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A complete workflow for high-throughput isolation of DNA and RNA from FFPE samples using FormaPure Total and the KingFisher™ technology: an application for robust and scalable cancer research and biomarker discovery

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Summary

Formalin-fixed paraffin-embedded (FFPE) tissues are an invaluable resource for histological and genetic testing for cancer. Current next-generation sequencing (NGS) technologies and extraction methods make it possible to study and identify cancer-causing alterations at the genomic and transcriptomic levels. However, challenges exist in most FFPE workflows, especially when high-throughput extractions are a requirement. First, FFPE samples generally provide low-quality nucleic acids, especially the RNA, as RNA undergo more severe degradation and chemical and covalent modifications due to the effects of formalin-fixation than DNA. Second, consistent results for most high-throughput and automated extraction methods for FFPE are difficult to achieve due to inherent differences in the variety of tissue and disease types. Without human interactions, it is almost impossible for automated methods to identify and resolve all of the challenges involved in FFPE extractions. A solution that offers the ability to apply high-throughput and reliable genomic applications to FFPE samples can help accelerate biomarker discovery and clinical assays.

This application note demonstrates the use of FormaPure Total, a total nucleic acid extraction kit for FFPE samples, in conjunction with the KingFisher Duo Prime automated protein purification system as a potential solution that mitigates some of the challenges with FFPE workflows. FormaPure Total has been verified to improve the quality of FFPE-derived nucleic acids compared to other commercially available extraction methods. Moreover, by producing higher integrity (higher molecular weight) nucleic acids, FormaPure Total significantly improves NGS end-points. Specifically, FormaPure Total isolates FFPE-derived RNA that provide 86% more fusion calls and 267% fusion supporting reads when using the TruSeq® Stranded Total RNA and the TruSight® Tumor 170 library preparation kits.[1] The workflow outlined in this study demonstrates proof of concept for using KingFisher technology with the FormaPure Total reagents, and illustrates considerations for the automation parameters. Lastly, data presented in this study suggests that an automated workflow can be utilized without compromising the performance of extractions from FFPE samples.

Materials and Methods

FFPE extractions were semi-automated on the KingFisher Duo Prime using the FormaPure Total kit. First, the samples are processed manually for sample deparaffinization, tissue digestion, decrosslinking and lysate splitting steps. Next, the binding, washing, and elute steps of the FormaPure Total protocol were automated using the KingFisher Duo Prime. The illustrated KingFisher Duo Prime workflow and optimized parameters can offer a proof of concept for larger KingFisher platforms that allow higher-throughput sample handling (up to 96 samples per run), as the automated magnetic-particle processing technology is the same across all models.

Manual processing

In this study, four different FFPE samples, representing three cancer tissues of lung, liver and breast, were processed. DNA and RNA were isolated from single 10 µm curl inputs. Incubation times, temperatures and volumes required for each step of the extraction process were followed as per the FormaPure Total protocol. Briefly, the samples were placed into 1.5 mL microcentrifuge tubes and deparaffinized in mineral oil. After complete deparaffinization of the samples, the lysis buffer and proteinase K were added to the samples and incubated for two hours on a heat block at 60°C. After tissue lysis, half of the lysates were pipetted into a 96-well KingFisher plate for RNA isolations. The remaining half of the lysates were transferred to an 80°C heat block for DNA decrosslinking. After 1 hour, the DNA are decrosslinked and the RNA isolations are completed on the KingFisher. DNA isolations can subsequently processed on the KingFisher or the lysates can be left at 60°C to incubate for up to overnight if tissues require further digestion.

Automated processing

The parameters for mix times and speeds, and the collect counts time for KingFisher Duo Prime were optimized for the magnetic beads, reagents and volumes used for FormaPure Total kit (Table 1 and 2). Different KingFisher models may require additional optimizations to ensure adequate resuspension of samples and complete collection and transfer of beads.

RNA Isolations:

Implementation of the KingFisher Duo Prime starts after the lysates are split for RNA isolations. Lysates are pipetted into Row B on a 96-KingFisher plate with the reagents pre-aliquoted into the appropriate wells of the KingFisher 96 Deep-Well plate **(Table 1)**. The first automated step is DNase I treatment. Afterward, the plate is removed from the instrument and the re-bind solution is manually pipetted into Row A to re-constitute the binding of RNA to the beads. The plate is loaded back on the instrument to automate the rest of the extraction process. Table 1 outlines the parameters of following steps involved in automating the RNA isolations: the two bind steps, two wash steps, DNase treatment and elution of RNA.

				Automation Parameters		
RNA purification step	**Plate row**	**Regent**	**Volume**	**Mixing time/Mixing speed (bottom mix)/Pause time**	**Collect count/ time [s]**	
DNase I treatment	A	DNase I solution	120 µL	10 s/medium/20 min	5/30 s	Heating 37°C
First Bind	B	Bind buffer Lysate	150 µL 100 µL	20 s/medium/5 min	5/30 s	
Wash 1	C	80% Ethanol	375 µL	20 s/medium/5 min	5/30 s	
Wash 2	E	80% Ethanol	375 µL	20 s/medium/5 min	5/30 s	
NA	F	Empty				
NA	F	Empty				
Comb Tip	G	KingFisher Duo 12-Tip Comb				
RNA Elution	H	Nuclease Free Water	40 µL	20 sec/medium/5 min	5/30 sec	

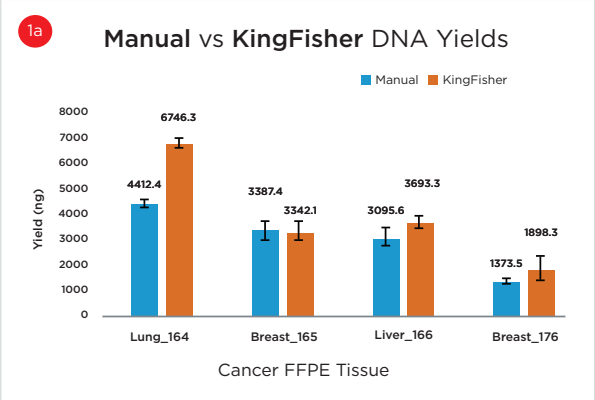
Table 1. KingFisher Duo Prime RNA isolation set-up.

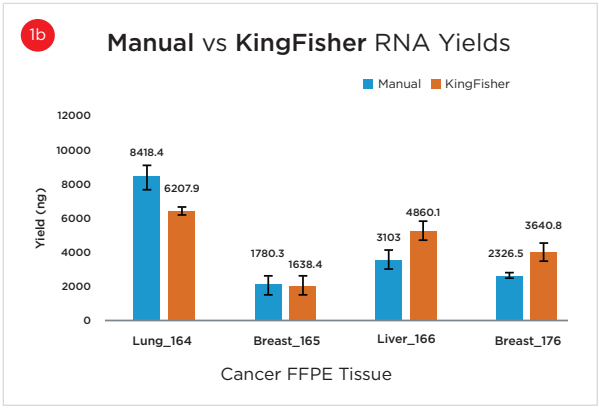
DNA Isolations:

After the RNA isolations are complete, the remaining half of the decrosslinked lysates are transferred to Row B on a separate KingFisher 96 Deep-Well plate. Before the plate is placed on the KingFisher, RNase A is added to the lysates, pipet mixed, and incubated at room temperature for 5 minutes. Then, the bind solution is added to the lysates to immobilize the DNA onto the beads. The required reagent are aliquoted into the appropriate wells **(Table 2)**. For DNA isolations, one bind step, two wash steps, and elution of DNA are performed on the KingFisher Duo Prime. The automated portion of the DNA isolations takes ~30 minutes to complete.

				Automation Parameters	
DNA purification step	**Plate row**	**Regent**	**Volume**	**Mixing time/Mixing speed (bottom mix)/Pause time**	**Collect count/time [s]**
DNA Elution	A	Nuclease Free Water	40 µL	20 s/medium/5 min	5/30 s
Bind	B	Bind buffer Lysate RNase A	150 µL 100 µL 2.5 µL	20 s/medium/5 min	5/30 s
Wash 1	C	Wash Buffer	200 µL	20 s/medium/5 min	5/30 s
Wash 2	D	80% Ethanol	375 µL	20 s/medium/5 min	5/30 s
Comb Tip	E	KingFisher Duo 12-Tip Comb	NA	NA	
NA	F	Empty			
NA	G	Empty			

Table 2. KingFisher Duo Prime DNA isolation set-up.





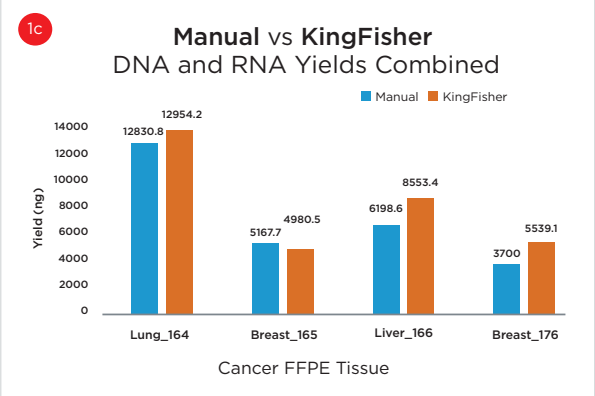


Figure 1. DNA (a), RNA (b), and total nucleic acid (c) yields from FFPE cancer tissues via manual and KingFisher semi-automated workflows. DNA and RNA yields were obtained using Quant-iT PicoGreen™ DNA and RiboGreen™ RNA Assay Kits (Thermo Fisher Scientific, Inc), respectively.

Results

The FormaPure Total chemistry was successfully automated using the KingFisher Duo Prime to isolate DNA and RNA from four types of FFPE cancer tissues. The performance of the semi-automated workflow was compared to a completely manual workflow using the same FFPE samples. Both the manual and semi-automated workflows recovered more than a microgram of DNA **(Figure 1a)** and RNA **(Figure 1b)** from single 10 µm curls. Some differences in DNA and RNA yields were observed for several FFPE samples between the two workflows. In order to determine the cause of the discrepancies in recoveries, the combined DNA and RNA yields were assessed **(Figure 1c)**. Similar amounts of total nucleic acids were recovered from the lung (164) and breast (165) FFPE samples, suggesting that the lysates may have been split unequally for the manual and semi-automated methods. Interestingly, the KingFisher workflow yielded ~38% and ~50% more nucleic acids from liver (166) and breast (176) FFPE samples, respectively, suggesting that less of the samples or beads may have been lost during the automated extraction process compared to the manual workflow. Nevertheless, both the manual and semi-automated workflows recovered ample amounts of DNA and RNA suitable for most downstream assays from single 10 µm curls.

Next, DNA and RNA integrities, or the measurement of nucleic acid degradation, were assessed via fragment analyses **(Figure 2)**. In order to evaluate the compatibility of the FFPE-derived nucleic acids for NGS applications, the DV_{200} values were determined. Illumina defines DV_{200} as the percentage of RNA fragments greater than 200 nucleotides. Since FFPE-derived RNA are typically severely degraded, the DV_{200} metric is considered for many NGS applications in lieu of the RNA Integrity Number (RIN). Using the FormaPure Total kit, all DV_{200} values were greater than 80%, which are well-suited for any FFPE-based NGS assays. For the RNA, the lung (164) and breast (165) samples exhibited essentially identical traces between the manual and semi-automated workflows **(Figure 2b)**. FFPE liver (166) and breast (176) samples showed minor differences in the traces albeit these difference rendered insignificant changes to the DV_{200} values. Lastly, as expected, DNA integrities were not affected by the two workflows conducted **(Figure 2a).**

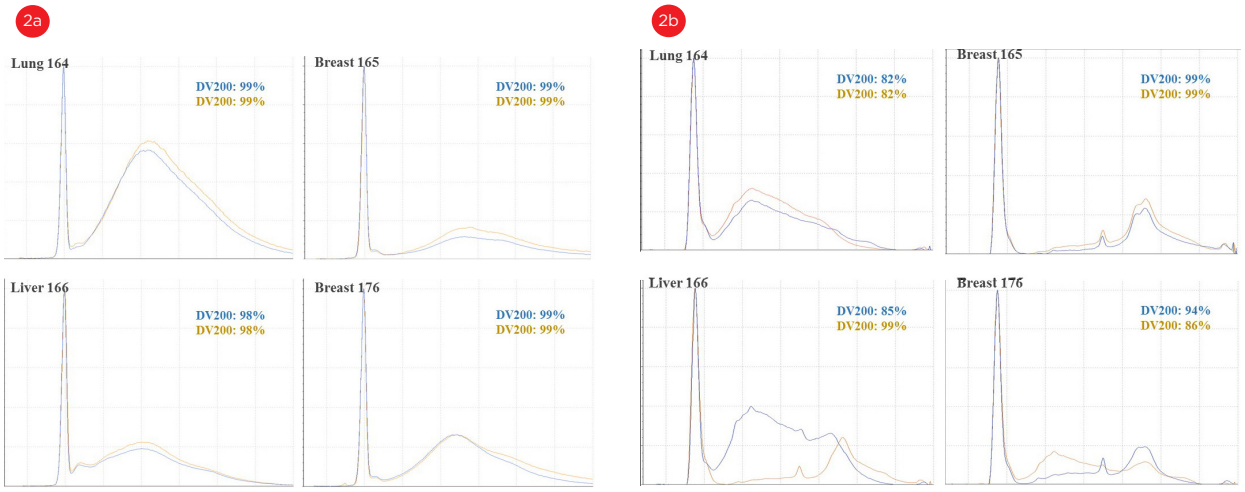


Figure 2. Fragment analyses of DNA (a) and RNA (b) isolated via manual vs. KingFisher Duo processing using FormaPure Total. DNA (2a) and RNA (2b) isolated from four FFPE samples were run on a TapeStation (Agilent Technologies) to determine the integrity of the nucleic acids. Blue and oranges traces represent nucleic acids that were isolated manually and with the KingFisher Duo, respectively. DV_{200} values were determined by the TapeStation software.

Conclusion

This study demonstrates a solution that enables a semi-automated workflow for reliable isolation of DNA and RNA from single FFPE samples. The data presented suggests that the yield and integrity of DNA and RNA extracted from FFPE samples are comparable between manual and semi-automated extractions using FormaPure Total. There are several advantages of using KingFisher technology to semi-automate the FormaPure Total chemistry.

First, the semi-automated workflow ensures the maximum yield and quality of DNA and RNA are achieved regardless of the inherent variations in sample types and input amounts. Up-front manual processing of sample deparaffinization, tissue digestion, decrosslinking and lysate splitting enables researchers to identify and resolve many of the challenges that typically lead to sample loss or drop-outs.

Second, once these upstream steps are meticulously performed, the latter half of the extractions, which involves the bind, wash and elute steps, can be automated in a fraction of the time that it takes to process manually. Semi-automated processing of FFPE samples using FormaPure Total and KingFisher technology provides a viable solution to accelerate biomarker discovery. **Table 3** outlines several differences between the manual vs. semi-automated workflows.

	FFPE DNA and RNA Extraction Comparison				
	Throughput per run	**Hands-on Time**	**Processing time**	**Instrument footprint (W × D × H)**	**Performance consistency**
Manual	24	~4.5 hours	6.5 hours	NA	Low due to potential manual bias.
Semi-automated KingFisher Duo	12	~1.5 hours	5 hours	40 × 46 × 35 cm	High

Table 3. FFPE extraction comparisons for manual vs. semi-automated workflows.

1 FormaPure Reagent Kits, <www.beckman.com>.

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