A Comparison of Active and Passive FFPE DNA Extraction Methods and Effects on Downstream Analysis Metrics

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Introduction

Formalin Fixation and Paraffin Embedding (FFPE) has been a mainstay of histology for over a century and remains a routine oncology practice due to the quality and stability of structural information preserved. However, the method was conceived primarily to facilitate microscopic analysis rather than molecular approaches such as targeted sequencing and whole genome sequencing which is becoming increasingly common in clinical settings. Chemical crosslinks, dehydrated tissue, and the paraffin matrix of FFPE preserved samples present significant challenges for the robust extraction of Next Generation Sequencing (NGS) quality DNA. Data presented here illustrate that Covaris truXTRAC® FFPE DNA Kit combined with Adaptive Focused Acoustics® (AFA®) technology is an uniquely effective method to take full advantage of the wealth of information contained in FFPE tissue samples.

Summary

This case study compares the quantity and quality of DNA extracted using the Covaris truXTRAC FFPE DNA kit to the QIAGEN® QIAamp® FFPE tissue kit. In the Covaris truXTRAC workflow, the focused, short wavelength acoustic field generated by Covaris AFA allows for active paraffin removal without the use of organic solvents, and enables efficient tissue rehydration. This results in a highly simplified workflow, which ensures high yield extraction of nucleic acids. In the QIAGEN QIAamp workflow, paraffin is passively removed by Xylene or other proprietary solvents. FFPE tissue sections from two different human tissue types were used and analyzed using the metrics of:

1. Fluorescence microscopy of FFPE sections to determine the extent of paraffin embedding and removal

2. Double stranded DNA specific fluorescence assay utilizing Qubit® to determine the yield of double stranded DNA

3. qPCR-based quality analysis utilizing a commercially available Human Genomic DNA Quantification and QC kit

4. Whole genome sequencing on an Illumina® HiSeq2500 to directly measure the representation of the genome in the extracted DNA.

Our data illustrate that the Covaris AFA technology for FFPE DNA extraction offers greater DNA extraction yield and significantly more robust and consistent whole genome sequencing results.

Materials and Methods

Tissue handling

FFPE kidney and uterus tissue blocks were stored at 4 °C upon delivery from CHTN, and frozen matched tissues were stored at -80 °C. Prior to sectioning, excess paraffin was trimmed from tissue blocks, and a microtome was used to section tissue to 20 μm scrolls. 25 mg of the matched frozen tissue were cut using a scalpel for DNA extraction.

Fluorescence Microscopy

After paraffin removal, samples were mounted on microscope slide (VWR) and dried for at least 30 min. A fluorescent microscope (Olympus Model IX73 with Fluorescence and EXI-BLU Camera) was used to record autofluorescence of the paraffin remaining in the tissues after the removal treatment.

DNA Extraction and Quantification (FFPE and Fresh Frozen Tissue)

Covaris truXTRAC FFPE DNA kit was used with 20 μm sections according to the kit protocol Option C on even numbered sections. DNA from odd numbered sections was extracted using QIAamp FFPE tissue kit according to the kit protocol. DNA from matched frozen tissue was extraction using QIAamp mini Kit, after cryofracturing the tissue using the Covaris cryoPREP.

DNA Quantity Normalization

In order to obtain 5 µg of total DNA using the QIAGEN QIAamp FFPE tissue kit, five 20 µm sections of kidney FFPE, and three sections of uterus tissue were processed. For Covaris FFPE extraction, three kidney sections, and one uterus sections were processed to achieve a 5 µg yield.

DNA Concentration and Quality Assessment

The concentration of extracted DNA was determined using the Qubit Quant-it dsDNA BR assay kit using either 5 or 10 µl of the extracted DNA sample. qPCR quantification of the DNA, and quality assessment, was carried out using KAPA® hgDNA Quantification and QC Kit according to the supplier instructions.

Sequencing and Data Analysis

5 µg of DNA from each sample type were submitted to Elim Biopharmaceuticals Inc. for 2x100bp whole genome sequencing using an Illumina HiSeq2500. Reads were aligned using BW software package. Coverage analysis of the aligned reads was carried out using BedTools software package. Whole genome coverage analysis and viewing was carried out using Broad Institute's IGV genome analysis software package.
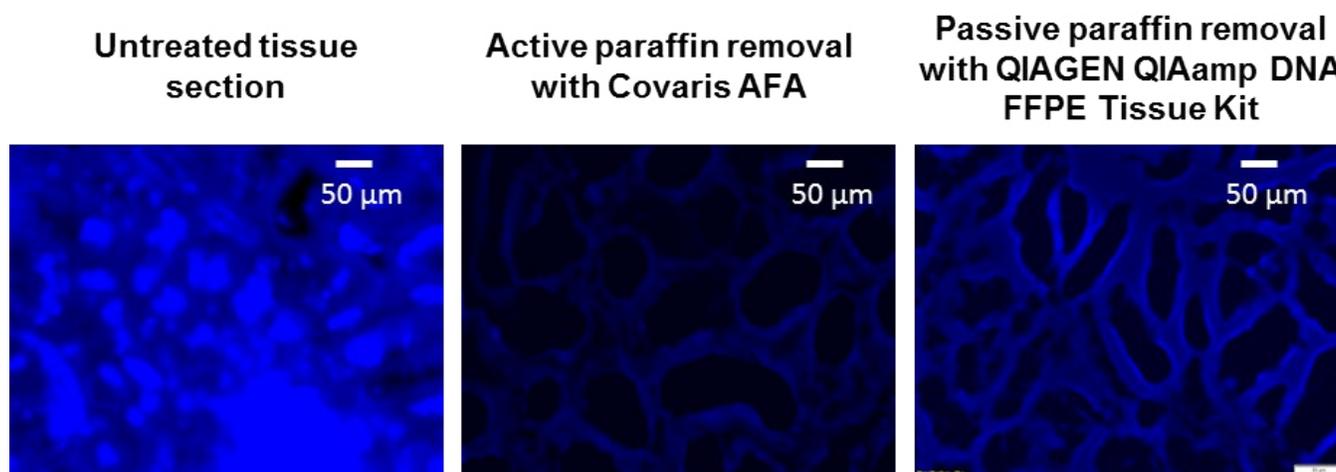

Figure 1: Active vs. passive paraffin removal. Comparison of active and passive paraffin removal. Images of fluorescence of FFPE tissue sections.

Analysis

Paraffin removal

Active paraffin removal involves the use of AFA energy to emulsify and remove paraffin from the tissue matrix. Passive methods utilize organic solvents or mineral oils to dissolve paraffin in a diffusion dependent manner. To compare the efficiency of AFA-based active paraffin removal to a passive removal with a chemical agent, three adjacent tissue sections were cut from the same tissue block. The first section was kept unprocessed as a negative control. A second section was processed with AFA technology and a third section was processed following the QIAGEN QIAamp DNA FFPE Tissue Kit protocol. The amount of paraffin present in the tissue (*Figure 1*) is proportional to intensity of the blue signal (the paraffin develops intrinsic auto-fluorescence properties during the embedding process). Active paraffin removal with AFA results in a much more efficient paraffin removal compared to a passive treatment. On average, the auto-fluorescence intensity of the remaining paraffin in the tissue is three times lower in samples treated with Covaris AFA than in samples treated with the QIAGEN QIAamp DNA FFPE Tissue Kit. Inefficient paraffin removal will have significant effect on tissue rehydration, proteinase K digestion, and reversing of the crosslinks thereby aversely affecting DNA yield and quality.

DNA Yield

NGS, qPCR and array based applications require varying amounts of DNA for analysis, so yield is often a metric used to determine success in extraction of DNA from FFPE tissues. Based on the results of Qubit analysis, both kidney and uterus FFPE sections extracted using Covaris truXTRAC Kit generated higher yields as compared to the QIAGEN QIAamp FFPE Tissue Kit (*Figure 3*). This is significant since in our hands, using the QIAGEN kit protocol, it took five 20 µm sections of kidney FFPE, and three sections of uterus tissue to obtain 5 µg of double stranded DNA required for

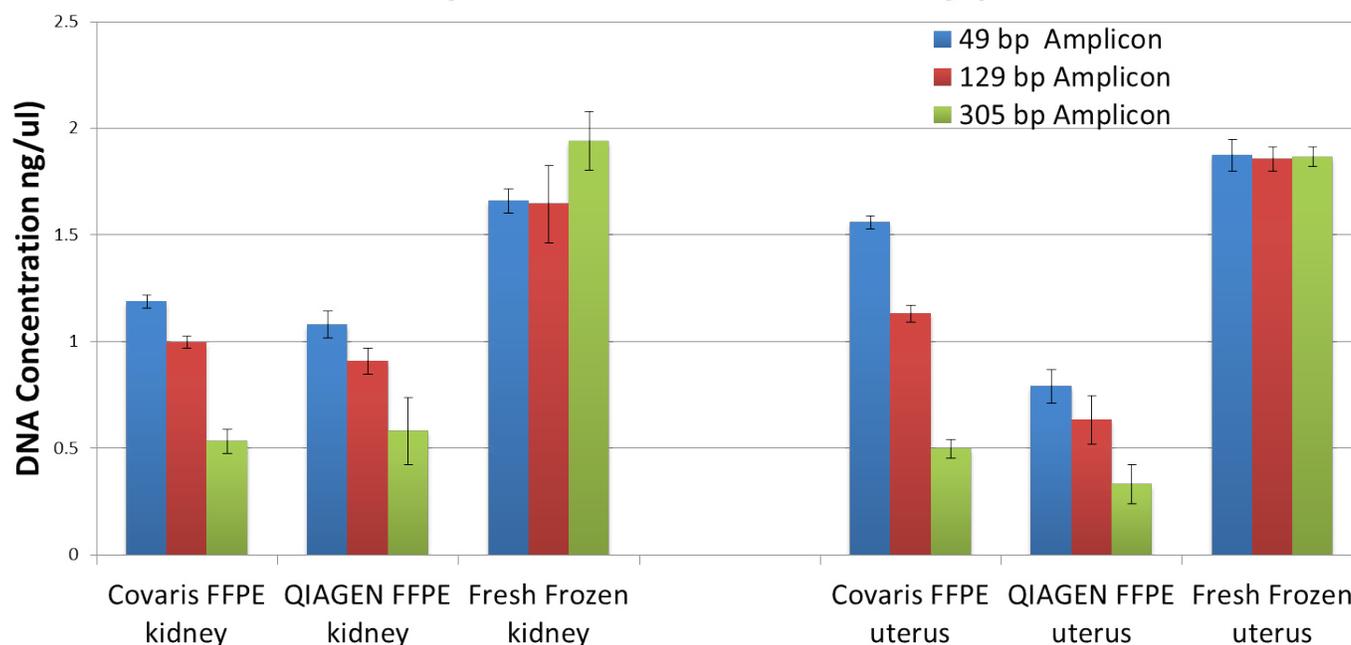## Quality and Concentration Assessment by qPCR

Figure 2: qPCR-based DNA concentration assessment fresh frozen tissue compared to Covaris and QIAGEN extracted FFPE DNA.

whole genome sequencing. Utilizing the Covaris truXTRAC Kit, we only had to use three kidney sections and one uterus sections to achieve the same yield. Since alternate sections from the same tissue blocks were used for extraction using both kits, the yield data clearly indicates that passive methods of DNA extraction from FFPE sections cannot maximize DNA yield. This is likely due to inefficient paraffin removal and tissue rehydration, rendering a significant portion of the tissue inaccessible for the extraction reagents.

qPCR-based Yield/Quality

To further analyze the extracted DNA, we utilized a commercially available qPCR based kit (KAPA hgDNA Quantification and QC Kit) that is commonly used for assessment of the quality of DNA prior to NGS library preparation. DNA isolated from matched fresh frozen kidney and uterus tissues was used as a control. Although the same amount of DNA was used for both extraction methods, the results (*Figure 2*) indicate a lower amount of amplifiable DNA for the QIAGEN extracted DNA. Prolonged formaldehyde fixation of tissues for FFPE preservation does cause damage to DNA, but the low extraction efficiency of the QIAGEN kit seems to amplify that effect. This is certainly significant since the amplicons for the KAPA kit are likely housekeeping gene, so the extraction of other genes of interest monitored for clinical applications are very likely similarly affected.
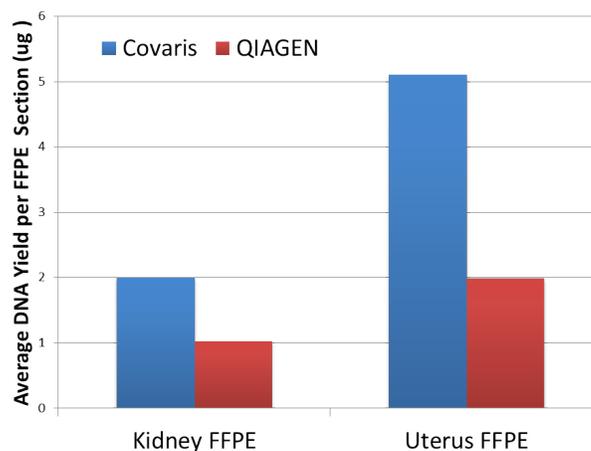

Figure 3: Qubit double stranded DNA yield analysis of FFPE sections extracted using Covaris truXTRAC FFPE DNA Kit and QIAGEN QIAamp FFPE Tissue Kit.

Whole Genome Sequencing Results

Although higher yield, and better qPCR results are metrics used by some to assess the quality of extracted DNA from FFPE tissues, information gathered from sequencing results provide the best indication of quality. qPCR analysis of FFPE DNA hinted at possible biased extraction from QIAGEN samples, so we submitted the DNA samples from both tissue types extracted by the passive QIAGEN method, and the active Covaris methods, as well as DNA from the matched fresh frozen tissues for full genome sequencing on an Illumina HiSeq2500 instrument. Analysis of the sequencing

results from QIAGEN FFPE, Covaris FFPE, and matched fresh frozen samples indicated a clear distinction in quality between the DNA extraction methods.

Carrying out a genome-wide coverage analysis **(Figure 4)**, QIAGEN extracted FFPE DNA samples had consistently lower coverage across the genome as compared to the Covaris extracted DNA and DNA from fresh frozen samples.

Close inspection of the coverage and comparison with Covaris extracted FFPE DNA, and DNA from fresh frozen tissue (**Figure 5**) also indicated inconsistent coverage across the genome for QIAGEN processed samples but coverage consistency of Covaris processed samples were remarkably similar to that of fresh frozen tissue.

Analysis of the coverage at chromosomal level (**Figure 6**) indicated a lack of significant coverage in QIAGEN extracted samples. Looking at several other chromosomes we noticed that gene rich areas of the chromosomes (**Figure 7**) were consistently underrepresented in QIAGEN processed samples. Our analysis of all chromosome indicated similar under representation for QIAGEN processed samples. The reason for coverage anomalies with QIAGEN processed samples is probably due to the inefficient passive method of paraffin removal and incomplete tissue rehydration preventing the full digestion of tissue by proteinase K. Reversal of crosslinks requires an aqueous environment present in hydrated tissue. Incomplete rehydration of the tissue will inhibit the reversal of crosslinks, thereby hindering the efficient purification of protein-bound DNA. Gene rich regions of the genome seem to lack consistent coverage depth in QIAGEN extracted samples, indicating a bias in the library representation for regions of the chromatin with low abundance of genes. This in our opinion is an indication that non-coding DNA regions are extracted at a higher efficiency by the QIAGEN FFPE DNA extraction, thereby effectively introducing a bias in the library preparation. Inconsistent and underrepresented coverage across clinically relevant genes or chromosomal regions is not acceptable in a clinical setting.

Conclusion

High yield, and high quality extraction of DNA from FFPE tissues for clinical applications is made possible by the use of AFA. Taking into consideration the results of the four analysis metrics used in this case study we can conclude that:

- Passive paraffin removal dependent on organic solvent diffusion is not efficient. Only active paraffin removal utilizing Covaris AFA, as indicated by direct fluorescence microscopy, is effective for maximal paraffin removal.
- Inefficient paraffin removal prevents adequate tissue rehydration, greatly affecting DNA yield as illustrated by fluorescence DNA yield, and qPCR quantification. Maximal DNA yield is directly linked to efficient paraffin removal and tissue hydration.
- Inefficient tissue hydration affects the reversal of the formaldehyde crosslinks, thereby biasing the DNA extraction to the chromosomal regions not extensively crosslinked as indicated by the coverage analysis of whole genome sequencing results.

As the utilization of FFPE extracted DNA in a clinical setting is becoming more prevalent, and the genetic analytic tools more sensitive, FFPE DNA extraction utilizing Covaris AFA technology is a uniquely positioned to match the sensitivity and analytical requirement of clinical applications by:

- Efficient removal of paraffin from FFPE cores, sections, and slides without the use of organic solvents or mineral oils.
- Enabling simultaneous tissue rehydration and paraffin removal.
- Promoting efficient tissue digestion by introducing proteinase K inside the tissue matrix thereby allowing tissue digestion from the inside out as well as outside in.
- Unbiased effective nucleic acid release from the remaining tissue matrix.
- Providing the choice of extracting tightly fragmented DNA ready for library preparation or large fragments up to 8kb.

Acknowledlgements and References

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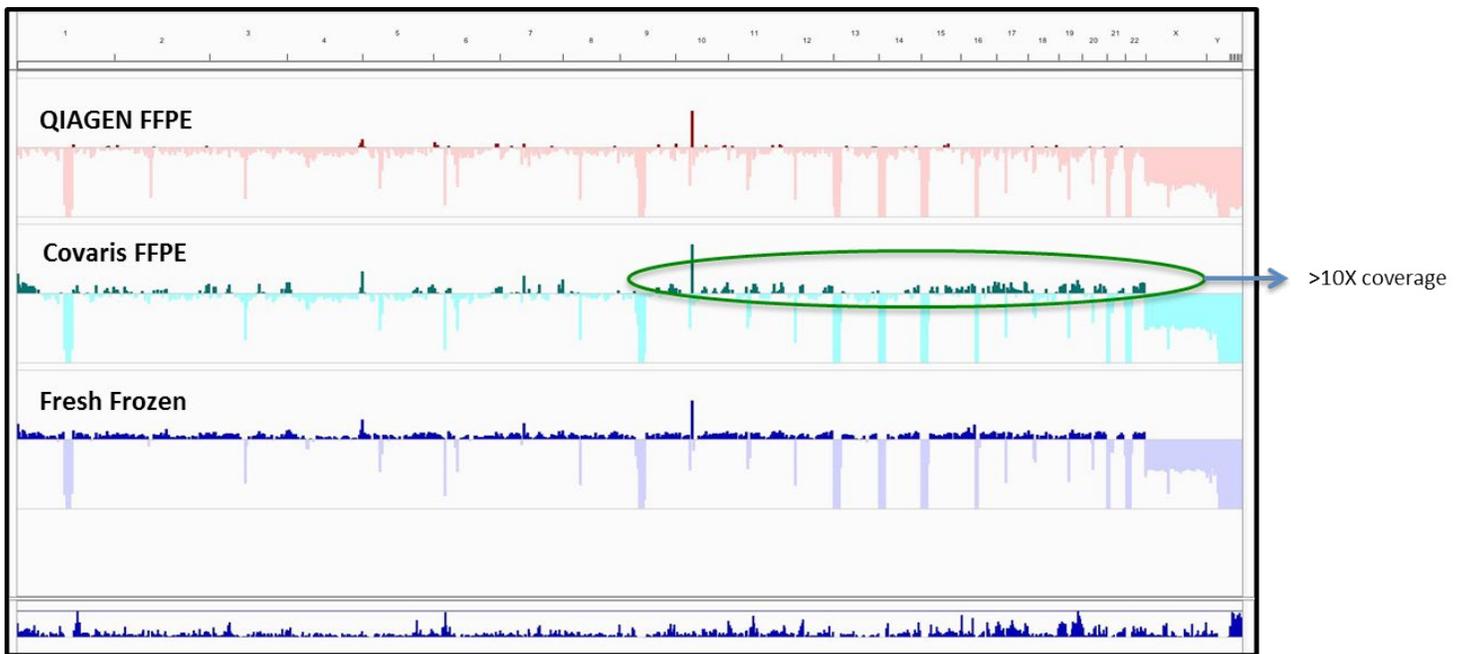
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Figure 4: Sequencing track from QIAGEN (red), Covaris (green) extracted FFPE kidney DNA, and fresh frozen (indigo) samples were loaded on the IGV viewer, and the coverage analyzed across the entire human genome using BedTools package. Coverage of >10x are indicated in dark colors, coverage of <10X are indicated in light colors. QIAGEN samples had consistently lower than 10X coverage across the genome as compared to the Covaris samples.
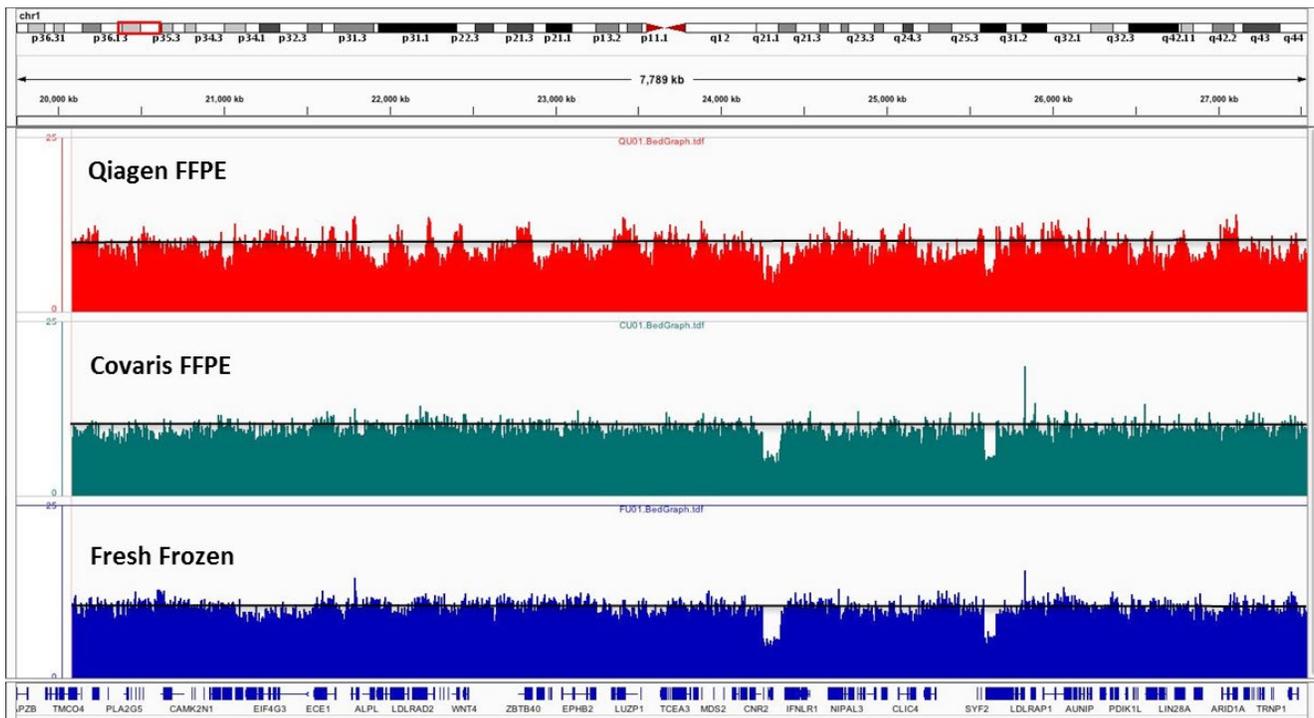

Figure 5: Sequencing track from QIAGEN (red), Covaris (green) extracted FFPE uterus DNA, and fresh frozen (indigo) samples were loaded on the IGV viewer, and the coverage analyzed for chromosome 1 with a baseline of 10X coverage. Chromosomal view of coverage indicates that Covaris extracted DNA uniformity resembles that of the DNA extracted from fresh frozen tissue quality resembles that of the DNA
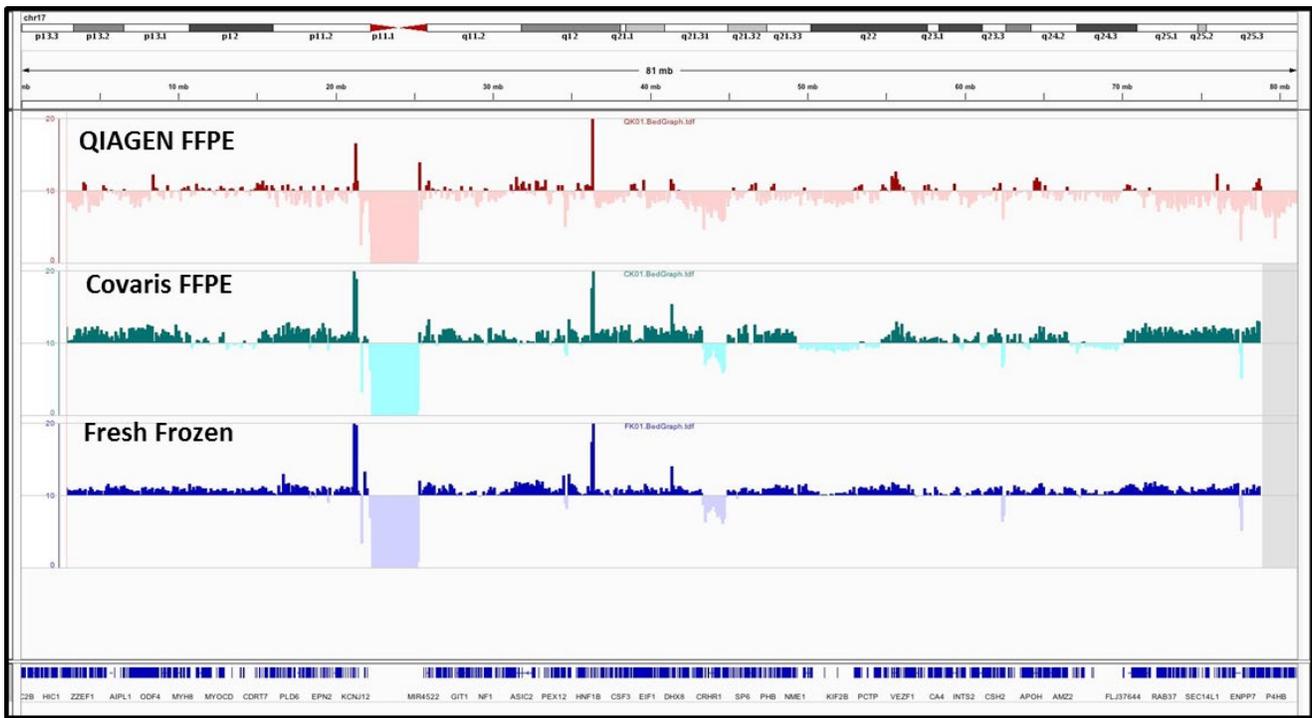
Figure 5: Sequencing track from QIAGEN (red), Covaris (green) extracted FFPE kidney DNA, and fresh frozen (indigo) samples were loaded on the IGV viewer, and the coverage analyzed for chromosome 17. Coverage of >10x are indicated in dark colors, coverage of <10X are indicated in light colors. Chromosomal view of coverage indicates that Covaris extracted DNA quality resembles that of the DNA extracted from fresh frozen tissue.

Figure 6: Sequencing track from QIAGEN (red), Covaris (green) extracted FFPE kidney DNA, and fresh frozen (indigo) samples were loaded on the IGV viewer, and the coverage analyzed for chromosome 19. Coverage of >10x are indicated in dark colors, coverage of <10X are indicated in light colors. Chromosomal view of coverage indicates that QIAGEN extracted DNA is significantly underrepresented in gene rich areas of chromosomes.