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Stephanie R. Weldon, PhD
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Dear Dr. Weldon

Thank you for inviting us to publish our manuscript "Making the most of FFPE-RNA – optimization for sequencing and analysis of degraded FFPE-RNA samples" in the Journal of Visualized Experiments.

RNA sequencing has emerged as the leading method for gene expression analysis of different types of samples, providing valuable insights into cellular pathways determining disease and treatment outcomes. However, the most widely used method for preserving tissues in clinical settings generates Formalin-Fixed Paraffin-Embedded (FFPE) samples, that, often being highly degraded, fall short of the usual quality requirements for next-generation sequencing. In the attached manuscript, we describe a method for generating good quality data from such suboptimal samples. We list the various steps and precautions for sample quality control, sequencing library preparation, sequencing, and data analysis, that, taken together, increase the chances of reliable gene expression analysis from FFPE-RNA samples. We demonstrate this method using an example dataset of FFPE-RNA samples. We have highlighted parts of the protocol that can be explained better using the visual media.

Given the wide availability of FFPE tissues as samples, this method would be of broad interest to researchers trying to generate good quality gene expression data to understand the molecular mechanisms at work in various diseases.

The contents of this manuscript are original, and all authors of this paper have read and approved the final version.

Thank you for considering this manuscript. We look forward to your comments.

Best Regards,
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TITLE:

Optimization for sequencing and analysis of degraded FFPE-RNA samples

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KEYWORDS:

RNA sequencing, formalin-fixed paraffin embedded, FFPE, next generation sequencing, NGS, RNA-seq analysis

SUMMARY:

This method describes the steps to improve the quality and quantity of sequence data that can be obtained from formalin-fixed paraffin-embedded (FFPE) RNA samples. We describe the methodology to more accurately assess the quality of FFPE-RNA samples, prepare sequencing libraries, and analyze the data from FFPE-RNA samples.

ABSTRACT:

Gene expression analysis by RNA sequencing (RNA-seq) enables unique insights into clinical samples that can potentially lead to mechanistic understanding of the basis of various diseases as well as resistance and/or susceptibility mechanisms. However, FFPE tissues, which represent the most common method for preserving tissue morphology in clinical specimens, are not the best sources for gene expression profiling analysis. The RNA obtained from such samples is often degraded, fragmented, and chemically modified, which leads to suboptimal sequencing libraries. In turn, these generate poor quality sequence data that may not be reliable for gene expression analysis and mutation discovery. In order to make the most of FFPE samples and obtain the best possible data from low quality samples, it is important to take certain precautions while planning experimental design, preparing sequencing libraries, and during data analysis. This includes the use of appropriate metrics for precise sample quality control (QC), identifying the best methods for various steps during the sequencing library generation, and careful library QC. In addition, applying correct software tools and parameters for sequence data analysis is critical in order to identify artifacts in RNA-seq data, filter out contamination and low quality reads, assess uniformity of gene coverage, and measure the reproducibility of gene expression profiles among biological replicates. These steps can ensure high accuracy and reproducibility for profiling of very heterogeneous RNA samples. Here we describe the various steps for sample QC, library preparation and QC, sequencing, and data analysis that can help to increase the amount of useful data obtained from low quality RNA, such as that obtained from FFPE-RNA tissues.

INTRODUCTION:

Use of next-generation sequencing approaches has enabled us to glean a wealth of information from various types of samples. However, old and poorly preserved samples remain unworkable for the commonly used methods of generating sequence data and often require modifications to well-established protocols. FFPE tissues represent such a sample type that has been widely utilized for clinical specimens[1-3]. While FFPE preservation maintains tissue morphology, the nucleic acids in FFPE tissues usually exhibit a wide range of damage and degradation, making it difficult to retrieve the genomic information that may lead to important insights about molecular mechanisms underlying various disorders.

Gene expression data generated by RNA sequencing is often instrumental in studying disease and resistance mechanisms and complements DNA mutation analysis. However, RNA is more susceptible to degradation, making it more challenging to generate accurate gene expression data from FFPE tissues. Furthermore, because the wide availability and affordability of sequencing is relatively recent, older specimens were often not stored in conditions required to preserve RNA integrity. Some of the issues for FFPE samples include degradation of RNA due to embedding in paraffin, chemical modification of RNA leading to fragmentation or refractoriness to enzymatic processes required for sequencing, and loss of the poly-A tails, limiting the applicability of oligo-dT as a primer for reverse transcriptase[4]. Another challenge is the handling/storage of FFPE samples under suboptimal conditions, which may lead to further degradation of labile molecules such as RNA in the tissues[5]. This is especially relevant for older samples that may have been collected at a time when gene expression analysis by RNA sequencing was not anticipated for the samples. All these lead to decreased quality and quantity of the extracted RNA available for generating useful sequence data. The low probability of success, combined with the high cost of sequencing, has dissuaded many researchers from trying to generate and analyze gene expression data from potentially useful FFPE samples. Some studies in recent years have demonstrated the usability of FFPE tissues for gene expression analysis[2,6-9], albeit for fewer and/or more recent samples.

As a feasibility study, we used RNA extracted from FFPE tumor tissue specimens from three Residual Tissue Repositories from Surveillance, Epidemiology, and End Results (SEER) cancer registries for RNA sequencing and gene expression analysis[10]. Procured from clinical pathology labs, the FFPE tissues from high-grade ovarian serous adenocarcinomas were stored from 7–32 years under varying conditions before RNA extraction. Because in most cases these blocks had been stored in different sites for years without the expectation of any sensitive genetic analysis in the future, not much care had been taken to preserve the nucleic acids. Thus, most of the samples exhibited poor quality RNA, with a large proportion of samples contaminated with bacteria. Nevertheless, we were able to perform gene quantification, measure the uniformity and continuity of gene coverage, and perform the Pearson correlation analysis among biological replicates to measure reproducibility. Based on a set of key signature gene panel, we compared the samples in our study with The Cancer Genome Atlas (TCGA) data and confirmed that approximately 60% of the samples had comparable gene expression profiles[11]. Based on the correlation between various QC results and sample metadata, we identified key QC metrics that have good predictive value for identifying samples that are more likely to generate usable sequence data[11].

Here we describe the methodology used for FFPE-RNA quality assessment, generation of sequencing libraries starting from extracted RNA samples, and bioinformatic analysis of the sequencing data.

PROTOCOL:

1. RNA quantity and quality assessment

1.1. Select the FFPE samples according to predefined criteria and extract RNA using an appropriate method (e.g., FFPE-nuclei acid extraction kit, **Table of Materials**).

NOTE: There are several different methods available for FFPE-RNA extraction, including the newer microdissection methods that can work with very little tissue and extract good quality RNA[12-14].

1.2. Utmost care should be taken to preserve the integrity of RNA at all stages. This includes working with RNase free deionised water, using RNase free plasticware, and cleaning all instruments that come in contact with the FFPE blocks with RNase decontamination reagents.

1.3. RNA should always be handled carefully and kept in ice unless otherwise specified to minimize degradation while handling.

1.4. If enough material is available, extract RNA from more than one region in the FFPE block to generate biological replicates from as many samples as possible. For some of the samples with ample RNA yield, divide the extracted RNA into two to process as technical replicates.

1.5. If possible, collect a small amount of sample separately after extraction for QC (i.e., a QC aliquot) to avoid repeated handling and freeze-thaw cycles of the sample that will likely lead to degradation of the RNA.

1.6. Check the quality of the RNA (preferably from the QC aliquot) by running it on an RNA QC system (e.g., Agilent Bioanalyzer system using an RNA Nano chip, **Table of Materials**) according to the manufacturer's instructions.

1.7. Analyze the distribution of RNA fragments in the samples (e.g., using the Bioanalyzer 2100 Expert software) by calculating the DV_{200} and DV_{100} values as the percent of fragments larger than 200 nt (DV_{200}) or 100 nt (DV_{100}) in size.

1.8. Among DV_{200} and DV_{100}, identify the metric that has a larger spread of values for the given sample set, and pick that for grouping the samples according to their degree of intactness.

NOTE: For sample sets with more intact RNA molecules (i.e., high DV_{200} values, all or most with DV_{200} > 40%), DV_{200} is likely to be a useful QC metric. However, for sample sets with more degraded transcripts (i.e., low DV_{200} values, all or most with DV_{200} < 40%), DV_{100} is more likely to be useful.

1.9. Based on the QC metrics, identify the samples that have DV_{100} < 40%. Because this degree of degradation is highly likely to not generate useful sequencing data[11], it is advisable to avoid processing such samples. If replacements for such samples are available, their quality should be checked to ideally only include samples with DV_{100} > 50%.

2. Sequencing library preparation

2.1. Based on the quality of the samples as assessed in section 1, identify an appropriate method for generating the sequencing libraries.

2.1.1. For sample sets with very low degradation and high DV_{200} values, use mRNA sequencing (i.e., capture of polyadenylated transcripts), targeted RNA sequencing (i.e., use of capture probes for specific genes of interest), RNA exome sequencing (i.e., use of capture probes to enrich for the coding transcriptome), or total RNA sequencing (i.e., use of random primers for reverse transcription to sequence the entire RNA population after removing ribosomal RNA from the samples). However, it is important to note that the fixation process may introduce bias in the extracted RNA. Thus, the capture approaches may not work well in all cases, even with high DV_{200} values.

2.1.2. If the sample set includes samples with high degradation (DV_{200} < 30%), use a total RNA library preparation method and not one that depends on the capture of specific regions of the transcripts, because those specific regions may be missing in degraded samples. The use of random primers for generation of cDNA leads to higher representation of usable RNA in the final library, and is, therefore, more suited for FFPE-RNA samples.

2.1.3. For ribosomal RNA depletion for sample sets with high degradation, use RNaseH-based methods. These are methods where rRNA-specific DNA probes bind to rRNA, double-stranded molecules are digested by RNaseH, and leftover probes are cleaned up by DNase (e.g., NEBNext rRNA depletion kit, **Table of Materials**). These methods work better for degraded samples than some other methods[8].

2.2. For generating sequencing libraries, use higher input amounts (if possible) for samples that have more degraded RNA (DV_{100} < 60%). While samples with reasonably good quality RNA (DV_{100} > 60%) may yield good sequence data even at lower input amounts (the lowest tested for this protocol with FFPE-RNA was ~20 ng), for more degraded RNA (DV_{100} < 60%), it is better to start with higher input amounts (e.g., >100 ng).

NOTE: If enough (e.g., >500 ng) sample is available, it is advisable to save at least half of the sample for repeating the library preparation, if needed. For low input samples (e.g., <100 ng), it is usually better to use the entire amount and generate a library of sufficient diversity.

2.3. After selecting a suitable library preparation kit for generating total RNA seq libraries from samples with high degradation (e.g., NEBNext Ultra II RNA Library Prep Kit for Illumina, see **Table of Materials**), follow the manufacturer’s instructions to generate the libraries.

NOTE: During library preparation, it is important to skip the RNA fragmentation step for degraded samples and to ensure the use of random primers for first strand cDNA synthesis.

2.4. For improving the efficiency and speed, especially for the low-input samples, use appropriate magnetic racks with strong fixed magnets for bead-based purification and size-selection steps (see **Table of Materials**).

2.5. For PCR enrichment of adapter ligated DNA, adjust the number of amplification cycles based on the amount of input DNA to ensure maximum representation while avoiding unnecessary duplication of the library molecules. For low input FFPE-RNA samples (<100 ng), we recommend 16–18 amplification cycles, while the high input samples (1,000 ng) usually generate enough library amounts in 12–14 rounds of amplification.

2.6. Following PCR amplification and cleanup per the manufacturer’s instructions, assess the library quality by analyzing library concentration and molecule distribution on an appropriate platform (e.g., Agilent Bioanalyzer DNA Chip, see **Table of Materials**). For samples with primer peaks (~80 bp) or adapter-dimer peaks (~128 bp), repeat the cleanup to remove those peaks.

2.7. Calculate the average library size for each library (e.g., using the Bioanalyzer 2100 Expert software).

3. Sequencing library QC

3.1. Once it has been ascertained that the libraries are free of excess primer and adapter-dimers and have sufficient concentration for subsequent sequencing, quantitate further by qPCR.

NOTE: Owing to the sensitivity of cluster generation towards library concentration, accurate quantification is vital to prevent costly sequencing runs from underperformance or overloading. Quantitative real-time PCR (qPCR) methods are useful for improving cluster density on Illumina platforms without resulting in overclustering. The qPCR method is more precise and more sensitive than the methods based on qualitative and/or quantitative analysis of all library molecules (e.g., Agilent Bioanalyzer), because it measures the templates that have both adapter sequences on either end that will form clusters on the flowcell. Library size must, however, be known in advance as a size correction must be applied to all samples so that results can be compared against a standard curve.

CAUTION: Lab coats and gloves must always be worn when performing qPCR, and the procedure must be performed in a biosafety cabinet following the manufacturer’s instructions.

3.1.1. Set up a 96 well plate with three replicates for each sample for error prevention using a suitable kit (e.g., KAPA SYBR FAST qPCR Master Mix for Illumina libraries, a part of Library Quantification kit, see **Table of Materials**), along with the standards, a positive control (e.g, PhiX control, see **Table of Materials**), and a no template control (NTC). The NTC is qPCR mix without DNA library. The positive control can be any library with known concentration and fragment size.

3.1.1.1. Prepare a minimum of six dilutions of the standards following the vendor protocol.

3.1.2. After adding all the components (i.e., qPCR master mix, libraries, standards), cover the plate with sealing film and use a squeegee to ensure the film makes even and secure contact with the plate.

3.1.3. Vortex and spin down the plate at 1,500 rpm for at least 1 min. Visually inspect the plate to make sure there are no air bubbles at the bottom of the wells.

3.1.4. Set up the plate on the thermal cycler (e .g. CFX96 Touch System, see **Table of Materials**) using the manufacturer's recommended settings.

3.1.5. Save the run folder where it can be accessed for data analysis.

3.1.6. During data analysis, check that the slope is in the -3.1 to -3.6 range, efficiency from 90% to 110% and the R^2 (coefficient of correlation obtained for the standard curve) no less than 0.98.

3.2. **Pooling**: Once the qPCR concentration of the sequencing ready libraries is obtained, pool equimolar amounts of each of the libraries, depending on the number of sequencing reads required per sample and the sequencing output of the instrument.

3.3. **QC of the pools**: Quantitate the library pools again by qPCR following the same protocol as described in step 3.1.

4. **Sequencing**

4.1. Depending on the run parameters, pull the sequencing reagent kits and thaw them following the user guide. Please check the Illumina website for the latest versions of all user guides for sequencing on Illumina instruments.

4.2. Make sure the reagents are completely thawed and place the reagents tray at 4 °C. The run should be started no later than 2 h after the reagents have been defrosted. Not doing that could affect quality of the run results.

4.3. Invert the cartridge 5x to mix reagents and gently tap on the bench to reduce air bubbles.

4.4. Set the unwrapped flow cell package aside at room temperature for 30 min.

4.5. Unwrap the flow cell package and clean the glass surface of the flow cell with a lint-free alcohol wipe. Dry the glass with a low-lint laboratory tissue.

4.6. Open the Illumina "**Experiment Manager**" application. Choose "**Create Sample Sheet**", then choose the **Sequencer** and click "**Next**".

4.7. Create and upload the sample sheet based on Illumina sequencer criteria (e.g., Illumina Experiment Manager, software guide).

4.8. At the prompts, scan in the reagent kit barcode and enter the run **Set Up Parameters** (e.g., for a single indexed PE 75 cycle run, enter **76-8-76**).

4.9. Denature and dilute the library pool based on the sequencer user guide recommendation (e.g., NextSeq 500 System guide from Illumina, see **Table of Materials**).

4.10. Denature and dilute the control library PhiX (see **Table of Materials**) to the appropriate concentration (e.g., 1.8 pM for NextSeq).

4.11. Mix sample library and PhiX control to result in a 1% PhiX control volume ratio.

4.12. Load denatured and diluted sample into the reagent cartridge in the designated reservoir.

4.13. Load the flowcell, buffer cartridge, and the reagent cartridge.

4.14. Perform an automated check and review to ensure that the run parameters pass the system check.

4.15. When the automated check is complete, select **Start** to begin the sequencing run.

5. Data analysis and quality assessment

NOTE: A typical RNA-seq data analysis workflow (**Figure 1**) includes preprocessing and QC, alignment to genome and post alignment QC, gene and transcript quantification, sample correlation analysis, differential analysis between different sample groups, treatment conditions, and gene set enrichment and pathway analysis.

The RNA-seq data may have quality issues that can affect the accuracy of gene profiling and lead to erroneous conclusions. Therefore, initial QC checks for sequencing quality, contamination, sequencing coverage bias, and other sources of artifacts are very important. Applying an RNA-Seq QC pipeline similar to the workflow described here is recommended to detect artifacts and apply filtering or correction before downstream analysis.

5.1. Preprocessing

NOTE: This includes demultiplexing, assessment of sequence read quality, GC content, presence of sequencing adapters, overrepresented *k*-mers, and PCR duplicated reads. This information helps to detect sequencing errors, PCR artifacts, or contamination.

5.1.1. Demultiplex Illumina sequencing run using the Illumina software tool **bcl2fastq2** to generate raw FASTQ files for each sample defined in the sample sheet. Allow one mismatch in the sample index barcodes to tolerate sequencing errors if there is no barcode collision.

5.1.2. Run the **FASTQC**[15] software tool to perform a quality check on raw FASTQ files to detect any poor quality or abnormalities in sequencing reads.

5.1.3. For adapter and low-quality bases trimming, trim the sequencing adapters and low quality bases using **Cutadapt**[16] or **Trimmomatic**[17] software tools. Save the trimmed reads in the pair-end fastq files.

5.1.4. Contamination screen

5.1.4.1. Run **FASTQ_screen**[18] to detect possible cross contamination with other species.

5.1.4.2. Run **miniKraken** of Kraken2[19] to identify the taxonomies of contaminating species.

5.2. Alignment to reference genome and post alignment QC

5.2.1. The trimmed reads can be aligned to a reference genome sequence (GRCh Build hg19 or hg38) using STAR aligner[20]. Apply the Gencode annotation GTF file to guide the spliced transcript alignment. It is recommended to run **STAR 2-pass** to increase sensitivity to novel splice junctions. In the second pass, all reads will be remapped using annotated gene and transcripts and novel junctions from the first pass.

5.2.2. Perform post-alignment QC.

5.2.2.1. Run Picard's[21] **MarkDuplicates** to evaluate the library complexity by determining the amount of unique or nonduplicated reads in the samples.

5.2.2.2. Run Picard's **CollectRnaSeqMetrics** program to collect mapping percentages on coding, intronic, intergenic, UTR regions, and gene body coverage.

5.2.2.3. Run **RSeQC**[22] to determine the read pair inner distance, read distribution among CDS exons, 5'UTR, 3'UTR, intron, TSS_up_1kb, TSS_up_5kb, TSS_up_10kb, TES_down_1kb, TES_down_5kb, TES_down_10kb, read GC content, junction saturation, and library strand information.

5.2.2.4. Run **multi-QC**[23] to generate an aggregated report in HTML format.

5.3. Gene quantification and correction analysis

5.3.1. Run **RSEM**[24] to get raw count as well as normalized read count on genes and transcripts. The read count measurement such as RPKM (reads per kilobase of exon model per million reads), FPKM (fragments per kilobase of exon model per million mapped reads), and TPM (transcripts per million) are the most often reported RNA-seq gene expression values. Genes expressed below a noised threshold (such as TPM < 1 or raw count <5) can be filtered.

5.3.2. Perform transcript quantification to aggregate raw counts of mapped reads to each transcript sequences using programs such as HTSeq-count or featureCounts.

5.3.3. Run **Principal Components Analysis** (PCA) using an **R script** to determine batch effects and assess a quality map of the given dataset[25]. Sample correlation analysis can be carried out using the Pearson correlation between different metrics.

5.4. **Differential gene expression analysis**

5.4.1. Perform gene differential analysis between sample conditions using the program **edgeR**[26,27] and/or **limma-Voom**[28] and use normalization methods including **TPM**, **TMM**, **DESeq**, or **UpperQuartile**.

5.4.2. It is recommended to run at least two differential analysis software tools in order to call two set of DEGs lists for comparison and get the final DEGs to improve detection sensitivity and accuracy.

5.5. **Gene set enrichment and pathway analysis**

5.5.1. Perform **Gene Set Enrichment Analysis** (GSEA)[29,30] based on ranking of transcripts according to a measurement of differentially expressed genes (DEGs) list to determine if the DEGs show statistically significant, concordant differences between biological conditions.

5.5.2. Perform function analysis using resources such as **Gene Ontology**[31], **DAVID**[32,33], or other available software tools.

REPRESENTATIVE RESULTS:

The methodology described above was applied to 67 FFPE samples that had been stored under a variety of different conditions for 7–32 years (the median sample storage time was 17.5 years). The dataset and analysis results presented here were previously described and published in Zhao et al.[11]. On checking the sample quality as described earlier (i.e., example traces in **Figure 2**), DV_{100} was found to be more useful than DV_{200} because it is more sensitive to accurately measure the proportion of smaller fragment sizes for highly degraded RNA samples.

In the given sample set, fewer than 10% of the samples (7 of 67) were above the DV_{200} cut off of 30%, as recommended by Illumina[34]. About 26% of the samples (19 of 67) had a DV_{100} > 60% (i.e., higher likelihood of generating good sequence data), 40% (27 of 67) were in the 40%–60% range for DV_{100} (i.e., acceptable, but with a lower likelihood of generating good sequence data), and about 10% (7 of 67) had a DV_{100} of <40% (i.e., very low likelihood of resulting in good sequence data). For 14 of 67 samples, the software was unable to determine the DV values. **Table 1** shows a summary of QC metrics for the samples in different DV_{100} categories. For detailed QC analysis and data correlation for all 67 samples, please see Zhao et al.[11].

Given the high degree of degradation in the sample set, a 'total RNA' library preparation method was chosen, and sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (**Table of Materials**). In order to improve the representation of the sequencing libraries in spite of the high degree of sample degradation, the maximum possible amount of RNA (1,000 ng when available) was used as input for library preparation. Additionally, the high degradation of the FFPE-RNA samples necessitated the rRNA depletion method, because the degraded transcripts were likely to not have the poly-A tails for mRNA capture. Following the depletion of ribosomal RNA by hybridization to specific probes and digestion of the hybridized transcripts using RNaseH, the remaining transcripts were converted into cDNA using random primers. Size selection was also avoided for libraries prepared from lower input samples. Example traces of final libraries are shown in **Figure 3**.

Highly degraded FFPE samples represent a great challenge for gene expression profiling in tumor samples. Thus, applying correct bioinformatics analysis methods and software tools is critical to detect artifacts or abnormalities in datasets to ensure high accuracy and reproducibility of gene quantification. The software tools used in this study are listed in the **Supplementary Table**. In the given sample set, we performed sequencing and library quality assessment, with some example metrics shown in **Figure 4**. An overview of raw fastq file sequencing quality and sample adapter content are shown in **Figures 4A** and **4B**, respectively. Fastqc screen can help detect contamination, such as bacterial and mouse contamination, in the samples as shown in **Figure 4C**. In the given sample set, 41 of 67 samples had 5%–48% bacterial contamination, and six samples had 4%–11% mouse contamination (**Figure 4C**). STAR alignment results (**Figure 4D**) showed the proportion of reads mapped to the reference genome, percentage of reads uniquely mapped to the reference genome, and proportion of reads that were not mapped or mapped to multiple loci. Picard CollectRNAStatistics was used to determine the percent mRNA, intronic, and intergenic bases present in the alignment files (**Figure 4E**). In order to assess the uniformity of read coverage on gene and transcripts, we used the Picard software tool to generate a gene body coverage plot, which measures the percentage of reads that cover each nucleotide position of all genes scaled into bins from 5′ UTR to 3′ UTR. **Figure 4F** shows that some degraded libraries had 3′ bias, where more reads are mapped closer to 3′ end than to the 5′ end.

FFPE samples usually have large variability in gene expression profiles that may arise due to variable degradation during sample storage, RNA extraction, or sample processing. It is important to use appropriate statistical methods to uncover the underlying patterns and measure the variation and correlation among samples. We applied Principal Component Analysis (PCA) for six pairs of biological replicates from a subset of the 67 FFPE samples. A PCA plot showed that 26% of total variation was captured by the first principal component and 19% from the second and third components combined (**Figure 5**). Among the six pairs of replicates, two pairs of replicates had higher variations (correlations below 0.22) than the last four samples (correlation values between 0.7–0.8) when comparing gene expression values between the replicate pairs. Because the replicates were generated by extracting RNA from two different tissue curls cut from the same FFPE blocks, the tissue age was not a factor in the higher variance here, and it was likely caused by the different amount of bacterial contamination (1%–55%) as well as different mRNA content (2–3 fold difference) between the replicates. The randomness of mRNA degradation after extraction could also contribute to the higher variance between samples of similar origin.

FIGURE AND TABLE LEGENDS:

Figure 1: RNaseq analysis workflow. The flowchart describes the analysis steps for preprocessing, quality assessment, mapping to reference, gene quantification, and differential analysis between different sample groups.

Figure 2: Example Bioanalyzer traces of six different FFPE-RNA samples. The horizontal axis denotes the molecular weight (bp) and fluorescence units (FU) and the vertical axis shows the concentration of different sized fragments. The RNA Integrity Numbers (RIN), DV_{200} (i.e., percent of fragments >200 bp), and DV_{100} (i.e., percent of fragments >100 bp) values are indicated on each profile. A 25 bp peak in each profile indicates the molecular weight marker.

Figure 3: Example Bioanalyzer traces of final libraries prepared from four different samples. The horizontal axis denotes the molecular weight (bp) and fluorescence units (FU) on the vertical axis indicate the concentration of different sized fragments. The lower (35 bp or 50 bp) and upper (10,380 bp) marker peaks are labeled in green and purple, respectively.

Figure 4: Example multi-QC report for preprocessing QC results. (**A**) Line chart showing the percentages of Q30 bases of all sequencing reads in each sample. (**B**) Sequencing adapter content in raw fastq files. (**C**) Contamination screen to check closely matched species. (**D**) Genome mapping statistics. (E) Read distribution based on Gencode gene annotation. (**F**) Gene body/transcript coverage

Figure 5: Example PCA analysis to show sample group concordance. PCA analysis for biological replicates. PCA plot with samples plotted in two dimensions using their projections onto the first two principal components. Biological replicates are shown in the same color.

Table 1: Summary of sample set QC metrics. The table shows the QC metrics of the samples, grouped according to their DV_{100} values. The number of samples in each group is listed, and median values for each metric are shown.

Supplementary Table: Analysis software tools, parameters, and software reference. The table lists the analysis software tools and parameters used in each step of the RNA-seq analysis. The software tool references are listed in the table.

DISCUSSION:

The method described here outlines the main steps required to obtain good sequence data from FFPE-RNA samples. The main points to consider with this method are: (1) Ensure that the RNA is preserved as best as possible after extraction by minimizing the sample handling and freezing and thawing cycles. Separate QC aliquots are very helpful. (2) Use a QC metric that is best for the given sample set. RIN values and DV_{200} are often not useful for degraded samples, and DV_{100} may be the metric of choice to assess the quality in a given sample set. (3) For more degraded samples, it is best to use a high sample input. Higher input amounts lead to better diversity and lower duplication in the final library, leading to improved data quality. Because not all RNA in FFPE-RNA samples is usable due to high degradation and refractoriness to enzymatic processes, these effects are more pronounced in FFPE-RNA compared to fresh frozen RNA. (4) Use random priming for the reverse transcription step as opposed to the use of oligo-dT or specific sequences as primers. Unless the set of specific probes is able to cover as much sequence as possible for all transcripts of interest, random primers are a safe bet to ensure the conversion of a maximum number of transcripts (or fragments thereof) into cDNA. Thus, total RNA library prep methods are more useful for degraded samples than mRNA methods, which rely on the presence of poly-A tails. (5) Accurate quantification of libraries by quantitative real-time PCR (qPCR) is important to avoid underperformance or overloading of the sequencers. (6) Assess potential contamination of the RNA as part of the standard post sequencing RNA-Seq QC protocols. Bacterial contamination and genomic DNA contamination are common for FFPE samples due to storage conditions and sample preparation procedures. Samples contaminated with foreign species can waste sequencing coverage, depending on the extent of contamination. In addition, internal contamination can arise from incomplete rRNA depletion, leading to a high percentage of reads mapping to rRNAs. Inefficient genomic DNA removal during DNase digestion could lead to false positive expression detection of transcripts or erroneous de novo assembly of transcripts. Adapter contamination introduced during library preparation is also a common problem for highly degraded RNAs with very short RNA fragments. Contamination can affect the gene and transcript profiling accuracy and lead to false discovery. Therefore, it is important to accurately identify the contamination sources and remove the contamination, if possible, during the sample or library preparation steps, or filter the contaminating reads during the data processing step. (7) Preprocessing and post-alignment quality control are important to detect bad quality and low mRNA content samples. Those samples should be eliminated from further analysis. Gene expression data from samples that generate low gene counts, poor coverage should be used with caution. (8) It is good practice to include biological replicates in order to measure samples variance and correlation to ensure data reproducibility.

FFPE samples represent a very valuable resource for a large number of diseases. The ability to obtain reliable sequence information from such samples would aid a lot of studies aimed at understanding the molecular mechanisms behind various disorders, resistance, and susceptibility. Though the limitations imposed by the frequently suboptimal quality of RNA extracted from such samples do hamper such efforts, the steps described here help to mitigate those limitations to some extent and enable us to make the most of FFPE-RNA to obtain reliable gene expression information.

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We are thankful to Dr. Danielle Carrick (Division of Cancer Control and Population Sciences, National Cancer Institute) for continued help, especially for initiating this study, providing us with the samples, and for helpful suggestions during data analysis. We sincerely thank all members of the CCR Sequencing Facility at the Frederick National Laboratory for Cancer Research for their help during sample preparation and sequencing, especially Brenda Ho for assistance in sample QC, Oksana German for library QC, Tatyana Smirnova for running the sequencers. We also would like to thank Tsai-wei Shen and Ashley Walton at Sequencing Facility Bioinformatics Group for helping with data analysis and RNA-seq pipeline implementation. We also thank CCBR and NCBR for assistance with RNaseq analysis pipeline and best practices development.

DISCLOSURES:

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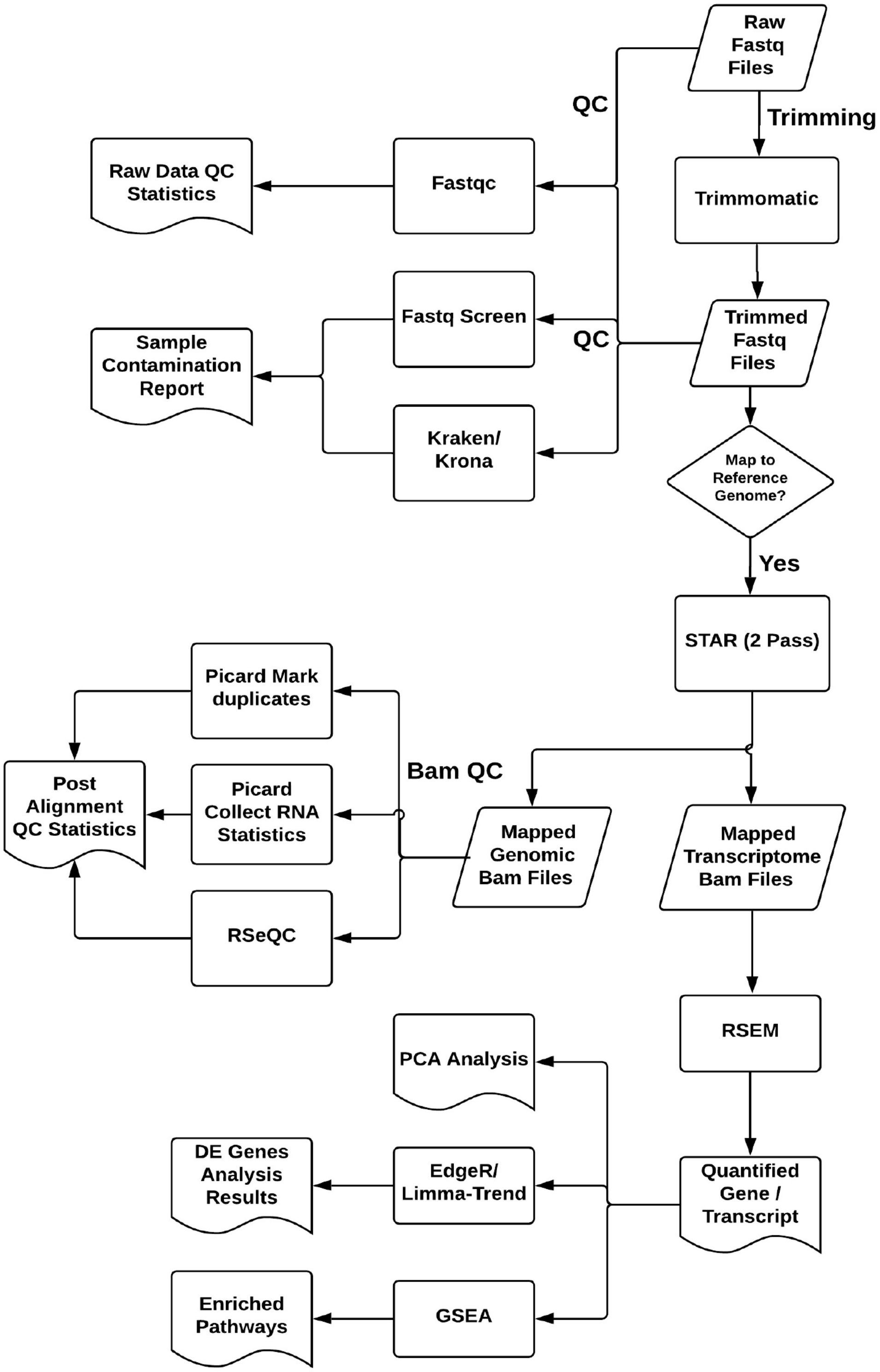
Raw Fastq Files
QC
Trimming
Raw Data QC Statistics
Fastqc
Trimmomatic
Fastq Screen
QC
Trimmed Fastq Files
Sample Contamination Report
Kraken/ Krona
Map to Reference Genome?
Yes
STAR (2 Pass)
Picard Mark duplicates
Bam QC
Post Alignment QC Statistics
Picard Collect RNA Statistics
Mapped Genomic Bam Files
Mapped Transcriptome Bam Files
RSeQC
RSEM
PCA Analysis
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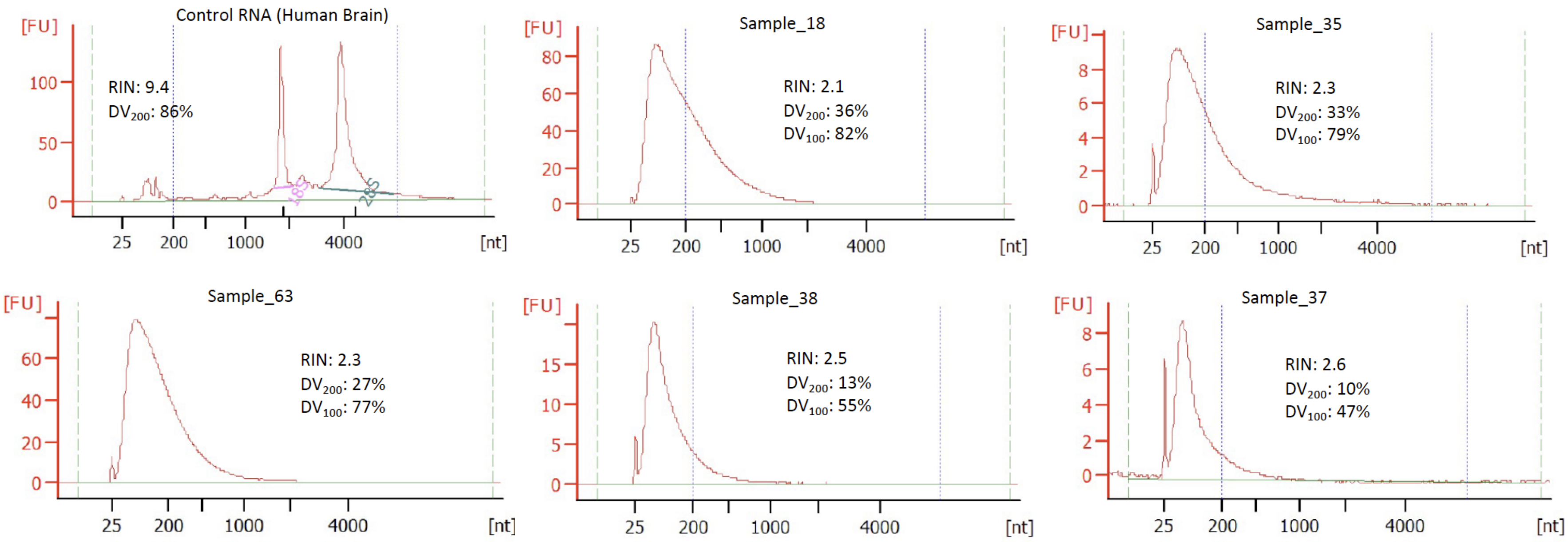
EdgeR/ Limma-Trend
Quantified Gene / Transcript
Enriched Pathways
GSEA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DV200: 86%
18S
28S
25
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1000
4000
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[FU]
80
60
40
20
0
RIN: 2.1
DV200: 36%
DV100: 82%
25
200
1000
4000
[nt]
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[FU]
8
6
4
2
0
RIN: 2.3
DV200: 33%
DV100: 79%
25
200
1000
4000
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60
40
20
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DV200: 27%
DV100: 77%
25
200
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4000
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15
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5
0
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DV200: 13%
DV100: 55%
25
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1000
4000
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Sample_37
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DV100: 47%
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1000
4000
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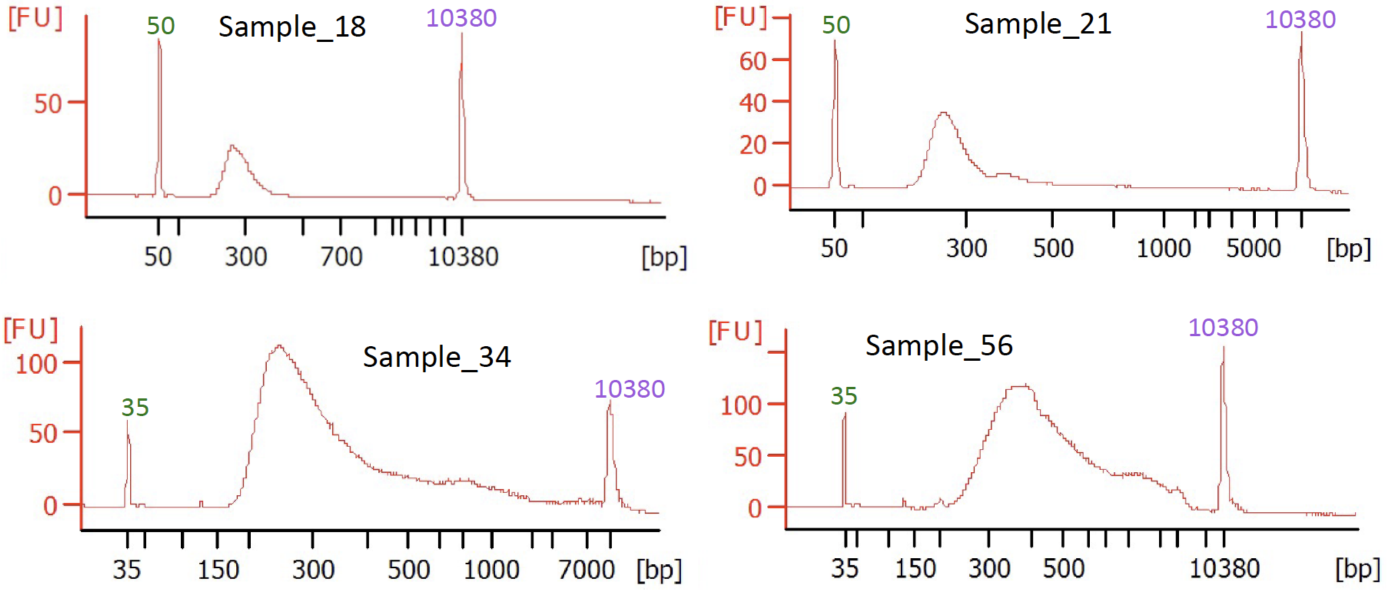
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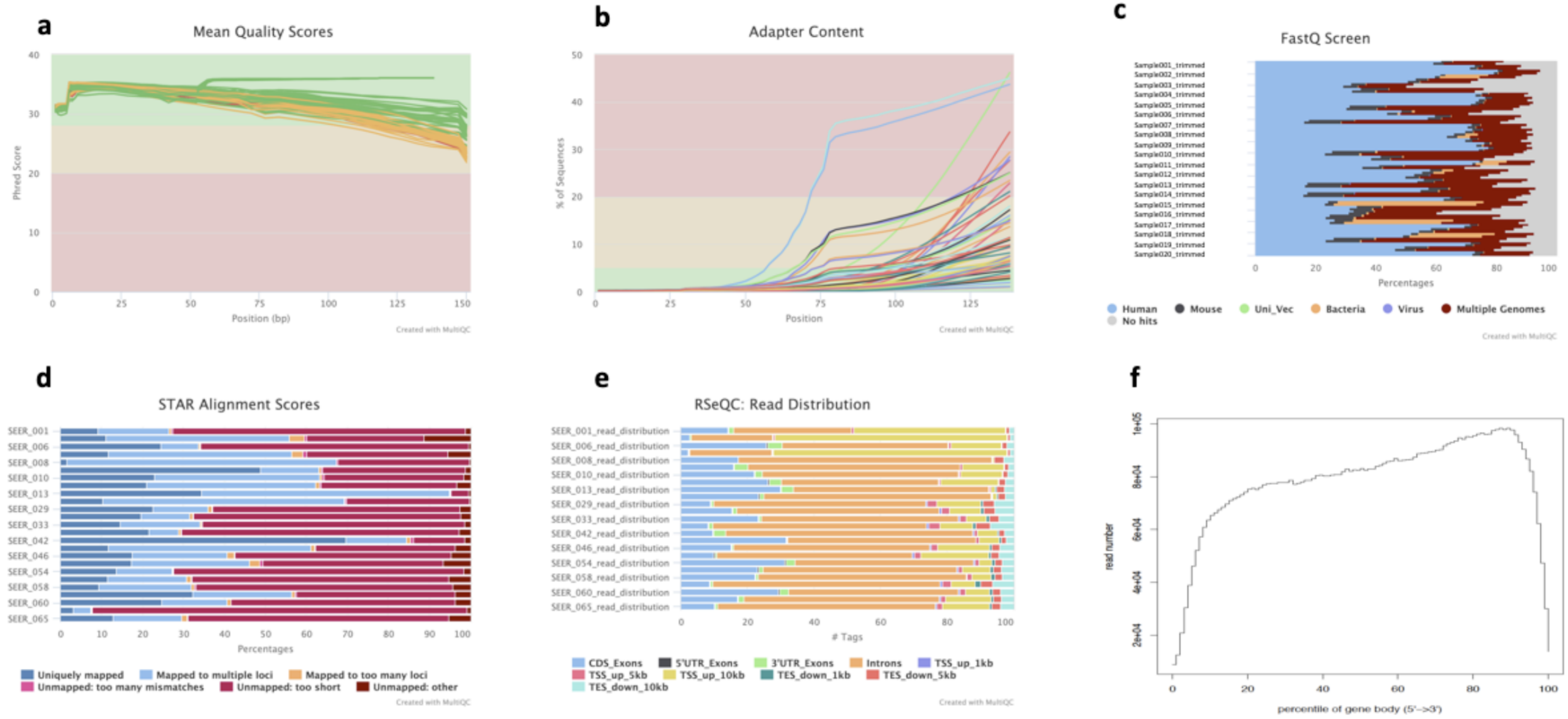
a
Mean Quality Scores
Phred Score
0
10
20
30
40
0
25
50
75
100
125
150
Position (bp)
Created with MultiQC
b
Adapter Content
% of Sequences
0
10
20
30
40
50
0
25
50
75
100
125
Position
Created with MultiQC
c
FastQ Screen
Sample001_trimmed
Sample002_trimmed
Sample003_trimmed
Sample004_trimmed
Sample005_trimmed
Sample006_trimmed
Sample007_trimmed
Sample008_trimmed
Sample009_trimmed
Sample010_trimmed
Sample011_trimmed
Sample012_trimmed
Sample013_trimmed
Sample014_trimmed
Sample015_trimmed
Sample016_trimmed
Sample017_trimmed
Sample018_trimmed
Sample019_trimmed
Sample020_trimmed
0
20
40
60
80
100
Percentages
● Human
● Mouse
● Uni_Vec
● Bacteria
● Virus
● Multiple Genomes
● No hits
Created with MultiQC
d
STAR Alignment Scores
SEER_001
SEER_006
SEER_008
SEER_010
SEER_013
SEER_029
SEER_033
SEER_042
SEER_046
SEER_054
SEER_058
SEER_060
SEER_065
0
10
20
30
40
50
60
70
80
90
100
Percentages
■ Uniquely mapped
■ Mapped to multiple loci
■ Mapped to too many loci
■ Unmapped: too many mismatches
■ Unmapped: too short
■ Unmapped: other
Created with MultiQC
e
RSeQC: Read Distribution
SEER_001_read_distribution
SEER_006_read_distribution
SEER_008_read_distribution
SEER_010_read_distribution
SEER_013_read_distribution
SEER_029_read_distribution
SEER_033_read_distribution
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SEER_058_read_distribution
SEER_060_read_distribution
SEER_065_read_distribution
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20
40
60
80
100
# Tags
■ CDS_Exons
■ 5'UTR_Exons
■ 3'UTR_Exons
■ Introns
■ TSS_up_1kb
■ TSS_up_5kb
■ TSS_up_10kb
■ TES_down_1kb
■ TES_down_5kb
■ TES_down_10kb
Created with MultiQC
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read number
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60
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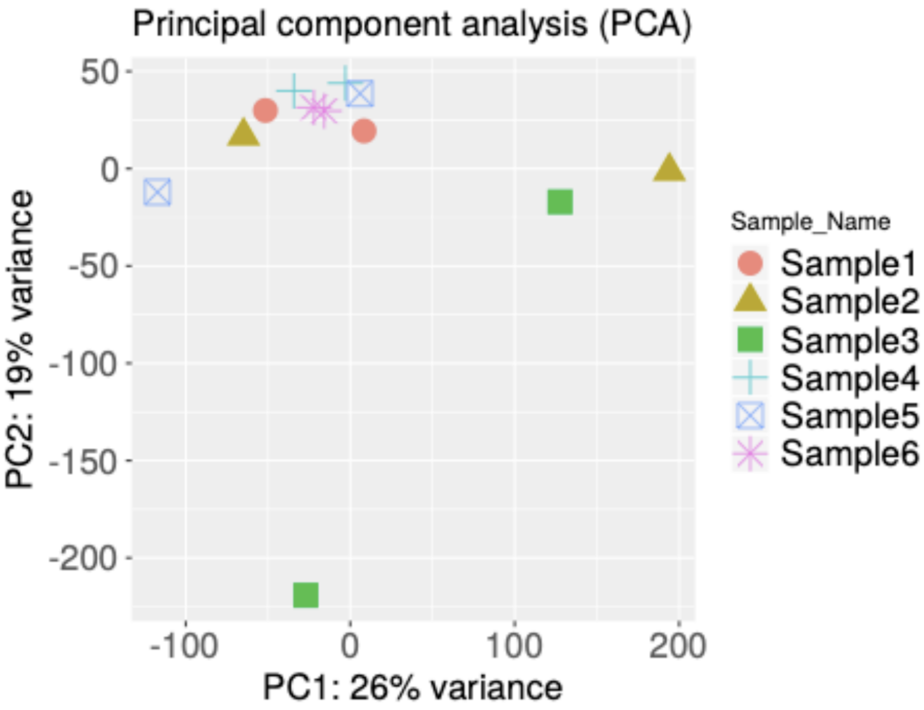
Principal component analysis (PCA)
PC2: 19% variance
50
0
-50
-100
-150
-200
-100
0
100
200
PC1: 26% variance
Sample_Name
Sample1
Sample2
Sample3
Sample4
Sample5
Sample6

	Number of samples	Median Input for lib prep (ng)	Median RIN	Median DV_{200}	Median DV_{100}	Median Lib size (bp)
DV100 <40%	7	237.6	2.5	6	34	445
DV100 40-60%	27	1000	2.5	12	51	408
DV100 >60%	19	1000	2.3	26	73	355

Median Lib yield (ng)	Median Lib Molarity (nM)	Median Specimen storage time (Years)	Median % contamination	Median Gene Count
24.5	7	22	27.4	14,759
19.8	5.9	18	9.9	10,202
84.9	24	13	3.2	9,993

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_Jan2020.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2100 Bioanalyzer	Agilent	G2939BA	
Agilent DNA 7500 Kit	Agilent	5067-1506	
Agilent High Sensitivity DNA Kit	Agilent	5067-4626	
Agilent RNA 6000 Nano Kit	Agilent	5067-1511	
AllPrep DNA/RNA FFPE Kit	Qiagen	80234	
CFX96 Touch System	Bio-Rad	1855195	
Library Quantification kit v2-Illumina	KapaBiosystems	KK4824	
NEBNext Ultra II Directional RNA Library Prep Kit for Illumina	New England Biolabs	E7765S	https://www.neb.com/protoc
NEBNext rRNA Depletion Kit (Human/Mouse/Rat)	New England Biolabs	E6310L	
NextSeq 500 Sequencing System	Illumina	SY-415-1001	NextSeq 500 System guide:
NextSeq PhiX Control Kit	Illumina	FC-110-3002	
NSQ 500/550 Hi Output KT v2.5 (150 CYS)	Illumina	20024907	
10X Genomics Magnetic Separator	10X Genomics	120250	
Rotator Multimixer	VWR	13916-822	
C1000 Touch Thermal Cycler	Bio-Rad	1851197	
Sequencing reagent kit	Illumina	20024907	
Flow cell package	Illumina	20024907	
Buffer cartridge and the reagent cartridge	Illumina	20024907	
Sodium hydroxide solution (0.2N)	Millipore Sigma	SX0607D-6	
TRIS-HCL Buffer 1.0M, pH 7.0	Fisher Scientific	50-151-871	

:ols/2017/02/07/protocol-for-use-with-ffpe-rna-nebnext-rrna-depletion-kit

https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/nextseq/nextseq-

-500-system-guide-15046563-06.pdf

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Phillip Steindel, Ph.D.
Review Editor
JoVE

Jan 17th, 2020

Dear Dr. Steindel

Thank you for the opportunity to submit a revised version of our manuscript, "Making the most of FFPE-RNA – optimization for sequencing and analysis of degraded FFPE-RNA samples" in the Journal of Visualized Experiments. We thank you and the reviewers for the careful review of our manuscript. We have responded to the editorial and reviewer comments, and submitted the updated manuscript with changes made in response to the comments marked in track.

All authors have read and approved the final version.

Thank you for considering this manuscript. We look forward to your comments.

Best Regards,
Monika Mehta, Ph.D. [C]
NCI CCR Sequencing Facility
Cancer Research Technology Program
Frederick National Laboratory for Cancer Research (FNLCR)
Leidos Biomedical Research, Inc.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have done this in the updated manuscript.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

The manuscript formatting adheres to the given parameters.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Agilent Bioanalyzer, Illumina

Thank you for letting us know. We have tried to eliminate the company names from the main text as much as we could. However, we have included the names within parenthesis as examples. Please let us know if that would be a problem.

Example:

"*Check the quality of RNA (preferably from the QC aliquot) by running it on a RNA QC system (e.g., Agilent Bioanalyzer system using an RNA Nano chip, Table of Materials) according to manufacturer's instructions*."

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted less than 2.75 pages for filming.

2. Please add more details to your protocol steps, especially those that are to be filmed. Please ensure you answer the "how" question (i.e., how is the step performed) sufficiently enough that someone new to this procedure could replicate it. Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have tried to make the protocol adhere to these directions as closely as we could.

Specific Protocol steps:

1. 2.1: This is especially vague for filming-are you intending to show all of these procedures? Please provide sufficient detail for the procedures you decide to film (if any).

Thank you for this comment. We have now removed this part from filming section.

Figures and Tables:
1. Please cite Figure 6 and Table 1 outside of the figure/table legends section.

Thank you for catching this. We have now referred to both in the main text (page 9 and 10, respectively). Please note that based on a reviewer suggestion, the earlier Table 1 is now Supplementary Table.

2. Please remove 'Figure 1' etc. from the figures themselves.

We were not aware of this requirement. We have now removed the titles from the figures.

References:
1. Please do not cite product manuals in the References; these should be in the text and/or in the Table of Materials.

Thank you for letting us know. We have now removed references to user guides from "References".

2. Please do not abbreviate journal titles.

We used the JoVE EndNote style file for EndNote to format the references, and have not changed any formatting. Please let us know which journal titles we need to change.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Thank you for the note. We rechecked the protocol and have added some more materials to the table, that we had previously missed. To our knowledge, we have now added all materials and equipment used in the protocol to the Table of Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript presented by Levin et al addresses the steps during preparation of RNA samples that are extracted from FFPE tissue to ensure adequate quality prior to next-generation sequencing.
While I think the article could be informative to some people using the methodology, the manuscript currently seems more like an iteration of the work that this group has published previously this year, and is missing the discussion and critical assessment of several important aspects, such as strengths and weaknesses of the proposed approach in respect to other published approaches in the field, and several key references in the work presented as it is. Hence, I would urge the authors to go over their manuscript and critically compare it to existing literature, include the pros and cons of each step. This would tremendously improve the quality of the manuscript to be a 'technical review' of the field for someone who has not been working with the approach yet, instead of only representing a mere repetition of their previously published manuscript.

Thank you for the helpful suggestions for improving the manuscript. We have tried to address the various issues below.

Major Concerns:
Some details in particular:
1. Line 81+: "albeit fewer and not very old samples": For instance Sinicropi et al (doi: 10.1371/journal.pone.0040092) have shown RNAseq for FFPE samples up to 12.4 years of age; and Hedegaard et al (doi:10.1371/journal.pone.0098187.s018) even for up to 20 years. These two papers should be referenced. Similarly, more recently, it has been shown feasible to perform RNAseq not only for full sections of FFPE tissue, but actually also microdissected tissue, as demonstrated by the Markannen group (Amini et al, BMC Mol Biol 18:22. doi: 10.1186/s12867-017-0099-7; Amini et al, Dis Model Mech. doi: 10.1242/dmm.040444). The NGS strategy they used in their paper makes use of much lower RNA inputs than what is suggested in this manuscript. The same is true for Wimmer et al, Sci Rep (DOI:10.1038/s41598-018-24781-6). Inclusion and discussion of these references would be very important to give a proper overview of the current state of the field.

Thank you for the suggestion to include these references. While we had referred to one of the abovementioned papers (Hedegaard et al, 2014, Reference #2) in our manuscript, we had missed the other 4. We have now added those too to provide a more wholesome overview of the field.

While all these papers did show the feasibility of using FFPE-RNA using different novel ways, we do, respectfully, stand by our claim that our study (Zhao et al, 2019) included samples that came from older FFPE blocks (7-32 years, median storage age 17.5 years) than most of the above-listed studies. Additionally, since we received pre-extracted RNA, we could not control the RNA extraction process. The RNA samples that we worked with, all represented bad quality samples, that were well below the quality thresholds usually put for RNA samples (61 out of 67 samples had $DV_{200} < 30\%$). In addition to this, the worst quality samples were also most limited in input quantity (summary in Table 1 in this manuscript, and detailed QC metrics in Table 2 in Zhao et al, 2019), making it more challenging to generate usable data from them. We do agree that some of the studies mentioned above did utilize lower input amounts than us, though they typically had good quality RNA to work with. As we have mentioned in our previous publication, our goal was to test the bad quality samples to see if any useful information can be obtained from these or if they should just not be used for sequence analysis at all.

2. Protocol: Before Step 1 I am missing any reference as to how to select cases, and what to keep in mind when processing them, and the fact that there are several different extraction procedures for RNA from FFPE. Here, for instance, it is extremely important to make sure to work with RNAse free dH2O when preparing samples, and always to clean all instruments that come in contact with the FFPE blocks with RNAse away. As a matter of fact, in the paper published by the authors earlier this year (REF 10), they had a significant amount of samples that showed contamination with murine RNA - this is a red flag indicating insufficient care when preparing samples before RNA extraction! If the authors do not want to discuss these aspects, I suggest they at least mention the importance of adhering to certain standards, and reference the following work which describes these aspects in detail: Amini et al, BMC Mol Biol 18:22. (doi: 10.1186/s12867-017-0099-7); Butler et al, J HIstotechnol (doi: 10.1080/01478885.2015.1106073); Wimmer et al, Sci Rep (DOI:10.1038/s41598-018-24781-6). Please revise the section accordingly.

Thank you for this suggestion. We have added the abovementioned precautions to the protocol (step 1.2), and also referred to the studies describing the extraction protocols. RNA extraction from FFPE blocks was performed by another group, and we started this project with pre-extracted RNA. We aimed to test the suboptimal quality samples to see if we could generate any usable information at all or not. Our focus for this protocol is to share the various steps that should be taken to increase the likelihood of generation of useful sequence data from bad quality FFPE-RNA samples.

3. Protocol Step 2.1.2: I am not sure whether it is advisable to use any methodology that depends on the capture of specific regions (such as PolyA) for RNA extracted from FFPE, regardless of its DV200 value, since the fixation step in itself may introduce a lot of bias in which kinds of targets can be recovered etc. I would suggest to put more emphasis here on why it would be adviseable to use a random priming approach.

Thank you for this suggestion. Though we do focus on the random priming approach in our protocol, have now added the following sentences (to steps 2.1.1 and 2.1.2, respectively) to emphasize this and advise against capture approaches:

"*However, it is important to note that the fixation process may introduce bias in the extracted RNA, and thus, the capture approaches may not work well in all cases, even with high* DV_{200} *values.*"

"The use of random primers for generation of cDNA leads to higher representation of usable RNA in the final library, and is, therefore, more suited for FFPE-RNA samples."

4. Representative results: The way the results are described here, the reader gets an impression that these were new results that were specifically made in the context of this manuscript. However, these are results that have been published by the authors elsewhere before (Ref 10). Please make sure to make this fact sufficiently clear.

We have taken your advice and made it is clear in the manuscript that the data and results described here were from previous study. The following was added to the manuscript (beginning of the "Representative Results" section):

"*The data set and analysis results presented here were previously described and published*[11]."

I am confident that the authors will be able to address these aspects in a very productive way and am looking forward to seeing a revised version of the manuscript.

Reviewer #2:

Manuscript Summary:

The authors describe an extensive and helpful protocol for RNA sequencing using formalin fixed and paraffin embedded tissue. In total 67 archival samples were analyzed with ages ranging between 7-32 years old. The authors compared the FFPE derived expression patterns to those described in the TCGA data and found comparable expression patterns in 60% of samples. Several useful tips are provided in the protocol, including a cutoff value for RNA quality when not to proceed to RNA sequencing.

Thank you for the questions and helpful suggestions. We have addressed the various questions below and also highlighted the appropriate changes made in the manuscript, that have improved the clarity and readability of the manuscript.

Major Concerns:
1. Interesting that the authors measure contamination of RNA. But can the authors describe the significance of the contamination that is present. Does it interfere with the gene expression profiles. And how relevant is this information for the result of the procedure. Please elaborate in the manuscript (preferably in the discussion section)
We thank the reviewer for this suggestion. We have taken your advice and added the following to the Discussion section to describe sources of contamination and their impact to gene expression profiling:
"*Samples contaminated with foreign species can waste sequencing coverage, depending on the extent of contamination. In addition, internal contamination can arise from incomplete rRNA depletion, leading to high percentage of reads mapping to rRNAs. Inefficient genomic DNA removal during DNase digestion could lead to false positive expression detection of transcripts or erroneous de novo assembly of transcripts. Adapter contamination introduced during library preparation is also a common problem for highly degraded RNAs with very short RNA fragments. Contamination can affect the gene and transcript profiling accuracy and lead to false discovery. Therefore, it is important to accurately identify the contamination sources and remove the contamination, if possible, during the sample or library preparation steps, or filter the contaminating reads during the data processing step.*"

2. Can the authors explain why they chose this particular RNA isolation kit? Can they comment on any other kits that were tested as well and which other kits would be suitable as well I their opinion.
Thank you for this question. The specific RNA isolation kit had been chosen since it usually performs well for FFPE tissues in the hands of the group that performed the RNA extraction. We started this study using the pre-extracted RNA. We have now added additional references to the protocol (step 1.1) to point the readers towards some other available methods for RNA extraction from FFPE blocks.

3. Addition of an overview table with information on the age of the tissue block, the RNA input, DV100 and DV200, sequence QC and the sequence result of all samples provides the reader with a better overview of the results.
Thank you for your suggestion. We have now included a summary table (Table 1) with QC metrics. The detailed QC table for all samples has been included in our previous publication (Zhao et al, 2019). We have added the following sentence in "Representative Results" (page 9):
"Table 1 shows a summary of QC metrics for the samples in different DV_{100} categories. For detailed metrics of all 67 samples, please see Zhao et al, 2019[11]."

4. Step 2.4: the authors mention that the number of amplification cycles should be based on the amount of input. Can they provide an indication of the number of cycles with certain amounts of RNA input?
Thank you for this suggestion. We have now added the following sentence to step 2.5:

“*For low input FFPE-RNA samples (<100 ng), we recommend 16 - 18 amplification cycles, while the high input samples (1000 ng) usually generate enough library amounts in 12-14 rounds of amplification.*”

5. Figure 6a: Can the authors speculate on why some samples show a larger variance? Is this due to tissue block age?
Thank you for this question and for giving us a chance to clarify. In addition to the tissue block age, there were other factors also responsible for the large variance among some samples, as highlighted by the replicates in our study. Among the 6 pairs of biological replicates, 4 pairs had correlation values between 0.7 - 0.8 when comparing gene expression values between the replicate pairs, while the remaining 2 pairs had correlations below 0.22. Although the tissue block age was different among different samples, for each pair of replicates, 2 separate sets of tissue curls were cut from the same FFPE blocks. Thus, each pair of replicates had the same block age. The high variation in these cases was likely due to different amounts of bacterial contamination (2- to 5-fold difference) as well as different mRNA content (2- to 3-fold difference) between the replicates. We have added the following text in the manuscript (end of Representative Results section) to elaborate this:

“*Among the six pairs of replicates, two pairs of replicates had higher variations (correlations below 0.22) than the rest four samples (correlation values between 0.7 - 0.8) when comparing gene expression values between the replicates pairs. Since the replicates were generated by extracting RNA from two different tissue curls cut from the same FFPE blocks, the tissue age was not a factor in the higher variance here, and it was likely caused by the different amount of bacterial contamination (1% – 55%) as well as different mRNA content (2 -3 fold difference) between the replicates The randomness of mRNA degradation after extraction could also contribute to the higher variance between samples of similar origin.”*

6. Figure 1 does not seem necessary, an explanation in the text on which controls etc should be included would suffice.

Thank you for this comment. We have now removed Figure 1, while retaining the explanation in step 3.1.3.

7. Figure 2: for this paper it makes sense that RNAseq data analysis is performed irrespective of the QC results, it might be useful that the authors comment on the fact that usually QC is performed before further analysis and interpretation of the data
Thank you for this suggestion. We have taken your advice and added the following text in the protocol (Step 5):
“The RNA-seq data may have quality issues that can affect the accuracy of gene profiling and lead to erroneous conclusions. Therefore, initial QC checks for sequencing quality, contamination, sequencing coverage bias and other sources of artifacts is very important. We recommend applying RNA-seq QC pipeline, similar to the workflow described here to detect artifacts and apply filtering or correction before downstream analysis.”

8. Can the authors comment on the effect of sample input on assay quality as they mention that the maximum amount of input available was used. What is in their experience the lower limit of the RNA input?

Thank you for this suggestion. We have now added the following sentence to the protocol (step 2.2):
"*While samples with reasonably good quality RNA (DV_{100} > 60%) may yield good sequence data even at lower input amounts (lowest that we have tested with FFPE-RNA was ~20 ng), for more degraded RNA (DV_{100} < 60%), it is better to start with higher input amounts (>100 ng).*"

Additionally, we added the following sentence to the discussion section:
"*Higher input amounts lead to better diversity and lower duplication in the final library leading to improved data quality. Since not all RNA in FFPE-RNA samples is usable (due to high degradation and refractoriness to enzymatic processes), these effects are more pronounced in FFPE-RNA as compared to fresh frozen RNA.*"

Minor Concerns:
1. Please add the median age of the tissue blocks.
Thank you for this suggestion. We have now modified the first sentence in "Representative Results" to:
"*The methodology described above was applied to 67 FFPE samples that had been stored under a variety of different conditions for 7 to 32 years (17.5 years as the median sample storage time).*" Additionally, the new Table 1 also mentions the median storage time for samples in different groups, grouped according to their DV_{100} values.

2. Consequently use adaptor or adapter throughout the manuscript.
Thank you for catching this. We have now changed all usages of the word in the manuscript to 'adapter'.

3. Comments/Description in table of materials can be left out as there are no comments or descriptions.
The updated Table of Materials has a couple of comments now, so we have kept the Comments/Descriptions column.

4. Consider to move the software parameters table to supplementary data
Thank you for the suggestion. We have moved the software table to supplementary data.

Analysis Step	Software Version
Demultiplexing	Bcl2fastq 2.17
Raw fastq quality check	fastqc 0.11.8
Filtering Adaptor and low quality bases	Cutadapt
	Trimmomatic 0.30
Fastq_screen	Fastq_screen 0.11.4
Contamination Check	Kraken 1.0
Alignment	STAR 2.7.0f / STAR 2 Pass

RNAStatistics	Picard 2.18.26
Duplication Statistics	Picard 2.18.26
Insert Size Statistics	RseQC 2.6.4
MultiQC	MultiQC 1.7
Gene Quantification	RSEM 1.3.1
DE Analysis	EdgeR 3.24.3

Limma-voom 3.38.3

Software Parameters
bcl2fastq -r 8 -p 8 -w 8 --no-lane-splitting -i $path_to_run_Intensities_basecalls_dir -R $run_dir_name --barcode-mismatches 1 --ignore-missing-bcls --ignore-missing-filter --ignore-missing-positions --ignore-missing-controls --sample-sheet $SampleSheet.csv -o $unaligned_dir
fastqc -o $prefix_name --noextract -k 5 -t $threads -f fastq $input_R1.fastq $input_R2.fastq
CutAdapt -j $threads -b $adapter_file --trim-n -m 20 -o $output_R1.fastq -p $output_R2.fastq $input_R1.fastq $input_R2.fastq
Trimmomatic PE -threads 16 -phred33 ILLUMINACLIP:adapters.fa:2:36:10 LEADING:10 TRAILING:10 MAXINFO:50:0.97 MINLEN:20
fastqc_screen --outdir $prefix_name --threads $threads --subset 0 --nohits --conf $program_config --aligner bowtie2 $input_R1.fastq $input_R2.fastq
kraken --fastq-input --threads $thread --db $krakendb --output $prefix --paired $input_R1.fastq $input_R2.fastq && cut -f2,3 $output_results > $output_krona_dir
STAR --genomeDir $star_genome --readFilesCommand zcat --readFilesIn $trimmed_R1.fastq.gz $trimmed_R2.fastq.gz --outSAMunmapped Within --outFilterType BySJout --outFilterMultimapNmax 20 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --sjdbScore 1 --runThreadN $threads --genomeLoad NoSharedMemory --outSAMtype BAM Unsorted --quantMode TranscriptomeSAM
STAR --genomeDir $star_genome --outSAMunmapped Within --outFilterType BySJout --outFilterMultimapNmax 20 --outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --alignSJoverhangMin 8 --limitSjdbInsertNsj 2500000 –s jdbFileChrStartEnd $input.sj --alignSJDBoverhangMin 1 --sjdbScore 1 --readFilesCommand zcat --readFilesIn $trimmed_R1.fastq.gz $trimmed_R2l.fastq.gz --outFileNamePrefix $params.prefix --runThreadN $clusterConfig_star2p $threads --outFilterMatchNminOverLread 0.66 --outSAMtype BAM Unsorted --quantMode TranscriptomeSAM --peOverlapNbasesMin 10 --alignEndsProtrude 10 ConcordantPair

```
CollectRnaSeqMetrics.jar REF_FLAT=annotation_refFlat.txt INPUT=sample.bam OUTPUT=
RnaSeqMetrics.txt RIBOSOMAL_INTERVALS= ribosome_interval_list.txt STRAND_SPECIFICITY=NONE
VALIDATION_STRINGENCY=LENIENT
```

```
MarkDuplicates.jar INPUT=sample.bam OUTPUT=sample.MKDUP.bam
METRICS_FILE=sample.bam.metric ASSUME_SORTED=true
MAX_FILE_HANDLES_FOR_READ_ENDS_MAP=1000 VALIDATION_STRINGENCY=LENIENT
```

```
inner_distance.py–i sample.bam –o ./ -r annotation.bed
infer_experiment.py $rseqc_file –i $sample.bam
read_GC.py –i $sample.bam
read_distribution.py –i $sample.bam
junction_saturation.py –i $sample.bam –r $annotation.bed
```

```
MultiQC -f -c $program.multiqc_conf -n $output.multiQC.html  $dir
```

```
rsem-calculate-expression –bam --paired-end --estimate-rspd   $Transcriptome.out.bam
$RSEM_Genome $Sample_Name
```

```
edgeR_script.R  $out_outdir  $input_sample_table  $input_raw_count_matrix_file  $contrasts_file
$refererence  $params_projectId $params.gtffile  $params.dtype  $params.karyobeds
```

```
Limma_script.R $DEG_outdir  $input_sample_table $input_raw_count_matrix_file  $contrasts_file
$params_refererence $params.projectId  $params.gtffile $params.dtype $params.karyobeds
```

Software Reference
https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/bcl2fastq/bcl2fastq_letterbooklet_15038058brpmi.pdf
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