OmniFusion™ RNA Lung Cancer Panel

User Guide

This user guide is for the following products for sequencing on Illumina® NGS platforms:

OmniFusion™ RNA Lung Cancer Panel
OmniFusion™ Custom RNA Panel

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For Research Use Only. Not for use in diagnostic procedures.

Revision History

Document	Date	Description of Change
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Overview

Product Information

OmniFusion™ RNA technology is a fast, robust, and versatile solution for target enrichment and library preparation for next-generation sequencing (NGS) on Illumina® sequencing platforms. OmniFusion RNA Panels generate highly accurate data from as little as 50 ng of RNA using a fast and simple workflow. OmniFusion RNA Panels are expertly optimized with predesigned primers to generate valuable insights in key cancer and disease research areas.

OmniFusion 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 OmniFusion Product line is one of two technologies tailored specifically to your RNA-seq needs. AccuFusion™ panels offers robust targeted fusion with known fusion partners. By contrast, the OmniFusion panels utilizes single ended amplification to also discover fusions with unknown fusion partners.

OmniFusion RNA Panels feature a fast and simple workflow that can be completed in about 7 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. OmniFusion 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 OmniFusion 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.

OmniFusion Target Enrichment and Library Preparation Workflow

7 hours of total assay time, 85 minutes of hands-on time

Applications

The OmniFusion RNA Panels can be used to detect gene fusions, splice variants and the typical SNVs and indels. 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 *Detection* panels are tailored towards highly specific panels for known fusions, whereas OmniFusion *Discovery* panels utilizes 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.

Kit Contents

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

- OmniFusion RNA Lung Cancer Panel
- CleanPlex Indexed PCR Primers
- CleanMag Magnetic Beads (or equivalent)
- (Optional) NEBNext® Magnesium RNA Fragmentation Module (catalog # E6150S). See Genomic RNA Fragmentation (pg 16)

Panel Specifications

Panel	Number of Primer Pools	Primer Pool Concentration	Number of Amplicons	Average Amplicon Length	Average Library Length
OmniFusion RNA Lung Cancer Panel	1	10X	61	Variable	200-600bp

OmniFusion RNA Panel — Kit Contents, Store at –20°C

Panel	SKU	Size (Reactions)	Components
			OmniFusion RNA Library Kit
OmniFusion RNA Lung Cancer Panel	917100	8	1-pool, 8 rxns
	917101	96	1-pool, 96 rxns
	917102	384	1-pool, 384 rxns

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

OmniFusion RNA Library Kit — Kit Contents, Store at –20°C (not sold separately)					
	Configuration		1-Pool		
		Size	8 Rxns	96 Rxns	384 Rxns
Component	**Cap Color**	**SKU**	917010	917011	917012
RT Primer Mix SP	Purple Striped		24 µl	288 µl	1152 µl
RT Enzyme	Blue Striped		8 µl	96 µl	384 µl
RT Buffer SP	Orange Striped		40 µl	480 µl	1920 µl
RT Clean Reagent	Yellow Striped		16 µl	192 µl	768 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)

- 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
CleanMag Magnetic Beads, 1 ml	718001	1 ml	~6
CleanMag Magnetic Beads, 5 ml	718002	5 ml	~33
CleanMag Magnetic Beads, 20 ml	718005	20 ml	~130
CleanMag Magnetic Beads, 60 ml	718003	60 ml	~400
CleanMag Magnetic Beads, 450 ml	718004	450 ml	~3,000

- RNA from cells lines or genomics sources needs to be fragmented before use. We suggest NEBNext® Magnesium RNA Fragmentation Module (catalog # E6150S) for quick and easy fragmentation.

- 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

OmniFusion Panels and CleanPlex Indexed PCR Primers are shipped on blue ice (ice packs). Upon receipt, immediately store OmniFusion 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.

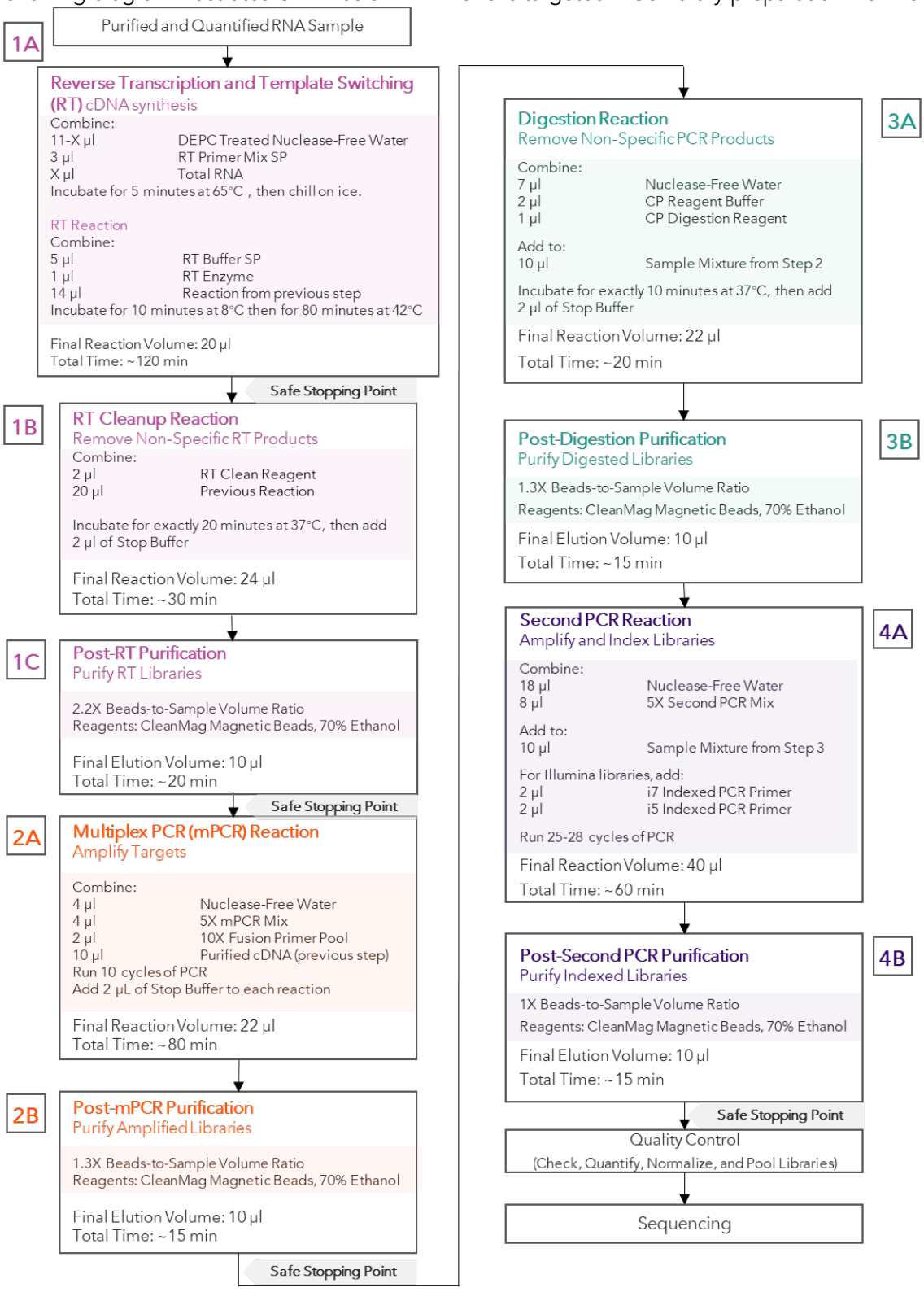
The components containing enzymes are viscous (10X Fusion pool, RT Enzyme Mix, RT Clean Reagent, 5X mPCR Mix, CP Digestion Reagent, 2nd PCR Mix) and specially formulated for storage at -20°C without freezing to avoid freeze-thaw cycles to retain their full activity. Please avoid storing these components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

OmniFusion 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 OmniFusion RNA Panel's targeted NGS library preparation workflow.



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 (10X Fusion pool, RT Enzyme Mix, RT Clean Reagent, 5X mPCR Mix, CP Digestion Reagent, 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 work day.

- 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 and 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 (10X Fusion pool, RT Enzyme Mix, RT Clean Reagent, 5X mPCR Mix, CP Digestion Reagent, 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 pipette or vortex 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 just 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 transfer in order to avoid or reduce sample loss. For the single-pool OmniFusion RNA Panel, the entire protocol is performed following a single-tube workflow, with no tube-to-tube transfers.

- 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 a 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. A master mix calculation sheet can be downloaded from the Product Documents page on the Paragon Genomics website at www.paragongenomics.com/product_documents/.

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 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	Recommended RNA Input	Recommended FFPE RNA Input
OmniFusion RNA Lung Cancer Panel	25–100 ng	25 ng	50 ng
OmniFusion RNA Custom Panel	25–100 ng	25 ng	50 ng

- The maximum volume of RNA input per 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 RT Reaction. Lower RNA input may result in biased detection of certain targets.
- RNA from **cell lines** or genomic sources needs to be fragmented before use. We suggest NEBNext® Magnesium RNA Fragmentation Module (catalog # E6150S).
- RNA from **FFPE** sources can be used directly without pretreatment.
- 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 RT. 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 combination (CleanPlex Dual-Indexed PCR Primers for Illumina) to each sample.
- Note the safe stopping points after RT Reaction, mPCR reaction, mPCR purification and 2nd PCR purification. After starting Digestion Step, samples must be carried through to 2nd PCR purification without stopping. Plan accordingly.
- Upon initial receipt, Stop Buffer can be thawed and stored continuously at room temperature.

Genomic RNA fragmentation

- FFPE RNA does not require additional fragmentation.
- RNA from **cell lines** or genomic sources needs to be fragmented before use. We suggest NEBNext® Magnesium RNA Fragmentation Module (catalog # E6150S) from New England Biolabs, Inc. for easy and quick fragmentation. Follow the associated instructions for use.
- After incubation, as instructed by the NEBNext® Magnesium RNA Fragmentation Module user guide, perform a 2.2X magnetic bead purification with 10 minutes room temperature incubation. Wash 2X with 70% ethanol and elute the fragmented RNA in DEPC treated water. See step 1C below for detailed magnetic bead purification instructions.
- Re-quantify the fragmented RNA by Qubit if needed, and proceed to step 1A Reverse Transcription and Template switching reaction.

1A. Reverse Transcription and Template Switching (RT) Reaction

1A.1. Thaw RT primer Mix and RT Buffer 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 Reverse Transcription and Template Switching 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 Random Primers. 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 RT Primer Annealing Reaction.*

Note: *Thaw and keep Stop Buffer and Magnetic Beads at room temperature for use at the end of step 1A and beginning of 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 SP	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 Random 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 RT primer annealing, continue to Reverse transcription and Template Switching Reactions.

1A.4. Keep the tubes/plate on ice. Open the tubes or carefully remove the adhesive film from the PCR plate. Prepare the Reverse Transcription (RT) and Template Switching (TS) 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 and Template Switching Reaction Mixture. Mix well, aliquot into individual tubes.*

RT and TS Reaction Mixture

Reagent	Cap Color	Volume per reaction
RT Buffer SP	Orange 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 temperature 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	∞	

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. RT Cleanup Reaction

Note: *After starting the RT Cleanup Reaction, the samples cannot be stored and must continue to Post-RT Cleanup Purification then mPCR Reaction. Plan accordingly.*

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

1B.1. Quick spin the tubes/plate to collect the liquid. Open the tubes or carefully remove the adhesive film from the PCR plate. Keep the samples on ice or on a cold block then add RT Cleaning Reagent to each sample.

RT Cleanup Reaction Mixture		
Reagent	**Cap Color**	**Volume per reaction**
Previous Reaction	--	20 µl
RT Clean Reagent	Yellow Striped	2 µl
Total Volume per reaction		22 µl

Important! RT Cleaning Reagent is viscous. Pipette slowly, remove any excess reagent on the outside of the pipette tip, and rinse tip in solution for quantitative transfer.

1B.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: *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.*

1B.3. Load the tubes/plate in the thermal cycler and run the following thermal cycling protocol to clean up cDNA transcripts.

Step	Temperature	Time	Preheat
Incubation	37 °C	20 min	37 °C
Hold	4 °C	∞	-

1B.4. Immediately transfer the tube onto ice or ice block after the 20-minute incubation. 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 sample is now approximately 24 µl.

Note: *Do not allow samples to hold at 10 °C for longer than 20 minutes before adding Stop Buffer.*

Important. Do not stop and store samples after the RT Cleanup Reaction. Proceed to next step, Post-RT Cleanup Purification immediately.

1C. 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.

1C.1. Vortex the magnetic beads suspension vigorously until homogeneous. Perform a **2.2X** bead-based purification by adding 53 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	24 µl
Magnetic Beads	53 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.

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

1C.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.*

1C.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 Cleanup *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.*

1C.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.

1C.6. Repeat step 1C.5.

1C.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 open 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.*

1C.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.

1C.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

2A.1. Thaw 10x Fusion Primer pool and vortex thoroughly before use. Prepare the mPCR Reaction Master Mixture by adding components in the following order on ice or a cold block directly into the same tubes (or 96-well PCR plate). No tube to tube transfer is needed and the magnetic beads from the previous reaction are carried onto the next steps.

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	—	4 µl
5X mPCR Mix	Green	4 µl
10X OmniFusion RNA Lung Cancer Primer Pool	—	2 µl*
Total Volume		10 µl

** Custom OmniFusion panels may benefit from primer pool titrations within a range of 0.5X-2X for best performance.*

Important! 5X mPCR Mix and 10X Fusion 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	10 µl
mPCR Master mix	10 µl
Total Volume per reaction	20 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.

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.3. 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 sample is now approximately 22 µl.

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

2A.4. 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. Quick 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 beads suspension vigorously until homogeneous. Perform a **1.3X** bead-based purification by adding 29 µl 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	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.

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 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 open 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 Reagent 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 RT

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 Reagent 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 1B	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 to Step 3B. Post-Digestion Purification immediately.

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

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 Digestion 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.

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 to Step 4A. Second PCR Reaction immediately.

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

4A. Second PCR Reaction

Note: *Remember to assign a specific index or unique combination of dual index 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
Second PCR Reaction Master Mix	26 µl
Purified Sample from Step 2B	10 µl
i5 Indexed PCR Primer for Illumina	2 µl
i7 Indexed PCR Primer for Illumina	2 µl
Total Volume per reaction	**40 µl**

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 for the Ready-to-Use NGS Panels and Custom NGS Panels.

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.*

Note*: For OmniFusion Custom NGS Panels, use the total number of amplicons to determine the appropriate Second 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	See table below
Annealing/Extension	60 °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/Ss..

OmniFusion NGS Panels — Second PCR Cycle Number		
Number of Amplicons	25 ng of High Quality RNA*	25 ng of Low Quality RNA*
OmniFusion RNA Lung Cancer Panel	22	25-28
7 - 50	22	26-29
51 - 100	22	25-28
101 - 200	21	24-27

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

Second PCR Reaction — Input Specific Cycle Number	
Input RNA	Change in Cycle Number
10 ng	+ 2
25 ng	-
50 ng	– 1
100 ng	– 2
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 ensures 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.

OmniFusion 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 OmniFusion panels due to the ability to detect novel fusions and depending on the genotype of the sample itself, and the fragment length of the starting RNA. The library peaks typically span between 200-600 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 OmniFusion RNA panels are designed to be compatible with PE 150 bp reads (2x150 bp). Custom Fusion panels are also 2x150 bp designs unless otherwise decided and communicated between the customer and the Paragon Genomics panel design team.

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)**
OmniFusion RNA Lung Cancer Panel	Novel and 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 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

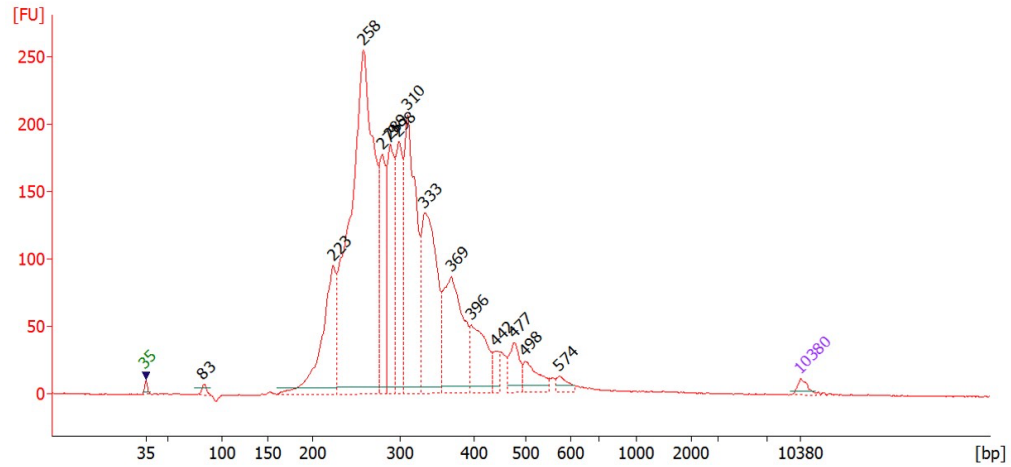
i5 Index	Index Sequence	i5 Bases for Sample Sheet	
		MiSeq, NovaSeq, HiSeq 2000/2500	MiniSeq, NextSeq, HiSeq 3000/4000
Q5001	AGCGCTAG	AGCGCTAG	CTAGCGCT
Q5002	GATATCGA	GATATCGA	TCGATATC
Q5007	ACATAGCG	ACATAGCG	CGCTATGT
Q5008	GTGCGATA	GTGCGATA	TATCGCAC
Q5009	CCAACAGA	CCAACAGA	TCTGTTGG
Q5010	TTGGTGAG	TTGGTGAG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

Troubleshooting Guide

Examples of Libraries Prepared with OmniFusion NGS Panels

Depending on the OmniFusion Panel used, library peak(s) may span between 200 and 600 bp. Below are representative Agilent Bioanalyzer traces generated with the OmniFusion RNA Lung Cancer Panel with 50 ng control fragmented genomic RNA input.

Example OmniFusion RNA Lung Cancer Panel



Due to the unique nature of the fusion chemistry, the distribution of amplicon sizes for the final library can vary significantly based on the fragment size and which fusion mutations are present in the sample.

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.

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 CleanPlex 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.

Technical Support

For technical assistance, please contact Paragon Genomics Technical Support.

Phone: 650-822-7545
Email: techsupport@paragongenomics.com
Website: www.paragongenomics.com

Paragon Genomics
5020 Brandin Court, FL 2
Fremont, CA 94538 USA
1-510-363-9918
contact@paragongenomics.com
www.paragongenomics.com