Application Note

Extraction of FFPE DNA for NGS

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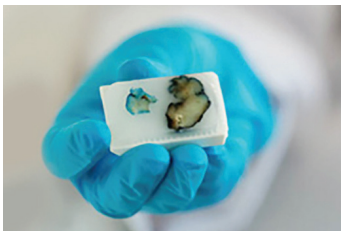
The KAPA NGS DNA Extraction Kit enables rapid and robust extraction of FFPE DNA for targeted next-generation sequencing

FFPE samples are an invaluable but challenging resource for the study of solid tumors using next-generation sequencing (NGS). The KAPA NGS DNA Extraction Kit offers a rapid and robust method for the extraction of NGS-ready DNA from FFPE curls for somatic oncology applications.

Introduction



Formalin-fixed, paraffin-embedded (FFPE) tissue blocks prepared from solid tumor biopsies are a ubiquitous and invaluable resource for the molecular characterization of cancers using PCR, sequencing, and other DNA/RNA analysis methods. Targeted next-generation sequencing (NGS) strategies are broadly used in basic, translational, and clinical research settings to survey the somatic mutations in specific regions of cancer genomes.

Targeted NGS workflows based on hybridization capture are widely employed as they enable the interrogation of all mutation classes (point mutations, short indels, gene fusions, and copy number variants). These protocols typically require the preparation of a genomic DNA fragment library from which regions of interest are captured with pre-defined probe panels. Mid- to high-nanogram quantities of input DNA (of sufficient quality to support efficient pre-capture amplification of 150 to >350 bp library fragments) are required for successful sequencing.

A wide range of methods are available for the extraction and purification of DNA from FFPE samples. However, not all methods yield DNA of sufficient quality for NGS analysis. High intra- and inter-method variability in DNA yield and quality is commonplace, and impacted by different tissue processing techniques, archiving time, and tissue type. In addition, molecular damage caused by fixation and deparaffinazation can result in a high proportion of molecules in an FFPE DNA preparation that does not support successful NGS library construction.

The KAPA NGS DNA Extraction Kit offers a rapid and robust method for the extraction of NGS-ready FFPE DNA, without the need for specialized equipment. In this Application Note, we compare yield and quality of FFPE DNA extracts generated with the KAPA NGS DNA Extraction Kit vs. an industry-leading, automated DNA extraction system. In addition, we demonstrate that the KAPA NGS DNA Extraction kit supports high-quality targeted cancer sequencing on the Illumina® platform.

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Materials and methods

Experimental design

To demonstrate the utility of the KAPA NGS DNA Extraction Kit for the preparation of NGS-ready DNA extracts, DNA was extracted from FFPE blocks prepared from four different tissue types. For comparison, DNA was extracted from the same samples with the QIAsymphony® SP automated DNA purification instrument (QIAGEN). After assessment of FFPE DNA yield and quality, hybrid capture libraries were prepared for targeted cancer sequencing on the Illumina® platform (Figure 1).

For the purpose of this study, library preparation, target enrichment, and data analysis were performed with the AVENIO Tissue Tumor Analysis Kit (Surveillance Panel) and AVENIO Oncology Analysis Software. However, FFPE DNA extracted with the KAPA NGS DNA Extraction Kit is broadly compatible with targeted NGS pipelines and has been validated for use in the KAPA HyperCap[1] and KAPA HyperPETE[2] workflows.

Tissue samples

FFPE blocks prepared from four different tissue types (breast, colon, lung, and prostate) were obtained from a commercial supplier (Discovery Life Sciences). Ten blocks, all ≤3 years old, were sourced per tissue type, and each was sectioned 100 times at 5 µM thickness. For each tissue type, six samples (4 x 5 µM curls each) were selected from each of three different blocks, for a total of eighteen samples per tissue type.

DNA Extraction and Cleanup

KAPA NGS DNA Extraction Kit: Half of the samples (nine sets of curls x four tissue types) were processed with the KAPA NGS DNA Extraction Kit (Roche PN: 09189823001 or 09190023001), according to the standard protocol.[3] In short, each set of four FFPE curls was transferred to a 1.5 mL tube, and submersed in 100 µL of freshly prepared Extraction Master Mix. Tubes were incubated at 75ºC for 1 hour with shaking, using an Eppendorf® ThermoMixer.® Reaction products were centrifuged to pellet cellular debris, after which clear DNA-containing extracts were transferred to fresh tubes, taking care to avoid carryover of cellular debris and wax. DNA was purified using KAPA HyperPure Beads (Roche PN: 08963835001, 08963843001, 08963851001, 08963878001, or 08963860001) using a 1.8:1 (1.8X) volumetric bead reagent-to-DNA extract ratio. Extracted DNA was recovered in 40 µL of DNA Elution Buffer (10 mM Tris-HCl, pH 8.0), transferred to fresh tubes and stored at -20ºC.

QIAsymphony system: The remaining samples were processed using a QIAsymphony SP instrument (software v5.0.4) and QIAsymphony DSP DNA Mini Kit (version 1), following the manufacturer's recommended protocol[4] with an elution volume of 50 µL.

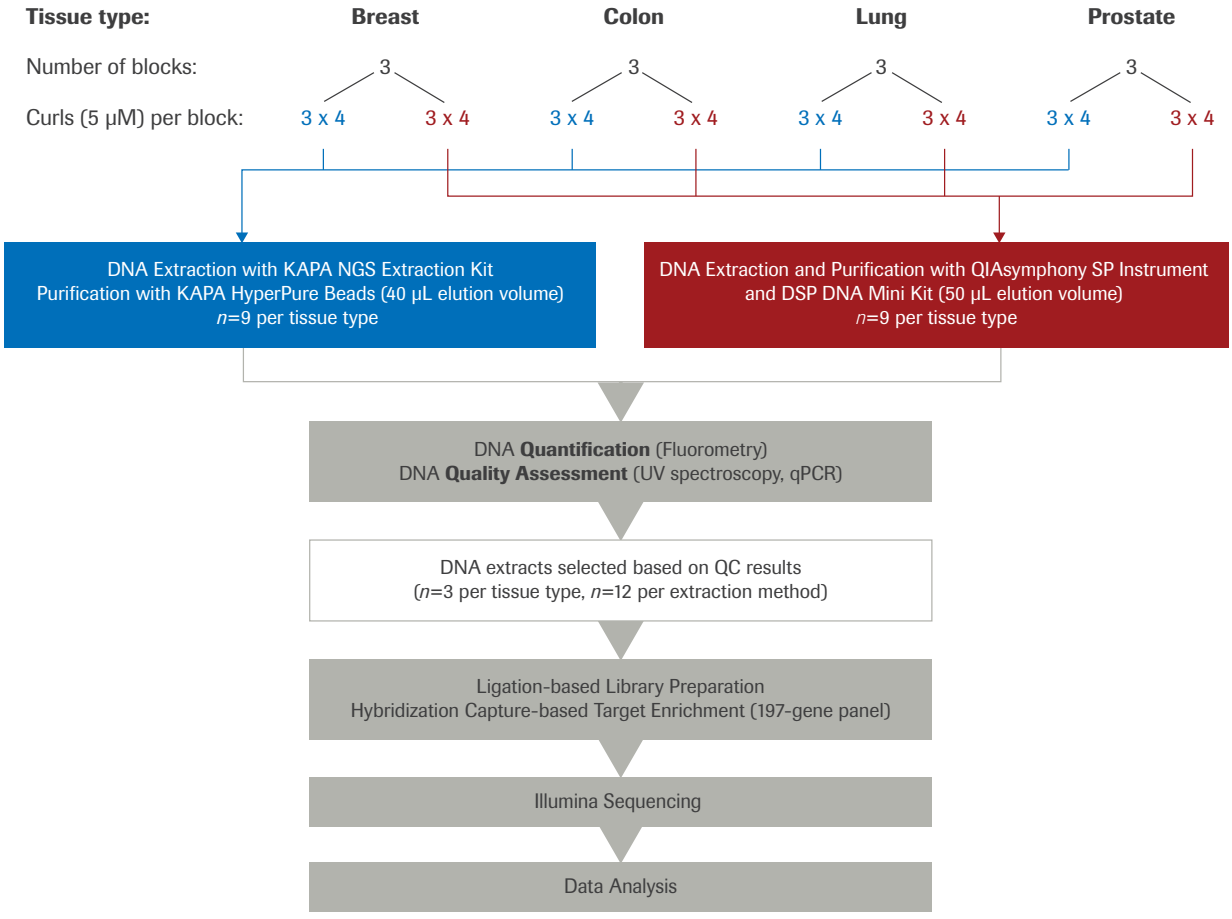

Figure 1. FFPE DNA extraction, library preparation, and sequencing workflow used in this study. FFPE tissue blocks (all ≤3 years of age) were obtained from a commercial supplier. Of ten available blocks per tissue type, three were selected for this experiment. Three sets of four 5 µM sections were obtained from each block, for a total of nine samples per tissue type or 36 samples per extraction method. DNA was extracted and assessed as described in Materials and Methods. After DNA quantification and QC analysis, three extracts from each tissue type/extraction method were selected for further processing. Library preparation, sequencing, and data analysis were performed as outlined in Materials and Methods.

Assessment of FFPE DNA concentration and quality

The concentration of dsDNA obtained from each extraction was determined with a Qubit® 3.0 Fluorometer and Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific). Two 5 µL aliquots were taken from each DNA extract, for duplicate measurements performed according to the manufacturer's instructions.[5,6]

DNA quality was assessed spectrophotometrically as well as with a qPCR-based method. For the spectrophotometric method, UV absorbance readings were taken at 230 nm, 260 nm and 280 nm, and absorbance ratios (A260/A280 and A260/A230) determined using a NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific). Two 2 µL aliquots were taken from each DNA extract, diluted 1/10, and analyzed according to the manufacturer's recommended Nucleic Acid protocol.[7]

The qPCR-based assay was performed with the KAPA NGS FFPE DNA QC Kit (Roche PN: 09217193001 or 09217207001), according to the standard protocol.[3] Briefly, one 1 µL aliquot was taken from each DNA extract, diluted 1/500 in nuclease-free water, and assayed in triplicate using a LightCycler® 480 Instrument II (Roche PN: 05015278001 or 05015243001) according to instructions in the LightCycler® 480 Instrument Operator's Manual Software Version 1.5.[8] Average Cp values for the two (66 bp and 191 bp) amplicons generated from each DNA extract, as well as from the QC PCR DNA Standard of known quality, were used were used to calculate the quality score (Q score) and normalized Q score for each DNA extract, using the following formulas:

$$Q\ score = 2^{(average(Cp66) - average(Cp191))}$$

Normalized Q score=Sample Q score/QC PCR DNA Standard Q score

Library Preparation, Sequencing, and Data Analysis

Of the nine FFPE DNA extracts generated from each tissue type with each of the two extraction methods, three samples were selected. The amount of input DNA needed for library construction from each DNA extract was determined using the formula:

Input mass (ng)=10/(normalized Q score)+10

Libraries were constructed and library QC was performed as described in the AVENIO Tumor Tissue Analysis Kit Reagent Workflow User Guide (Version 1.1, Software Version 2.0.0).[9] Enrichment was performed with the 197-gene Surveillance Panel. Libraries were pooled for multiplexed, paired-end (2 x 150 bp) sequencing on an Illumina® NextSeq™ 500/550 instrument. Data analysis was performed as described in the AVENIO Oncology Analysis Software User Guide, Software Version 2.0.0.[10]

Results and discussion

DNA concentration and quality

Results from the fluorometric quantification of the 72 DNA extracts generated in this study are shown in Figure 2. Results confirmed an impact of tissue type on DNA extraction efficiency, with colon samples performing best and prostate samples performing worst with both extraction methods.

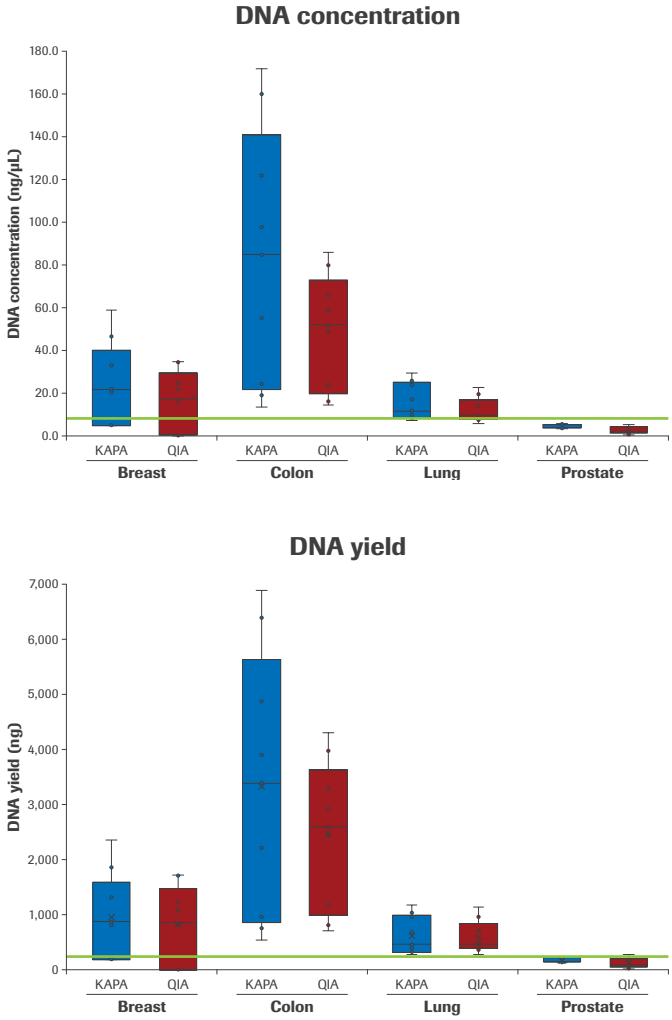

Figure 2. DNA concentration (top) and yield (bottom) for FFPE DNA extracts prepared from four different tissue types, using the KAPA NGS DNA Extraction Kit (blue) or QIAsymphony system (red). DNA was extracted and quantified as described in Materials and Methods. The green lines represent a DNA concentration of 8.5 ng/µL and a DNA yield of 260 ng, respectively.

DNA extracts prepared with the KAPA kit were 33 – 77% more concentrated, and total DNA yields were 6 – 43% higher (depending on tissue type), as compared to results obtained with the QIAsymphony® system. This indicated that FFPE DNA extraction with the KAPA NGS DNA Extraction Kit is intrinsically more efficient than with the automated system.

Results from the quality assessment of DNA extracts (using absorbance ratios and a qPCR-based method) are summarized in Figure 3 on the next page. Average A260/A280 absorbance ratios ranged between 1.85 and 2.16 for KAPA DNA extracts (after removal of the obvious outlier—attributed to operator error—from the colon samples), and between 1.63 and 1.82 for extracts generated with the QIAsymphony system. A260/A230 ratios were much lower for all tissue types, and lower overall for the QIAsymphony system (average ratios of 0.12 – 0.57 for different tissue types) as compared to the KAPA kit (average ratios of 0.58 – 1.22). This suggested a higher degree of carry-over of salts and/or solvents from the automated chemistry.

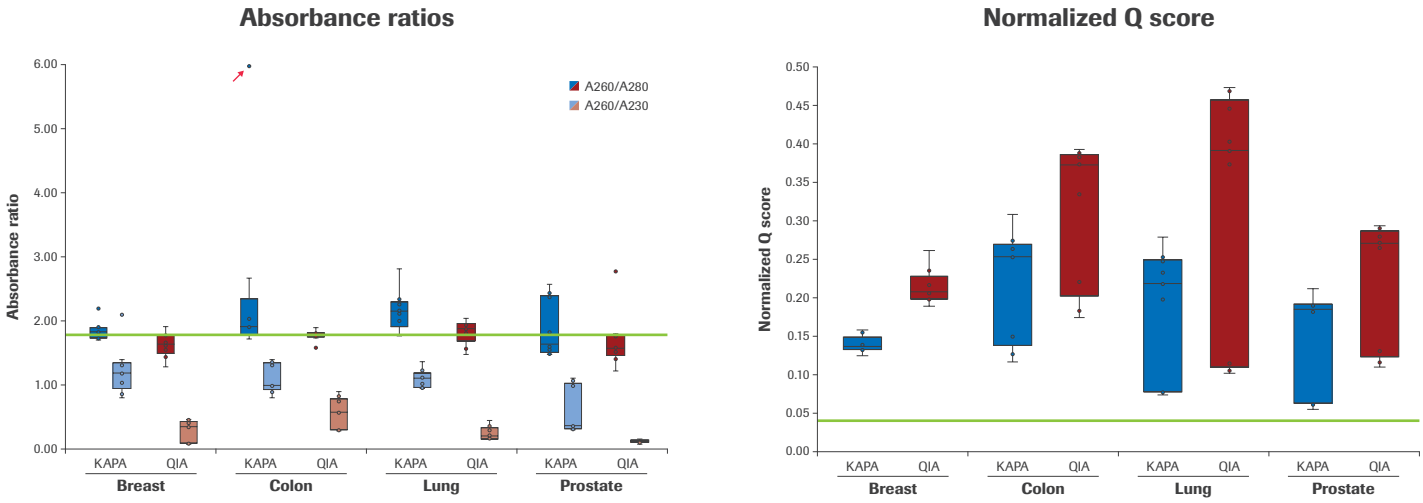

Figure 3. Absorbance ratios (left) and normalized Q score (right) for FFPE DNA extracts prepared from four different tissue types, using the KAPA NGS DNA Extraction Kit (blue) or QIAsymphony system (red). DNA was extracted and quality assays were performed as described in Materials and Methods. Green lines represent an absorbance ratio of 1.8 and normalized Q score of 0.04, respectively. The A260/A280 outlier (indicated with the arrow; attributed to operator error) was omitted from the calculation of average A260/A280 ratio for the colon DNA extracts prepared with the KAPA kit.

Normalized Q scores calculated using the qPCR-based assay (Figure 3, right) were higher for FFPE DNA extracts generated with the QIAsymphony® system (average of 0.21 – 0.32, depending on tissue type) than those for KAPA extracts (0.14 – 0.22), but also more variable. Both extraction methods produced DNA extracts with normalized Q scores >0.04 for all replicates of all tissue types. Although not applicable to all protocols for the preparation of FFPE libraries, this is the minimum cut-off value for library construction with the AVENIO kit.

It is important to note that DNA concentration and yield are not reliable predictors of DNA quality and sequencing outcomes. As a case in point, breast tissue samples ranked second with respect to DNA concentration and yield with both extraction methods, but produced the lowest quality DNA (with both extraction and analysis methods), and the worst sequencing results (see below).

Library construction metrics

Three replicates from each set of nine FFPE DNA extracts generated per tissue type and extraction method—deemed to be representative of DNA quantity and quality across each set—were selected for library construction. The mass of input DNA required from each extract was calculated from the normalized Q score. Actual input masses (second column, Table 1) corresponded to calculated values, except in two cases where DNA concentrations were too low to achieve the desired input using the maximum DNA input volume of 30.5 µL.

Key library construction metrics are given in the remainder of Table 1. All FFPE DNA extracts supported successful adapter ligation, as evidenced by an average pre-enrichment library fragment size of approximately 300 bp (or larger). Average pre-enrichment library fragment sizes were longer for KAPA vs. QIAsymphony libraries (350 ±26 bp vs. 286 ±11 bp) across the entire sample set.

One pre-enrichment library (Breast-KAPA 1) returned a concentration lower than recommended for target enrichment with the workflow used in this study (<5 ng/µL). Since the actual mass of input DNA for this library was relatively high (80 ng vs. the average input mass of 62 ng across all 24 samples), and equal to the input mass calculated from the normalized Q score, operator error during library construction was identified as the likely cause for the low pre-enrichment library concentration. Since the post-enrichment concentration for this library was also lower than desired, this library was excluded from sequencing and subsequent data analysis.

Two additional enriched libraries (Breast-QIA 1 and Breast-QIA 3) also returned post-enrichment library concentrations outside of the desired range (0.5 – 15 ng/µL). These two libraries were also excluded from sequencing data analysis.

All enriched libraries displayed an average fragment size of approximately 300 bp or higher (optimal for 2 x 150 bp paired-end sequencing). Average fragment sizes for KAPA libraries were again longer than for QIAsymphony libraries (370 ±37 bp vs. 304 ±31 bp). Enriched libraries had slightly longer average fragment sizes than pre-enriched libraries, suggesting that amplification with uracil-intolerant, high-fidelity enzymes may select for longer and less-damaged molecules during the pre-enrichment amplification.

Sequencing results

On-target rate, coverage depth and uniformity, and error rate, are important primary sequencing analysis metrics in targeted NGS, and were assessed to evaluate the quality of FFPE DNA extracts generated with the KAPA NGS DNA Extraction Kit and QIAsymphony system. Results are summarized in Table 2 and Figure 4 on pp. 6 – 7. Desired values for each metric (listed in Table 2) specifically apply to the AVENIO Workflow used in this study, and may be different for other target enrichment workflows and/or capture panels.

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Table 1. Library construction parameters and QC metrics

Sample	Input (ng)	Pre-enrichment library conc. (ng/µL)	Average fragment size of pre-enrichment library (bp)	Enriched library conc. (ng/µL)	Average fragment size of enriched library (bp)
Breast–KAPA 1	80	2.83[b]	319	0.25[c]	303
Breast–KAPA 2	85	27.9	342	2.80	357
Breast–KAPA 3	71	27.1	328	2.26	355
Breast–QIA 1	9[a]	8.19	285	0.43[c]	322
Breast–QIA 2	58	38.6	274	1.13	276
Breast–QIA 3	49	35.9	303	0.26[c]	387
Colon–KAPA 1	42	20.8	362	1.29	400
Colon–KAPA 2	48	18.0	363	1.05	400
Colon–KAPA 3	77	23.4	337	1.05	362
Colon–QIA 1	35	14.4	280	0.74	280
Colon–QIA 2	37	22.9	294	1.56	315
Colon–QIA 3	56	19.5	297	1.41	324
Lung–KAPA 1	46	10.2	388	1.46	434
Lung–KAPA 2	53	9.93	401	1.38	411
Lung–KAPA 3	138	10.4	344	1.18	337
Lung–QIA 1	31	34.7	277	1.58	291
Lung–QIA 2	35	38.5	281	2.10	296
Lung–QIA 3	98	25.1	276	1.29	285
Prostate–KAPA 1	173	15.7	314	6.70	331
Prostate–KAPA 2	64[a]	9.94	345	4.94	367
Prostate–KAPA 3	57	6.26	351	3.34	386
Prostate–QIA 1	64	28.5	290	4.12	289
Prostate–QIA 2	44	24.3	275	3.30	284
Prostate–QIA 3	46	29.0	302	5.60	293

[a] Actual input lower than calculated input (9 ng vs. 60 ng for Breast–QIA 1, and 64 ng vs 86 ng for Prostate–KAPA 2) due to low DNA concentrations and input volume constraints.
[b] Concentration of pre-enrichment library lower than desired (<5 ng/µL).
[c] Concentration of post-enrichment library lower than desired (0.5 – 15 ng/µL).

On-target rate: On-target rate is an important indicator of capture efficiency and sequencing economy. On-target rates were significantly higher for libraries constructed from KAPA DNA extracts (average of 89.5 ±4.04% vs. 73.9 ±7.46% across all tissue types). Less variation was also observed between tissue types for KAPA vs. QIAsymphony® extracts, with QIAsymphony DNA extracts yielding on-target rates below the desired value of 70% for two of the four tissue types (breast and prostate). Higher on-target rates for KAPA libraries were partially attributed to longer (more optimal) library fragment/insert sizes, confirmed by both library QC (Table 1) and sequencing (Table 2, last column).

Coverage depth and uniformity: Coverage depth and uniformity are critical, inter-related sequencing metrics. A minimum coverage depth is required to call variants with confidence. Coverage uniformity, or the evenness of coverage across diverse portions of the capture target (e.g., regions of high GC or AT content), determines whether coverage criteria can be achieved from the amount of sequencing data that is collected in a study.

Libraries prepared from KAPA FFPE DNA extracts returned better overall coverage metrics. Average, deduplicated coverage depth across all four tissue types was higher for KAPA libraries (849 ±368X, vs. 779 ±246X for QIAsymphony libraries). The two extraction methods performed comparably in terms of the percentage of targets covered ≥300X, with prostate samples performing significantly worse than the other three tissue types both extraction methods. With respect to the other coverage uniformity metric (90th/10th coverage percentile ratio), the KAPA NGS DNA Extraction Kit performed better overall, and more consistently across tissue types, as compared to the QIAsymphony system.

Table 2. Summary of sequencing results

Sample	% reads on target	Deduplicated coverage depth (X)	% of targets covered ≥300X	90th/10th coverage percentile ratio	Error rate (%)	Mean insert length (bp)
Desired values	**>70**	**>300**	**>90**	**1.5 – 4.0**	**<0.01**	**Larger is better**
Breast–KAPA (*n*=2)	86.6 ±1.98	1,306 ±291	99.3 ±0.300	2.6 ±0.17	*0.011 ±0.0023	159 ±2
Colon–KAPA (*n*=3)	93.5 ±1.11	1065 ±154	98.3 ±0.624	2.9 ±0.12	0.0083 ±0.0012	190 ±19
Lung–KAPA (*n*=3)	90.5 ±1.12	720 ±34	94.9 ±1.42	3.0 ±0.15	0.0096 ±0.0047	198 ±36
Prostate–KAPA (*n*=3)	86.2 ±5.31	459 ±275	*63.5 ±28.0	*5.8 ±3.2	0.0079 ±0.0017	165 ±14
All tissues–KAPA (*n*=11)	**89.5 ±4.04**	**849 ±368**	**88.1 ±20.2**	**3.7 ±2.0**	**0.0091 ±0.0027**	**179 ±26**
Breast–QIA (*n*=1)	*67.0	628	92.3	*4.4	*0.015	85
Colon–QIA (*n*=3)	81.4 ±6.66	891 ±319	95.1 ±6.56	3.5 ±0.44	*0.010 ±0.0022	113 ±19
Lung–QIA (*n*=3)	76.1 ±1.16	927 ±126	95.6 ±2.57	*4.6 ±0.81	0.0083 ±0.0036	94 ±7
Prostate–QIA (*n*=3)	*66.6 ±2.74	570 ±157	*76.5 ±17.3	*9.3 ±5.0	0.0086 ±0.0030	96 ±0.3
All tissues–QIA (*n*=10)	**73.9 ±7.46**	**779 ±246**	**89.4 ±13.0**	***5.6 ±3.5**	**0.0096 ±0.0031**	**99 ±14**

Sequencing data for Breast-KAPA 1, Breast-QIA 1 and Breast-QIA 3 libraries not taken into account in calculation of average values, as pre-enrichment library concentrations for these libraries were indicative of sub-optimal sequencing results.

*Average value lower than desired value or outside of desired range.

Error rate: Libraries prepared from KAPA FFPE DNA extracts displayed lower overall and average error rates for all tissues, except for lung.

Mean insert length: Library insert lengths in the last column of Table 2 were calculated from sequencing data (after adapter and quality trimming), and are always shorter than average library fragment sizes (inclusive of adapters) determined with an electrophoretic method (Table 1). Insert sizes are intrinsically limited by the quality (degree of degradation) of input DNA, but may also be impacted by the efficiency of DNA extraction and/or library preparation. Insert sizes generally corresponded to Q-ratios (i.e., were shortest for breast and prostate samples), and were 1.7- to 2.1-fold longer for libraries prepared from KAPA FFPE DNA extracts as compared to those prepared from DNA extracted with the automated platform.

Conclusions

In this study, FFPE DNA was extracted from four different tissue types with the KAPA NGS DNA Extraction Kit. DNA yield and quality was assessed before using the DNA in a targeted sequencing (NGS) workflow. For comparison, DNA was extracted with the automated QIAsymphony® system, and processed using the same workflow.

Results from the direct analysis of DNA extracts, QC assessment of libraries, and primary analysis of sequencing data collectively demonstrate the following:

- The KAPA NGS DNA Extraction Kit is intrinsically more efficient than the QIAsymphony system, producing higher yields of FFPE DNA, more concentrated FFPE DNA preparations, and fewer carry-over contaminants. This is expected to translate to higher overall success rates when extracting DNA from cohorts of real-life FFPE blocks prepared from different tissue types, with different fixation methods, and/or stored under different conditions for different periods of time.
- FFPE DNA prepared with the KAPA NGS DNA Extraction Kit is highly suitable for the construction of libraries for targeted, short-read sequencing. DNA extracts exceeded the minimum required normalized Q score (an NGS-relevant quality metric), and supported successful ligation-based library preparation and hybridization-based target enrichment—yielding pre- and post-enrichment libraries that met QC requirements (concentration and fragment length) for subsequent sequencing.
- FFPE DNA extracted with the KAPA NGS Extraction Kit supports high-quality targeted sequencing on the Illumina® platform, producing higher on-target rates, better overall coverage metrics (depth and uniformity) and comparable error rates than DNA prepared with the automated QIAsymphony system.
- As demonstrated in this study, tissue type can have a material impact on the efficiency of DNA extraction—irrespective of the extraction method used—and, consequently, on library construction and sequencing success. Half of the breast FFPE DNA extracts selected for library construction and sequencing failed to produce libraries that met pre-sequencing QC metrics (pre- and/or post-enrichment library concentration). Libraries produced from both KAPA and QIAsymphony prostate DNA passed library QC, but produced low-quality sequencing data. For this reason, it is critical to assess the quality of FFPE DNA with an NGS-relevant assay (e.g., the qPCR assay used here), and not rely on DNA concentration and/or UV absorbance ratios to predict library construction and sequencing outcomes.

The KAPA NGS DNA Extraction Kit offers a rapid and robust method for the extraction of NGS-ready DNA from FFPE samples. Used in combination with the KAPA HyperCap or KAPA HyperPETE Workflows, it offers a complete sample preparation solution for somatic oncology research.

Data on file.

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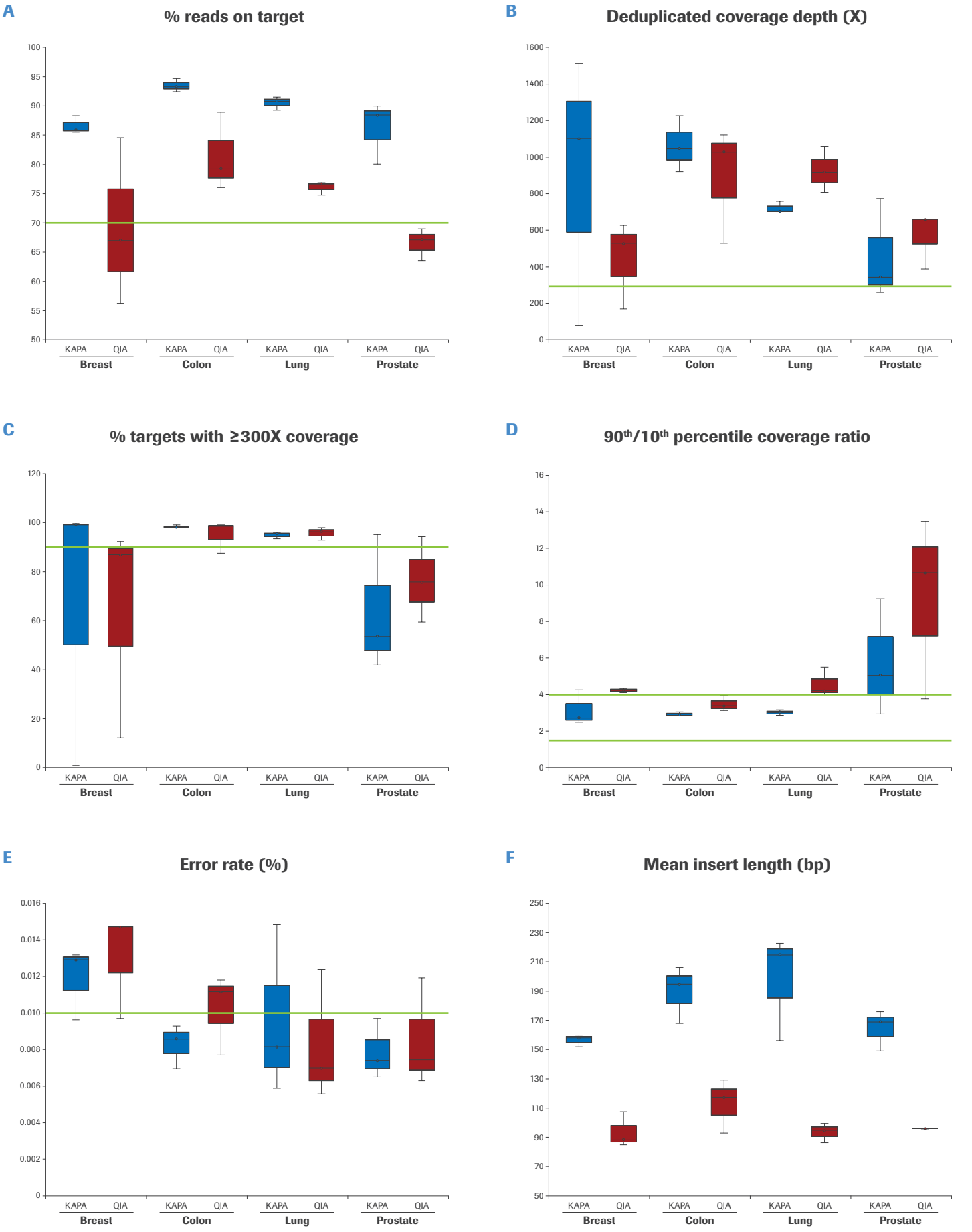

Figure 4. Sequencing metrics for libraries prepared from FFPE DNA extracted with the KAPA NGS DNA Extraction Kit (blue) and QIAsymphony system (red). DNA was extracted and analyzed, libraries were prepared, sequencing was performed, and data were analyzed as described in Materials and Methods. Green lines indicate desired results for the AVENIO Tumor Tissue Analysis Kit: **(A)** on-target rate: >70%, **(B)** coverage depth: ≥300X, **(C)** coverage uniformity (% of targets covered at ≥300X): >90%, **(D)** coverage uniformity (ratio of samples in the 90th coverage depth percentile/samples in the 10th coverage depth percentile): 1.5 – 4, and **(E)** error rate: <0.01%. For mean insert length (determined by sequencing, **(F)**) no upper or lower values are indicated. Longer inserts are preferred and are expected to improve overall sequencing metrics and sequencing economy. The libraries excluded from average metric calculations in Table 2 (Breast-KAPA 1, Breast-QIA 1 and Breast-QIA 3) are **included** in these analyses.

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