**Solid Tumor Fusion Panel Procedure**

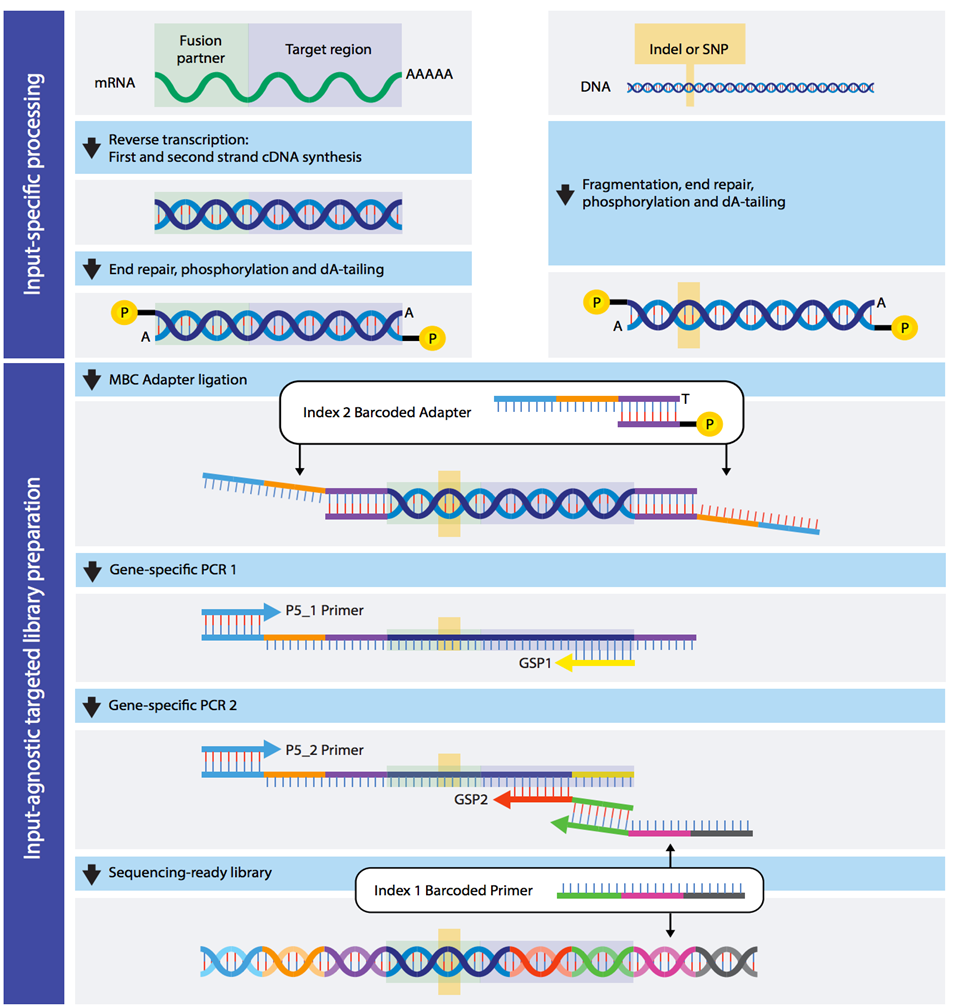
1. **PRINCIPLE:** 
   1. Chromosomal translocations, interstitial deletions, and inversions that lead to gene fusions are common in solid tumors and sarcomas. However, in the clinical laboratory, the detection of gene fusions can be difficult due to the heterogeneous nature of most tumor tissue and the limited amount of clinical sample available for testing. As a result, sensitive methodologies (such as Next-Generation Sequencing (NGS)) are useful for the detection of gene fusions.
   2. The Comprehensive Solid Tumor Fusion Panel is a robust sequencing assay designed to detect and identify gene fusions involving 105 target genes. This NGS panel is a custom Archer®DX FusionPlex® assay, which performs targeted RNA sequencing using Anchored Multiplex PCR (AMP™). This chemistry utilizes unidirectional gene-specific primers to detect fusion genes by targeting one of the fusion partners (e.g., *ALK* or *EWSR1*); therefore, it is particularly suitable for the identification of fusion genes when there is alternative splicing and/or possibility of multiple partner genes.
   3. The assay is also offered clinically for tumor-specific subsets (Sarcoma; Neuro, Bladder; and Lung, Thyroid, Salivary Gland) using the same reagents.
      1. Tables 1-4 below shows the genes and exons targeted by the Fusion Panels.
      2. The remainder of this procedure shall refer to all of these panels as “Fusion Panel”.
   4. AMP™ is an enrichment method that generates cDNA libraries isolated from the RNA of FFPE tumor specimens. This chemistry allows for the simultaneous detection of both known recurrent fusions as well as previously unidentified fusions at key fusion points in target genes. Some key features of AMP™ technology include:
      1. **Molecular barcodes (MBCs)** – Adapters ligated to molecules before amplification carry MBCs that enable unique molecule counting and error correction for confident mutation calling.
      2. **Unique start sites** – Open-ended amplification gives flexibility for novel fusion detection in RNA.
      3. **Low-input FFPE** – AMP™ allows for successful library preparation of highly fragmented, low-yield sample types such as FFPE.
      4. **Strand-specific priming** – Dual, independent coverage across target regions ensure that some reads are retained when one primer drops out due to primer-blocking SNVs.
   5. The Illumina NextSeq® 500 System (NextSeq®) is an NGS instrument that measures fluorescence signals of labeled nucleotides using instrument-specific reagents, flow cell, and imaging hardware. High-throughput sequencing of Fusion Panel libraries is performed by the NextSeq® (see the NextSeq®500 Instrument Procedure).
   6. The data generated by the NextSeq® are analyzed by multiple vendor-developed and custom Lifespan bioinformatics platforms. This results in highly-confident fusion detection supported by relevant literature and databases.
   7. The Solid Tumor Fusion Panels consist of these broad parts:
      1. Archer® FusionPlex® Kit for Illumina Platform
         1. FusionPlex Reagents, for Illumina
         2. Molecular Bar Code (MBC) Adapters
         3. Panel-specific Gene Specific Primers (FusionPlex Lifespan Solid Tumor version 2.0).
      2. KAPA Library Quantification Kit for Illumina Platform.
      3. Illumina NextSeq® 500/550 Mid Output Sequencing Kit (version 2.5).
      4. Archer® Analysis Software (version 6.2.2).
      5. Custom Lifespan Fusion Reporter Bioinformatics Pipeline (version 1.0).
      6. Custom Lifespan Review Tool (version 1.0).
      7. Custom Lifespan All\_Fusions Tool (version 1.0).
   8. The Fusion Panel follows a simplified **NGS workflow**:
      1. **Extraction** – Total nucleic acid (TNA) extraction from the specimen is performed prior to library preparation.
      2. **Library Preparation** – Construction of target-enriched libraries is performed using the Archer® FusionPlex® Kit (Lifespan Solid Tumor version 2.0). Library preparation follows a series of reaction steps using Reaction Tubes that are provided with the FusionPlex® Kit. The Reaction Tubes are 0.2mL PCR tube-strips that contain reaction-specific lyophilized pellets. Each reaction step serves an essential role and must be processed in the following order to generate sequencing-ready libraries:
         1. Random Priming 2.0 – Random hexamers serve as priming sites on the RNA fragments to initiate polymerization by Reverse Transcriptase (RT).
         2. First Strand cDNA Synthesis – Complementary DNA (cDNA) strands of different lengths and start positions for each transcript are synthesized.
         3. Second Strand cDNA Synthesis – Incomplete RNA digestion by Ribonuclease H (RNase-H) leaves small RNA fragments, which serve as primers for 2nd strand synthesis.
         4. PreSeq RNA QC Assay – The PreSeq RNA QC Assay (PreSeq Assay) is a SYBR® -based qPCR assay designed to assess the quality of the input RNA. The PreSeq Assay amplifies the universally expressed transcript, Valosin-containing Protein (*VCP*), to determine the amount of amplifiable RNA in the sample (fragments greater than 100 bp in length). iTaq Universal SYBR green Supermix, 10X VCP Primer mix, and diluted First-Strand cDNA are combined. PCR is performed, producing a 113bp VCP amplicon.
         5. End Repair – The ends of cDNA fragments are enzymatically blunted, phosphorylated, and A-tailed. This prepares the cDNA fragments for adapters to be ligated.
         6. Ligation 1 – Adapters are ligated onto random start sites of cDNA fragments that have been end-repaired.
         7. MBC Adapter Incorporation – The adapters contain a P5 primer, Index 2 (sample multiplexing), Read 1 site for Illumina sequencing, and an MBC for molecule-specific identification. A random 8-mer Molecular Barcode is part of the adapter.
         8. Ligation 2 – A second round of Ligation is performed.
         9. First PCR – The first round of PCR creates amplicons containing the regions between Gene-Specific Primers (GSP1) at the 3’ end, amplified against the universal P5 primer ligated to the 5’ end.
         10. Second PCR – The second PCR incorporates a nested Gene-Specific Primer 2 (GSP2), which contains a tail composed of a Read 2 region, Index 1 sequence, and the P7 sequencing primer.
      3. **Quantification of the Purified Final Libraries** – Final Library concentration is calculated by a highly-sensitive qPCR assay. The KAPA Library Quantification Kit (KAPA qPCR Assay) is a SYBR®-based assay designed specifically for Illumina platforms. The KAPA qPCR Assay performs absolute quantification of final libraries flanked by the P5 and P7 flow cell oligo sequences.
      4. **Sequencing** – Paired-end sequencing is performed on the NextSeq® using the NextSeq® 500/550 Mid Output 300 Cycles Kit (version 2.5).
      5. **Analysis** – The sequencing data is analyzed, and the results are reported. These steps are performed by a Bioinformatician, Pathologist/Director, or other appropriately designated personnel.
   9. **Figure 1** below summarizes the steps and subcomponents of the Fusion Panel.
   10. **Figure 2** below summarizes Archer® AMP™ technology.

**Figure 1.** Summary of the Fusion Panel Wet-Bench Steps



**Figure 2.** Anchored Multiplex PCR (AMP™)

**TNA/RNA DNA**



**Table 1.** Comprehensive Solid Tumor Fusion Panel Targets

|  |  |  |  |
| --- | --- | --- | --- |
| **Target\_Name** | **NCBI\_Reference\_Sequence** | **Target\_Exons** | **Direction** |
| *AKT1* | NM\_005163 | 2,3,4,5 | 5 |
| *AKT2* | NM\_001626 | 5 | 5 |
| *AKT3* | NM\_005465 | 1,2,3 | 5 |
| *ALK* | NM\_004304 | 2,4,6,10,16,17,18,19,20,21,22,23,26 | 5 |
| *ARHGAP26* | NM\_015071 | 2,10,11,12 | 5 |
| *AXL* | NM\_021913 | 18,19 | 3 |
| *BCL7A* | NM\_020993 | 3 | 3 |
| *BCOR* | NM\_001123385 | 6,7,12,14,15 | 3 |
| *BCOR* | NM\_017745 | 8 | 5 |
| *BCOR* | NM\_001123385 | 7,8,15 | 5 |
| *BRAF* | NM\_004333 | 1,3,7,8,10,13 | 3 |
| *BRAF* | NM\_004333 | 2,7,8,9,10,11,12,15,16 | 5 |
| *BRD3* | NM\_007371 | 9,10,11,12 | 3 |
| *BRD4* | NM\_058243 | 10,11 | 3 |
| *CAMTA1* | NM\_015215 | 8,9,10 | 5 |
| *CAMTA1* | NM\_015215 | 3 | 3 |
| *CBFA2T3* | NM\_005187 | 4 | 5 |
| *CCNB3* | NM\_033031 | 3,4,5,6 | 5 |
| *CCNB3* | NM\_033031 | 2 | 5 |
| *CD274* | NM\_014143 | 7 | 3 |
| *CD274* | NM\_014143 | 2,3,4,5 | 3 |
| *CD274* | NM\_014143 | 7 | 5 |
| *CDKN2A* | NM\_000077 | 1,2 | 3 |
| *CDKN2A* | NM\_000077 | 3 | 3 |
| *CDKN2A* | NM\_000077 | 3 | 5 |
| *CDKN2A* | NM\_000077 | 1 | 5 |
| *CIC* | NM\_015125 | 17,18,19 | 3 |
| *CIC* | NM\_015125 | 20 | 3 |
| *CLIP2* | NM\_003388 | 3 | 5 |
| *CRTC3* | NM\_022769 | 3 | 5 |
| *CSF1* | NM\_172212 | 9 | 3 |
| *CSF1* | NM\_000757 | 5,6,7 | 3 |
| *CSF1* | NM\_000757 | 8 | 3 |
| *CSF1* | NM\_000757 | 6 | 5 |
| *DNAJB1* | NM\_006145 | 1,2 | 3 |
| **Target\_Name** | **NCBI\_Reference\_Sequence** | **Target\_Exons** | **Direction** |
| *EGFR* | NM\_005228 | 7,8,9,16,19,20 | 5 |
| *EGFR* | NM\_005228 | 1,24,25 | 3 |
| *EPC1* | NM\_025209 | 9,10,11 | 3 |
| *ERBB2* | NM\_004448 | 23,24,25,26 | 3 |
| *ERBB2* | NM\_004448 | 3,4,5 | 5 |
| *ERBB4* | NM\_005235 | 2,3,4,14,15,16,17,18,23 | 5 |
| *ERG* | NM\_004449 | 4,5,6,7,8,9,10,11 | 5 |
| *ERG* | NM\_004449 | 2,3 | 5 |
| *ESR1* | NM\_000125 | 8 | 5 |
| *ESR1* | NM\_001122742 | 3,4,5,6 | 3 |
| *ESR1* | NM\_000125 | 6 | 3 |
| *ESRRA* | NM\_004451 | 2,3 | 3 |
| *ETV1* | NM\_004956 | 3,4,5,6,7,8,9,10,11,12,13 | 5 |
| *ETV4* | NM\_001986 | 2,4,5,6,7,8,9,10 | 5 |
| *ETV5* | NM\_004454 | 2 | 5 |
| *ETV5* | NM\_004454 | 3,7,8,9 | 5 |
| *ETV6* | NM\_001987 | 2,3,4,5,6,7 | 5 |
| *ETV6* | NM\_001987 | 1,2,3,4,5,6 | 3 |
| *EWSR1* | NM\_005243 | 4,5,6,7,8,9,10,11,12,13,14 | 3 |
| *FGFR1* | NM\_015850 | 2 | 5 |
| *FGFR1* | NM\_015850 | 3,4,5,6,7,8,9,10,11,17 | 5 |
| *FGFR1* | NM\_015850 | 12,17 | 3 |
| *FGFR2* | NM\_000141 | 5,7,8,9,10 | 5 |
| *FGFR2* | NM\_000141 | 16,17 | 3 |
| *FGFR2* | NM\_000141 | 2 | 5 |
| *FGFR3* | NM\_000142 | 3,5,8,9,10 | 5 |
| *FGFR3* | NM\_000142 | 16,17 | 3 |
| *FGR* | NM\_005248 | 2 | 5 |
| *FOSB* | NM\_006732 | 1 | 5 |
| *FOSB* | NM\_006732 | 2 | 5 |
| *FOXO1* | NM\_002015 | 2,3 | 3 |
| *FOXO1* | NM\_002015 | 1 | 5 |
| *FOXO1* | NM\_002015 | 2,3 | 5 |
| *FOXO1* | NM\_002015 | 1 | 3 |
| *FUS* | NM\_004960 | 3,4,5,6,7,8,9,10,11,13,14 | 3 |
| *GLI1* | NM\_005269 | 4,5,6,7 | 5 |
| *GLI1* | NM\_005269 | 4,5,6,7 | 3 |
| **Target\_Name** | **NCBI\_Reference\_Sequence** | **Target\_Exons** | **Direction** |
| *HMGA2* | NM\_003483 | 1,2,3,4,5 | 3 |
| *HMGA2* | NM\_003483 | 5 | 3 |
| *INSR* | NM\_000208 | 20,21,22 | 3 |
| *INSR* | NM\_000208 | 12,13,14,15,16,17,18,19 | 5 |
| *JAK2* | NM\_004972 | 6,7,8,9,10,11,12,13,14,15,16,17,18,19,20 | 5 |
| *JAK2* | NM\_004972 | 9,10,11,12 | 3 |
| *JAZF1* | NM\_175061 | 2,3,4 | 3 |
| *LPP* | NM\_005578 | 7 | 3 |
| *MAML2* | NM\_032427 | 2,3 | 5 |
| *MAML2* | NM\_032427 | 2 | 3 |
| *MAP3K3* | NM\_002401 | 2 | 5 |
| *MAST1* | NM\_014975 | 7,8,9,18,19,20,21 | 5 |
| *MAST2* | NM\_015112 | 2,3,5,6 | 5 |
| *MDM2* | NM\_002392 | 2,4,6,7,8 | 3 |
| *MDM2* | NM\_002392 | 5,7,11 | 5 |
| *MEAF6* | NM\_001270875 | 4,5 | 3 |
| *MET* | NM\_000245 | 2 | 5 |
| *MET* | NM\_000245 | 2,13 | 3 |
| *MET* | NM\_000245 | 4,5,6,13,14,15,16,17,21 | 5 |
| *MKL2* | NM\_014048 | 11,12,13 | 5 |
| *MN1* | NM\_002430 | 1,2 | 3 |
| *MSMB* | NM\_002443 | 2,3,4 | 3 |
| *MUSK* | NM\_005592 | 7,9,10,12,13,14,15 | 5 |
| *MYB* | NM\_001130173 | 7,8,9,11,12,13,14,15,16 | 3 |
| *MYBL1* | NM\_001080416 | 8,9,10,11,12,13,14,15 | 3 |
| *NCOA2* | NM\_006540 | 11,12,13,14,15,16 | 5 |
| *NOTCH1* | NM\_017617 | 24,25,26,27,28,29 | 5 |
| *NOTCH1* | NM\_017617 | 2,4,24,29,30,31 | 3 |
| *NOTCH2* | NM\_024408 | 26,27,28 | 5 |
| *NOTCH2* | NM\_024408 | 5,6,7 | 3 |
| *NRG1* | NM\_013957 | 1 | 5 |
| *NRG1* | NM\_004495 | 1,2,3,6 | 5 |
| *NRG1* | NM\_013957 | 4,8 | 5 |
| *NRG1* | NM\_013962 | 1 | 3 |
| *NTRK1* | NM\_002529 | 2,4,6,8,10,11,12,13 | 5 |
| *NTRK2* | NM\_006180 | 5,7,9,11,12,13,14,15,16,17 | 5 |
| *NTRK3* | NM\_002530 | 13,14,15 | 3 |
| **Target\_Name** | **NCBI\_Reference\_Sequence** | **Target\_Exons** | **Direction** |
| *NTRK3* | NM\_002530 | 4,7,10,12,13,14,15,16 | 5 |
| *NTRK3* | NM\_001007156 | 15 | 5 |
| *NUMBL* | NM\_004756 | 3 | 5 |
| *NUP107* | NM\_020401 | 5 | 3 |
| *NUP214* | NM\_005085 | 29 | 3 |
| *NUTM1* | NM\_175741 | 3,4,5 | 5 |
| *NUTM2A* | NM\_001099338 | 2,3,4,5,6,7 | 5 |
| *PDGFB* | NM\_002608 | 2,3 | 5 |
| *PDGFRA* | NM\_006206 | 10,11,12,13,14,15 | 5 |
| *PDGFRA* | NM\_006206 | 7 | 3 |
| *PDGFRB* | NM\_002609 | 8,9,10,11,12,13,14 | 5 |
| *PIK3CA* | NM\_006218 | 2 | 5 |
| *PIK3CD* | NM\_005026 | 3 | 5 |
| *PKN1* | NM\_002741 | 10,11,12,13 | 5 |
| *PLAG1* | NM\_002655 | 1,2,3,4 | 5 |
| *PPARG* | NM\_015869 | 1,2,3 | 5 |
| *PRKACA* | NM\_002730 | 2 | 5 |
| *PRKCA* | NM\_002737 | 4,5,6 | 5 |
| *PRKCB* | NM\_002738 | 3 | 5 |
| *PSIP1* | NM\_033222 | 2 | 3 |
| *PTCH1* | NM\_001083602 | 1 | 3 |
| *RAF1* | NM\_002880 | 4,5,6,7,8,9,10,11,12 | 5 |
| *RAF1* | NM\_002880 | 4,5,6,7,9 | 3 |
| *RELA* | NM\_021975 | 3,4 | 5 |
| *RET* | NM\_020630 | 2,4,6 | 5 |
| *RET* | NM\_020975 | 8,9,10,11,12,13,14 | 5 |
| *ROS1* | NM\_002944 | 2,4,7,31,32,33,34,35,36,37 | 5 |
| *RSPO2* | NM\_178565 | 1,2 | 5 |
| *RSPO3* | NM\_032784 | 2 | 5 |
| *SPECC1* | NM\_152904 | 2 | 3 |
| *SS18* | NM\_001007559 | 4,5,6,8,9,10 | 3 |
| *SS18* | NM\_001007559 | 10,11 | 5 |
| *STAT6* | NM\_001178078 | 3,4,5,6,7,15,16,17,18,19,20 | 5 |
| *STAT6* | NM\_001178078 | 1,2 | 5 |
| *TAF15* | NM\_139215 | 6,7 | 5 |
| *TAF15* | NM\_139215 | 5,6,7 | 3 |
| *TCF12* | NM\_207036 | 4,5,6 | 3 |
| **Target\_Name** | **NCBI\_Reference\_Sequence** | **Target\_Exons** | **Direction** |
| *TERT* | NM\_198253 | 2 | 5 |
| *TFE3* | NM\_006521 | 2,3,4,5,6,7,8 | 5 |
| *TFE3* | NM\_006521 | 2,3,4,5,6 | 3 |
| *TFEB* | NM\_007162 | 1,2,3 | 5 |
| *TFEB* | NM\_007162 | 4 | 5 |
| *TFG* | NM\_006070 | 3,4,5,6,7 | 3 |
| *TFG* | NM\_006070 | 6 | 5 |
| *THADA* | NM\_022065 | 24,25,26,27,28,29,30,36,37 | 3 |
| *TMPRSS2* | NM\_001135099 | 1,2 | 3 |
| *TMPRSS2* | NM\_005656 | 3,4,5,6 | 3 |
| *TMPRSS2* | NM\_005656 | 1 | 3 |
| *TMPRSS2* | NM\_001135099 | 2 | 5 |
| *TOP1* | NM\_003286 | 3 | 5 |
| *USP6* | NM\_004505 | 1,2 | 5 |
| *USP6* | NM\_004505 | 3 | 5 |
| *VAV1* | NM\_005428 | 27 | 5 |
| *YWHAE* | NM\_006761 | 5 | 3 |

**Table 2.** Sarcoma Fusion Panel Targets

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *ALK* | *ETV6* | *MKL2* | *PDGFRA* | *TFE3* |
| *BCOR* | *EWSR1* | *NCOA2* | *PDGFRB* | *TFEB* |
| *CAMTA1* | *FOXO1* | *NTRK1* | *PLAG1* | *TFG* |
| *CCNB3* | *FUS* | *NTRK2* | *RET* | *USP6* |
| *CIC* | *GLI1* | *NTRK3* | *ROS1* | *YWHAE* |
| *EGFR* | *HMGA2* | *NTRK3* | *SS18* |  |
| *EPC1* | *JAZF1* | *NUP107* | *STAT6* |  |
| *ETV1* | *LPP* | *NUTM1* | *TAF15* |  |
| *ETV4* | *MDM2* | *NUTM2A* | *TCF12* |  |
| *ETV5* | *MEAF6* | *PDGFB* | *TERT* |  |

**Table 3.** Neuro-oncology Fusion Panel Targets

|  |  |  |
| --- | --- | --- |
| *AKT1* | *MYBL1* | *RELA* |
| *AKT2* | *NTRK1* |  |
| *AKT3* | *NTRK2* |  |
| *BRAF* | *NTRK3* |  |
| *EGFR* | *PDGFB* |  |
| *EWSR1* | *PDGFRA* |  |
| *FGFR1* | *PDGFRB* |  |
| *FGFR2* | *PRKCA* |  |
| *FGFR3* | *PRKCB* |  |
| *MYB* | *RAF1* |  |

**Table 4.** Lung, Thyroid, Salivary Gland Fusion Panel Targets

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *AKT1* | *EGFR* | *KRAS* | *NUTM1* | *SLC5A5* |
| *ALK* | *ERBB2* | *KRT20* | *PIK3CA* | *TFG* |
| *AXL* | *ERBB4* | *KRT7* | *PKN1* | *THADA* |
| *BRAF* | *FGFR1* | *MAP2K1* | *PPARG* | *TTF1* |
| *BRD3* | *FGFR2* | *MET* | *PRKCA* | *EWSR1* |
| *BRD4* | *FGFR3* | *NRAS* | *PRKCB* | *MAML2* |
| *CALCA* | *GNAS* | *NRG1* | *PTH* | *MN1* |
| *CCND1* | *HRAS* | *NTRK1* | *RAF1* | *MYB* |
| *CTNNB1* | *IDH1* | *NTRK2* | *RET* | *MYBL1* |
| *DDR2* | *IDH2* | *NTRK3* | *ROS1* | *ETV6* |

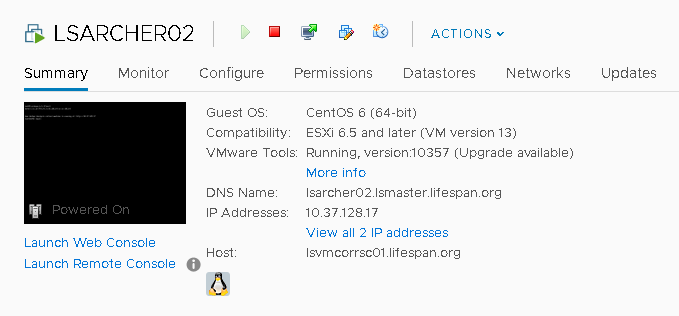
1. **SPECIMENS:**
   1. Sample types: FFPE unstained slides, FFPE scrolls, FFPE cell blocks, reference control cell line and cytology preparation specimens (such as smear and liquid-based cytology slides) are acceptable specimen types for the Fusion Panel.
      1. Although not standard specimens for the Fusion Panel, fresh and frozen tissue may be accepted with Director/Pathologist approval. If all QC passes, results may be determined to be acceptable at the discretion of the Director/Pathologist.
   2. Total nucleic acid can be extracted from FFPE Specimens using either:
      1. The Promega Maxwell® Rapid Sample Concentrator (RSC); see the Maxwell® RSC Instrument Procedure.
      2. The FFPE Organic Extraction method. See the TNA (RNA), Tissue Manual Extraction Procedure.
   3. Cytology preparation specimens are processed according to the Cytology Slide Preparation method. See the TNA, Cytology Manual Extraction Procedure.
   4. After extraction of total nucleic acid from the specimen, RNA concentration is measured by the Qubit 3.0 Fluorometer. See the Qubit 3.0 Fluorometer Instrument Procedure.
   5. Specimen acceptability criteria:
      1. The Fusion Panel is optimized for RNA input of up to 250 ng in a total volume of 20 µL. Accordingly, samples must have a minimum RNA concentration of 2.1 ng/µL.
      2. Samples with RNA concentration <2.1 ng/µL should be discussed with the Director/Pathologist. At the discretion of the Director/Pathologist, testing may be attempted with the possibility that the sample will be insufficient for testing based on the results of the PreSeq Assay or the KAPA qPCR Assay (see the PreSeq Assay and KAPA qPCR Assay requirements).
      3. To enable more sensitive fusion detection, it is recommended to input the maximum allowable mass for a sample.
      4. In addition to input mass, sample quality is also paramount to obtaining high quality libraries that produce reliable sequencing data.
      5. Sample quality is based on the Cycle Threshold (Ct) value of the PreSeq Assay:
         1. Samples should have a Ct value of <30.
         2. Samples with Ct ≥30 have the lowest probability of successful library generation and are least likely to pass Archer® Analysis Software QC (see Bioinformatics section on Archer® Analysis Software below).
         3. In consultation with a Director/Pathologist, samples with Ct ≥30 may be continued through library preparation. Based on the final concentration from the KAPA qPCR, the sample may then be excluded from sequencing.
      6. Before proceeding with sequencing on the NextSeq®, the purified final libraries are quantified (See Test Procedure: Fusion Panel Step 11 – KAPA qPCR).
         1. The KAPA qPCR must pass the Run Metrics as outlined in Fusion Panel Step 11 – KAPA qPCR.
         2. The KAPA qPCR must be repeated if a Run Metric fails.
         3. In addition, each Standard and sample must pass the Cycle Threshold (Ct) criteria of the KAPA qPCR, as outlined in Fusion Panel Step 11 – KAPA qPCR.
         4. The KAPA qPCR must be repeated if the Standards do not meet the analysis criteria.
         5. Libraries that do not meet the analysis criteria must be excluded from the final library pool or be repeated.
         6. Finally, libraries must have a final concentration ≥50nM to be sequenced.
         7. At the discretion of the Director/Pathologist, sequencing may be performed for samples with final concentration <50nM but ≥4nM (which is the normalized concentration of the final pool).
2. **MATERIALS AND EQUIPMENT:**
   1. REAGENTS:
      1. Archer® FusionPlex Reagents for Illumina Platform. Store at 2 to 8°C.
      2. Archer® FusionPlex Solid Tumor Gene Specific Primers (Lifespan Custom Panel version 2.0). Store at -15 to -30°C.
      3. Archer® MBC Adapters for Illumina Platform. Store at 2 to 8°C.
      4. 70% Ethanol (Pharmco, or equivalent Molecular Grade Ethanol). Store at room temperature in flammable cabinet.
      5. iTaq Universal SYBR Green Supermix. Store at -15 to -30°C.
      6. Tris-HCl Buffer (1M, pH 8.0). Store at room temperature in the acid cabinet
         1. Fisher Scientific, cat# AAJ22638AE.
      7. Agencourt® AMPure XP Beads. Store at 2 to 8°C.
      8. 1N Sodium Hydroxide. Store at room temperature in the acid cabinet.
      9. KAPA Universal Library Quantification Kit (Illumina Platform). Store at -15 to -30°C.
      10. Tris-HCl Buffer (0.2M, pH7.0). Store at room temperature in the acid cabinet.
          1. Boston BioProducts, cat# C-8255R or Teknova, cat# T2260.
      11. PhiX Control. Store at -15 to -30°C.
      12. Illumina NextSeq 500/550 Mid Output Sequencing Kit (version 2.5):
          1. NextSeq® 500/550 Mid Output Reagent Cartridge version 2.0 (300 Cycles). Store at -15 to -30°C.
          2. NextSeq Accessory Box 2. Store at -15 to -30°C.
          3. NextSeq 500/550 Mid Output Flow Cell version 2.5. Store at 2 to 8°C.
          4. NextSeq 500/550 Buffer Cartridge version 2.0. Store at 15 to 30°C.
      13. The Archer® FusionPlex® Kit Components with part numbers, quantities, and storage conditions are listed in Table 5 below:

**Table 5.** Components of the Archer® FusionPlex® Kit

|  |  |  |  |
| --- | --- | --- | --- |
| **Description** | **Part Number** | **Quantity** | **Storage Temperature** |
| Random Priming 2.0 | SA0194 | 1 foil pouch (8 reaction tubes-strip) | Store at 2°C to 8°C |
| First Strand cDNA Synthesis | SA0002 |
| Second Strand cDNA Synthesis | SA0003 |
| End Repair | SA0204 |
| Ligation Step 1 | SA0196 |
| Ligation Step 2 | SA0197 |
| First PCR (Illumina®-P) | SA0109 |
| Second PCR Reactions 1 thru 8 (Illumina®-P) | SA0110 |
| Ligation Cleanup Beads | SA0210 | 1 tube (sufficient for processing 8 samples) |
| Ligation Cleanup Buffer | SA0209 |
| FusionPlex GSP1  (Lifespan Solid Tumor version 2.0) | dSA11432082 | 1 tube (sufficient for processing 8 samples) | Store at -30°C to -10°C |
| FusionPlex GSP2  (Lifespan Solid Tumor version 2.0) | dSA11432082 |
| 10X VCP Primer Mix | SA0126 | 1 tube (sufficient for processing 8 samples) |

* 1. CONTROLS:
     1. Sensitivity (Positive) Control:
        1. FFPE Tonsil RNA with known fusions **OR**
        2. MilliporeSigma *EWSR1*-*FLI1* Positive Control (Purified RNA Cell Line A673). Single use aliquots, store at -80°C.
           1. Each new lot of Cell Line A673 will be tested on a clinical run for validation of the new lot.
           2. Cell line A673 RNA is diluted in cell line SW480 at a ratio of 1:300 to serve as a sensitivity control.
           3. In addition, this sample is negative for all other fusions targeted by this assay and serves as a negative control.
     2. RSC Blank: this serves as a control of the extraction process. The blank is tested in the PreSeq assay. Any blank with a Ct <30 indicates potential contamination and should be investigated.
     3. No Template Controls:
        1. FUS\_NTC\_PreSeq: This NTC (consisting of water in place of patient sample) is tested in the PreSeq assay. Any NTC with a Ct <30 indicates potential contamination and should be investigated.
        2. FUS\_NTC\_KAPA: This NTC (consisting of water in place of patient sample) is tested in the KAPA assay. Any NTC with a Ct that is less than 3 cycles of the Ct of Standard 5 indicates potential contamination and should be investigated.
  2. MAJOR EQUIPMENT:
     1. Illumina NextSeq® 500
     2. QuantStudio™ 7 Flex Real-Time PCR System (with Real-Time PCR Software v1.7)
     3. Promega Maxwell RSC Extraction Platform
     4. Applied Biosystems (ThermoFisher) Veriti Thermal Cycler
     5. Standard Lifespan Desktop Computer
     6. Dell Server (lsmplinux2) with Red Hat Enterprise Linux 7
     7. Dell Server with VMware ESXi (v6.5.0, Build 13932383) and Archer Analysis (v6.2.2)
        1. Host: ESXi Server (vSphere) - lsvmcorrsc01.lifespan.org
        2. Virtual Machine
           1. DNS Name: lsarcher02.lsmaster.lifespan.org
           2. IP Addresses: 10.37.128.17
           3. See **Figure 3**.

**Figure 3.** Archer Analysis Server Architecture



1. **QUALITY CONTROL:**
   1. Quality control is monitored throughout the procedure. See relevant sections in procedure below.
2. **TEST PROCEDURE – FUSION PANEL:**
   1. **NOTE:** This Test Procedure section contains many complex, critical, detailed-oriented steps and conditions that must be followed accordingly. Failure to adhere to or any deviation from the stated procedure can result in damage to the product, decreased assay performance, and/or assay failure.
   2. The Test Procedure also highlights numerous SAFE STOPPING POINTS. This is an indication that the test can be safely stopped, stored (-30°C to -10°C) and resumed later without risk of compromised assay performance. Take note of these and plan the workflow accordingly.
   3. **NOTE:** Reaction steps that do not have a safe stopping point must be continued **immediately** when the specific thermal cycler program ends.
   4. The Test Procedure steps are performed by a molecular laboratory technologist, unless otherwise indicated.
   5. Preliminary processing:
      1. Total Nucleic Acid (TNA) extraction from the sample should be performed according to the relevant procedure for the specimen type. See separate procedures.
      2. If the specimen contains potential PCR inhibitors or interfering substances (such as melanin or hemoglobin), discuss with a Director/Pathologist, and perform the following steps:
         1. If appropriate, the sample may be tested as per usual protocol and then discussed with a Director/Pathologist for further processing.
         2. Alternatively, the specimen may be diluted with BSA or water after extraction.
         3. Refer to the PCR Interfering Substances BSA Method Procedure for more information.
   6. Best practices to observe during Test Procedure:
      1. Follow the procedure accordingly.
      2. When applicable, carefully detach the desired number of Reaction Tubes using a clean scissors or razor blade. Unused Reaction Tubes must be placed back in the foil pouch with the desiccant and stored in the FusionPlex Kit in the refrigerator. Label the pouch with the date opened and the number of Reaction Tubes remaining.
      3. Reaction Tubes with lyophilized contents must equilibrate to room temperature prior to use (allow Reaction Tubes to sit at room temperature for at least 5 minutes prior to use).
      4. Reaction Tubes that have products added become Reaction Mixtures and must be placed on ice as soon as possible unless stated otherwise.
      5. During transfers of Reaction Mixtures and discarding of supernatant, multiple attempts might be needed due to pipetting issues. Use extreme caution and good technique when dealing with pipetting issues to prevent the loss of reaction product.
      6. WORKING WITH LYOPHILIZED REACTION PELLETS: Never touch the lyosphere with the pipette tip. The lyosphere must be allowed to dissolve before mixing.
      7. WORKING WITH AMPure XP BEADS: Reaction cleanup with AMPure XP beads is performed at room temperature (20°C to 25°C) and is used repeatedly throughout the Fusion Panel workflow. To achieve optimal reaction cleanup, the beads must be equilibrated to room temperature and fully resuspended prior to each use. Never use a multi-channel pipette when handling the AMPure XP beads.
      8. It is acceptable to prepare single aliquots of 70% Ethanol, AMPure XP beads, and 10mM Tris-HCl, pH 8.0, with sufficient volumes to be used throughout library preparation. Label/date aliquots appropriately.
      9. Plan ahead for an efficient workflow: prepare reagents, workstations, and pre-start thermal cycler programs when applicable.
   7. Step 1 – Random Priming 2.0:
      1. Place the Sensitivity Control, the RSC Blank, and the Samples on ice.
      2. **Create your Random Priming worksheet: FUSBENCH - Test Worksheet Builder.**
         1. Open FUSBENCH- Test Worksheet Builder by using the tile on the dashboard.
         2. Select **Find**.
         3. If applicable, on the Found Activities tab, click **OK** or double click on any row.
         4. If applicable, select **New** in the Pending Worksheets window.
         5. Click the Barcode# field. Scan product label in the Barcode# field and select **Enter** on the keyboard to add the samples to the worksheet.
         6. Check for internal notes on each of the samples being run.
            1. Highlight the correct patient sample.
            2. Select **Tools**. Then, click **Internal Notes**.
         7. If a sample requires modification, note the modification in the Comment field of the FUSBENCH Test Worksheet Builder screen.
         8. Verify control lot number by clicking on the **Sample ID** field.
            1. If the control needs to be changed, click on the dropdown arrow and select the correct control lot number in the window that appears.
         9. Enter control concentrations for FUSSENS and RSC\_BLANK in the Qubit Concentration Field.
         10. Verify that the Test Code for the controls matches the sample test code.
             1. **Note: The control test code only needs to match the test from one sample if there are multiple different test types on the run.**
             2. **Note**: **The controls must only have one Test Code. Verify the controls only have one Test Code when the worksheet prints.**
         11. Verify the reagent lot numbers by clicking on the vertical **Settings** tab on the left side of the screen.
             1. If the reagent lot needs to be changed, click on the dropdown arrow in the **Stock#** field and select the correct lot in the window that appears.
         12. Mark the **Completed** checkbox and select **Save**. Worksheet Print Preview window will automatically open.
             1. **Note:** Q Numbers will not generate for the controls until the Tasklist.
         13. Select the **printer** icon, verify the correct printer is selected, and click **Print**.
         14. Close the preview window.
         15. Select **Back** in the FUSBENCH – Test Worksheet Builder screen.
         16. Exit Soft Molecular application.
      3. Pre-heat the thermal cycler by starting the Random Priming 2.0 program on a Veriti thermal cycler with the following parameters and the heated lid option on:

|  |  |  |
| --- | --- | --- |
| **Random Priming 2.0** | | |
| **Use a heated lid (≥100°C)** | | |
| **Step** | **Temp** | **Time** |
| 1 | 65°C | Hold |
| 2 | 65°C | 5 min |
| 3 | 4°C | Hold |

* + 1. Remove the Random Priming 2.0 foil pouch from the FusionPlex Kit in the refrigerator.
    2. Label the appropriate number of Reaction Tubes with the corresponding Reaction Tube #s.
    3. Centrifuge the Reaction Tubes briefly to pull the lyophilized contents down. Then, place in a rack.
    4. Perform the 25 µL sample dilution according to the Random Priming 2.0 Worksheet:
       1. Obtain and label the appropriate number of new 0.2 mL PCR tubes-strip with the corresponding Reaction Tube #s.
       2. Make the dilutions in the newly labeled 0.2 mL tubes-strip by combining the appropriate volumes of laboratory PCR-grade water and purified total nucleic acid (TNA) found on the Worksheet.
       3. **NOTE:** For samples or controls with RNA concentration ≤12.5 ng/µl, no dilution is required. Pipette 20 µl of the Sample directly into the corresponding Random Priming 2.0 Reaction Tube
    5. Briefly vortex and spin down the **diluted** samples.
    6. Transfer 20 µL of each **diluted** sample and control to the appropriate Random Priming 2.0 Reaction Tubes.
    7. Allow 7 seconds for the pellet to dissolve.
    8. Pipette up and down 8 times to mix after the lyosphere has dissolved.
    9. Briefly centrifuge and place the Random Priming 2.0 Reaction Mixtures on ice.
    10. Transfer the Reaction Mixtures to the pre-heated Veriti thermal cycler and resume the program by selecting **SKIP**.
    11. The program will run for approximately 6 minutes.
    12. After the program has reached 4°C, **place the Reaction Mixtures on ice for 2 minutes.**
    13. **Process your worksheet: Random Priming**
        1. Open Random Priming - Test Worksheet Processing by using the tile on the dashboard.
        2. Scan the barcode of the Random Priming Worksheet into the Worksheet# field or select **Find**.
        3. Use the dropdown under **Used Instrument:** to select the correct thermal cycler used.
        4. Complete the Fusion Random Priming action by marking the **Completed** checkbox and selecting **Save**.
        5. Click on the **Build Next Worksheet** button.
        6. Verify FUSCDNASYN **To Build**, Select all tests, and Transfer Controls are selected.
        7. Click **OK**.
        8. The system will ask the user if they want to open the worksheet. User clicks **No**.
        9. Select **Back** in the Random Priming – Test Worksheet Processing worksheet.
  1. Step 2 – First Strand cDNA Synthesis:
     1. Remove the First Strand cDNA Synthesis foil pouch from the FusionPlex Kit in the refrigerator.
     2. Label the appropriate number of Reaction Tubes with the corresponding Reaction Tube #s.
     3. Centrifuge the tubes briefly to pull the lyophilized contents down. Then, place in a rack.
     4. **After the two minutes on ice**, spin down the Random Priming 2.0 Reaction Mixtures and transfer the entire contents (20 µL) to the corresponding First Strand cDNA Synthesis Reaction Tubes.
     5. Allow 7 seconds for the pellet to dissolve.
     6. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     7. Briefly centrifuge and place the First Strand cDNA Synthesis Reaction Mixtures on ice. Discard the empty Random Priming 2.0 tubes-strip.
     8. Transfer the First Strand cDNA Reaction Mixtures onto a Veriti thermal cycler and initiate the First Strand cDNA Synthesis program with the following parameters and the heated lid option on:

|  |  |  |
| --- | --- | --- |
| **First Strand cDNA Synthesis** | | |
| **Use a heated lid (≥100°C)** | | |
| **Step** | **Temp** | **Time** |
| 1 | 25°C | 10 min |
| 2 | 42°C | 30 min |
| 3 | 80°C | 20 min |
| 4 | 4°C | Hold |

* + 1. The program will run for approximately 61 minutes.
    2. **Process your worksheet: cDNA Synthesis**
       1. Open cDNA Synthesis- Test Worksheet Processing by using the tile on the dashboard.
       2. Select **Find** to search for your worksheet and, if applicable, select correct worksheet.
       3. Use the dropdown under **Used Instrument:** to select the correct thermal cycler used.
       4. Complete 1st Strand cDNA synthesis action by marking the **Completed** checkbox and selecting **Save**.
       5. Complete 2nd Strand cDNA synthesis action by marking the **Completed** checkbox and selecting **Save**.
       6. Click on the **Build Next Worksheet** button.
       7. **Mark FUSPRESEQ** **To Build** and verify Select all tests and Transfer Controls are selected.

**NOTE: The system may default to marking FUSLIBPREP as the next worksheet to build. Please ensure FUSPRESEQ is checked.**

* + - 1. Click **OK**.
      2. The system will ask the user if they want to open the worksheet. Click **Yes** to open the PreSeq worksheet immediately**.**
    1. **Prepare PreSeq Worksheets**
       1. Open PreSeq- Test Worksheet Processing by using the tile on the dashboard, if not already open.
       2. If applicable, select **Find** to search for your worksheet and, if applicable, select the correct worksheet.
       3. Click on the **Print Plate View** button to open the Plate View Preview window.
       4. Select **OK** to save.
       5. Select the **printer** icon, verify the correct printer is selected, and click **Print**.
       6. Close the Print Preview window.
       7. Click on the **Settings** button. Verify the correct printer is populated in the dropdown field. Select **Print**.
       8. Close the Print Preview window.
       9. Select **Back** in the PreSeq – Test Worksheet Processing window.
    2. After the program has reached 4°C, briefly spin down the Reaction Mixtures and place in a rack.
    3. Prepare a 1:10 dilution of the First Strand cDNA Synthesis Reaction Mixtures for the PreSeq RNA QC Assay:
       1. Obtain and label the appropriate number of new 0.2 mL PCR tubes-strip.
       2. Pipette 9 µL of laboratory PCR-grade water into each new tube.
       3. Pipette 1 µL of each First Strand cDNA Synthesis Reaction Mixture into the corresponding tubes with water.
       4. Briefly vortex and centrifuge the 10 µL diluted cDNA samples and then, place on ice for use in the PreSeq Assay.
    4. **NOTE: The diluted cDNA samples are unstable and must be used in the PreSeq RNA QC Assay within 30 minutes.**
       1. Remove the iTaq SYBR Green Supermix and the 10X VCP Primer Mix from the freezer and allow to thaw on ice, while kept away from light.
  1. Step 3 – Second Strand cDNA Synthesis:
     1. Bring the thermal cycler to 16°C by starting the Second Strand cDNA Synthesis program on a Veriti.
     2. Remove the Second Strand cDNA Synthesis foil pouch from the FusionPlex Kit in the refrigerator.
     3. Label the appropriate number of Reaction Tubes with the corresponding Reaction Tube #s.
     4. Centrifuge the tubes briefly to pull the lyophilized contents down and then, place in a rack.
     5. Place the tubes containing the **First Strand cDNA Synthesis Reaction Mixture** in a rack and add 21 µL of laboratory PCR-grade water to each tube for a total reaction volume of 40 µL (19 µL of First Strand cDNA Synthesis Reaction Mixture + 21 µL of laboratory PCR-grade water).
     6. Pipette up and down several times to mix and then, briefly spin down.
     7. Transfer the entire 40 µL First Strand cDNA Synthesis Reaction Mixtures into the corresponding Second Strand cDNA Synthesis Reaction Tubes.
     8. Allow 7 seconds for the pellet to dissolve.
     9. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     10. Briefly centrifuge and place the Second Strand cDNA Reaction Mixtures on ice. Discard the empty First Strand cDNA Synthesis tubes-strip.
     11. Transfer the Reaction Mixtures to the Veriti thermal cycler and resume the program at 16°C by selecting **SKIP**.
     12. The Second Strand cDNA Synthesis program is as follows:

|  |  |  |
| --- | --- | --- |
| **Second Strand cDNA Synthesis** | | |
| **Use a heated lid (≥100°C)** | | |
| **Step** | **Temp** | **Time** |
| 1 | 16°C | Hold |
| 1 | 16°C | 60 min |
| 2 | 75°C | 20 min |
| 3 | 4°C | Hold |

* + 1. The program will run for approximately 1 hour and 21 minutes.
    2. During this program, proceed to set-up of Step 4 – PreSeq RNA QC Assay.
    3. After the Second Strand cDNA Synthesis program has reached 4°C, briefly spin down the Reaction Mixtures and place on ice **or** store at -30°C to -10°C.
    4. Place the Reaction Mixtures on ice if proceeding directly to Step 5 – End Repair.
    5. If stopping the workflow, observe the Safe Stopping Point.
    6. SAFE STOPPING POINT: After the Second Strand cDNA Synthesis program ends, briefly spin down and place the Reaction Mixtures in a cooler tube rack. Then, store in the freezer. Resume the workflow the next day.
  1. Step 4 – PreSeq RNA QC Assay:
     1. **NOTES:**
        1. Each sample is run in duplicate reactions.
        2. For quality control of the PreSeq Assay, a No Template Control (NTC) is run in duplicate reactions.
     2. Gently finger-flick to mix. Then, centrifuge the thawed iTaq SYBR Green Supermix and the 10X VCP Primer Mix.
     3. Prepare the appropriate amount of PreSeq RNA QC Assay master mix in a clean microcentrifuge tube according to the PreSeq Worksheet.
        1. **NOTE:** If needed, the PreSeq Assay master mix is prepared as follows:

|  |  |
| --- | --- |
| **Reagent** | **Vol/Rxn** |
| iTaq SYBR Green Supermix | 5µl |
| 10x VCP Primer mix | 1µl |
| Total | 6µl |

* + 1. Briefly vortex and centrifuge the qPCR master mix.
    2. Pipette 6 µl of the master mix into each assigned well of a new 96-well optical PCR plate (USA Scientific 1402-9300).
    3. For the NTC, add 4 µl of laboratory PCR-grade water into wells A1 and B1 of the 96-well plate.
    4. Add 4 µl of the 1:10 diluted cDNA samples into the corresponding assigned wells with master mix according to the PreSeq Plate View.
    5. Mix by slowly pipetting and avoid introducing bubbles.
    6. Seal the plate with an optical seal (USA Scientific 2921-7800).
    7. Centrifuge for 1 minute at 1500 x g.
    8. Centrifuge again if there is a bubble or as needed.
    9. Perform the PreSeq Assay qPCR using the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System (QuantStudio™).
       1. Ensure that the QuantStudio™ is turned on.
       2. Load the reaction plate onto the QuantStudio. Use Real-Time PCR Software v1.3 to setup the QuantStudio™ run:
          1. Click on the QuantStudio™ Real-Time PCR Software icon located on the desktop of the connected computer.
          2. Under the Set-Up panel, select Template.
          3. In the dialog box, open the RIH Templates folder.
          4. Select the “PreSeq\_RNA\_QC\_Assay.edt” file.
          5. In the **Experiment Name** field, scan the PreSeq Plate View. Then, change any dash or period to an underscore.
          6. **Experiment Properties** parameters should be as follows:

Instrument Type: QuantStudio™ 7 Flex System

Block Type: 96-well (0.2mL)

Experiment Type: Melt Curve

Detection Reagent: SYBR® Green Reagents

Instrument Properties: Standard

* + - * 1. Under the **Setup** panel on the far left of the screen, select **Define**. The **Define** parameters should be as follows:

Target Name: Target 1

Reporter: SYBR

Quencher: None

Passive Reference: ROX

* + - * 1. Create a sample list.
        2. Under the **Samples** section located on the right side of the screen, select **New**.
        3. Click **New** once for each sample or control on the run (e.g., click **New** 7 times for a run of 6 patient samples and 1 control).
        4. Enter each Sample Name (Soft Molecular Order#) by clicking on a “Sample #” text field and entering the Sample Name.
        5. Repeat for all samples and controls on the run.
        6. Under the **Setup** panel on the far left of the screen, select **Assign**.
        7. In the Plate Layout tab on the right side of the screen, the NTC will be pre-filled with “N” (Negative).
        8. Complete the Plate Layout by assigning a Sample Name to the corresponding wells:

Click and drag to highlight the desired wells.

Assign the appropriate Sample Name by clicking the Soft Molecular # from the **Sample** section on the left side of the screen.

The highlighted wells will populate with the selected Sample Name.

Repeat for all samples and controls until the Plate Layout is complete.

* + - * 1. Highlight all assigned wells on the Plate Layout and select Target 1 from the **Targets** panel located on the left side of the screen.

Assign “Unknown” to all samples and the positive control by selecting “U” under the **Task** drop-down window.

Target 1 is used to determine the RNA QC score of the sample, which is equivalent to the Ct. value.

* + - * 1. Under the **Setup** panel on the far left of the screen, select **Run Method**. The **Run Method** parameters should be as follows:

Reaction Volume per well = 10 µl.

Cycling program:

|  |  |  |  |
| --- | --- | --- | --- |
| **PreSeq RNA QC Assay** | | | |
| **Step** | **Temp** | **Time** | **Cycles** |
| Activation | 95°C | 20 seconds | 1 |
| Denaturation | 95°C | 15 seconds | 35 |
| Primer Annealing & Extension | 60°C | 60 seconds |
| Melt-curve gradient | 60-95°C | 0.5°C/sec | 1 |

* + - * 1. On the far-left side of the screen, select **Run**. The Run panel should open once selected.
        2. Select the green “Start Run” button on top left corner of the screen.

Select the QuantStudio™ instrument.

Save the run using the correct format by scanning the PreSeq Plate View. (This will save the run file to the default on-board computer).

* + - * 1. The assay will run for approximately 1 hour and 20 minutes. Once the run is complete, the plate can be discarded.
        2. It is recommended to review the PreSeq Assay results before proceeding to Step 5 – End Repair.
    1. **Process your worksheet: Fusion PreSeq**
       1. Open PreSeq – Test Worksheet Processing by using the tile on the dashboard.
       2. Scan the barcode of the PreSeq Plate View into the Worksheet# field and select **Find**.
       3. Use the dropdown under **Used Instrument:** to select the QuantStudio used.
       4. Complete PreSeq Load QuantStudio action by marking the **Completed** checkbox and selecting **Save**.
       5. Select **Back** in the PreSeq – Test Worksheet Processing window.
       6. Exit Soft Molecular application.
    2. While PreSeq assay is running, find next MBC Adapter codes (Index 2 P5) and Index 1 P7 codes that will be used and fill out Fusion Index Code log appropriately.
    3. Once the run is complete, on a Lifespan computer, navigate to the RICMBLAB$ shared drive.
       1. Go to the CMB\_Tests folder and navigate to the appropriate month folder.
       2. Create a subfolder for the new Fusion run.
       3. Name the folder by scanning the FUSBENCH worksheet.
          1. Example: 06.27.22-fusbench-1
    4. Export the \*.eds file from the QuantStudio.
       1. Insert a Lab-approved Flash Drive into the QuantStudio computer.
       2. On the far-left side of the screen, select **Export**.
       3. Select the blue **Browse** button located at the top of the screen.
       4. Select **Computer**.
       5. Select the appropriate **Flash Drive**.
       6. Select **Open**.
       7. Export the **Results** tab only as an \*.xls file.
       8. Select **Start Export** button located at the bottom of the screen.
       9. Safely eject the flash drive and remove from the computer.
       10. Insert the flash drive and open this exported file on any Lifespan computer.
           1. In the Fusion run folder located on the RICMBLAB$ network drive, create a new subfolder entitled “**QuantStudio Files**.”
           2. Move the exported \*.xls file from the flash drive to this folder.
    5. On the RICMBLAB$ network drive, go to the \\PCR\_Worksheets\_Current\Fusion\_Solid\_Tumor subfolder and open the FUS\_PreSeq\_ImportDocument\_v3 file.
    6. In the FUS\_PreSeq\_Clean Import Document, select **File** followed by **Save As**.
    7. Select **Browse** to locate the Fusion run folder.
    8. Name the file in the File Name field by scanning the PreSeq Plate View.
    9. Open the Clean PreSeq Import Document and the Quant Studio Raw Data file.
    10. On the QuantStudio Raw Data file, highlight the header row containing column names (sample name, etc.).
        1. From the Home screen in Excel, select “Sort and Filter”, then “Custom Sort”.
        2. A box will pop up. Click “Sort”.
        3. Sort by **Task**.
        4. Add a level.
        5. Then, sort by **Sample Name**.
        6. Click “OK”.
        7. Highlight the data under the header row, exclude the column titles, and copy the data.
    11. Paste the data from the QuantStudio Raw file starting in the A2 well of the PreSeq Import Document. Select **Save** and close both Excel files.
        1. **NOTE:** The acceptable range for RNA quality score is Ct ≤30. For more information, refer to the Specimen Acceptability criteria mentioned above.
    12. **Process your worksheet: PreSeq Import**
        1. Open FUS PreSeq – Test Worksheet Processing by using the tile on the dashboard.
        2. Scan the barcode of the PreSeq Plate View into the Worksheet# field and select **Find**.Select the ‘**…**’ button to the right of the Import button to import the PreSeq Clean Document that was created.
        3. Change the radial button from **XML** to **Excel**.
        4. Select the ‘**…**’ button next to the File Name field to select the edited PreSeq import document.
        5. Click on the **Import** button.
        6. Review the data to ensure all data imported appropriately.
           1. If any results yield a “Consult Director” comment in the PS Sample QC column, see a Pathologist or Director.
        7. Verify Worksheet is populated in the Image Type field.
        8. Select **Images** button.
        9. On the window that opens, select the **Add File** tab. Then, select the **add file (folder)** icon.
        10. Find and select the PreSeq import file to be added from the Windows Explorer window. Select **Open**.
        11. Choose **Instrument Documents** from the Template dropdown.
        12. Select the **green check** icon to add the files.
        13. Close the window.
        14. Complete PreSeq Import action by marking the **Completed** checkbox and selecting **Save**.
        15. Click on the **Build Next Worksheet** button.
        16. Select **OK** to Save changes.
        17. **Mark FUSLIBPREP** **To Build** and verify Select all tests and Transfer Controls are selected.

**NOTE: The system may default to marking FUSKAPA as the next worksheet to build. Please ensure FUSLIBPREP is checked.**

* + - 1. The system will ask the user if they want to open the new worksheet. Click **No**.
      2. Select **Back** in PreSeq – Test Worksheet Processing window.
      3. Exit Soft Molecular application.
  1. Step 5 – End Repair:
     1. Remove the AMPure XP beads from the refrigerator.
     2. Completely resuspend the AMPure beads by vortexing vigorously for 10 seconds.
     3. Make an aliquot of AMPure beads needed for End Repair Cleanup and set it aside at room temperature until cleanup (aliquot volume can be found on the FUSLIBPREP Settings).
        1. **NOTE:** If the Second Strand cDNA Synthesis Reaction Mixtures were stored, remove from the freezer and thaw at room temperature.
     4. **Print your worksheets:** **FUSLIBPREP End Repair through cDNA Synthesis Worksheet Builder and Worksheet Processing**
        1. Open FST LIBPREP – Test Worksheet Builder by using the tile on the dashboard.
        2. Select **Find**.
        3. Open the vertical **Plate View tab**.
        4. Delete the replicates of controls by highlighting the well(s) and clicking **Delete**.
        5. Delete controls that will be dropped at the end of the plate (e.g., H7 and H8).
        6. If necessary, rearrange samples to go in positions A1-A8, B1-B8, etc. **Do not put samples in any well position 9-12 (e.g., A9-A12 through H9-H12).**
        7. Open the vertical **Worksheet View tab**.
        8. Select both MBC Adapter (Index 2 P5) and Index 1 P7 codes for patient samples and controls based on what was recorded in the Fusion Index Code Log using the dropdown menu in each appropriate I7 and I5 column.
           1. The Fusion Index Code Log can be found on the RICMBLAB$ network drive, in the \\PCR\_Worksheets\_Current\Fusion\_Solid\_Tumor subfolder.
        9. Select Print Worksheet. Select **OK** to Save.
        10. Select the **printer** icon, verify the correct printer is selected, and click **Print**.
        11. Close the Print Preview window.
        12. Click on the **Settings** button. Verify the correct printer is populated in the dropdown field. Select **Print**.
        13. Close the Print Preview window.
        14. Select **Back** in the FST LIBPREP – Test Worksheet Processing window.
        15. Exit Soft Molecular application.
     5. Remove the End Repair foil pouch from the FusionPlex Kit in the refrigerator.
     6. Label the appropriate number of End Repair Reaction Tubes with the corresponding Reaction Tube #s.
        1. RSC\_BLANK will not proceed in the run. **DO NOT transfer RSC\_BLANK** Second Strand cDNA strip tube product to End Repair strip tube.
        2. Physically cut out the RSC\_BLANK to avoid transferring the Second Strand cDNA product to the End Repair tube.
     7. Centrifuge the tubes briefly to pull the lyophilized contents down. Then, place on a rack.
     8. Briefly spin down the Second Strand cDNA Synthesis Reaction Mixtures.
     9. Pipette the entire content (40 µL) into the corresponding End Repair Reaction Tubes.
     10. Allow at least 7 seconds for the pellet to dissolve.
     11. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     12. Briefly centrifuge and place on ice.
     13. Transfer the Reaction Mixtures onto a Veriti thermal cycler and start the End Repair program with the following parameters and the heated lid option **OFF**:

|  |  |  |
| --- | --- | --- |
| **End Repair** | | |
| **Heated Lid Off** | | |
| **Step** | **Temp** | **Time** |
| 1 | 25°C | 30 min |
| 2 | 4°C | Hold |

* + 1. The program will run for approximately 31 minutes.
    2. During this program, make an aliquot of 70% ethanol needed for End Repair Cleanup.
    3. Also, make a fresh 10mM Tris-HCl, pH 8.0 elution buffer to be used throughout the set-up in the PCR room.
    4. Make the 10mM Tris-HCl, pH 8.0 by mixing 15 µL of 1M Tris-HCl, pH 8.0 with 1485 µL of laboratory PCR-grade water.
    5. Obtain an aliquot of 1M NaOH to be used during the set-up in the PCR room.
       1. Optional: To prepare for Ligation 2, combine 5 µL of 1M NaOH with 995 µL of laboratory PCR-grade water to create 5mM NaOH solution.
    6. When the End Repair program has completed, briefly spin down the Reaction Mixtures and place in a rack. Then, proceed to cleanup.
    7. **Process your worksheet: FST LIBPREP, End Repair**
       1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
       2. Press **Find** and if, applicable, double click the correct worksheet.
       3. Use the dropdown menu under **Used Instrument:** to select the instrument used.
       4. Complete End Repair action by marking the **Completed** checkbox and selecting **Save**.
       5. Select **Back** in the FST LIBPREP – Test Worksheet Processing.
       6. Exit Soft Molecular application.
    8. Reaction Cleanup after End Repair:
       1. Vortex the aliquot of AMPure beads for 10 seconds.
       2. Add 100 µL of beads to each End Repair Reaction Mixture.
       3. Vortex for 8 seconds to mix and visually inspect the color of the Reaction Mixture to ensure a homogenous mixture.
       4. Incubate the Reaction Mixtures for 5 minutes at room temperature.
       5. After incubation, briefly spin down the Reaction Mixtures and then place them on the magnet for 5 minutes to fully pellet the beads against the tube walls.
       6. Without disturbing the bead pellet, use a pipette set to 150 µL to remove and discard the supernatant.
          1. **NOTE:** If the pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return the contents back to the tube and repeat the 5-minute magnet incubation.
       7. With the supernatant removed and the tubes still on the magnet, add 200 µL of 70% ethanol to each pellet.
       8. Incubate for 30 seconds to allow the bead to fully pellet against the side of the tubes.
       9. Without disturbing the bead pellet, use a pipette set to 200 µL to remove and discard the supernatant.
       10. Repeat (steps g.– i.) for a total of two washes in 70% ethanol.
       11. After the final wash, use a pipette (≤20 µL capacity) to completely remove visible supernatant residue.
       12. Allow the bead pellets to dry for 8 minutes at room temperature on the magnet with the tube lids open. If needed, allow more time for the beads to properly dry.
           1. **NOTE:** Take care not to over-dry the beads as this will significantly decrease overall recovery (yield) of nucleic acid.
       13. Observe the appearance of the beads: a “glossy” appearance indicates that the bead is not properly dry. A “cracked” appearance indicates over-drying.
       14. Elute the DNA by resuspending the beads in 20 µL of 10mM Tris-HCl pH 8.0 solution.
       15. Vortex briefly and spin down.
       16. Place the tubes on the magnet for 2 minutes.
  1. Step 6 – Ligation 1:
     1. Remove the Ligation 1 foil pouch from the FusionPlex Kit in the refrigerator.
     2. Label the appropriate number of Ligation 1 Reaction Tubes with the corresponding Reaction Tube #s.
     3. Centrifuge the tubes briefly to pull the lyophilized contents down. Then, place on a rack.
     4. After 2 minutes on the magnet, transfer 20 µL of the purified End Repair Reaction Mixtures into the corresponding Ligation 1 Reaction Tubes.
     5. Allow at least 7 seconds for the pellet to dissolve.
     6. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     7. Briefly centrifuge and place the Ligation 1 Reaction Mixtures on ice.
     8. Place the Reaction Mixtures on a Veriti thermal cycler and start the Ligation 1 program with the following parameters and the heated lid option **on**:

|  |  |  |
| --- | --- | --- |
| **Ligation 1** | | |
| **Use a heated lid (≥100°C)** | | |
| **Step** | **Temp** | **Time** |
| 1 | 37°C | 15 min |
| 2 | 4°C | Hold |

* + 1. The program will run for approximately 16 minutes.
    2. After the program has reached 4°C, briefly spin down the Reaction Mixtures and place on ice.
    3. Proceed to reaction cleanup.
    4. Reaction Cleanup after Ligation 1:
       1. Completely resuspend the AMPure beads by vortexing vigorously for at least 10 seconds.
       2. Make a sufficient aliquot of beads for Ligation 1 cleanup.
       3. Make an aliquot of 70% ethanol needed for Ligation 1 cleanup.
       4. Add 50 µL of beads to each Ligation 1 Reaction Mixture.
       5. Vortex for 8 seconds to mix and visually inspect the color of the Reaction Mixture to ensure a homogenous mixture.
       6. Incubate the Reaction Mixtures for 5 minutes at room temperature.
       7. After incubation, briefly spin down and place the Reaction Mixtures on the magnet for 5 minutes to fully pellet the beads against the tube walls.
       8. Without disturbing the bead pellet, use a pipette set to 80 µL to remove and discard the supernatant.
          1. **NOTE:** If the pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return the contents back to the tube and repeat the 5-minute magnet incubation.
       9. With the supernatant removed and the tubes still on the magnet, add 200 µL of 70% ethanol to each pellet.
       10. Incubate for 30 seconds to allow the bead to fully pellet against the side of the tubes.
       11. Without disturbing the bead pellet, use a pipette set to 200 µL to remove and discard the supernatant.
       12. Repeat (steps i. – k.) for a total of two washes in 70% ethanol.
       13. After the final wash, use a pipette (≤20 µL capacity) to completely remove visible supernatant residue.
       14. Allow the bead pellets to dry for 8 minutes at room temperature on the magnet with the tube lids open. If needed, allow more time for the beads to properly dry.
           1. **NOTE:** Take care not to over-dry the beads as this will significantly decrease overall recovery (yield) of nucleic acid.
       15. Observe the appearance of the beads: a “glossy” appearance indicates that the bead is not properly dry. A “cracked” appearance indicates over-drying.
       16. Elute the DNA by resuspending the beads in 42 µL of 10mM Tris-HCl pH 8.0 solution.
       17. Vortex briefly and spin down.
       18. Place the tubes on the magnet for 2 minutes.
  1. Step 7 – MBC Adapter Incorporation:
     1. Remove the MBC Adapter foil pouch from the refrigerator.
        1. **NOTE:** Open only one MBC Adapter foil pouch at a time. When an MBC Adapter foil pouch is opened for the first time, immediately label **ALL** 8 Reaction Tubes (left to right) with the corresponding sample index tag letter (A, B, or C) and number (1-48) from the MBC Adapters pouch label.
        2. **NOTE:** Unused MBC Adapter Reaction Tubes must be labeled before returning to the pouch for storage in the refrigerator.
     2. This step incorporates your index tag for sample-level tracking. Therefore, be sure to record which MBC Adapter is being used for each sample. This is done in the Fusion Index Code Log.
     3. Orient the lid hinges to the back as illustrated:



* + 1. Use a permanent marker and label the side of the MBC Adapter Reaction Tubes with the corresponding P5 Adapter Index tag (e.g., A8, A9, A10, A11, A12, A13, A14, A15, A16).
    2. Now, label the appropriate number of MBC Adapter Reaction Tubes with the corresponding Reaction Tube #s. Ensure the label matches the FST LIBPREP Worksheet.
    3. Centrifuge the tubes briefly to pull the lyophilized contents down and then, place on a rack.
    4. After the 2 minutes on the magnet, transfer 40 µL of the purified Ligation 1 Reaction Mixtures into the corresponding MBC Adapter Reaction Tubes.
    5. Allow at least 7 seconds for the pellet to dissolve.
    6. Pipette up and down 8 times to mix after the lyosphere has dissolved.
    7. Spin down the tubes and place on ice.
    8. **Immediately proceed to Step 8 – Ligation 2**.
  1. Step 8 – Ligation 2:
     1. Remove the Ligation 2 foil pouch from the FusionPlex Kit in the refrigerator.
     2. Label the appropriate number of Ligation 2 Reaction Tubes with the corresponding Reaction Tube #s.
     3. Centrifuge the tubes briefly to pull the lyophilized contents down. Then, place on ice.
     4. Transfer the entire volume (40 µL) of the MBC Adapter Reaction Mixtures to the corresponding Ligation 2 Reaction Tubes.
     5. Allow at least 7 seconds for the pellet to dissolve.
     6. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     7. Briefly centrifuge and return the Ligation 2 Reaction Tubes to ice.
     8. Place the Reaction Mixtures onto a Veriti thermal cycler and start the Ligation 2 program with the following parameters and the heated lid option **OFF**:

|  |  |  |
| --- | --- | --- |
| **Ligation 2** | | |
| **Heated Lid Off** | | |
| **Step** | **Temp** | **Time** |
| 1 | 22°C | 5 min |
| 2 | 4°C | Hold |

* + 1. The program will run for approximately 6 minutes.
    2. After the program has reached 4°C, briefly spin down the Reactions Mixtures and place on ice **or** store at -30°C to -10°C.
    3. Place the Reaction Mixtures on a rack if proceeding directly to Reaction Cleanup after Ligation 2.
    4. If stopping the workflow, observe the Safe Stopping Point.
    5. SAFE STOPPING POINT: After the Ligation 2 program ends, briefly spin down and place the Reaction Mixtures in a cooler tube rack. Then, store in the freezer. Resume the workflow the next day. Complete appropriate Soft Molecular steps.
       1. **Process your worksheet: FST LIBPREP**
          1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
          2. Press **Find** and double click on the correct worksheet, if applicable.
          3. **Complete** and **Save** Ligation 1 and MBC Adapters action.
          4. **Complete** and **Save** Ligation 2 action.
          5. Select **Back** in the FST LIBPREP – Test Worksheet Processing.
          6. Exit Soft Molecular application.
    6. Reaction Cleanup after Ligation 2:
       1. Remove the **Ligation Cleanup Beads** and the **Ligation Cleanup Buffer** from the FusionPlex Kit in the refrigerator.
       2. Allow the beads and the buffer to equilibrate to room temperature.
       3. Completely resuspend the **Ligation Cleanup Beads** by vigorously vortexing for 10 seconds.
       4. Obtain and label the appropriate number of a new 0.2mL PCR tubes-strip with the Reaction Tube #s.
       5. For each sample, pipette 50 µL of **Ligation Cleanup Beads** into the new tubes-strip.
       6. Briefly spin down and place the tubes on the magnet for 1 minute.
       7. Without disturbing the bead pellet, use a pipette set to 60 µL to remove and discard the supernatant.
          1. **NOTE:** If the pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return contents to the tube and repeat the 1-minute magnet incubation.
       8. With the supernatant removed, place the tubes in a rack and resuspend the beads by pipetting 50 µL of **Ligation Cleanup Buffer** into each tube.
       9. Briefly vortex and quick spin the ready-to-use beads.
       10. If the Ligation 2 Reaction Mixtures were stored, remove from the freezer and thaw at room temperature.
       11. Briefly spin down the Ligation 2 Reaction Mixtures.
       12. Pipette the entire content (40 µL) into the corresponding tubes with the ready to-use **Ligation Cleanup Beads**.
       13. Place fingers firmly on all lids and mix the samples by vigorously vortexing for 5 seconds.
       14. Inspect the color of the Reaction Mixture to ensure a homogenous mixture.
       15. Incubate at room temperature for 5 minutes.
       16. After 5 minutes, mix the samples again by vigorously vortexing for 5 seconds.
       17. Incubate at room temperature for 5 minutes.
       18. After 5 minutes, briefly spin down the Reaction Mixtures and place on the magnet for 2 minutes.
       19. Without disturbing the pellet, use a pipette set to 100 µL and carefully remove the supernatant.
           1. **NOTE:** If the bead pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return the contents back to the tube and repeat the 2-minute magnet incubation.
       20. With the supernatant removed, wash the beads **two times** by resuspending in **Ligation Cleanup Buffer**.
       21. Add 200 µL of **Ligation Cleanup Buffer** to each bead pellet.
       22. Vortex for 5 seconds, briefly spin down, and place on the magnet for 1 minute.
       23. Once the mixture has cleared, use a pipette set to 200 µL and remove the supernatant.
       24. Repeat steps w.-y. for a second wash.
       25. After the second wash with **Ligation Cleanup Buffer**, wash the beads **once** with laboratory PCR-grade water.
       26. Add 200 µL of **laboratory PCR-grade water** to each bead pellet.
       27. Vortex for 5 seconds, briefly spin down, and place on the magnet for 1 minute.
       28. Make a fresh 5mM NaOH solution by mixing 5 µL of 1M NaOH with 995 µL of laboratory PCR-grade water, if not prepared previously.
       29. After 1 minute on the magnet, carefully remove and discard the supernatant.
       30. Elute the DNA from the ligation cleanup beads by adding 18 µL of 5mM NaOH to each pellet.
       31. Vortex briefly and spin down. Ensure that the pellet is completely resuspended. Vortex again if needed.
       32. Transfer the beads to a Veriti thermal cycler and start the Bead Elution program with the following parameters and the heated lid option **on**:

|  |  |  |
| --- | --- | --- |
| **Bead Elution** | | |
| **Use a heated lid (≥100°C)** | | |
| **Step** | **Temp** | **Time** |
| 1 | 75°C | 10 min |
| 2 | 4°C | Hold |

* + - 1. The program will run for approximately 11 minutes.
      2. During this time, proceed to Step 9 – First PCR and prepare First PCR Reaction Tubes and GSP1.
      3. After the program has reached 4°C, briefly spin down, and transfer the tubes to the magnet. Incubate on the magnet for 2 minutes.

* 1. Step 9 – First PCR:
     1. Remove the GSP1 primer from the freezer, thaw at room temperature and place on ice.
     2. Each GSP1 tube contains approximately 18 µL of primer, which is enough to process 8 samples.
        1. When a new tube is opened, label with the date and if applicable, record the number of samples that can be processed with the remaining GSP1.
     3. Remove the First PCR foil pouch from the FusionPlex Kit in the refrigerator.
     4. Label the appropriate number of First PCR Reaction Tubes with the corresponding Reaction Tube #s.
     5. Centrifuge the tubes briefly to pull the lyophilized contents down and then, place in a rack.
     6. Pipette **2 µL** of the GSP1 into each First PCR Reaction Tube.
        1. **NOTE:** The primer may be pipetted against the wall of the Reaction Tube to visually confirm that primer was added.
     7. Briefly spin down the tubes.
     8. Without disturbing the beads, pipette 18 µL of the purified Ligation 2 products into the corresponding First PCR Reaction Tubes.
     9. Allow at least 7 seconds for the pellet to dissolve.
     10. Pipette up and down 8 times to mix after the lyosphere has dissolved.
     11. Briefly centrifuge and return to ice.
     12. Immediately transfer the Reaction Mixtures onto a Veriti thermal cycler and start the First PCR program with the following parameters and the heated lid option **on**:

|  |  |  |  |
| --- | --- | --- | --- |
| **First PCR Reaction**  **Use a heated lid (≥100°C)** | | | |
| **Step** | **Temp** | **Time** | **Cycles** |
| 1 | 95°C | 3 minutes | 1 |
| 2 | 95°C | 30 seconds | 15 |
| 3 | 65°C | 5 minutes (100% ramp rate) |
| 4 | 72°C | 3 minutes | 1 |
| 5 | 4°C | Hold | 1 |

* + 1. The program will run for approximately 1 hour and 37 minutes.
    2. After the program has ended, proceed to Reaction Cleanup after First PCR, **or** safely stop the workflow.
       1. If proceeding directly to Reaction Cleanup after First PCR, briefly spin down the Reaction Mixtures and place in a rack.
       2. If stopping the workflow, observe the Safe Stopping Point.
    3. SAFE STOPPING POINT: After the First PCR program is completed, briefly spin down and place the Reaction Mixtures in a cooler tube rack. Then, store in the freezer. It is also acceptable to leave the Reaction Mixtures on the thermal cycler at 4°C overnight.
    4. **Process your worksheet: FST LIBPREP**
       1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
       2. Press **Find** and double click on the correct worksheet, if applicable.
       3. **Complete** and **Save** Ligation 1 and MBC Adapters action, if applicable.
       4. **Complete** and **Save** Ligation 2 action, if applicable.
       5. **Complete** and **Save** First PCRaction.
       6. Select **Back** in the FST LIBPREP – Test Worksheet Processing.
       7. Exit Soft Molecular application.
    5. Reaction Cleanup after First PCR:
       1. Completely resuspend the AMPure XP beads by vortexing vigorously for 10 seconds.
       2. Make an aliquot of beads needed for First PCR Cleanup.
       3. If the First PCR Reaction Mixtures were stored, remove from the freezer and thaw at room temperature.
       4. Add 24 µL of AMPure beads to each First PCR Reaction Mixture.
       5. Vortex for 8 seconds to mix and visually inspect the color to ensure a homogenous mixture.
       6. Incubate the Reaction Mixtures for 5 minutes at room temperature.
       7. After incubation, briefly spin down and place the Reaction Mixtures on the magnet for 5 minutes to fully pellet.
       8. Without disturbing the bead pellet, use a pipette set to 50 µL to remove and discard the supernatant.
          1. **NOTE:** If the pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return the contents back to the tube and repeat the 5 minutes magnet incubation.
       9. With the supernatant removed and the tubes still on the magnet, add 200 µL of 70% ethanol to each pellet.
       10. Incubate for 30 seconds to allow the beads to fully pellet against the side of the tubes.
       11. Without disturbing the bead pellet, use a pipette set to 200 µL to remove and discard the supernatant.
       12. Repeat (steps i. – k.) for a total of two washes in 70% ethanol.
       13. After the final wash, use a pipette (≤20 µL capacity) to completely remove visible supernatant residue.
       14. Allow the bead pellets to dry for 6 minutes at room temperature on the magnet with the lids open. If needed, allow more time for the beads to properly dry.
           1. **NOTE:** Take care not to over-dry the beads as this will significantly decrease overall recovery (yield) of nucleic acid.
       15. Observe the appearance of the beads: a “glossy” appearance indicates the bead is not properly dry. A “cracked” appearance indicates over-drying.
       16. Elute the DNA by resuspending the beads in 24 µL of 10mM Tris-HCl pH 8.0.
       17. Vortex briefly and spin down.
       18. Place the tubes on the magnet for 2 minutes.
       19. After 2 minutes, store the purified products **or** proceed directly to Step 10-Second PCR setup.
       20. Store the purified products by transferring 22 µL to a new, labeled 0.2mL PCR tube-strip.
           1. **NOTE:** Avoid transferring beads with the purified products.
       21. At this time, the workflow can be stopped by observing the Safe Stopping Point.
       22. SAFE STOPPING POINT: Place the purified First PCR products in a cooler tube rack and store in the freezer. Resume the workflow the next day. Complete appropriate Soft Molecular steps.
       23. **Process your worksheet: FST LIBPREP**
           1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
           2. Press **Find** and double click on the correct worksheet, if applicable.
           3. **Complete** and **Save** Clean up after First PCR action.
           4. Select Back in the FST LIBPREP – Test Worksheet Processing.
           5. Exit Soft Molecular application.
       24. If proceeding directly to Second PCR, keep the First PCR Reaction Mixtures on the magnet while the Second PCR Reaction Tubes are prepared (see Step 10 – Second PCR below).
  1. Step 10 – Second PCR:
     1. Remove the GSP2 primer from the freezer, thaw at room temperature and place on ice.
     2. Each new GSP2 tube contains approximately 18 µL of primer, which is enough to process 8 samples.
        1. When applicable, record the number of samples that can be processed with the remaining GSP2.
     3. Remove the Second PCR foil pouch from the FusionPlex Kit in the refrigerator.
     4. The P7 Index tag is incorporated during this step. Therefore, be sure to record which Index tag is being used for each sample in the Fusion Index Code Log prior to starting.
     5. **NOTE:** When a Second PCR foil pouch is opened for the first time, immediately label **ALL** 8 Reaction Tubes with the corresponding Index tag.
        1. Unused Second PCR Reaction Tubes must be labeled before returning to the pouch for storage in the refrigerator.
     6. Use a permanent marker and label the sides of the Second PCR Reaction Tubes as shown below (1 to 8; from left to right). **Confirm** these numbers match the FST LIBPREP Worksheet.



* + 1. Now, label the appropriate number of Second PCR Reaction Tubes with the corresponding Reaction Tube #s.
    2. Centrifuge the tubes briefly to pull the lyophilized contents down. Then, place in a rack.
    3. Pipette **2 µL** of the GSP2 into each Second PCR Reaction Tube.
       1. **NOTE:** The primer may be pipetted against the wall of the Reaction Tube to visually confirm that primer was added.
    4. Spin down the tubes briefly.
    5. Pipette 18 µL of the purified First PCR products into the corresponding Second PCR Reaction Tubes.
    6. Allow 7 seconds for the pellet to dissolve.
    7. Pipette up and down 8 times to mix after the lyosphere has dissolved.
    8. Briefly centrifuge and return to ice.
    9. Immediately transfer the Reaction Mixtures onto a Veriti thermal cycler and start the Second PCR program with the following parameters and the heated lid option on:

|  |  |  |  |
| --- | --- | --- | --- |
| **Second PCR Reaction**  **Use a heated lid (≥100°C)** | | | |
| **Step** | **Temp** | **Time** | **Cycles** |
| 1 | 95°C | 3 minutes | 1 |
| 2 | 95°C | 30 seconds | 20 |
| 3 | 65°C | 5 minutes (100% ramp rate) |
| 4 | 72°C | 3 minutes | 1 |
| 5 | 4°C | Hold | 1 |

* + 1. The program will run for approximately 2 hours and 7 minutes.
    2. **Process your worksheet: FST LIBPREP**
       1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
       2. Press **Find** and double click on the correct worksheet, if applicable.
       3. **Complete** Second PCR action and **Save**.
       4. Select **Back** in the FST LIBPREP – Test Worksheet Processing.
       5. Exit Soft Molecular application.
    3. After the program has ended, proceed to Reaction Cleanup after Second PCR **or** safely stop the workflow.
       1. If proceeding directly to Reaction Cleanup after Second PCR, briefly spin down the Reaction Mixtures and place in a rack.
       2. If stopping the workflow, observe the Safe Stopping Point.
    4. SAFE STOPPING POINT: After the Second PCR program is completed, briefly spin down and place the Reaction Mixtures in a cooler tube rack. Then, store in the freezer. It is also acceptable to leave the Reaction Mixtures on the thermal cycler at 4°C overnight.
    5. Reaction Cleanup after Second PCR:
       1. Completely resuspend the AMPure XP beads by vortexing vigorously for 10 seconds.
       2. Make an aliquot of beads needed for Second PCR Cleanup.
       3. If the Second PCR Reaction Mixtures were stored, remove from the freezer and thaw at room temperature.
       4. Add 24 µL of AMPure beads to each Second PCR Reaction Mixture.
       5. Vortex for 8 seconds to mix and visually inspect the color to ensure a homogenous mixture.
       6. Incubate the Reaction Mixtures for 5 minutes at room temperature.
       7. After incubation, briefly spin down and place the Reaction Mixtures on the magnet for 5 minutes to fully pellet.
       8. Without disturbing the bead pellet, use a pipette set to 50 µL to remove and discard the supernatant.
          1. **NOTE:** If the pellet becomes dislodged from the magnet or a portion is drawn into the pipette tip, return the contents back to the tube and repeat the 5-minute magnet incubation.
       9. With the supernatant removed and the tubes still on the magnet, add 200 µL of 70% ethanol to each pellet.
       10. Incubate for 30 seconds to allow the beads to fully pellet against the side of the tubes.
       11. Without disturbing the bead pellet, use a pipette set to 200 µL to remove and discard the supernatant.
       12. Repeat (steps i. – k.) for a total of two washes in 70% ethanol.
       13. After the final wash, use a pipette (≤20 µL capacity) to completely remove visible supernatant residue.
       14. Allow the bead pellets to dry for 6 minutes at room temperature on the magnet with the lids open. If needed, allow more time for the beads to properly dry.
           1. **NOTE:** Take care not to over-dry the beads as this will significantly decrease overall recovery (yield) of nucleic acid.
           2. Observe the appearance of the beads: a “glossy” appearance indicates the bead is not properly dry. A “cracked” appearance indicates over-drying.
       15. Elute the DNA by resuspending the beads in 24 µL of 10mM Tris-HCl pH 8.0.
       16. Vortex briefly and spin down.
       17. Place the tubes on the magnet for 2 minutes.
       18. After 2 minutes, transfer 22 µL of the purified Second PCR products (Final Libraries) to a new, labeled 0.2mL PCR tube-strip.
           1. **NOTE:** Avoid transferring beads with the final libraries.
           2. **NOTE:** If stopping the workflow, observe the Safe Stopping Point.
       19. At this point, you may store the purified products **or** proceed directly to Step 11-KAPA qPCR setup.
       20. SAFE STOPPING POINT: Place the purified final libraries in a cooler tube rack and store in the freezer.
           1. **NOTE:** The purified final libraries can be stored for up to 2 weeks at a temperature of -30°C to -10°C and used for sequencing.
    6. **Process your worksheet: FST LIBPREP**
       1. Open FST LIBPREP – Test Worksheet Processing by using the tile on the dashboard.
       2. Press **Find** and double click on the correct worksheet, if applicable.
       3. **Complete** Clean up after Second PCR action and **Save**.
       4. Select **Build Next Worksheet**.
       5. Mark the **To Build** FUSKAPA checkbox. Verify Transfer Controls and Select all tests are checked. Click on the **OK** button.
       6. The system will ask the user if they want to open the new worksheet. Select **No**.

NOTE: If a sample is being re-run to KAPA, this will allow you to see it pending in Worksheet Builder.

* + - 1. Open FST – KAPA Test Worksheet Builder.
      2. Select **Find** and double click on the correct worksheet, if applicable.
      3. If there any pending patient samples, add the sample to the run by scanning the label in the Barcode# field and pressing Enter.
      4. Click on the **Print Plate View** button to open the Plate View Preview window.
      5. Select **OK** to save.
      6. Select the **printer** icon, verify the correct printer is selected, and click **Print**.
      7. Close the Print Preview window.
      8. Click on the **Settings** button. Verify the correct printer is populated in the dropdown field. Select **Print**.
      9. Select **Back** in the FST KAPA – Test Worksheet Processing.
      10. Exit Soft Molecular application.
    1. If proceeding directly to KAPA qPCR, place the final libraries on ice while the KAPA qPCR setup is performed (see Step 11 – KAPA Library Quantification below).
  1. Step 11 – KAPA Library Quantification and Normalization
     1. **NOTE: Before starting this step, check Soft Molecular for any pending samples to see if any samples from prior runs have been sent for rerun from the KAPA worksheet. For repeat samples, the I5 and I7 indices do not transfer to later worksheets. When exporting the sample sheet, manually add this information from the prior run.**
     2. Remove the KAPA qPCR kit from the freezer.
     3. The components of the kit are:
        1. Six DNA Standards (80 µL each): Standard 1 (20pM), Standard 2 (2 pM), Standard 3 (0.2 pM), Standard 4 (0.02 pM), Standard 5 (0.002 pM), and Standard 6 (0.0002 pM).
        2. KAPA SYBR FAST qPCR Master Mix (5 mL).
        3. Primer Mix (1 mL).
        4. 50X ROX High and 50X ROX Low (200 µL each).
           1. **NOTE:** The ROX High is not used for the Fusion Panel workflow.
     4. Place the Standards, SYBR FAST qPCR Master Mix, Primer Mix and 50X ROX Low on ice and allow to thaw for 1 hour away from light. Allow more time if needed.
        1. **NOTE:** The ROX High is not used in this assay.
        2. **NOTE:** The components of the kit are light sensitive and susceptible to degradation. Therefore, they must be kept on ice and away from direct light exposure as much as possible.
        3. **NOTE:** The KAPA qPCR kit is stable through 30 freeze-thaw cycles.
     5. If opening the kit for the first time, prepare a ready-to-use master mix. Combine the following components by pipetting each directly into the the 5 mL bottle of 2X KAPA SYBR FAST qPCR Master Mix:
        1. 1 mL of the 10X Primer Premix.
        2. 0.2 mL of the 50X ROX Low.
     6. Label the bottle with the date to document when the kit was opened and the master mix prepared.
     7. Vortex for 10 seconds to mix, place on ice, and proceed to KAPA qPCR setup.
        1. **NOTE:** The KAPA master mix is now ready for use. For subsequent runs, remove and thaw only this master mix and the Standards.
     8. The Fusion Panel libraries are very concentrated and must be diluted for quantification when using the KAPA qPCR Assay.
     9. Dilute the purified final libraries at a 1:250,000 dilution:
        1. Prepare a sufficient volume of 10mM Tris-HCl, pH 8.0 to be used as the diluent (see KAPA Worksheet for appropriate volumes).
        2. Prepare the 1:250,000 dilution according to the following 3-step sequence of serial dilutions: (**2:198**, **1:99**, and **2:48**).
           1. First, transfer 198 µL of 10mM Tris-HCl, pH 8.0 to a new set of labeled, 0.2 mL tube-strip.
           2. Add 2 µL of each purified final library to the appropriate tube containing the 198 µL diluent.
           3. **NOTE:** Place the final libraries back on ice while the KAPA qPCR is performed.
           4. Mix the diluted samples by vortexing for 5 seconds or by pipetting up and down 10 times.
           5. Briefly spin down the tubes. This results in a 1:100 dilution of the final libraries.
           6. Next, transfer 99 µL of 10mM Tris-HCl, pH 8.0 to a new set of labeled, 0.2 mL tube-strip.
           7. Add 1 µL of each 1:100 diluted library to the appropriate tube containing the 99 µL diluent.
           8. Mix by vortexing for 5 seconds or by pipetting up and down 10 times.
           9. Briefly spin down the tubes. This results in a 1:10,000 dilution of the final libraries.
           10. Finally, transfer 48 µL of 10mM Tris-HCl, pH 8.0 to a new set of labeled, 0.2 mL tube-strip.
           11. Then add 2 µL of each 1:10,000 diluted library to the appropriate tubes containing the 48 µL diluent.
           12. Mix by vortexing for 5 seconds or by pipetting up and down 10 times.
           13. Briefly spin down the tubes. This results in the 1:250,000 dilution for quantification.
           14. Place the 1:250,000 diluted libraries on ice until needed. The 1:100 and 1:10,000 dilutions can be discarded.
     10. All DNA Standards, controls, and diluted libraries are run in duplicate reactions.
     11. Vortex the master mix.
     12. Briefly vortex and centrifuge the Standards.
     13. Place the master mix and the Standards on ice.
         1. **NOTE:** Immediately return each component on ice after use.
     14. Pipette 6 µl of master mix into the appropriate wells of a 96-well optical PCR plate (USA Scientific 1402-9300). Refer to the corresponding Plate Layout of the KAPA Worksheet.
     15. Add 4 µl each of DNA Standards, laboratory PCR-grade water, and diluted libraries into the corresponding wells containing master mix.
     16. Mix by pipetting and avoid introducing bubbles.
     17. Seal plate with an optical seal (USA Scientific 2921-7800).
     18. Centrifuge the plate for 1 minute at 1500 x g.
     19. Centrifuge again if there are bubbles present.
     20. The KAPA qPCR Assay is performed on the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System (QuantStudio™).
     21. Ensure that the instrument is turned on (see the QuantStudio™ 7 Flex Instrument Procedure for more info).
     22. Load the reaction plate onto the QuantStudio™.
     23. Use Real-Time PCR Software v1.3 to setup the QuantStudio™ run:
         1. Open the QuantStudio™ Real Time PCR Software icon located on the desktop of the connected computer.
         2. Under the Set-Up panel, select Template.
         3. In the dialog box, open the RIH Templates folder.
         4. Select the “KAPA\_qPCR.edt” file.
         5. In the Experiment Name field, input the run name by scanning the KAPA Plate View. Then, change all periods to underscores.
         6. **Experiment Properties** parameters should be as follows:
            1. Instrument Type: QuantStudio™ 7 Flex System
            2. Block Type: 96-well (0.2mL)
            3. Experiment Type: Melt Curve
            4. Detection Reagent: SYBR® Green Reagents
            5. Instrument Properties: Standard
         7. Under the **Setup** panel on the far left of the screen, select **Define**. The **Define** parameters should be as follows:
            1. Target Name: Target 1
            2. Reporter: SYBR
            3. Quencher: None
            4. Passive Reference: ROX
            5. Create a sample list.
         8. Under the **Samples** section located on the right side of the screen, select **New**.
         9. Click **New** once for each sample on the run (e.g., click **New** 7 times for a run of 7 patient samples).
         10. Enter each Sample Name (Soft Molecular Order #) by clicking on a “Sample #” text field and entering the Sample Name.
         11. Repeat for all samples on the run.
         12. Under the **Setup** panel on the far left of the screen, select **Assign**.
         13. In the Plate Layout tab on the right side of the screen, the NTC will be pre-filled with “N” (Negative) and the FUS\_SENS with “U” (Unknown).
         14. Complete the Plate Layout by assigning a Sample Name to the corresponding wells:
             1. Click and drag to highlight the desired wells.
             2. Assign the appropriate Sample Name by clicking the Soft Molecular # from the **Sample** section on the left side of the screen.
             3. The highlighted wells will populate with the selected Sample Name.
             4. Repeat for all samples until the Plate Layout is complete.
         15. Highlight all assigned wells on the Plate Layout and select Target 1 from the **Targets** panel located on the left side of the screen.
         16. Assign “Unknown” to all samples and the positive control by selecting “U” under the **Task** drop-down window.
         17. Under the **Setup** panel on the far left of the screen, select **Run Method**. The **Run Method** parameters should be as follows:
             1. Reaction Volume per well = 10 µl.
             2. Cycling program:

|  |  |  |  |
| --- | --- | --- | --- |
| **KAPA qPCR (Standard)** | | | |
| **Step** | **Temp** | **Time** | **Cycles** |
| Initial denaturation | 95°C | 5 min | 1 |
| Denaturation | 95°C | 30 sec | 35 |
| Annealing/Extension/Data | 60°C | 45 sec |
| Melt curve analysis | 1. - 95 °C | | |

* + - 1. On the far-left side of the screen, select **Run**. The Run panel should open once selected.
      2. Select the green “Start Run” button on top left corner of the screen.
         1. Select the QuantStudio™ instrument.
         2. Save the run using the correct format by scanning the Fusion KAPA Plate View.
      3. The program will run for approximately 1 hour and 20 minutes.
    1. **Process your worksheet: FST KAPA – Test Worksheet Processing**
       1. Open FST KAPA – Test Worksheet Processing by using the tile on the dashboard.
       2. Scan the barcode of the KAPA Plate View into the Worksheet# field and select **Find**.
       3. Use the dropdown under **Used Instrument:** to select the correct QuantStudio.
       4. Complete KAPA Load QuantStudio action by marking the **Completed** checkbox and selecting **Save**.
       5. Select Back in the KAPA – Test Worksheet Processing window.
       6. Exit Soft Molecular application.
    2. When the run is complete, discard the plate. Then, export the \*.eds file from the QuantStudio.
       1. Insert a Flash Drive into the QuantStudio computer.
       2. On the far-left side of the screen, select **Export**.
       3. Select the blue **Browse** button located at the top of the screen.
       4. Select **Computer**.
       5. Select the appropriate **Flash Drive**.
       6. Select **Open**.
       7. Export the **Results** tab only as an \*.xls file.
       8. Select **Start Export** button located at the bottom of the screen.
       9. Open this exported file on any Lifespan computer.
          1. In the Fusion run folder located on the RICMBLAB$ network drive, create a new subfolder entitled “**QuantStudio Files**.”
          2. Move the exported \*.xls file from the flash drive to this folder.
    3. On the RICMBLAB$ network drive, go to the \\PCR\_Worksheets\_Current\Fusion\_Solid\_Tumor subfolder and open the FUS\_KAPA\_ImportDocument\_v9 file.
    4. In the FUS\_KAPA\_Clean Import Document, select **File** followed by **Save As**.
    5. Select **Browse** to locate the Fusion run folder.
    6. Name the file in the File Name field by scanning the KAPA Plate View.
    7. Open the Clean KAPA Import Document and the Quant Studio Raw Data file.
    8. On the QuantStudio Raw Data file, highlight the header row containing column names (sample name, etc.).
    9. From the Home screen in Excel, select “sort and filter”, then “custom sort”.
       1. A box will pop up. Click “Sort”.
       2. Sort by **Task**.
       3. Add a level.
       4. Then, sort by **Sample Name**.
       5. Click “OK”.
    10. Highlight the data under the header row, exclude the column titles, and copy the data.
    11. Paste the data from the QuantStudio Raw file starting in the A2 well of the KAPA Import Document. Select **Save** and close both Excel files.
        1. Review the results/metrics and ensure that the following criteria are met:
           1. Baseline settings for the run Start at cycle 1 and End at cycle 3.
           2. R2 value for the run is >0.99.
           3. Efficiency is in the range of 90-110%.
           4. ΔCt. between replicate data points of Samples and Standards are ≤1.0 cycle.
           5. ΔCt. between Standards and NTC are in the range of 2.0 – 3.8 cycles.
    12. Repeat the KAPA qPCR Assay if any of the following is true.
        1. Two or more Standards fail QCs.
           1. If one standard fails QC, see Director/Pathologist and/or repeat testing.
        2. R2 value is not within the acceptable range.
        3. Run Efficiency is not within the acceptable range.
        4. The NTC is contaminated.
        5. If the ΔCt for the sample replicates is >1.0, discuss with the Director/Pathologist.
           1. If appropriate, repeat the KAPA qPCR assay for that sample.
           2. Alternatively, if the value for one of the replicates appears out of range, exclude that value for that sample. In the workbook, make a copy of the worksheet and edit accordingly so that the original data is still visible on the original worksheet.
    13. Libraries quantified with the KAPA qPCR Assay must meet the dynamic range of the assay:
        1. No library should amplify before Standard 1 and no library should amplify after Standard 6.
        2. The calculated final concentration of each library should be ≥50 nM.
        3. Samples that do not meet the criteria described above may be repeated on the KAPA qPCR Assay.
        4. Samples that do not meet the minimum final concentration should be excluded from the sequencing.
           1. **NOTE:** At the discretion of the Director/Pathologist, samples that do not meet the minimum final concentration may be sequenced. This rare exception process could be based on factors such as: tissue size of the specimen, RNA concentration (Qubit), or RNA quality (PreSeq QC Assay). Sequencing may be performed for samples with final concentration <50nM but ≥4nM (which is the normalized concentration of the final pool).
    14. **Process your worksheet: KAPA Import**
        1. Open FST KAPA – Test Worksheet Processing by using the tile on the dashboard.
        2. Scan the barcode of the KAPA Plate View into the Worksheet# field and select **Find**.
        3. Select the ‘**…**’ button to the right of the Import button to import the KAPA Clean Document created.
        4. Change the radial button from **XML** to **Excel**.
        5. Select the ‘**…**’ button next to the File Name field to select the edited KAPA import document.
        6. Click on the **Import** button.
        7. Verify Worksheet is populated in the Image Type field.
        8. Select **Images** button.
        9. On the window that opens, select the **Add File** tab. Then, select the **add file (folder)** icon.
        10. Find and select the file to be added from the Windows Explorer window. Select **Open**.
        11. Choose **Instrument Documents** from the Template dropdown.
        12. Select the **green check** icon to add the files.
        13. Close the window.
        14. Complete KAPA Import action by marking the **Completed** checkbox and selecting **Save**.
        15. Click on the **Build Next Worksheet** button.
        16. Mark FUSNORMLOD **To Build**.
        17. Verify Transfer Controls and Select all tests are selected. Click on the **OK** button.
        18. The system will ask the user if they want to open the new worksheet. Click **No**.
        19. Select **Back** in FST KAPA – Test Worksheet Processing window.
        20. Open FST Normalization and Loading – Test Worksheet Builder by using the tile on the dashboard.
        21. Press **Find** and double click the correct worksheet.
        22. Open the vertical **Plate View tab**.
        23. Delete the replicates of standards and controls by highlighting the well and clicking **Delete**.
        24. Select Print Worksheet.
        25. Select **Yes** to save.
        26. Close the Print Preview box.
        27. Select **Back** in FST Normalization and Loading – Test Worksheet Builder window
        28. Exit Soft Molecular application.
    15. Normalization:
        1. After quantification, each final library is normalized to a concentration of 4nM.
        2. Label a clean 1.5 mL microcentrifuge tube for each sample or an appropriate size tube for dilution.
        3. Perform the 4nM dilution by combining 5 µL of final library with the corresponding volume of laboratory PCR-grade water into the appropriate tube (see the Normalization Worksheet).
        4. Once the dilutions are made, place the final libraries back on ice.
        5. Vortex and briefly centrifuge the 4nM diluted libraries.
        6. Label a clean 1.5 mL microcentrifuge tube with “4nM Big Pool Library” and the date.
        7. Create a “4nM Big Pool Library” by pipetting **5 µL** of each 4nM diluted library into this tube.
        8. Vortex and briefly centrifuge, then place the 4nM Big Pool Library on ice.
        9. Discard the individual 4nM diluted libraries.
           1. NOTE: The 4nM Big Pool is available for repeats. In rare cases, if necessary, the individual 4nM libraries can be remade from the final libraries.
        10. After normalization, proceed to Sequencing Setup and Loading.
  1. Sequencing Setup and Loading
     1. This section of the procedure covers the preparation of the Big Pool Library, the PhiX Control, and the NextSeq® instrument prior to starting sequencing.
     2. Prepare the NextSeq® 500/550 Mid Output kit components.
        1. **NOTE:** For more detailed information regarding use of the sequencing kit or the NextSeq® instrument, refer to the NextSeq® 500 Instrument Procedure.
     3. Remove the 10nM PhiX Control from the freezer and place on ice until ready to use.
        1. **NOTE:** The PhiX Control is derived from the bacteriophage genome (PhiX) and is provided as a ready-to-use library at a concentration of 10nM. The PhiX Control is used to monitor the quality of sequencing runs on Illumina platforms.
     4. Once the HT1 is thawed, prepare (denature and dilute) the Big Pool Library and PhiX Control as follows:
        1. Make a fresh aliquot of 0.2N NaOH by combining 4 µL of 1N NaOH with 16 µL of laboratory PCR-grade water.
        2. Obtain a tube of 200mM Tris-HCl, pH7.0.
        3. Label a clean 1.5 mL microcentrifuge tube for “Library Denature and Dilute”.
        4. Pipette 10 µL of the previously made Big Pool Library into this tube and set aside at room temperature.
        5. Return the Big Pool Library stock to ice.
        6. Finger flick and briefly spin down the thawed PhiX Control.
        7. Label a clean 1.5 mL microcentrifuge tube for “PhiX Denature and Dilute”.
        8. Pipette 2 µL of the 10nM PhiX Control and 3 µL of laboratory PCR-grade water into this tube.
        9. This results in a 5 µL PhiX Control at a 4nM concentration.
        10. Denature the 10 µL Library Denature and Dilute tube and the 5 µL PhiX Control by respectively adding 10 µL and 5 µL of 0.2N NaOH to each tube.
        11. Vortex both tubes briefly to mix and centrifuge.
        12. Incubate the tubes at room temperature for 5 minutes.
        13. After 5 minutes, stop the denaturation by adding 10 µL of 200mM Tris-HCl, pH 7.0 to the Library Denature and Dilute tube and 5 µL of 200mM Tris-HCl, pH 7.0 to the PhiX Control.
        14. Vortex both tubes briefly to mix and centrifuge.
        15. Dilute the Library Denature and Dilute tube by adding 970 µL of ice-cold HT1.
        16. Dilute the PhiX Control by adding 985 µL of ice-cold HT1.
        17. Vortex both tubes to mix, spin down, and place on ice
        18. This result in a 40pM denatured Library Denature and Dilute tube and a 20pM denatured PhiX Control.
        19. Label a clean 1.5 mL microcentrifuge tube “Final Loading Pool”.
        20. Pipette the following into this tube to create the Final Loading Pool:
            1. 39.0 µL of the 40pM denatured and diluted Library Denature and Dilute tube.
            2. 19.5 µL of the 20pM denatured and diluted PhiX Control.
            3. 1241.5 µL of ice-cold HT1.
        21. This results in a 1.3 mL Final Loading Pool at a concentration of 1.2pM with 20% PhiX.
        22. Place the Final Loading Pool on ice until ready to use.
        23. Inspect the thawed Reagent Cartridge to make sure the reagents are completely thawed.
            1. **NOTE:** Allow more time if the cartridge reagents are not completely thawed.
        24. Invert the cartridge 5 times to mix the reagents.
        25. Gently tap the cartridge on a hard surface to remove bubbles and dislodge water from the base of the cartridge.
        26. Use a dry Kimwipe and clean the foil seal covering the well designated ‘Load Library Here’ (well #10).
        27. Pierce the foil seal with a clean 1 mL pipette tip.
        28. Pipette the entire Final Loading Pool (1.3 mL) into the Library Load well.
            1. **NOTE:** Take care to avoid introducing bubbles into the well as this could affect cluster generation and the performance of the Flow Cell.
        29. Set the Reagent Cartridge aside in the hood until ready to load.
        30. On the Home Screen of the NextSeq®, select the **Sequence** button.
        31. Click **Manual** in the window that appears.
        32. BaseSpace Sequence Hub Settings: Leave unchecked
        33. On the Run Setup screen, enter the run parameters as follows:
            1. Run Name: “Worksheet Name”, (e.g., 06.23.22-fusnormlod-1).
            2. Library ID: Leave blank.
            3. Recipe: Leave as Mid Output.
            4. Read Type: Select Paired End.
            5. Read Length:

Enter **151** in the text fields for both Read 1 and Read 2.

Enter **8** in the text fields for both Index 1 and Index 2.

* + - * 1. Custom Primer: Leave all options “**unchecked**”.
        2. Output Folder: Select or enter a specified output location for the sequencing data:

Option 1 (preferred): Enter or select [**\\192.168.1.16\nextseq\runs**](file:///\\192.168.1.16\nextseq\runs) for automatic transfer of the data to the lsmplinux2 server.

Option 2: Enter or select **D:\Output** for local storage of data on the NextSeq hard drive.

NOTE: If using option 2, a manual transfer step is required after sequencing is complete. Please refer to the Data Transfer Process section of the NextSeq 500 Instrument Procedure for more information.

* + - 1. When completed, select **Next**.
      2. Remove the used Flow Cell and discard.
      3. Gently remove the new Flow Cell from its packaging.
      4. Inspect the Flow Cell and place on the stage.
      5. Select **Load.**
      6. Select **Next** once the Flow Cell loads properly.
      7. When prompted, remove the used Buffer Cartridge or Wash Buffer Cartridge and discard accordingly.
      8. Then, remove the Spent Reagents Container and discard waste accordingly.
      9. Remove the new Buffer Cartridge from its packaging.
      10. Invert 5 times to mix.
      11. Gently tap the Buffer Cartridge on a hard surface to remove bubbles.
      12. Insert the new Buffer Cartridge into the instrument.
      13. Insert the empty Spent Reagents Container back into the instrument.
      14. Close the compartment door and select **Next**.
      15. When prompted, open the Reagent Cartridge compartment door and insert the Reagent Cartridge.
      16. Select **Load** and then, **Next**.
      17. The instrument will perform pre-run System Checks for all run components, disk space, and network connections.
          1. **NOTE:** If any checklist item fails during the System Check, an error notification will appear on the screen with instruction on how to correct the error.
          2. **NOTE:** If the error requires technical support, leave the loaded NextSeq® kit components on the instrument and contact Illumina Technical Support. The kit components are stable at room temperature for several hours (see the NextSeq® 500 Instrument Procedure for more info on Troubleshooting).
      18. When the System Check is successfully completed, the program should begin sequencing automatically.
          1. **NOTE:** The run takes approximately 27 hours to complete.
    1. **Process your worksheet: FST Normalization and Loading – Test Worksheet Processing**
       1. Open FST Normalization and Loading – Test Worksheet Processing by using the tile on the dashboard.
       2. Select **Find**.
       3. Complete the Normalization and Loading activity by marking the **Completed** checkbox and selecting **Save**.
       4. Select **Back** in FST Normalization and Processing – Test Worksheet Processing window.
    2. After the run is complete, perform the appropriate wash, according to the NextSeq Instrument Procedure.
  1. Sample Sheet
     1. A Sample Sheet is required to process and analyze the sequencing data from the NextSeq®. The Sample Sheet must be created and saved as a ‘comma delimited values’ file (.csv file).
     2. **Create Sample Sheet: FST Normalization and Loading – Test Worksheet Processing**
        1. Open FST Normalization and Loading – Test Worksheet Processing by using the tile on the dashboard.
        2. Select **Find**.
        3. Make sure the sub-template is set to “Sample Sheet”.
        4. Select the ‘**…**’ button to the right of the Export button to export the Sample Sheet.
        5. Change the radial button from **XML** to **Excel**.
        6. Select the ‘**…**’ button next to the Directory field and navigate to the run folder.
        7. Click on the **Export** button.
        8. On the RICMBLAB$ network drive, go to the \\PCR\_Worksheets\_Current\Fusion\_Solid\_Tumor subfolder and open the SampleSheetTemplate file.
        9. In the SampleSheetTemplate file, select **File,** followed by **Save As**.
        10. Select **Browse** to locate the Fusion run folder.
        11. Name the file in the File Name field by scanning the FUSNORMLOD Worksheet after Sample Sheet, replacing the word “Template”.
        12. Open the Sample Sheet and the exported FUSNORMLOD file.
        13. Copy the appropriate information from the exported FUSNORMLOD file into the template file. If applicable, delete any duplicated information (such as index codes). Save the Sample Sheet Excel file and then close the FUSNORMLOD file.
            1. **NOTE**: If a sample was re-run from KAPA, the Index code will be missing and will need to be manually entered. Find this information from the original FUSNORMLOD worksheet (or Index Code Log).
            2. **Sample ID**: enter the Soft Molecular number of each sample and control.
            3. **Sample Name:** enter the same information as the Sample ID column (e.g., control identifier and molecular numbers).
            4. **Index1\_P7\_ID:** obtain this information from the FUSNORMLOD file.
            5. **Index1\_P7\_Sequence:** the Excel workbook will calculate this information.
            6. **Index2\_P5\_ID:** obtain this information from the FUSNORMLOD file.
            7. **Index2\_P5\_Sequence:** the Excel workbook will calculate this information.
            8. **Sample Project:** Enter the project name using the format [DDMMYYYY]\_FusionPlex\_ST\_Fastq, where the date is the day that the instrument is loaded.
        14. In Soft Molecular, complete the Sample Sheet action by marking the **Completed** checkbox and selecting **Save**.
        15. When completed, inform the Bioinformatics team that the run was started, and the Sample Sheet is ready.
        16. This step is to be completed by the Bioinformatics team:
            1. Copy and Paste the information from the Excel file appropriately into the Sample Sheet .csv file template (received from Illumina). Save in the run folder.
            2. Close both the Excel and .csv files.
  2. Post Sequencing
     1. After sequencing is completed, an automatic post-run wash is performed using the wash solution provided in the Buffer Cartridge and the NaOCl provided in the Reagent Cartridge.
     2. On the NextSeq® Maintenance Sheet, document the date of the post-run wash.
     3. Transfer the archived (\*.zip) SAV file.
        1. Select the **Illumina Sequencing Analysis Viewer** icon located on the desktop of the NextSeq instrument.
        2. Select **Browse** on the top left.
        3. Select **Computer**.
        4. Select the **Data (D:)** drive.
        5. Select the **Illumina** folder.
        6. Select the **NextSeq Output** folder.
        7. Select the run (i.e., based on the date – example: 20220321).
        8. Select **OK**.
        9. Click on the **Summary** tab.
        10. Select the **Zip my Run** button on the bottom of screen.
        11. Save to the Fusion run folder on the **nextseq** drive.
            1. Nextseq\Runs
     4. Save a screenshot of the completed run screen on the nextseq server.
        1. Open the Snipping Tool program.
        2. Click the dropdown arrow next to Delay and select 3 seconds.
        3. If necessary, return to the completed run screen.
        4. In the Snipping Tool window, click **New**.
        5. Click on the completed run screen. A screenshot of the window should open.
        6. In the Snipping Tool window, click **File** followed by **Save As**.
        7. In the Save As window, navigate to the Runs folder on the nextseq server, then click **Save**.
     5. **Process your worksheet: FST Data Transfer**
        1. On a Lifespan computer, open FST Normalization and Loading – Test Worksheet Processing by using the tile on the dashboard.
        2. Select **Find**.
        3. Complete the Data Transfer action by marking the **Completed** checkbox and selecting **Save**.
        4. Select **Back** in the FST Normalization and Loading – Test Worksheet Processing.
        5. Open FST Tasklist by using the tile on the dashboard.
        6. Change the date range to one month.
        7. Scan FST Normalization and Loading Worksheet into the Worksheet# field and select **Find**.
        8. Click **Select All**.
        9. Click **OK**.
        10. Document SAV metrics in the Fusion Tasklist.
            1. % > Q30 – SAV Summary tab, Total
            2. Aligned % – SAV Summary tab, Read 1
            3. Cluster Density – NCS Screenshot
            4. % Cluster Passing Filter – NCS Screenshot
            5. Yield Total (G) – SAV Summary tab, Total
            6. Leave Reads (M) and Reads PF (M) blank, as they are not used for this assay
        11. Verify the Sample ID field is populated for each control. This indicates that a lot number is selected, and the Q numbers will generate appropriately.
            1. If a Sample ID field is empty for any of the controls, click the dropdown arrow in the Sample ID field.
            2. In the window that appears, select the correct lot number.

**Note:** Lot numbers can be found in the Sample ID fields of the printed worksheets.

* + - * 1. Repeat i-ii for each applicable control.
      1. Click **Select All**, then **Coll/Exp.** Button.
      2. Complete SAV Parameters QC action by marking the **Completed** checkbox located on the patient sample line and select **Save**.
         1. **Note:** Q numbers will generate for all controls.
      3. Select **Back** in the Tasklist Entry window.
      4. Exit Soft Molecular application.
    1. Document SAV metrics in the NextSeq\_Run\_Metrics\_FusionST log.
       1. RICMBLAB$\NGS\NextSeq\_Run\_Metrics\NextSeq\_Run\_Metrics\_FusionST

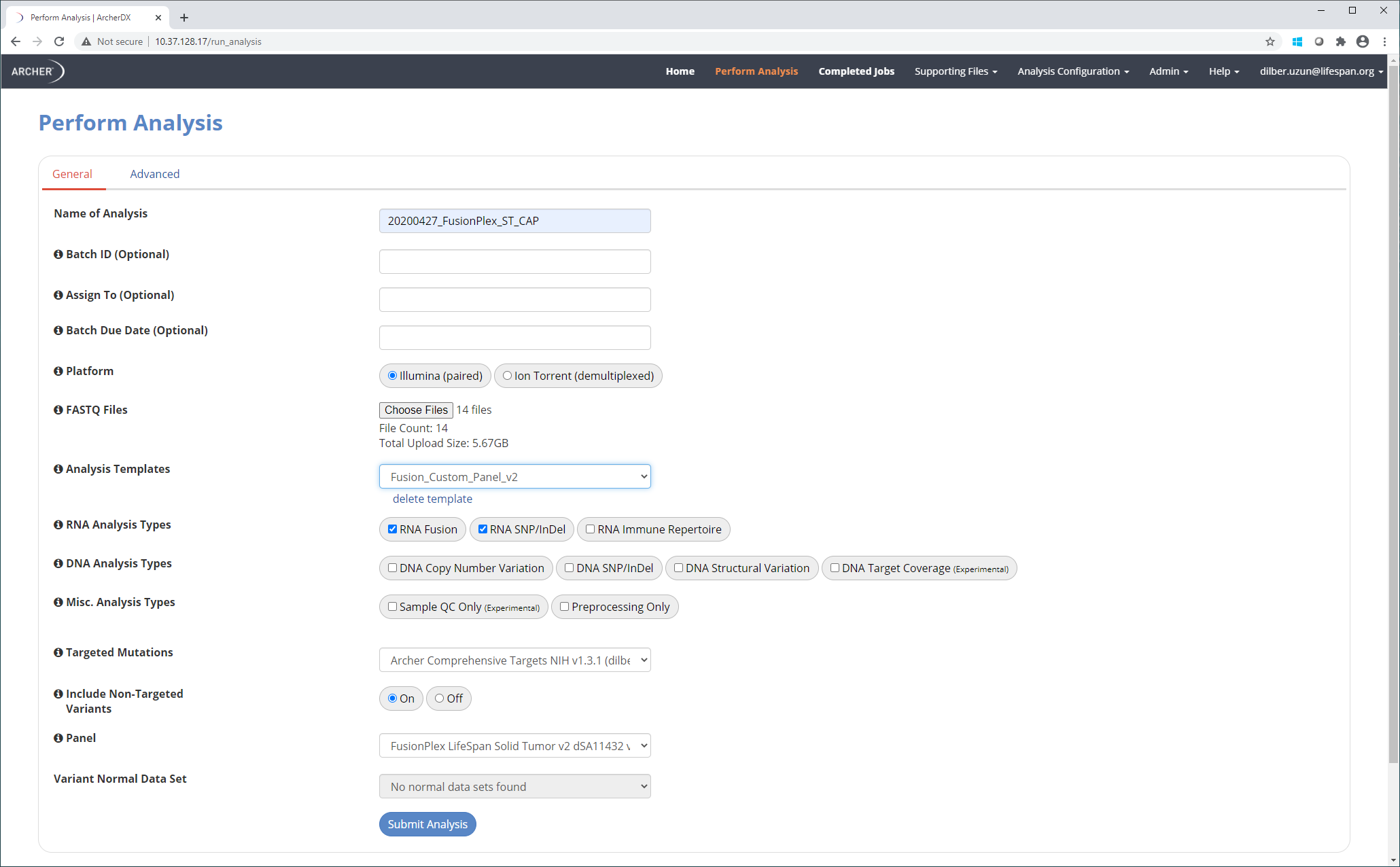
1. **TEST PROCEDURE – DATA TRANSFER PROCESS:**
   1. After the completion of sequencing, the data may be transferred automatically from the NextSeq to the Lifespan network shared drive or may have to be manually transferred.
   2. The Director of Clinical Bioinformatics will determine the appropriate methodology.
      1. To manually transfer the data:
         1. On a Lifespan networked computer, map the nextseq server.
            1. In the file finder, highlight **This PC** on the left side of the screen.
            2. Click the **Computer** tab at the top of the window.
            3. In the menu that appears, click **Map network drive**.
            4. Select the drive from the Drive dropdown.
            5. In the Folder field, enter [\\lsmplinux2\nextseq](file:///\\lsmplinux2\nextseq), then click **Finish**.
            6. In the Windows Security pop-up that appears, enter the username and password for the network drive.
            7. Click **OK**.
         2. Once the nextseq server is mapped, open the Runs folder.
         3. Locate the run folder, right-click then select **Cut** from the dropdown that appears.
         4. Navigate to the NextSeq\_Files folder on the MGPGenomicData$ drive, then right-click and select **Paste** from the dropdown that appears.
         5. Return to the Runs folder on the nextseq server, highlight the SAV zipped file and the NextSeq run screenshot, right-click then select **Cut** from the dropdown that appears.
         6. Navigate to the NextSeq\_Run\_Metrics folder following the file path: RICMBLAB$/NGS/NextSeq\_Run\_Metrics.
         7. Right-click and select **Paste** in the dropdown that appears.
2. **TEST PROCEDURE – BIOINFORMATICS ANALYSIS:**
   1. **NOTE**: These steps are performed by the Director of Clinical Bioinformatics, Bioinformatics Analyst, or other appropriately designated personnel.
   2. Make the **FASTQ Files** on lsmplinux02.
      1. Transfer the bcl2fastq\_command\_line.sh and sample sheet on the Lifespan Windows Computer to lsmplinux02.
         1. On a Lifespan computer, navigate to and open the run folder located in RICMBLAB$ Drive > CMB\_Tests folder.
         2. Copy the sample sheet and name the copy as “SampleSheet.csv”
         3. Navigate to **Computer** > **nextseq**
            1. This is mapped network drive that is shared from the onboard NextSeq computer.
         4. Locate the NextSeq run folder (the file name starts with the latest date).
         5. Copy bcl2fastq\_command\_line.sh which is located under \nextseq\Runs and the sample sheet into the NextSeq run folder.
      2. Alternative method for transferring FASTQ files:
         1. Open WinSCP.
         2. Enter 10.229.9.25 in Host name window.
         3. Enter the appropriate username and password.
         4. Click **Login**. The program will display two windows.
         5. In the left window, navigate to the run folder on the RICMBLAB$ drive.
         6. In the right window, navigate to the Runs folder on the nextseq server.
         7. Highlight the sample sheet file in the RICMBLAB$ run folder and drag to the run folder on the nextseq server.
         8. Wait until the file transfer is completed.
      3. Running the bcl2fastq program on lsmplinux02:
         1. On a Lifespan Windows computer, open Putty.
         2. Type [10.229.9.2](mailto:sbsuser@10.229.9.25)5 in Host name (or IP address) window.
            1. This is the IP address for lsmplinux2.
         3. Enter username/password.
         4. Type “cd /nextseq/Runs”
         5. Locate the run folder with the latest date.
         6. Type “cd [the run folder named with the latest date]”
         7. Type “scp /nextseq/Runs/bcl2fastq\_command\_line.sh ./” and press Enter on the keyboard.
            1. This will copy the bcl2fastq\_command\_line.sh file to the run folder

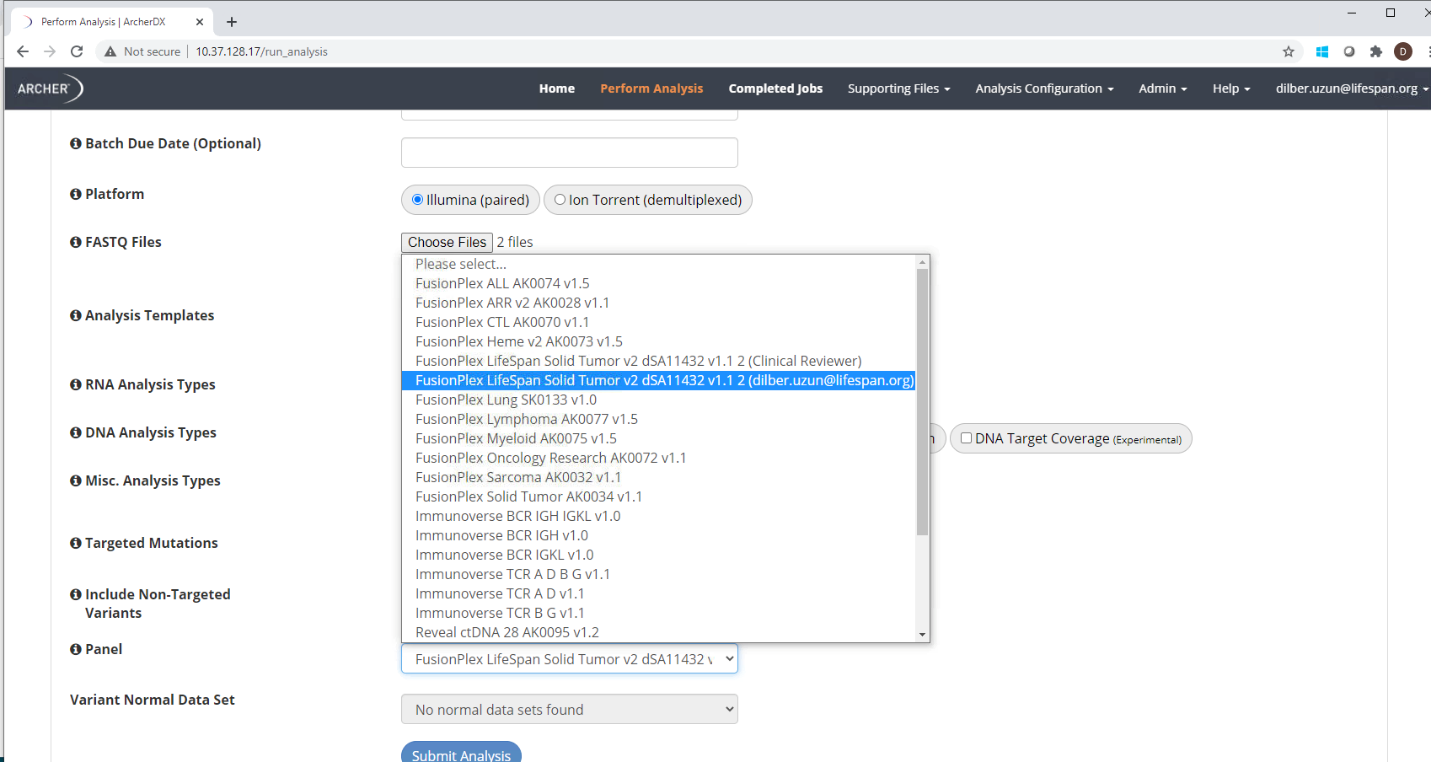
h. Type “sh bcl2fastq\_command\_line.sh”

* 1. Transfer FASTQ files from the NextSeq to the Lifespan desktop computer:
     1. On the Lifespan computer, click on **Windows start button** on the bottom left of screen.
     2. Navigate to **Computer>nextseq**.
     3. Enter username and password.
     4. Open **Runs** folder.
     5. Locate the run folder with the latest date.
        1. The FASTQ files are located in Data > Intensities > BaseCalls
        2. FASTQ folder is named with the format “[Date]\_FusionPlex\_ST\_Fastq”.
        3. Locate and copy that folder.
     6. Navigate to RICMBLAB$ **> CMB\_Tests**.
     7. Locate the Fusion run folder and open.
     8. Paste the Fastq folder into this folder.
     9. Alternative method for transferring FASTQ files:
        1. Create a folder in the run folder on the RICMBLAB$ drive using the format “[Date]\_FusionPlex\_ST\_Fastq’.
        2. If applicable, open WinSCP.
           1. Enter 10.229.9.25 in Host name window.
           2. Enter the appropriate username and password.
           3. Click **Login**. The program will display two windows.
        3. In the left window, navigate to the run folder on the RICMBLAB$ drive.
        4. In the right window, navigate to the Runs folder on the nextseq server.
        5. Highlight all the FASTQ files in the run folder on the nextseq server and drag them to the run folder on the RICMBLAB$ drive.
        6. Wait until the file transfer is completed and close WinSCP.

1. **TEST PROCEDURE – ARCHER® ANALYSIS SOFTWARE (version 6.2.2):**
   1. Background:
      1. The Archer® Analysis Software consists of a vendor-provided bioinformatics pipeline and variant/fusion calling software. The software is used to detect unique sequence fragments, enable error correction, read deduplication, and make high-confidence alignment and variant calling.
      2. The Archer® Analysis Software is supported by Archer® Quiver Fusion Database (Quiver). Quiver is a database of 9,481 published gene fusion partners and 10,660 unique breakpoints derived from 3,112 peer-reviewed publications (Quiver v.5.1.18). Archer® Quiver allows users to search for individual genes or gene fusions, distinguish synonymous gene names in the literature, search for published data on specific gene fusions, review disease states associated with particular gene fusions, and access links to existing gene-specific resources, such as PubMed, OMIM, NCBI, and UCSC. The database is comprised of information from 6 publicly available sources:
         1. COSMIC
         2. Mitleman Database
         3. ChimerDB
         4. dbCRiD
         5. TICdb
         6. ChiTars
   2. Set-up of the Archer**®** Analysis software:
      1. Open the appropriate web browser.
      2. Type http://10.37.128.27/auth/login
      3. Enter your username and password.
      4. Select **Log In**.
      5. Select the **Perform Analysis** located on the top middle of the screen.
      6. In the **Name of Analysis**, enter the Run name using the format [Date]\_fusbench\_1.
      7. In **FASTQ Files**, click on **Choose Files**.
      8. Select the FASTQ files saved on the RICMBLAB$ network drive.
      9. Select **Open**.
      10. Import the FASTQ files.
      11. Once all the samples have imported, in **Analysis Templates** select **Fusion\_Custom\_Panel\_v2**.
          1. This template is created by the Director of Clinical Bioinformatics.
          2. **NOTE:** At the discretion of the Director of Clinical Bioinformatics or Pathologist, the **Archer** modules may also be used.
          3. Please see the screenshot in **Figure 4** below for the template
      12. This should automatically select the following panel:
          1. **FusionPlex LifeSpan Solid Tumor v2 dSA11432 v1.1 2** in **Target Regions AND Archer Comprehensive Targets NIH v1.3.1 in Targeted Mutations**. It will also select RNA Fusion and RNA SNP/InDel.
      13. When analysis is done, open the Sign-out Batches to review the results.
   3. Delete Runs from the Archer**®** Analysis software:
      1. Open a web browser.
      2. Type http://10.37.128.27.
      3. Enter username and password.
      4. Select **Log In**.
      5. Select **Completed Jobs**.
      6. Select the runs to be deleted
      7. Save the run number to be used to delete the FastQ files from the Virtual Machine (steps 9a-i )
      8. Click on the trash can icon to delete the runs
      9. Archer**®** Analysis software keeps the FastQ files after the runs are deleted. The FastQ files need to be deleted from the Virtual Machine.
         1. Start Putty on a Lifespan Windows-based computer to open a Terminal.
         2. Type the IP address for the Archer server, lsarcher02 (10.37.128.27) on the “Host Name (or IP address)” window.
            1. Director of Clinical Bioinformatics and Bioinformatics Analyst have the username and password for the server.
         3. Type cd var/www/analysis/RunNumber to navigate to the run directory
         4. Type ls -lrt to list the FastQ files under the directory. Each FastQ file has a symlink. Copy the path to the symlink.
         5. Repeat steps a-b.
         6. Type cd the path to the symlink in step d.
         7. Delete the FastQ files under that directory using rm command.
         8. Go back to the Terminal with var/www/analysis/RunNumber.
         9. Type ls -lrt to list the FastQ files. You will see that the link to the symlink which was deleted in step g is broken. Delete that file using rm command.

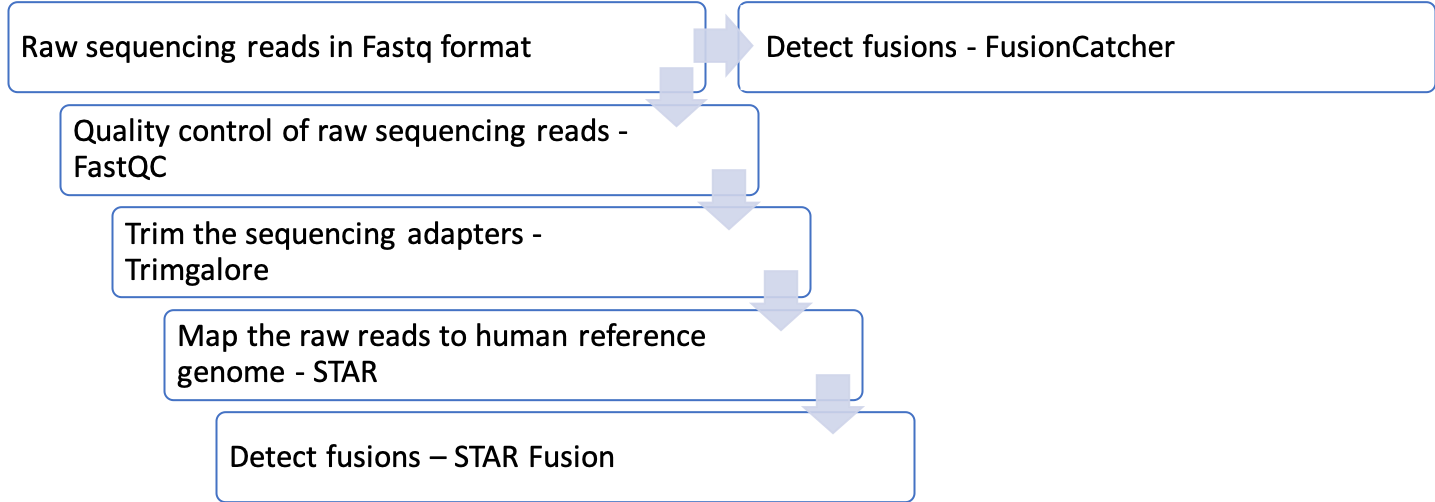
**Figures 4A-B.** Archer program Perform Analysis template

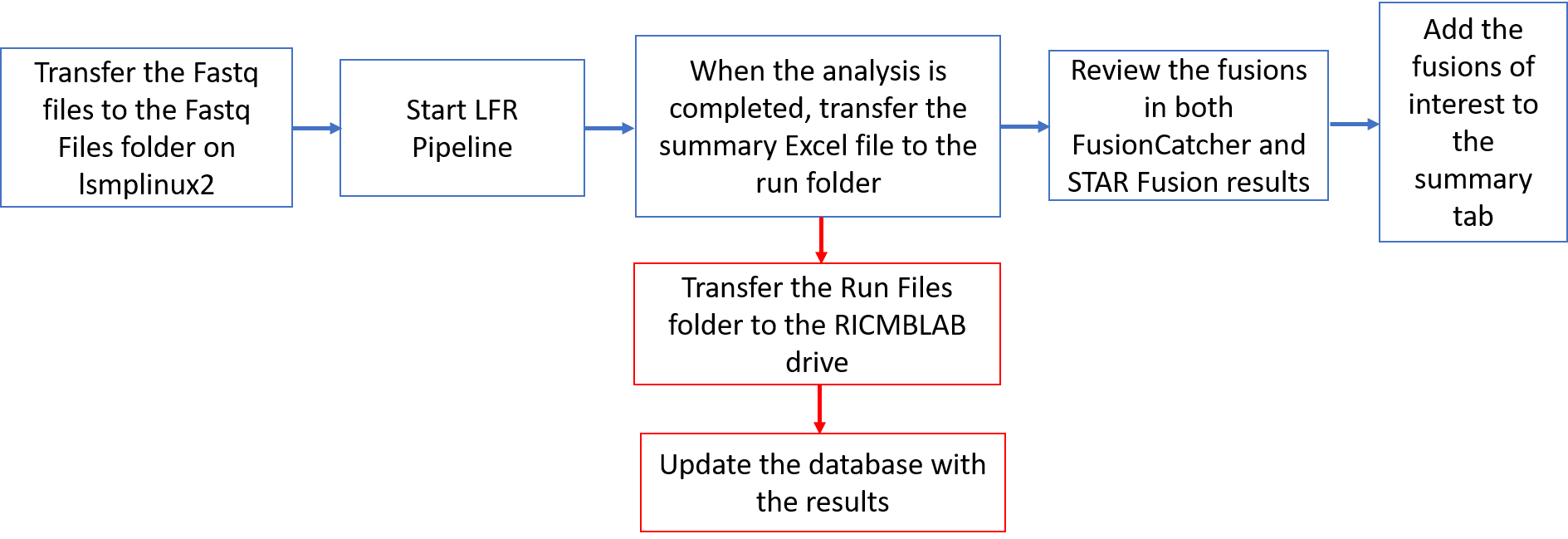




1. **TEST PROCEDURE – LIFESPAN FUSION REPORTER PIPELINE:**
   1. PRINCIPLE:
      1. **NOTE**: These steps are performed by the Director of Clinical Bioinformatics, Bioinformatics Analyst or other appropriately designated personnel.
      2. The Lifespan Fusion Reporter (LFR) is a data analysis (bioinformatics) pipeline which combines multiple open-source programs to analyze NGS data produced by the NextSeq® 500 instrument. The pipeline has been optimized to detect gene fusions in clinical samples.
      3. LFR includes 5 open-source programs: TrimGalore, FastQC, STAR, STAR-Fusion and FusionCatcher. In addition to these programs, the pipeline includes custom Perl scripts that create shell scripts in order to run the programs and perform upstream analysis. Please see **Figure 5** below for the pipeline flowchart.
         1. The Pipeline is performed on a desktop server running Red Hat Enterprise Linux 7.6 using Perl v5.16.3.
         2. TrimGalore is a program which trims the adapter sequences from the raw reads produced by NextSeq.
            1. LFR includes TrimGalore 0.4.5.
         3. FastQC is a program designed to perform quality control checks on raw sequencing data. It provides average quality scores for every base in each read. In addition, there are graphs indicating run metrics, including a tile graph that shows quality of the reads in the results. FASTQ files produced by the NextSeq are used as input.
            1. LFR includes FastQC version 0.11.7.
         4. STAR is a program which maps raw sequencing reads in FASTQ format produced by the NextSeq against human reference genome. STAR produces a BAM file with mapped reads.
            1. LFR includes STAR version 2.5.3a and GRCh37\_v19\_CTAT\_lib\_Feb092018 as the human reference file.
         5. STAR-Fusion is a program designed to detect fusions. It uses the output files from TrimGalore which are the trimmed FASTQ files.
            1. LFR includes STAR-Fusion version 1.2.0 and GRCh37\_v19\_CTAT\_lib\_Feb092018 as the human reference file.
         6. FusionCatcher is a program that detects fusions without trimming the adapter sequences.
            1. LFR includes FusionCatcher version 1.00 and human\_v90 as the human reference file.
      4. LFR includes custom Perl and shell scripts to automatically run the pipeline. There are two main scripts in the pipeline:
         1. Perl script “ListDetect\_Fusion.pl” creates shell scripts for each sample to run TrimGalore, FastQC, STAR, STAR-Fusion and FusionCatcher.
         2. Perl script “clean.pl” creates a shell script “clean.sh” to organize the results obtained by STAR-Fusion and FusionCatcher. The shell script “clean.sh” is run after the analyses are done and performs the following steps:
            1. It combines the results for all samples for STAR-Fusion and FusionCatcher.
            2. It creates separate summary files for STAR-Fusion and FusionCatcher results of all samples.
            3. It removes the shell scripts created by “ListDetect\_Fusion.pl” and FASTQ files.
            4. It moves the summary files to the run folder.
         3. Shell script “start\_code.sh” runs the scripts “ListDetect\_Fusion.pl”, “clean.pl” and creates separate folders for STAR, STAR-Fusion, FastQC and FusionCatcher results.
         4. Shell script “Final\_code.sh” runs the shell script “clean.sh”, adds headers to the summary files, merges summary files in a single Excel file removes the meta-files.
         5. Shell script “Indexed\_bam\_files.sh” sorts and indexes the aligned BAM files to prepare inputs for IGV.
         6. Please refer to **Appendix A** for more details, including the source code for the pipeline.

**Figure 5A-B.** Lifespan Fusion Reporter pipeline flowchart.





* 1. Data Processing:
     1. Start Putty on a Lifespan Windows-based computer to open a Terminal.
        1. Type the IP address for the Linux server, lsmplinux2 (10.229.9.25) on the “Host Name (or IP address)” window.
        2. The first time Putty is used, the IP address can be saved under “Saved Sessions”.
        3. If the IP address has been previously saved, click on the IP address under “Saved Sessions” window and press **Load**. Then, press **Open**.
        4. Enter your username/password.
        5. In the Terminal window:
           1. Type “cd /molpath/Analysis\_Directory/sh\_files”
           2. To transfer the FASTQ files to the Fastq\_Files folder in the Analysis Directory, type “scp /nextseq/Runs/[Date]\_[Illumina\_Run\_Number]/Data/Intensities/BaseCalls/ArcherDx\_ST\_Fastq/\* /molpath/Analysis\_Directory/Fastq\_Files”
           3. Type “cd ..”
           4. Type “cd /molpath/Analysis\_Directory/sh\_Files”
           5. Type “sh start\_code.sh”

start\_code.sh is a shell script which runs the ListDetect\_Fusion.pl script and the clean.pl script, which will create a shell script called “clean.sh”.

start\_code.sh also creates directories for metafiles produced by FastQC, STAR, STAR-Fusion and FusionCatcher.

* + - * 1. Type “ls –lrt”.

This will list all shell scripts for each sample. Check if all samples were included.

* + - * 1. Type “cd ..”
        2. Type “sh ./sh\_files/[sample\_ID].sh”.

This will run the shell script for the sample.

* + 1. Start Putty again to open another Terminal.
       1. Type “cd /molpath/Analysis\_Directory/”
       2. Type “sh ./sh\_files/<sample\_sh\_file\_name>”.
       3. File names can be determined from step 3.e.(vi).(1) above.
    2. Repeat step 2 for each sample.
    3. Lock the computer and leave it overnight as this step will take several hours (1-2 hours/sample).
    4. When the program is done running, the last line should read: “[User\_ID@lsmplinux2 Analysis\_Directory]$”
    5. Close all Terminal windows except one.
    6. In the Terminal window, type “sh Final\_Code.sh”.
       1. This will run another script to run the shell script clean.sh which will create the summary files from the STAR-Fusion and FusionCatcher results. It will run each shell script automatically and, therefore, this step should only be performed once.
    7. When it is done, the FASTQ files and shell scripts created for all samples will be removed. The summary files will be moved to /molpath/Analysis\_Directory/Run\_Files.
    8. Move the Run\_Files folder to the RICMBLAB$ shared drive.
       1. Using WinSCP, open the RICMBLAB$ run folder and copy the Run\_Files folder from the server into the RICMBLAB$ parent folder.
       2. Rename the Run\_Files folder using the format [YYYYMMDD]\_[Assay#]\_Lifespan\_Fusion\_Reporter\_Files (e.g., 20170508\_\*\*\*\_Lifespan\_Fusion\_Reporter\_Files).
    9. Go back to the Terminal and type “rm –r ./Run\_Files”.
       1. This will remove the Run\_Files folder from the Linux server.
    10. As an alternative LFR can be directly run on lsmplinux2 server
        1. Open a Terminal on the server and follow steps 1-10.
    11. FusionCatcher and STAR-Fusion results are stored in an SQLite based database under RICMBLAB$\Bioinformatics\MolDB\Fusion.db in their respective tables.
        1. In order to load the data onto the database, open Spyder (Python environment) on the desktop computer CO3MOLWIN10 and run the FusionImport.py script.
  1. Data Analysis
     1. Review the fusions according to the following steps:
        1. Open Lifespan\_Fusion\_Reporter\_Files folder.
        2. Rename the LFR\_Summary file using the format [YYYYMMDD]\_[Assay#]\_LFR\_Summary (e.g., 20170508\_\*\*\*\_LFR\_Summary).
        3. In FusionCatcher worksheet, filter out-of-frame fusions, fusions with Spanning\_Unique\_Reads < 5 and artifacts.
           1. After step c., review all fusions for any potential positive fusions with “out-of-frame” mark.
        4. In STAR\_Fusion worksheet, filter fusions with “INCL\_NON\_REF\_SPLICE which are out-of-frame and artifacts.
           1. After step d., review all fusions for any potential positive fusions with “INCL\_NON\_REF\_SPLICE” mark.
           2. Fusions with Junction\_Read\_Count < 5 are typically filtered but should be evaluated on a case-by-case basis.
        5. Add all potential fusions to “Summary” Excel worksheet.

1. **TEST PROCEDURE – Archer Analysis Program (INTERPERTATION):**
   1. Principle: Archer Analysis Program has a graphical user-interface which allows the user to analyze the data and mark the fusions to be reported.
   2. Filter Fusions:
      1. When the analysis is done, open the **Sign-out Batches** and select the last run.
      2. Click on the first sample.
      3. Review all fusions under the **Strong Fusions and Oncogenic Isoforms** filter-set.
         1. In the column headers, click on **SS** and open the filter.
            1. Select “Greater than or Equal to” and type “5” under “Value”.
         2. In the column headers, click on **Reads** and open the filter.
            1. Select “Greater than or Equal to” and type “5” under “Value”.
            2. Steps 3-4 will set a threshold value of 5 for SS (Supporting Sites) and Reads.
         3. In the column headers, click on **InFrame** and type “True” under “Value”.
         4. For any fusion calls, under the **Actions** column header, click on the graph icon.
            1. The target icon indicates that the exact fusion point for that fusion is known and included in the Archer Analysis Program database, Quiver.
            2. The check icon under **Filters** indicates that the fusion passed all strong evidence filter criteria.
            3. On the top left of the browser window, there is another graph icon which toggles the visualization for all isoforms.
         5. Mark the “Report” checkbox for fusions to be including in the Soft Molecular database.
            1. If a fusion has multiple isoforms reported, mark them as “Report” so that they can be included in the Excel summary file for review.
            2. Fusions with a value of 1 in “TO” (Total Observed) which are seen only once should be reviewed carefully as those might be potential true fusions.
         6. Mark the “Artifact” checkbox for artifacts.
      4. Click on the **Low Confidence Fusions** filter-set button to review fusions with low confidence.
         1. Repeat steps a.-f. from Step 3.
   3. Export the Fusion Data:
      1. The Bioinformatician will export the fusion data to prepare an Excel file that will aid in interpretation. This file includes:
         1. All high and low confidence fusions
         2. Hyperlinks to NCBI Genome Browser
         3. Graphs showing the frequency of fusions based on Supporting Sites (SS) and %Reads.
      2. On the RICMBLAB$ drive, navigate to the Fusion run folder.
         1. Create a subfolder called “TSV\_Files”.
         2. Within that folder, create a subfolder called “Low\_Confidence”.
      3. Return to Archer Analysis and navigate to the current run.
      4. For each sample, perform the following steps:
         1. Select the **Strong Fusions and Oncogenic Isoforms** filter-set.
            1. Click on the **TSV** hyperlink at the bottom right of the table to export the filtered data to TSV\_Files folder.
         2. Select the **Low Confidence Fusions** filter-set.
            1. Click on the **TSV** hyperlink at the bottom right of the table to export the filtered data to the TSV\_Files\Low\_Confidence

folder.

* + - * 1. Add “\_low\_confidence” at the end of the file name (i.e. Order#\_isoforms\_low\_confidence).
        2. Create folders called “Sample\_Reports” and “RNA\_Expression” under the run directory.
        3. Click on the Sample Report hyperlink on the left side of the program user interface to open Reports tab.
        4. Click on Sample Summary hyperlink and open the report.
        5. Download the report using the download icon on the top right and save the report under Sample Reports folder.
        6. Click on Job Options on the top right and open Download Files.
        7. Navigate to “All” tab and select summaries and rna\_expression under the run number to download RNA Expression data.
        8. Save the data under RNA\_Expression folder.
  1. Generate the All\_Fusions file using the All\_Fusions Tool:
     1. **NOTE:** This step is performed by the Bioinformatician.
     2. Fusion data is stored in an SQLite-based database under RICMBLAB$\Bioinformatics\MolDB\Fusion.db
     3. There are several Python scripts used to generate the All\_Fusions file, including the graphs and hyperlinks.
  2. FusionImport.py, ResultsFusion.py, FusionReadCountGrapher.py, FusionRead%Grapher.py
     1. Open Spyder on the desktop computer CO3MOLWIN10.
        1. Run ResultsFusion.py. This script creates the All\_Fusions file by using both the strong and low confidence CSV files generated by the Archer Analysis program.
           1. Make a new folder under “G:\Bioinformatics\Fusion\_Database\_Directory” using the name format MM.DD.YY\_fusbench-1 and copy the CSV files from the run directory into this folder.
           2. Open ResultsFusion.py under G:\Bioinformatics\MolDB\Fusion\_DB\_Graph\_Scripts\ResultsFusion.py on Spyder.
           3. Click on the green play button or press F5 to run the script.
           4. The program will ask the user to select files. Select the pre-review strong and low confidence CSV files from the folder created in Step 4a (i).
           5. This script will run FusionReadCountGrapher.py and FusionRead%Grapher.py to generate graphs and create hyperlinks to the graphs, NCBI as well as UCSC Genome browsers in All\_Fusions file.
           6. Name the file according to the following format: DDMMYYY\_FusionPlex\_ST\_All\_Fusions.
           7. Transfer the All\_Fusions file to the run directory.
        2. Run FusionImport.py, which imports the run data summarized and contained within the All\_Fusions file.
           1. Run the script under G:\Bioinformatics\MolDB\Fusion\_DB\_Graph\_Scripts\ FusionImport.py by repeating steps 4a(ii)-(vi)
           2. Select the All\_Fusions file to load hyperlinks to the database when asked.
  3. Export Read Statistics Data:
     1. **NOTE:** This step is performed by the Bioinformatician.
     2. On the RICMBLAB$ drive, navigate to the Fusion run folder.
        1. Create a subfolder called “ReadStatistics”.
     3. On the left side of the Archer Analysis program panel, click on **Read Statistics**.
        1. Click on **Export Data (TSV)** under DNA/RNA Statistics to export Read Statistics files to the ReadStatistics folder.
        2. Copy the file to the previously created ReadStatistics folder.
        3. Repeat this process for all applicable samples and controls.
     4. ReadStatsImport.py script imports the run statistics data into the SQLite database under RICMBLAB$\Bioinformatics\MolDB\Fusion.db in order to load the Read Statistics data to the database.
        1. Open Spyder on the desktop computer CO3MOLWIN10.
           1. Run the script under G:\Bioinformatics\MolDB\Fusion\_DB\_Graph\_Scripts\ ReadStatsImport.py
           2. Select the read statistics files when asked.
     5. Export Sample Reports:
        1. **NOTE:** This step is performed by the Bioinformatician or designee.
        2. On the RICMBLAB$ drive, navigate to the Fusion run folder.
           1. Create a subfolder labelled “Sample\_Reports”.
        3. On the left side of the Archer Analysis program, click **Sample Reports**.
           1. Click **Sample Summary**, which will create the PDF report.
           2. Click the download icon on the right side to download all files to the previously created Sample\_Reports folder.
           3. Repeat this process for all applicable samples and controls.
     6. Export RNA Expression Data:
        1. **NOTE:** This step is performed by the Bioinformatician or designee.
        2. On the RICMBLAB$ drive, navigate to the Fusion run folder.
           1. Create a subfolder labelled “RNA\_Expression”.
        3. On the right side of the Archer Analysis program, click **Job Options**.
           1. Click **Download Results**, click the **All** tab then open +**summaries** then mark the checkbox next to:

Summary.expression\_summary\_table

Summary.per\_sample\_columns\_expression\_summary\_table

* + - * 1. Under the All tab, open the **+4773** then mark the checkbox next to:

rna\_expression\_visualization\_0/1/2/3/4/5/6

rna\_expression\_visualization.pdf

rna\_expression\_visualization\_sample\_sheet.tsv

* + - 1. Once all the appropriate files have been marked, click **Download** on the top right of the window.
      2. Save all downloaded files to the previously created RNA\_Expression folder.
    1. Follow the steps below if using the FusionApp to generate the read statistics and Fusion Summary files:
       1. On the appropriate computer, navigate to the Bioinformatics folder on the RICMBLAB$ shared drive.
       2. Open the FusionApp folder and double click **FusionManager- Shortcut** to open the program.
       3. Login with the appropriate username and password, then click **Connect**.
       4. Click the ellipsis next to the Select Normal TSV files field.
       5. In the file finder, navigate to the TSV files saved in the Fusion run folder. Highlight all TSV files, then click **Open**.
       6. Click the ellipsis next to the Select Low Confidence TSV files field.
       7. In the file finder, navigate to the Low Confidence TSV files saved in the Fusion run folder. Highlight all Low Confidence TSV files, then click **Open**.
       8. Click the ellipsis next to the Select Read Statistics field.
       9. In the file finder, navigate to the XYZ files saved in the Fusion run folder. Highlight all Read Statistics files, then click **Open**.
       10. Click **Upload and Process**. Once complete, the comment “Files successfully processed” will appear.
       11. Once the files have been generated, verify the TSV\_Files subfolder contains the following documents:
           1. Fusion Summary: This should be moved to the parent Fusion run folder.
           2. readstatistics: This should be moved to the ReadStatistics subfolder.
       12. Rename the Fusion Summary file using the format [Worksheet#] \_Fusion\_Summary.
           1. Example: 06.21.22-fusbench-1\_Fusion\_Summary
  1. **BIOINFORMATICS REVIEW - TASKLIST**
     1. In Soft Molecular, open My Orders by using the icon on the dashboard.
     2. Click on the **Bioinformatician** tab.
     3. Click two times on tasklist number.
     4. Click on **No** button.
     5. In the tasklist, review the SAV Parameters for the run.
        1. A passing run on the NextSeq 500 shows:
           1. >= 75% of bases with a quality score of Q30
           2. >= 75% of Clusters Passing Filter
     6. Click the ‘**…**’ button to the right of the Import button.
     7. Click **OK**.
     8. Select the Excel radial button, choose the file path (directory and file name) for the Fusion\_Summary file and click **Import**.
     9. Repeat steps 5-7 for the readstatistics file.
     10. **NOTES**:
         1. Remember to import both files.
         2. If multiple tests are ordered on the same sample (e.g., sarcoma and lung panels), each test will show up on the tasklist as separate entries. When results are imported, they will only show up on the first test. Therefore, results must be manually copied to the additional test(s).
         3. If multiple test types are included on this run, separate sets of controls will be present on the tasklist for each test. However, the results will only be imported into the first control.
     11. Review fusions calls.
         1. Review all fusion calls for patient samples and controls by clicking the ‘+’ button to expand the child level results.
     12. Review QC Summary data for patient samples and controls by reviewing the “Avg Unique RNA SS per GSP2” and “Avg Unique RNA SS per GSP2 Ctrl” fields at the parent level.
     13. For each applicable patient sample, complete the 1st Bioinformatics action by marking the Completed checkbox located on the sample line.
     14. Select **Save**.
     15. Select **Back** in the Tasklist Entry window.
     16. Exit Soft Molecular application.
  2. After initial review by the bioinformatician, a second review will be performed by a pathologist, repeating Step B. above.
     1. Review Fusion Summary and All\_Fusions files.
     2. Guidelines to aid in fusion interpretation:
        1. In order to be called a strong fusion, the following criteria must be satisfied:
           1. Minimum number of reads: at least 5 breakpoint spanning reads that support the candidate.
           2. Percent GSP2: at least 10%
           3. Minimum unique start sites: Within the population of breakpoint spanning reads that support the candidate, there will be a distribution of unique start sites. There must be at least 3.
           4. Presence of fusion in Quiver (this over-rides all other criteria).
        2. Negative Evidence Criteria
           1. Exon-Intron Fusion
           2. Mispriming
           3. Paralogs
           4. Low Confidence
           5. Cross-Contamination
           6. Transcriptional Read-Through

**NOTE:** Transcriptional read-through events are placed in the STRONG category by default because by representing actual molecules produced in cells, they are technically not false positives.

* + - 1. For additional details, refer to the Archer Analysis User Manual.
    1. In Archer Analysis, mark the “Review Complete” box at the left side of the window or in the main Sample Summary page.

1. **Fusion Review Tool Interpretation:**
   1. Principle: This software is a Perl- and shell-script based tool that is used to review variants, review some of the read statistics info for the samples, and aid in the reporting of the results. Please see **Figure 7** below for the Archer QC flowchart.
      1. Fusion Review Tool has 2 modules:
         1. Fusion data module includes custom made Perl – and shell-scripts to:
            1. Parse, extract, add sample IDs from the CSV files to the list of fusions.
            2. Create a summary of all fusions which were marked as “Report”.
            3. Convert the summary file to Excel format.
         2. Read Statistics module includes custom made Perl-scripts to:
            1. Parse, extract Read Statistics data from CSV files.
            2. Create a summary file.
            3. Convert the summary file to Excel format.
   2. Data Processing:
      1. Start Putty on a Lifespan Windows-based computer to open a Terminal.
         1. Type the IP address for the Linux server, lsmplinux2 (10.229.9.25) on the “Host Name (or IP address)” window.
         2. Type “cd /molpath/Analysis\_Directory/Fusion\_Review\_Tool”
         3. Using WinSCP, transfer the TSV and Low Confidence TSV files in the run folder to the TSV\_Files and LC\_TSV\_Files folders, respectively.
         4. Type “sh ./FRT\_Command.sh” and click Enter.
            1. This will run the Perl- and shell-scripts to:

Parse, extract fusion data from the TSV files.

Add sample IDs to the list of fusions from the file names.

Create a summary file with all fusions which were marked as “Report” in Excel format.

* + - 1. Type “cd ..”
         1. This will change the directory to /molpath/Analysis\_Directory
      2. Using WinSCP, transfer the Fusion\_Summary file to the run folder and name it using the format [DDMMYYY]\_FusionPlex\_ST\_Summary\_Fusion.
      3. Using WinSCP, copy the Read Statistics file to the /molpath/Analysis\_Directory/Read\_Statistics folder
      4. Type “sh ./ReadStatisticsTool/readstatistics\_tool.sh”
         1. This will run the Perl-scripts to:

Parse, extract Read Statistics data from the TSV files.

Create a summary file in Excel format.

* + - 1. Using WinSCP, copy the “readstatistics” file to the ReadStatistics folder in the run folder. Rename the file using the format [DDMMYYY]\_FusionPlex\_ST\_ReadStatistics

1. **PATHOLOGIST REVIEW - TASKLIST**
   1. In Soft Molecular, open My Orders by using the icon on the dashboard.
   2. Click on the **Molecular Pathologist** tab.
   3. Click two times on tasklist number.
   4. Click on **No** button.
   5. In the tasklist, review the SAV Parameters for the run.
      1. A passing run on the NextSeq 500 shows:
         1. >= 75% of bases with a quality score of Q30
         2. >= 75% of Clusters Passing Filter
   6. Review fusions calls.
      1. Review all fusion calls for patient samples and controls by clicking the ‘+’ button to expand the child level results.
   7. Review QC Summary data for patient samples and controls by reviewing the “Avg Unique RNA SS per GSP2” and “Avg Unique RNA SS per GSP2 Ctrl” fields at the parent level.
      1. For each patient sample, in the Sample Quality Field, use the dropdown to select “Pass” or “Fail” based on the criteria:
         1. Average Unique RNA Start Sites Per GSP2 Control >= 10.0
      2. On the PreSeq assay, samples should have a Ct value of <30; however, this is only used as additional information and, by itself, should generally not be used to determine the Sample Quality result.
      3. Archer QC should be used to determine sample acceptability, especially when no Strong fusion calls are seen. However, if a Strong fusion call is made in a sample that fails the QC threshold, the fusion may be reported at the discretion of the pathologist with an interpretive comment about the QC results. Results should be interpreted with caution. If appropriate, testing may be repeated for the sample.
   8. For all fusion genes:
      1. To display the fusion on the report, mark the ‘Print on Report’ checkbox.
      2. Click the dropdown arrow in the “Report?” field.
         1. To display the fusion on the report, Select a Tier level of I-IV.
         2. For fusions that will not be reported, select “No” or “Artifact”, if appropriate.
         3. Tier designation is determined during the clinical interpretation review above. The following Fusion Tiering system shall be used:
            1. Tier I Fusions (Strong Clinical Significance)
            2. Tier II Fusions (Potential Clinical Significance)
            3. Tier III Fusions (Uncertain Clinical Significance)
            4. Tier IV Fusions (Benign or Likely Benign)
      3. If possible, ensure that variant nomenclature is consistent with Human Genome Variation Society (HGVS) guidelines and HUGO Gene Nomenclature Committee gene name. Reference transcript information should be included, as appropriate.
      4. The typical fusion call thresholds are:
         1. Number of unique start sites supporting the event: >= 5
            1. If the fusion call shows 3-5 start sites, is an otherwise Strong fusion call, is in an appropriate histology type, and all other QC criteria are appropriate, a call can be made with caution and a note on the report.
         2. Number of unique reads supporting the event: >= 5
         3. %Reads: There is not cutoff for this metric. However, >=5% can be used as a general guideline, which strong fusion calls showing <5% being evaluated on a case-by-case basis.
      5. Appropriate fusion calling practice and guidelines should be followed.
         1. For example, fusions may be manually visualized in Integrative Genomics Viewer or JBrowse to assess for artifacts, strand bias, and other sources of potential error.
   9. At the parent level, use the drop-down menu to select the Fusion Final Results for all patients and controls.
      1. **Note:** Controls must be resulted as pass or fail.
   10. For each applicable patient sample, complete the 2nd Bioinformatics action by marking the Completed checkbox located on the sample line, followed by **Save**. Then click **Edit** and repeat step 10 for the 1st Review and 2nd Review actions.
   11. **NOTES**:
       1. If multiple tests are ordered on the same sample (e.g., sarcoma and lung panels), each test will show up on the tasklist as separate entries. When results are imported, they will only show up on the first test. Therefore, results must be manually copied to the additional test(s).
       2. If multiple test types are included on this run, separate sets of controls will be present on the tasklist for each test. However, the results will only be imported into the first control.
       3. When samples have multiple tests, the controls need to be resulted separately.
   12. Select **Save**.
   13. Select **Back** in the Tasklist Entry window.
   14. Exit Soft Molecular application.
   15. After fusion calls are classified:
       1. Return to Archer Analysis and navigate to the current run.
       2. For each sample, perform the following steps:
          1. Select the **Strong Fusions and Oncogenic Isoforms** filter-set.
             1. Click on the **CSV** hyperlink at the bottom right of the table to export the filtered data to CSV\_Files\CSV\_2nd\_Strong folder in the run folder.
          2. Select the **Low Confidence Fusions** filter-set.
             1. Click on the **CSV** hyperlink at the bottom right of the table to export the filtered data to

CSV\_Files\CSV\_2nd\_Low folder in the run folder.

* + - * 1. Add “\_low\_confidence” at the end of the file name (i.e. Order#\_isoforms\_low\_confidence).
      1. On the left side of the Archer analysis program panel, click on “Sample Reports”.
         1. In the pop-up window, click on the “Sample Summary” hyperlink to open the PDF file. Save this file to the RICMBLAB$ shared drive Fusion run folder using the name format: Order#\_sample\_summary\_date\_time (When the file is downloaded, it automatically adds the date and time of download to the file name).

1. **TEST PROCEDURE – SIGN OUT ENTRY**
   1. Open My Orders by using the icon on the dashboard.
   2. Verify the **Molecular Pathologist** tab is displayed.
   3. Click two times on order.
   4. Click on **No** button.
   5. Verify whether RBS rules are triggered correctly (Result, Interpretation sections are filled appropriately).
   6. Overwrite Interpretation window may display.
      1. The pop-up window will display original text.
      2. Click **Skip** unless a new result was manually generated.
   7. In the Test Result tab, review the assay results.
      1. Make changes, as needed.
      2. **NOTE**: Review the Test Code to determine which Solid Tumor Fusion Panel was ordered. Classify and report only those results/genes included in that Panel.
      3. At the parent level, review the Fusion Final Results. If necessary, use the drop-down to change the result.
      4. **Note**: If the Final Result was changed, click on **Generate** button to fire reporting rules which populate interpretation sections with correct/updated data.
      5. Review and update the “Print on Report” and “Report?” fields, as appropriate.
   8. In the Final Test Specific Interpretation section:
      1. Update the Final Result comments.
      2. For each Tier I-IV that has a variant call, enter an interpretive comment in the appropriate section, indicating the clinical significance of each fusion.
         1. To aid in the interpretation, review sample information (specimen type, diagnosis, tumor percentage in the sample, etc.)
      3. NOTE: If there are any Tiers that do not have a variant call, leave the box blank. The Report will automatically indicate “NONE.”
      4. Check the QC and Methodology tab, as needed.
   9. Mark Completed checkbox.
   10. Select **Sign Out** button.
   11. Select **Sign Out** in the window that appears.
   12. Make sure that information on the report is correct or edit as needed.
   13. Complete sign out by selecting **Complete Sign Out** button.
   14. Select **Back** in Sign Out Entry.
   15. Exit Soft Molecular application.
2. **LIMITATIONS:**
   1. False positive or negative results may occur if there is low tumor content or genetic heterogeneity in the tumor.
   2. This is a targeted NGS assay designed to detect fusion genes. Therefore, the results do not exclude the possibility of other fusions or other alteration types that are not targeted by this assay.
   3. The Limit of Detection of the assay is:
      1. Input RNA: samples with as low as 40 ng RNA can be tested routinely. However, testing can be performed at the discretion of the Director/Pathologist for samples with below 40 ng RNA. In this case, sample QC should be carefully evaluated for determination of acceptability of the results.
      2. Fusion calling (Archer Analysis criteria):
         1. Start Sites: at least 5
            1. If a fusion call shows 3-5 Start Sites, is an otherwise Strong fusion call, is in an appropriate histology type, and all other QC criteria are appropriate, the call can be made with caution and a note on the report.
         2. Reads: at least 5.
         3. %Reads: a cut-off for positivity cannot be established for this metric; at least 5% is a general guideline.
   4. Paired germline sequencing is not routinely performed; therefore, this assay is not designed to distinguish somatic from germline variants.
   5. Deviations from procedure:
      1. For any assay runs that deviate from this Procedure or any samples that do not meet the acceptability criteria, indicate appropriately in Soft Molecular.
      2. Add this information, as well as any associated issue and/or corrective actions, to the NGS\_ExceptionLog.xlsx file on the RICMBLAB$ network drive. This information should be reviewed by a Director or designee, as appropriate.
3. **INTERFERING SUBSTANCES:**
   1. Hemoglobin and melanin are known to inhibit PCR amplification and may be present in clinical samples submitted for testing.
   2. If you suspect a PCR inhibitor is present in Formalin-fixed paraffin-embedded and other tissues, the following options may be attempted upon discussion with the Director:
      1. Run the sample as-is and evaluate sample QC after testing.Re-extract the sample using the organic extraction protocol found in the TNA Manual Extraction Procedure. Then, repeat testing.
      2. The sample can be diluted with water and testing can be repeated.
      3. The sample can be diluted with BSA and testing can be repeated. See the PCR Interfering Substances: BSA Method Procedure for additional details.
   3. Discuss with Director/Pathologist for other options, as appropriate for the potential inhibitor in a sample.
4. **REFERENCE RANGE:**
   1. The reference range for samples is wild type/negative for pathogenic fusions. Benign fusions may be identified and will be indicated as such on the clinical report.
5. **REPORTABLE RANGE:**
   1. All fusions otherwise identified may be reported, as clinically relevant and according to the Tier designation indicated on the clinical report.
6. **QUALITY MANAGEMENT PROGRAM**
   1. Refer to the NGS Quality Management Program document (Next-Generation Sequencing Infrastructure Policies) for the following policies and procedures:
      1. Monitoring, implementing, and recording upgrades to components of the assay (including wet bench and bioinformatics)
      2. Revalidation of the assay upon any changes made
      3. NGS wet bench processes
      4. NGS bioinformatics processes
      5. Variant database, reassessment, and reclassification
      6. Reporting of incidental findings
      7. Logging of positive results
   2. For the Fusion Solid Tumor assays, the controls, metrics, and quality control parameters used to monitor and assess each analytical run are described in the appropriate sections above.
   3. Refer to the Bioinformatics Policy document (NGS Infrastructure Policies) for information regarding:
      1. Management of NGS bioinformatics pipelines
      2. Versioning of pipelines
      3. Reference sequence utilized in the Lifespan pipelines
   4. Refer to the Data Management Policy document (NGS Infrastructure) for policies and procedures related to storage and transfer of electronic data relevant to this assay.
   5. Version Traceability
      1. For Fusion Solid Tumor assay runs in Soft Molecular, the Tasklist contains version number for software used.
7. **TROUBLESHOOTING:**
   1. For issues with networking (of the NextSeq and/or Lifespan computer), see Director or Bioinformatician.
   2. For issues related to bioinformatics, see Bioinformatician.
   3. Performing a System Check on the NextSeq.
      1. Refer to the NextSeq Instrument Procedure.
   4. Pausing a run:
      1. Refer to the NextSeq Instrument Procedure.
   5. Stopping a run:
      1. Refer to the NextSeq Instrument Procedure.
   6. Resolving RFID Read failures:
      1. Refer to the NextSeq Instrument Procedure.
   7. Regenerating FASTQ files:
      1. Refer to the NextSeq Instrument Procedure.
   8. For other Instrument troubleshooting concerns, refer to Appendix A of the MiSeq System Guide or contact technical support.
   9. For troubleshooting related to the FusionPlex kit, contact Asuragen technical support.
   10. TECHNICAL SUPPORT CONTACT INFORMATION:
       1. ArcherDX, Incorporated
          1. Phone: 1-877-771-1093
          2. Website: [www.archerdx.com](http://www.archerdx.com)
       2. KAPA Biosystems
          1. Phone: 1-855-527-2246
          2. Technical Support: [Support.SeqLS@roche.com](mailto:Support.SeqLS@roche.com)
       3. Illumina
          1. Phone: 1-800-809-4566
          2. Fax: 1-858-202-4766
          3. Sales and Info: [info@illumina.com](mailto:info@illumina.com)
          4. Technical Support: [techsupport@illumina.com](mailto:techsupport@illumina.com)
8. **REPEAT TESTING**
   1. During the testing process, testing for some samples must be repeated for a variety of technical or analytical reasons. The specimen can be sent back to one of multiple prior steps in the workflow.
   2. In Soft Molecular, repeating a sample to a prior action can be accomplished from a variety of steps in the test workflow and places in the system. Please see the Soft Molecular Rerun Procedure for the specific steps to perform when requesting rerun testing.
9. **REFERENCES:**
   1. Archer® FusionPlex® Protocol for Illumina, LA135.G (Released 06-06-2018).
   2. Archer® FusionPlex® Library Preparation: Best Practices and Troubleshooting Guide, APM011.A.
   3. Archer® Analysis User Manual, Software version 6.0 (Released 2019-03-22).
   4. Archer® Technical Note: PreSeq RNA QC Assay, APM029.A.
   5. Jennings LJ, et al. Guidelines for Validation of Next-Generation Sequencing-Based Oncology Panels: A Joint Consensus Recommendation of the Association for Molecular Pathology and College of American Pathologists. J Mol Diagn. 2017 May; 19(3):341-365.
   6. Winters JL, et al. Development and Verification of an RNA Sequencing (RNA-Seq) Assay for the Detection of Gene Fusions in Tumors. J Mol Diagn. 2018 July; 20(4):496-511.
   7. Illumina Sequencing Overview.
   8. NextSeq® 500 System Guide, Document # 15046563 version 02 (March 2016).
   9. NextSeq® System Denature and Dilute Libraries Guide, Document # 15048776 version 02 (January 2016).
   10. Illumina Sequencing Overview, Part # 15045845\_Rev.C (2013).
   11. Patel NR, Chrisinger JSA, Demicco EG, Sarabia SF, Reuther J, Kumar E, Oliveira AM, Billings SD, Bovée JVMG, Roy A, Lazar AJ, Lopez-Terrada DH, Wang WL. USP6 activation in nodular fasciitis by promoter-swapping gene fusions. Mod Pathol. 2017 Nov;30(11):1577-1588.
   12. Aziz N, et al. College of American Pathologists’ Laboratory Standards for Next-Generation Sequencing Clinical Tests. Arch Pathol Lab Med. 2015 Apr;139(4):481-93.
   13. Rehm H, et al. ACMG clinical laboratory standards for next-generation sequencing. Genet Med. 2013 Sep;15(9):733-47.
   14. Gargis AS, et al. Assuring the Quality of Next-Generation Sequencing in Clinical Laboratory Practice. Nat Biotechnol. 2012 Nov;30(11):1033-6.
   15. Li MM, Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. J Mol Diagn. 2017 Jan;19(1):4-23.
10. **REVISIONS:**
    1. 4/28/2021: Updated informatics steps and clarified naming conventions of Library Denature and Dilute tube.
    2. 7/6/2021: Added acceptable product information for TRIS-HCL and updated instructions post sequencing to ensure the correct sample ID is chosen so the correct lot number is selected.
    3. 11/18/2022: Updated several steps for Soft Molecular upgrade and updated analysis section to reflect new workflow.