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LETTER TO THE EDITOR

A High-Temperature, Pre-Incubation Step Before Proteinase K Treatment Notably Improves Recovery of Genomic DNA in Formalin-Fixed, Paraffin-Embedded Tissue Samples

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LIST OF ABBREVIATIONS

Cp - crossing point
FFPE - formalin fixation and paraffin embedding
gDNA - genomic DNA
qPCR - quantitative PCR

LETTER TO THE EDITOR

Formalin-fixed paraffin-embedded (FFPE) tissue is used for tissue analysis and as a source to archive biological material [1,2]. The technology currently exists to extract nucleic acid from FFPE tissue in clinical laboratories.

FFPE, however, causes chemical damages and nucleic acid degradation due to nucleic acid-protein crosslinking [2-4]. Therefore, sufficient amounts of high-quality genomic DNA (gDNA) cannot be effectively extracted from FFPE tissues [5,6].

The extraction of nucleic acids from FFPE samples can be described in three steps including: deparaffinization, degradation of proteins using proteinase K, and the high temperature removal of crosslinks [7]. These steps are followed by extraction and clean up using e.g., a spin column or magnetic beads. We examined the effect of a pre-incubation heating step, using high temperature before the proteinase K treatment, to see how it affected the gDNA concentration. This experiment was first performed using different fixation times as variables.

Table 1. Comparison of the data obtained from two methods in various fixation times.

Table 1A.

Fixation time (hour)	Pre-incubation method		Conventional method	
	Concentration (ng/μL)	R260/280	Concentration (ng/μL)	R260/280
24	61.7 ± 13.6	1.75 - 1.79	15.1 ± 1.6	1.75 - 1.80
48	58.4 ± 5.2	1.78 - 1.80	12.6 ± 3.1	1.79 - 1.82
72	48.3 ± 5.4	1.78 - 1.81	10.2 ± 0.7	1.77 - 1.82
120	45.6 ± 3.9	1.76 - 1.78	4.2 ± 0.3	1.75 - 1.78
192	42.1 ± 4.9	1.76 - 1.78	3.7 ± 0.5	1.75 - 1.78

Table 1B.

Fixation time (hour)	Pre-incubation method		Conventional method	
	Concentration (ng/μL)	R260/280	Concentration (ng/μL)	R260/280
24	61.7 ± 3.8	1.78 - 1.80	25.4 ± 1.7	1.80 - 1.84
48	43.0 ± 5.2	1.81 - 1.83	18.9 ± 1.0	1.89 - 1.94
72	41.4 ± 3.0	1.84 - 1.88	14.3 ± 0.5	1.86 - 1.89
120	41.0 ± 3.4	1.84 - 1.88	12.5 ± 0.4	1.88 - 1.93
192	37.0 ± 4.8	1.80 - 1.85	11.4 ± 0.5	1.87 - 1.94

Table 1C.

Fixation time (hour)	Pre-incubation method		Conventional method	
	Concentration (ng/μL)	R260/280	Concentration (ng/μL)	R260/280
24	31.7 ± 2.9	1.76 - 1.79	24.6 ± 1.4	1.77 - 1.85
48	28.0 ± 2.5	1.75 - 1.78	18.5 ± 1.8	1.75 - 1.84
72	26.9 ± 4.0	1.74 - 1.77	15.4 ± 2.1	1.70 - 1.80
120	26.3 ± 3.1	1.71 - 1.73	15.2 ± 1.9	1.70 - 1.73
192	25.4 ± 2.0	1.70 - 1.72	14.6 ± 1.6	1.70 - 1.75

Concentrations for gDNA are shown as the mean ± SD.
A. Rat lung. B. Pig liver. C. Pig aortas.

Table 2. Comparison of the data obtained from two methods using human colorectal cancer.

	Pre-incubation method	Conventional method
Concentration, ng/μL		
Mean	52.3	40.9
Median	56.8	39.2
Maximum	81.0	88.4
Minimum	20.6	16.0
R260/280	1.68 - 1.74	1.71 - 1.78
Cp value for the GAPDH gene		
60 nt	26.83 ± 0.38	26.89 ± 0.62
100 nt	26.98 ± 1.38	26.99 ± 1.09
200 nt	30.62 ± 1.68	30.72 ± 1.71

Cp values for each GAPDH gene data point are shown as the mean ± SD.

Aortas and liver from a pig and lungs from a rat were exposed to formalin fixation times of 24, 48, 72, 120, and 192 hours. Each tissue sample was cut into pieces with approximate side lengths of 20 x 10 mm, and pieces were embalmed in vessels of ten volumes of formalin. Once the tissue samples had completed their allocated fixation times, they were placed in 0.1 M phosphate buffer at pH 7.4. Each sample was thinly sliced to perform the FFPE treatment; tissue specimens were serially sectioned (5 µm-thick) and prepared for each test.
All DNA extractions were done using the High Pure PCR Template Preparation Kit (Roche Molecular Systems, Pleasanton, CA, USA). Five samples from each fixation time (24, 48, 72, 120, and 192 hours) were sampled and the mean gDNA concentration measured. The effect of the 1 hour/90°C heating step before proteinase K treatment was evaluated using these measurements. The manufacturer's instructions specified that heating at 90°C should be performed after proteinase K treatment. In this study, however, this heating step for FFPE tissues was performed before proteinase K treatment (pre-incubation method) and according to the manufacturer's instructions. The two methods were compared for both gDNA yield and quality. The Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the gDNA concentration according to the manufacturer's instructions, and the purity (260/280 ratio) of the DNA was measured using a DS-11FX series spectrophotometer/fluorometer (DeNovix, Wilmington, DE, USA).
Table 1 indicates the yields of gDNA and the 260/280 ratios. These results show that the average gDNA concentration recovered using the protocol containing a pre-incubation heating step, and a long fixation time, was higher than that recovered with the conventional method.
Once the results were confirmed, the same comparison was done using clinical samples; FFPE tissue samples from 15 patients with human colon cancer were analyzed using both protocols as described above.
Primers of different nucleotide lengths were designed for the GAPDH gene and the Cp values compared via real-time quantitative PCR (qPCR), performed using a LightCycler 96 instrument (Roche Molecular Systems) with LightCycler 1.1 software (Roche Molecular Systems). The following primers were used for amplification:
sense (5'- cagccagacgaggacaca-3')
and antisense (5'-cctttctgggattgcctttc-3') for 60 bp;
sense (5'- ggtacgtaaggcagcctgtg-3')
and antisense (5'-aaaaggcaggaaaacgtgatt-3') for 100 bp;
sense (5'-aggtggagcgaggctagc3')
and antisense (5'-ttttgcggtggaaatgtcct-3') for 200 bp.
A solution containing 10 ng gDNA, 0.2 µM of each primer, and 0.2 µM of the Universal ProbeLibrary (Roche Molecular Systems) - no. 21 for 60 bp, no. 54 for 100 bp and no. 83 for 200 bp. PCR conditions included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation (95°C for 10 seconds, 4.4°C/second), and annealing (60°C for 30 seconds, 2.2°C/second).
Table 2 indicates the gDNA concentrations, 260/280 ratios, and Cp values at different nucleotide lengths of the GAPDH gene for human colorectal cancer.
The pre-incubation method shows an approximately 1.5 times greater recovery compared to the conventional method. Furthermore, Cp values from the pre-incubation method were no larger than the conventional method.
Whether this effect can be observed in all extraction reagents optimized for gDNA extraction of FFPE tissue sample should be a topic of further research. However, we believe that the pre-incubation step should not be downregulated. Therefore, this step is worth performing in cases where the desired extraction amount cannot be obtained.
Molecular oncology assays are increasingly used to determine therapies for primary and metastatic tumors, and they can also provide important diagnostic and prognostic information [8,9]. We thought that the importance of the assay related to area increases using FFPE tissue.
The pre-incubation method showed that tissues dissolved more easily and rapidly in the proteinase K step compared to the conventional method (data not shown). The effectiveness of this method was confirmed with a preheating step of as little as ten minutes. In this study, the pre-incubation method did however need a longer reaction time than the conventional method of one hour or more. However, it might be possible to obtain a yield exceeding the conventional method in terms of quantity and purity, in less time by using this pre-heating step.
Degradation of FFPE samples and recovering gDNA are largely influenced by tissue composition and fixation time [1,10]. Clinical laboratories offer various analytical methods to analyze various sample types. The pre-incubation method described in this study has been used in routine clinical work within our laboratory. This result could aid other laboratories facing similar challenges regarding efficient gDNA extraction.

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Declaration of Interest:
The authors declare no conflict of interests.

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