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Robust gene expression analysis of FFPE samples from a wide range of tissues and qualities using CORALL FFPE whole transcriptome sequencing

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ABSTRACT

Formalin-fixed paraffin-embedded (FFPE) tissues provide a valuable but little exploited resource for molecular analysis of mammalian tissue biopsies. Promising applications include the characterization of human patient samples from hospitals and biobanks for analysis of known and identification of novel tissue biomarkers. Gene expression analysis of FFPE samples is particularly challenging because of the highly degraded nature of FFPE RNA, owing to the tissue fixation process and prolonged sample storage. Although human FFPE material often lacks detailed information about the sampling and storage history, we investigated the influence of selected FFPE sample parameters on the quality of RNA sequencing results in a benchmark study.

Here, we prepared FFPE blocks of mouse liver, lung and colon tissues under highly controlled conditions to evaluate the performance of our RNA-seq workflow on samples of varying quality. We followed standard tissue fixation protocols for the generation of high quality FFPE blocks, and additionally prepared FFPE blocks under suboptimal conditions, including delay to fixation and overfixation samples, to simulate clinical settings. FFPE RNA was extracted and subjected to RiboCop rRNA depletion and CORALL FFPE library preparation for total RNA sequencing, followed by gene expression analysis. In parallel, RNA extracted from matched fresh tissue served as reference material.

We found that both, tissue type and tissue preparation process, impacted the quality of the FFPE RNA which enabled us to compare gene expression results across a broad range of sample qualities. The CORALL FFPE whole transcriptome library preparation kit delivered robust and high-quality gene expression results for all samples analyzed, even for challenging tissues and low-quality samples at low input amounts. CORALL FFPE is therefore well suited for the analysis of challenging FFPE samples, particularly human patient material.

Experimental Setup

Preparation of FFPE blocks under highly controlled conditions

We dissected mouse liver, lung, and colon tissues and prepared FFPE blocks according to 3 different fixation conditions to generate FFPE samples of varying quality (Fig. 1). For standard conditions, samples were fixed in 10 % Neutral Buffered Formalin at 4 °C overnight. That procedure was modified either by storing tissue pieces on ice for 2 hours before fixation (delay to fixation) or by extending the fixation time to 72 hours (overfixation). Matched fresh tissue was stabilized in RNA/DNA Defender solution and used for comparison.

Figure 1 | Scheme of FFPE block preparation. Liver, lung, and colon tissues were isolated from mice and processed into FFPE blocks following either standard fixation conditions (STD) or suboptimal conditions, which included a 2 hour delay to fixation (DTF) and prolonged fixation for 72 hours (OF). Tissue pieces were then dehydrated and infiltrated with paraffin in an automated tissue processor, followed by embedding in paraffin. Examples of FFPE blocks are shown on the right.

FFPE RNA was isolated with our new SPLIT One-step FFPE RNA Extraction Kit (Fig. 2). RNA-seq libraries were prepared with our RiboCop rRNA Depletion Kit for Human/Mouse/Rat (HMR V2) and CORALL FFPE Whole Transcriptome Kit (Fig. 3).

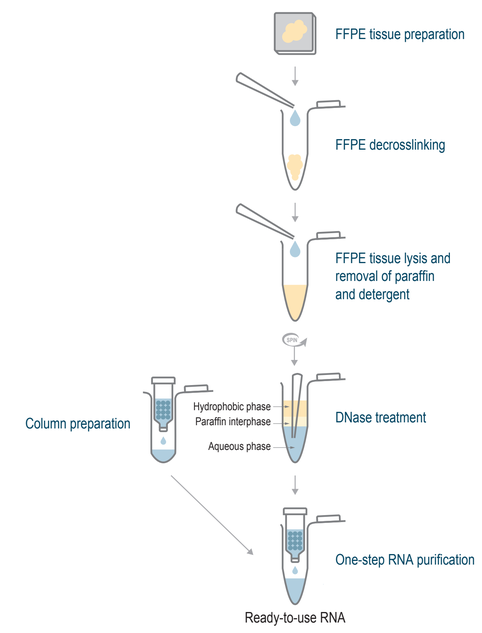

Figure 2 | SPLIT One-Step FFPE RNA Extraction workflow.

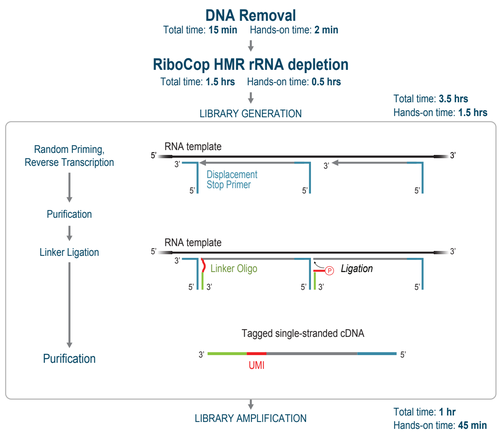

Figure 3 | CORALL FFPE library preparation workflow.

Results

Tissue type and preparation process have an impact on FFPE RNA quality

Blocks were sectioned with a microtome and 1x10 µm curl was extracted per sample (Fig. 4).

Figure 4 | Quality of extracted RNA and FFPE RNA. A) - B) Electropherogram of RNA isolated from fresh tissue (A) or from STD FFPE tissue (B). **C) - D)** Quality of RNA isolated from fresh tissue (C) or from FFPE tissue (D). Average and standard deviation of 3 replicates is shown. STD = std. fixation, DTF = delay to fixation, OF = overfixation.

- FFPE RNA quality varied between the three STD FFPE tissues, even though RNA quality was very high for all matched fresh tissue samples -> tissue-inherent differences in FFPE RNA quality.
- FFPE RNA quality varied between fixation conditions; particularly delayed fixation impacted negatively on FFPE RNA length.

Robust gene expression data from high-quality and low-quality FFPE RNA

Ribosomal RNA was depleted from 50 ng total RNA, and 1/10th was used for CORALL library prep (Fig. 5).

- FFPE libraries were only slightly shorter than respective fresh tissue libraries.
- FFPE libraries had similar sizes for all three tissues despite differences in FFPE RNA fragment sizes.

Libraries were sequenced on an Aviti instrument, followed by quality control and gene expression analysis of sequencing data (Fig. 6-7).

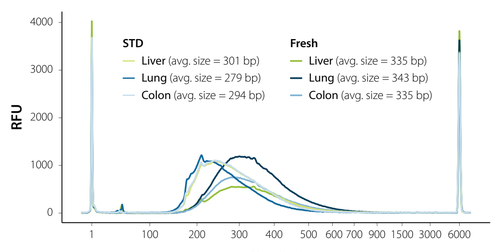

Figure 5 | Electropherograms of CORALL libraries. Representative examples of libraries prepared from fresh tissue samples and STD FFPE tisssue samples. Average library size is shown in parentheses.

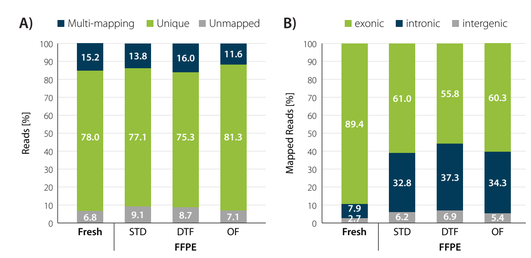

Figure 6 I RNA-seq read statistics. A) Mapping stats for trimmed reads. **B)** Feature attribution. CORALL FFPE libraries were generated from fresh and FFPE mouse liver tissues. Samples were sequenced at 3 M reads (2x76 bp) and plotted as average of 2 replicates. STD = std. fixation, DTF = delay to fixation, OF = overfixation.

- High-quality data with >75 % uniquely mapping reads for all samples (Fig. 6A).
- Data from FFPE samples is ideal for gene expression analysis with ~60 % of reads mapping to exons (Fig. 6B).
- Excellent library complexity with >80 % of mapped reads remaining for downstream analyses after UMI collapsing (not shown).

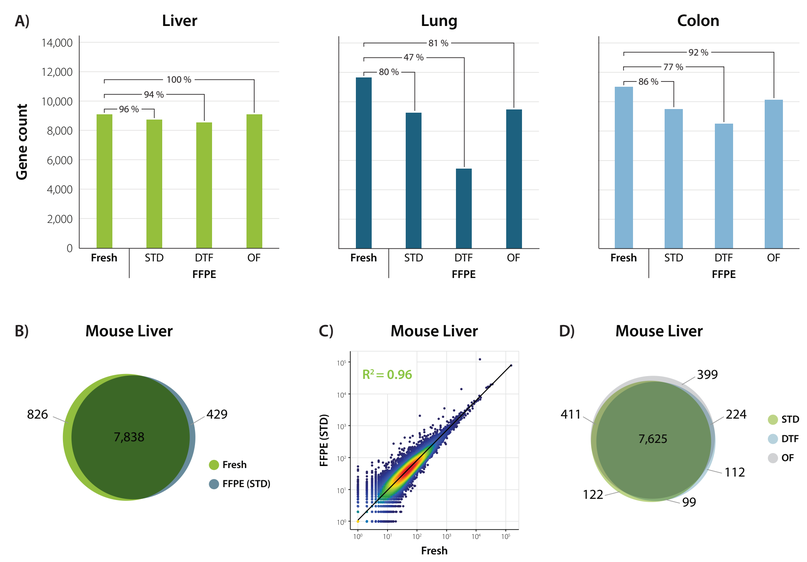

Figure 7 | Gene expression analysis. A) Number of protein-coding genes detected for liver (left), lung (center) and colon (right) samples at a threshold of 10 reads. Analysis based on 3 M reads. Average of 2 replicates. Percentages indicate the number of detected genes in FFPE tissue samples relative to respective fresh tissue samples. **B)** Overlapping genes between fresh and FFPE STD samples. **C)** Correlation between fresh and FFPE STD samples. **D)** Overlapping genes between FFPE fixation conditions. **B) - D),** Data from liver shown as example. STD = std. fixation, DTF = delay to fixation, OF = overfixation.

- Excellent gene detection rate for high-quality FFPE samples (FFPE STD), even for challenging tissues with lower DV200 values (Fig. 7A).
- Delay in tissue fixation negatively affects gene detection, particularly for challenging tissues such as lung.
- The CORALL FFPE Whole Transcriptome workflow is robust towards extended fixation times.
- Highly similar gene expression profiles of FFPE samples and fresh tissue samples (Fig. 7B-D).

Conclusions

- Quality of FFPE RNA tissues is highly variable and depends on pre-analytical factors such as tissue type and the block preparation process.
- Quality of CORALL FFFPE RNA sequencing data is very high for all FFPE tissues fixed under optimal conditions, even for challenging samples which show higher levels of RNA degradation. While CORALL is robust towards prolonged fixation of tissues, delayed fixation can result in lower gene detection rates depending on the tissue analyzed.
- Our SPLIT One-step FFPE RNA Extraction kit and CORALL FFPE Whole Transcriptome workflow with RiboCop rRNA depletion deliver robust results and are therefore well suited for the molecular analysis of challenging FFPE material and precious FFPE samples at low input amounts.

Acknowledgements:

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For more information please visit our website:
www.lexogen.com/corall-ffpe-rna-seq