pool requires one reaction of mPCR Reaction Master Mix and 5 µl of the purified RT reaction product. When working with a 1-pool panel, the remaining 5 µl of the purified RT reaction can be stored at −20°C for future use or discarded. When working with the 2-pool workflow, split the 10 µl of the purified RT reaction into two 5 µl tubes (1 tube for each pool), beads included.

2A.1. Thaw 10x AccuFusion Primer pool(s) and vortex thoroughly before use. Prepare the mPCR Reaction Master Mixture by adding components in the following order on ice or a cold. For fusion panels with multiple primer pools, prepare an individual reaction for **each** primer pool.

Note: *When working with multiple reactions, prepare a master mix. Mix well, then add 10 µl of master mix to each sample.*

mPCR Reaction Master Mix

Reagent	**Cap Color**	**Volume per reaction**
Nuclease-Free Water	—	2 µl
5X mPCR Mix	Green	2 µl
10X AccuFusion RNA Lung Cancer Pool 1 or 10X AccuFusion RNA Lung Cancer Pool 2	—	1 µl
Total Volume		**5 µl**

Important! 5X mPCR Mix and 10X AccuFusion Primer Pool(s) are viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution when handling these reagents.

mPCR Reaction Mixture

Reagent	**Volume per reaction**
Purified RT Sample from 1B	5 µl
mPCR Reaction Master Mix for Pool 1 or Pool 2	5 µl
Total Volume per reaction	**10 µl**

2A.2. Close the caps of the PCR tubes or seal the PCR plate with adhesive film, spin briefly to bring down the liquid, mix thoroughly by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogeneous. Avoid prolonged vortexing. Spin briefly for 3 seconds to collect the liquid.

Note*: It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can cause decreased yield and increase nonspecific product formation.*

***Note**: If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during thermal cycling.*

2A.3. Load the tubes/plate in the thermal cycler and run the following thermal cycling protocol.

mPCR Thermal Cycling Protocol

Step	Temperature	Time	Ramping*	Cycles
Initial Denaturation	95 °C	10 min	-	-
Denaturation	98 °C	15 sec	3 °C/s	10
Annealing/Extension	60 °C	5 min	2 °C/s	
Hold	10 °C	∞		

* For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/S.

2A.4. Immediately add 2 µl of Stop Buffer (red cap) into each tube/well and mix by spinning briefly then vortexing. Spin again briefly for 3 seconds to collect the liquid. The volume of each pool is now approximately 12 µl.

***Note**: Do not allow samples to hold at 10 °C for longer than 30 minutes before adding Stop Buffer.*

2A.5. Proceed to Step 2B. Post-mPCR Purification.

⚠

Important. Do not stop and store samples after mPCR Reaction. Proceed to Step 2B, Post-mPCR Purification immediately.

2B. Post-mPCR Purification

⚠

Important! Ensure the Magnetic Bead Solution has come to room temperature before use.

⚠

Important! Use freshly prepared 70% ethanol. Lower concentration ethanol may result in lower yields.

2B.1. Open the tubes or carefully remove the adhesive film from the PCR plate.
  - When using 1-pool workflow, add 10 µl of TE Buffer to each sample for a total of 22 µl volume.
  - When using 2-pool workflow, combine the 12 µl multiplex PCR products for each sample for a total of 24 µl volume.

2B.1. Close the caps of the PCR tubes or seal the PCR plate with adhesive film. Quickly spin the tubes/plate to collect the liquid. Open the tubes or carefully remove the adhesive film from the PCR plate.

2B.2. Vortex the magnetic bead suspension vigorously until homogeneous. Perform a **1.3X** bead-based purification by adding the appropriate volume of magnetic beads to each sample as described in the table below. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-mPCR Purification — 1.3X Beads-to-Sample Volume Ratio

Reagent	1-Pool workflow	2-Pool workflow
Total Sample Volume	22 µl (12 µl of sample + 10 µl of TE)	24 µl
Magnetic Beads to add	29 µl	31 µl

⚠

Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

2B.3. Incubate the mixture for **5 minutes** at room temperature.

2B.4. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/well. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.

Note: *If the magnetic beads are aspirated into the pipette tip at any point during the purification process, dispense the solution back into tube, remove the tip, allow the beads to migrate toward the magnet again, then repeat.*

2B.5. Cap/seal the tubes/plate and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

Important! Removing all residual supernatant from the Multiplex PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin-and-remove step ensures complete removal of supernatant.

Note: *A strong compatible magnetic rack is essential. If your magnetic rack or plate cannot collect the magnetic beads effectively, please look into a replacement.*

2B.6. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

2B.7. Repeat step 2B.6.

2B.8. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnet rack to gather the beads. Carefully remove the residual ethanol in each tube/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: *Over-dried beads can dislodge from the side of the tube and lead to cross contamination. They are also more difficult to resuspend during elution. Residual ethanol inhibits PCR and will result in reduced yield. Especially when working with 96-well PCR plates, make sure all residual ethanol is dried (may take longer than 5 minutes) before going to the next step.*

2B.9. Add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

2B.10. Proceed to Step 3A. Digestion Reaction.

Safe Stopping Point. Purified products may be stored at 10°C for up to 1 hour or at −20°C for up to 24 hours.

3A. Digestion Reaction

Note: *After starting the Digestion Reaction, the samples cannot be stored and must continue to Post-Digestion Purification, then Second PCR Reaction. Plan accordingly.*

Note: *When thawing CP Digestion Buffer, bring the tube to room temperature. Ensure all visible precipitate is dissolved by vortexing as needed.*

Note: *Pre-warm a thermal cycler or heating unit to 37°C before beginning the Digestion Reaction.*

Note: *If not already, keep magnetic beads and freshly prepared 70% ethanol ready at room temperature.*

3A.1. Prepare Digestion Reaction Master Mix and add to each purified sample from Step 2B. The magnetic beads in the sample mixture do not affect the Digestion Reaction.

Note: *When working with multiple reactions, prepare a master mix. Mix well, then add 10 µl of master mix to each sample.*

Digestion Reaction Master Mix

Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	7 µl
CP Digestion Buffer	White	2 µl
CP Digestion Reagent	Yellow	1 µl
Total Volume per reaction		**10 µl**

Digestion Reaction Mixture

Reagent	Volume per reaction
Digestion Reaction Master Mix	10 µl
Purified Sample from Step 2B	10 µl
Total Volume per reaction	**20 µl**

3A.2. Mix by pipetting up and down at least 5 times or vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds to collect the liquid.

Note: *It is crucial that the reaction mixture is homogeneous prior to incubation. Incomplete mixing can cause digestion of the library or under-digestion of nonspecific products.*

Note: *If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during incubation.*

3A.3. Incubate at 37°C for exactly 10 minutes. **Do not** incubate shorter or longer than 10 minutes.

3A.4. Immediately add 2 µl of Stop Buffer (red cap) to each tube/well and mix by spinning briefly then vortexing. Spin again briefly for 3 seconds to collect the liquid. The volume of each sample is approximately 22 µl.

3A.5. Proceed immediately to Step 3B. Post-Digestion Purification.

⚠

Important! Do not stop and store samples after the Digestion Reaction. Proceed immediately to Step 3B. Post-Digestion Purification.

3B. Post-Digestion Purification

3B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1.3X** bead-based purification by adding **29 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or vortex vigorously for at least 5 seconds until homogeneous.

Post-Digestion Purification — 1.3X Beads-to-Sample Volume Ratio

Reagent	**Volume per reaction**
Digestion Reaction Product	22 µl
Magnetic Beads	29 µl

⚠

Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

3B.2. Incubate the mixture for **5 minutes** at room temperature.

3B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/wall. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.

3B.4. Cap/seal the tubes/plate and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

⚠

Important! Removing all residual supernatant from the Post-Digestion Purification *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin and remove step ensure complete removal of supernatant.

3B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

3B.6. Repeat step 3B.5.

3B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tube/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: *Over-dried beads can dislodge from the side of the tube and lead to cross contamination, and they are also more difficult to resuspend during elution. Residual ethanol inhibits PCR and will result in reduced yield. Especially when working with 96-well PCR plates, make sure all residual ethanol is dried (may take longer than 5 minutes) before going to the next step.*

3B.8. Add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads.

3B.9. Proceed immediately to Step 4A. Second PCR Reaction.

⚠

Important! Do not stop and store samples after Post-Digestion Purification. Proceed immediately to Step 4A. Second PCR Reaction.

4A. Second PCR Reaction

Note: *Remember to assign a specific index or unique combination of dual indexes to each sample before starting this step.*

4A.1 Thaw Indexed PCR Primers, vortex thoroughly, then spin briefly to collect the liquid. Prepare Second PCR Reaction Master Mix and add to each purified sample from Step 3B. Then add a unique (combination of) Indexed PCR Primer(s) to each sample.

Note: *When working with multiple samples, prepare a master mix. Mix well, then add 26 µl of master mix to each sample.*

Second PCR Reaction Master Mix

Reagent	Cap Color	Volume per reaction
Nuclease-Free Water	—	18 µl
5X 2nd PCR Mix	Blue	8 µl
Total Volume per reaction		**26 µl**

Second PCR Reaction Mixture

Reagent	Illumina Volume per reaction	Ion Torrent Volume per reaction
Second PCR Reaction Master Mix	26 µl	26 µl
Purified Sample from Step 3B	10 µl	10 µl
i5 Indexed PCR Primer for Illumina	2 µl	—
i7 Indexed PCR Primer for Illumina	2 µl	—
Single-Indexed PCR Primer v2 for Ion Torrent	—	4 µl
Total Volume per reaction	**40 µl**	**40 µl**

Note: *For Illumina indexes in plates, the i5 and i7 indexes are already combined. Use 4 µl of the pooled index per sample. For separate indexes in tubes, use 2 µl each as shown above.*

Important! When handling Indexed PCR Primers, take extra care to prevent cross contamination by opening one tube at a time and changing pipette tips and gloves as necessary. Avoid touching the opening and inside of the tubes with your hands, pipette channel, or anything non-disposable.

4A.2 Close the caps of the PCR tubes or seal the PCR plate with adhesive film. Spin briefly to collect the liquid, then mix thoroughly by pipetting up and down at least 5 times or vortexing vigorously for at least 5 seconds until homogeneous. Avoid unnecessarily prolonged vortexing. Spin briefly for 3 seconds to collect the liquid.

Note: *It is crucial that the reaction mixture is homogeneous prior to thermal cycling. Incomplete mixing can cause decreased yield and increase non-specific product formation.*

Note: *If using a PCR plate, use an applicator tool to firmly secure each reaction well and around the perimeter of the plate to prevent evaporation during thermal cycling.*

4A.3 Load the tubes/plate in the thermal cycler, and run the following thermal cycling protocol to amplify and index the libraries. Use the three tables below for thermal cycling protocol and cycle number suggestions.

Note*: The Second PCR thermal cycling protocol depends on the starting RNA input amount and RNA quality. Generally, lower quality RNA, lower RNA input amount, or fewer amplicons in a panel requires more PCR cycles.*

Second PCR Reaction — Thermal Cycling Protocol

Step	Temperature		Time	Ramping*	Cycles
Initial Denaturation	95 °C		10 min	-	1
Denaturation	98 °C		15 sec	3 °C/s	Refer to table below for cycle numbers
Annealing/Extension	Illumina 60 °C	Ion Torrent 68 °C	75 sec	2 °C/s	
Hold	10 °C		∞		

** For thermal cyclers without adjustable ramp speed, the default setting can be used if max ramping speed is ≤5°C/s*

AccuFusion NGS Panels — Second PCR Cycle Number

Number of Amplicons	10 ng of High Quality RNA*	10 ng of Low Quality RNA*
AccuFusion RNA Lung Cancer Panel	21	25-28

***** *For optimal yield, AccuFusion panels may benefit from adjustment to Second PCR cycle numbers based on the sample quality, and application.*

Second PCR Reaction — Input Specific Cycle Number

Input RNA	Change in Cycle Number
5 ng	+ 1
10 ng	-
20 ng	– 1
50 ng	– 2
100 ng	– 3
Low Quality RNA	+ 3 to + 5

4A.4 Proceed to the next step, Post-Second PCR Purification.

Safe Stopping Point. Second PCR Reaction products may be stored at 10°C overnight or at −20 °C for up to 24 hours.

4B. Post-Second PCR Purification

4B.1. Vortex the magnetic beads suspension to disperse beads. Perform a **1X** bead-based purification by adding **40 µl** of magnetic beads to each sample. Mix by pipetting up and down at least 5 times or by vortexing vigorously for at least 5 seconds until homogeneous.

Post-Second PCR Purification — 1X Beads-to-Sample Volume Ratio

Reagent	Volume per reaction
Magnetic Beads	40 µl
Second PCR Reaction Product	40 µl

Important! Magnetic bead volume is critical to the purification process. Always dispense slowly and carefully. Keep the outside of the pipette free from droplets **AND** make sure the entire volume is added to the sample (residual from inside pipette tips) before discarding the tip.

Ensure the bead + sample solution is thoroughly mixed before incubation, especially when working in a 96-well PCR plate format. Inadequate mixing can result in lowered yields and/or increased background in the final library.

4B.2. Incubate the mixture for 5 minutes at room temperature.

4B.3. Briefly spin the tubes/plate. Place the tubes/plate on a magnetic rack and incubate for at least **2 minutes until the liquid is clear**. The beads will be drawn onto one side of each tube/wall. While keeping the tubes/plate on the magnetic rack, carefully remove and discard the supernatant without disturbing the beads.

4B.4. Cap/seal the tubes/plate and briefly spin again to bring down the remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Using a 10 µl pipette tip, carefully remove all residual supernatant from the bottom of the tube/well without disturbing the beads.

Important! Removing all residual supernatant from the Second PCR Reaction *prior* to ethanol washing is critical to obtaining a clean, high-quality library. The above spin and remove step ensure complete removal of supernatant.

4B.5. Add 180 µl of freshly prepared 70% ethanol to each tube/well. Reposition the tubes/plate on the magnetic rack by placing the clear side of the tubes/wells (the side without beads) against the magnet. Allow the beads to completely migrate through the ethanol to the other side. **Do not vortex.** Carefully remove and discard the supernatant without disturbing the beads.

4B.6. Repeat step 4B.5.

4B.7. After the second wash, briefly spin the tubes/plate to bring down all remaining liquid. Place the tubes/plate on the magnetic rack to gather the beads. Carefully remove the residual ethanol left behind in each tubes/well. Keeping the tubes/plate on the magnetic rack, air-dry the beads at room temperature for 5 minutes. **Do not over or under dry.**

Note: *Over-drying and under-drying the beads can lead to reduced yield.*

4B.8. Add 10 µl TE buffer directly onto the pellet for each tube/well. Briefly spin and vortex to resuspend the dried beads completely. The DNA will be immediately released from the beads as long as all beads are in solution. Spin briefly to collect the liquid to the bottom. There is no need to remove the beads. At this point the library is complete and can be stored with beads at −20°C.

Note: *To perform QC and sequencing, use a magnetic rack to separate the beads. Avoid transferring the beads when pipetting the clear supernatant for QC or sequencing.*

Safe Stopping Point. Purified products may be stored with beads at −20°C until ready to sequence. When taking the library out for QC and sequencing, vortex briefly and place the tubes or plate on a magnetic rack to pull the beads to one side. Avoid pipetting the beads, which will affect QC and sequencing.

Quality Control Prior to Sequencing

Check library quality and concentration using a high sensitivity fragment analyzer such as Agilent 2100 Bioanalyzer Instrument and Agilent High Sensitivity dsDNA Kit, or a qPCR-based method. The final library can also be quantified using a Qubit Fluorometer or equivalent. However, this method will only give you the absolute yield and not differentiate potential background from the actual library. To confirm the quality of the DNA, it is highly recommended that a high sensitivity fragment analyzer be used to visualize the peak shape, size, and potential background concentration.

AccuFusion Ready-to-Use panels typically have a yield of 6nM to 30nM, depending on RNA sample quantity/quality and cycle numbers. Custom panel yield might be more variable. Library quantity is not indicative of library quality. Additional input or 2nd PCR cycles can be adjusted accordingly for optimal yield for your downstream processing or sequencing needs.

Library peak size and shape can vary for AccuFusion panels depending on the genotype of the sample itself. The library peaks typically span between 200-400 bp for a typical design.

Please see troubleshooting guide towards the end of this document if you observe nonspecific peaks or unexpected yield.

After confirmation of library quality, the libraries can be normalized using library peak(s) concentrations only, not including the nonspecific product(s) concentration(s). Then samples with different index combinations can be pooled for sequencing together.

Recommended Sequencing Length and Depth

All AccuFusion RNA panels are designed to be compatible with Illumina PE 150 bp reads (2x150 bp) and 200bp on Ion Torrent.

For detecting somatic mutations down to 1% minor allele frequency, the recommended average sequencing read depth is 5,000X. Based on results, depth can be further reduced to maintain sufficient coverage but also increase sample multiplexing efficiency.

Recommended Sequencing Depth

Panel	Application	Average Read Depth (Paired End Reads)
AccuFusion RNA Lung Cancer Panel	Known Fusion Detection	2,000—5,000X

For recommended sample multiplexing on various Illumina NGS instruments, refer to panel-specific product sheets at https://www.paragongenomics.com/customer-support/product_documents/

Supporting Information

Dual-Indexed PCR Primers for Illumina

Primer Sequences

Each sample is indexed by a pair of Indexed PCR Primers for sequencing on Illumina platforms. **XXXXXXXX** denotes the index region of the primer. Index sequences are listed below.

i5 Indexed Primer
5'-AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**ACACTCTTTCCCTACACGACGCTCTTCCGATCT**-3'**

i7 Indexed Primer
5'-CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**-3'**

Set A and Set B have mutually exclusive sequences and allow use together to form 384 combinatorial index pairs for sequencing up to 384 samples on one Illumina flow cell.

CleanPlex Targeted RNA Library Kits and Panels are not compatible with most other commercially available index primer kits. Please use CleanPlex PCR Primers for Illumina for best results.

Index Sequences

CleanPlex Dual-Indexed PCR Primers for Illumina Set T
3 x 3 indexes, 9 reactions (SKU 716021)

A section of three i5 Indexes and three i7 Indexes from Illumina Set A to create 9 unique index combinations. Index selection may vary from kit to kit.

CleanPlex Dual-Indexed PCR Primers for Illumina Set A
12 x 8 indexes, 96 reactions (SKU 716006)
12 x 8 indexes, 384 reactions (SKU 716017)

i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems
A701	GTCGTGAT	ATCACGAC
A702	ACCACTGT	ACAGTGGT
A703	TGGATCTG	CAGATCCA
A704	CCGTTTGT	ACAAACGG
A705	TGCTGGGT	ACCCAGCA
A706	GAGGGGTT	AACCCCTC
A707	AGGTTGGG	CCCAACCT
A708	GTGTGGTG	CACCACAC
A709	TGGGTTTC	GAAACCCA
A710	TGGTCACA	TGTGACCA
A711	TTGACCCT	AGGGTCAA
A712	CCACTCCT	AGGAGTGG

i5 Index	Index Sequence	i5 Bases for Sample Sheet	
		MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
A501	TGAACCTT	TGAACCTT	AAGGTTCA
A502	TGCTAAGT	TGCTAAGT	ACTTAGCA
A503	TGTTCTCT	TGTTCTCT	AGAGAACA
A504	TAAGACAC	TAAGACAC	GTGTCTTA
A505	CTAATCGA	CTAATCGA	TCGATTAG
A506	CTAGAACA	CTAGAACA	TGTTCTAG
A507	TAAGTTCC	TAAGTTCC	GGAACTTA
A508	TAGACCTA	TAGACCTA	TAGGTCTA

CleanPlex Dual-Indexed PCR Primers for Illumina Set B
12 x 8 indexes, 96 reactions (SKU 716018)
12 x 8 indexes, 384 reactions (SKU 716019)

i7 Index	Index Sequence	I7 Bases for Sample Sheet All Illumina Systems
Q7005	ATATTCAC	GTGAATAT
Q7006	GCGCCTGT	ACAGGCGC
Q7007	ACTCTATG	CATAGAGT
Q7008	GTCTCGCA	TGCGAGAC
Q7015	AGTAGAGA	TCTCTACT
Q7016	GACGAGAG	CTCTCGTC
Q7017	AGACTTGG	CCAAGTCT
Q7018	GAGTCCAA	TTGGACTC
Q7023	AATTCTGC	GCAGAATT
Q7024	GGCCTCAT	ATGAGGCC
Q7025	ATCTTAGT	ACTAAGAT
Q7026	GCTCCGAC	GTCGGAGC

i5 Index	Index Sequence	i5 Bases for Sample Sheet	
		MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q5002	GATATCGA	GATATCGA	TCGATATC
Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q5009	CCAACAGA	CCAACAGA	TCTGTTGG
Q5010	TTGGTGAG	TTGGTGAG	CTCACCAA
Q5013	AACCGCGG	AACCGCGG	CCGCGGTT
Q5014	GGTTATAA	GGTTATAA	TTATAACC

Single-Indexed PCR Primers v2 for Ion Torrent

Primer Sequences

Each sample is indexed by uniquely designed Indexed PCR Primers for sequencing on Ion Torrent platforms. Only CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent are compatible with CleanPlex workflow for generating libraries intended for Ion Torrent sequencing.

Index Sequences

CleanPlex Single-Indexed PCR Primers v2 for Ion Torrent Sets C, D, E, and F contain index sequences identical to the sequences of the equivalent IonCode™ barcodes. These indexed PCR primers are provided in sealed 96-well PCR plates and arrange in ascending order in columns.

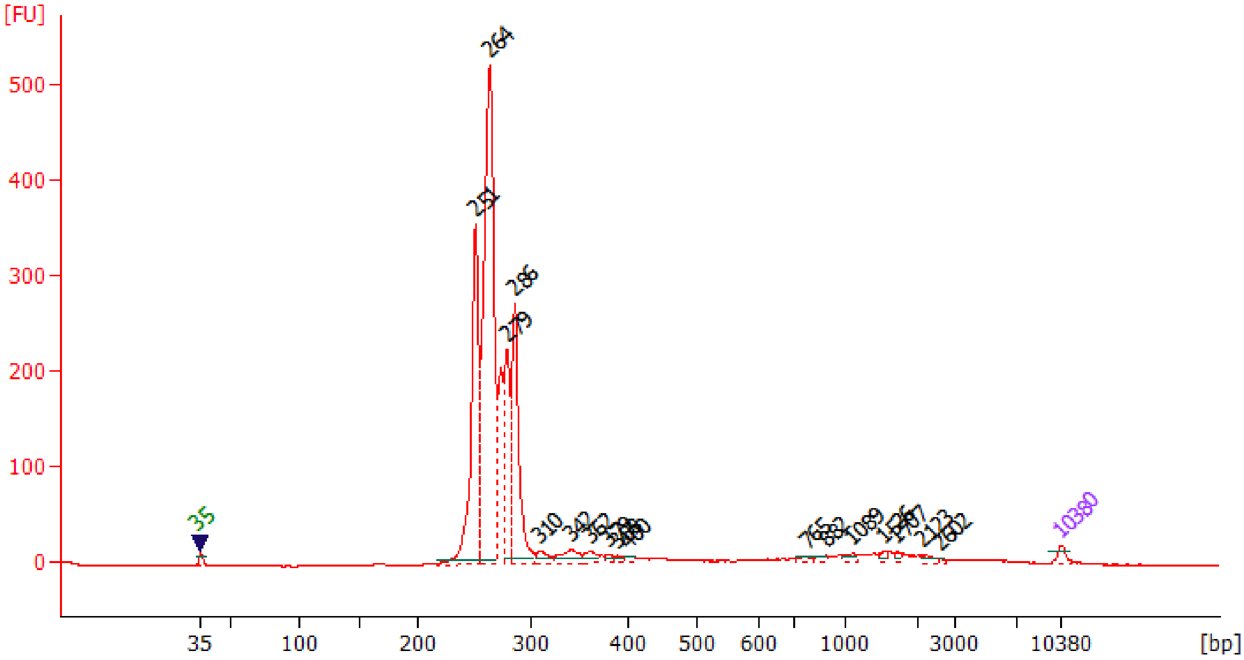
For plated indexes v2 sets C, D, E, and F, plate map and sequences information can be downloaded from Product Document Page under Indexes.

Troubleshooting Guide

Examples of Libraries Prepared with AccuFusion NGS Panels

Depending on the AccuFusion Panel used, library peak(s) may span between 200 and 500 bp. Below are representative Agilent Bioanalyzer traces generated with the AccuFusion RNA Lung Cancer Panel with 10 ng control genomic RNA input.

Example AccuFusion RNA Lung Cancer Panel



Potential Causes for Extra Peaks and Suggested Solutions

Peaks around 70–90 bp are index primer dimers from the Second PCR Reaction and result from incomplete removal of low molecular weight DNA fragments during the final magnetic bead purification (Post-Second PCR Purification). When these peaks are high, it usually indicates one or more of the following:

- Inaccurate pipetting of magnetic beads when making a large number of libraries in a short period of time.
- Insufficient removal of supernatant and/or ethanol washes during the last purification.
- Adding more than the specified amount of indexed primers to Second PCR Reaction.

Peaks around 150–190 bp are residues of digested non-specific amplification products and adapter dimers. They come from incomplete removal of low molecular weight DNA fragments during the Post-Digestion Purification or nonspecific products formed during or after the Multiplex PCR (mPCR) Reaction due to deviation from protocol or high concentration of the panel. The digestion reagent degrades non-specific amplification products into small pieces, which are then removed during magnetic bead purification. These peaks are usually caused by one or more of the following:

- Inaccurate pipetting of magnetic bead volume.
- Insufficient mixing of reaction solutions or bead and sample mixture.
- Insufficient removal of supernatant and ethanol washes during purification.
- Allowing mPCR products to sit for too long (and form nonspecific products) before purification.
- CleanPlex Custom NGS Panels may require panel titration for optimal performance. Often dimers will decrease by reducing the panel concentration in the mPCR Reaction Mixture.
- Incorrect annealing time, specifically unnecessarily longer annealing time.
- Cross contamination of Pool 1 and Pool 2 primers in mPCR reactions.

Broad peaks spread across 500 – 10,000 bp range are nonspecific products due to overamplification. Double check that the correct mPCR and Second PCR cycle numbers were used. PCR cycles are determined based on the amplicon count per pool of your panel, RNA input amount, and RNA quality. Try reducing the Second PCR cycles by 2-3 cycles if this issue persists. If you’re working with a custom panel for organisms with a much smaller genome than human’s, consider reducing the RNA input and Second PCR cycles accordingly.

Potential Reasons for No Peaks

- 30% ethanol instead of 70% ethanol was used in DNA or cDNA purification with magnetic beads.
- Magnetic beads were not added for one or more of the purification steps.
- Stop Buffer was not added or was added too late after Digestion Reaction, resulting in over-digestion of the samples. This may happen when handling a large number of samples.
- RNA quantification was inaccurate, especially if using spectrophotometric methods, such as the NanoDrop instrument. Try using more input RNA.
- RNA quality is extremely degraded. Try using more input RNA.
- Incompatible indexed PCR primers were used in the Second PCR Reaction. Only use CleanPlex Index primers with CleanPlex library prep.
- A weak or incompatible magnetic rack was used to perform magnetic bead purification, resulting in significant bead loss. Do **not** use magnetic racks designed for 1.5 ml tubes.

Additional Resources

Please visit www.paragongenomics.com/product/faq/ for additional troubleshooting resources.

Data Analysis Recommendations for Illumina

Please refer to the following recommended steps for analyzing AccuFusion NGS libraries sequenced on Illumina platforms.

1. **Trim Leftover Adapter Sequences.** Trim Illumina adapter sequences using a sequence trimming software such as cutadapt (https://cutadapt.readthedocs.io/en/stable/). Following are the adapter sequences to be trimmed from 3' end of the reads.

   R1 reads: AGATCGGAAGAGCACACGTCTGAA
   R2 reads: AGATCGGAAGAGCGTCGTGTAGG

2. **Map Reads to Reference Genome.** We recommend map reads to reference genome using STAR (https://www.ncbi.nlm.nih.gov/pubmed/23104886) with the following parameters (parameter values in bold italic are those to be customized):

```
STAR --genomeDir Path_to_STAR-indexed_Genome \
--readFilesIn Read1_Fastq_File Read2_Fastq_File \
--outFilterScoreMinOverLread 0 \
--outFilterMatchNminOverLread 0 \
--outFilterMatchNmin 0 \
--outFilterIntronStrands None \
--outFilterMismatchNmax 100 \
--outSAMunmapped Within \
--readFilesCommand zcat \
--runThreadN Num_Threads \
--outFileNamePrefix Output_Name_base \
--outSAMtype BAM Unsorted \
--quantMode TranscriptomeSAM GeneCounts \
--quantTranscriptomeBan Singleend \
--twopassMode Basic \
--outSAMstrandField intronMotif \
--chimSegmentMin 12 \
--chimOutType Junctions \
--chimJunctionOverhangMin 12 \
--chimOutJunctionFormat 1 \
--alignSJDBoverhangMin 10 \
--alignMatesGapMax 100000 \
--alignIntronMax 100000 \
--alignSJstitchMismatchNmax 5 -1 5 5 \
--outSAMattrRGline ID:GRPundef \
--chimMultimapScoreRange 3 \
--chimScoreJunctionNonGTAG -4 \
--chimMultimapNmax 20 \
--chimNonchimScoreDropMin 10 \
--peOverlapNbasesMin 12 \
--peOverlapMMp 0.1
```

3. **Process STAR Output Files to Make Fusion Calls.** Confirm splice junctions called by STAR by comparing SJ.out.tab and Chimeric.out.junction files to reference genome GTF annotations. This could be accomplished using STAR-Fusion (https://github.com/STAR-Fusion/STAR-Fusion)

   ```
   STAR-Fusion --no_remove_dups \
   --genome_lib_dir /CTAT_for_STAR-Fusion/GRCh37_gencode_v19_CTAT_lib_Oct012019.plug-n-play/ctat_genome_lib_build_dir/ \
   -J.Chimeric.out.junction \
   --output_dir Output_Directory
   ```

   The genome_lib_dir content can be downloaded from https://data.broadinstitute.org/Trinity/CTAT_RESOURCE_LIB/. If a graphical user interface is preferred over command line, users are encouraged to explore the STAR Fusion Caller tool on DNAnexus.

4. **Map Reads to Construct Fusion Transcripts.** As an alternative to step 2 and 3, reads can be mapped to construct fusion transcripts (provided by Paragon Genomics) using bwa mem or other sequence aligners. Mapped reads can be assigned to amplicons based on mapping position and counted. Amplicon BED files will be provided by Paragon Genomics.

Data Analysis Recommendations for Ion Torrent

Please refer to the following recommended steps for analyzing AccuFusion NGS libraries sequenced on Ion Torrent platforms.

The fusion reference file available is available for download with the design BED files after purchase from the My Download Page on the Paragon website and can be directly loaded to Torrent Suite for launching coverage/analysis plugin or to Ion Reporter for launching a fusion workflow.

For further technical questions specific to either Torrent Suite software or Ion Reporter, please refer to ThermoFisher's User Guides that are specific for each version: https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/next-generation-sequencing-support-center/ngs-software-support.html

Please contact Techsupport@paragongenomics.com for additional questions or information.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

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