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Enhancement of Pathologist's Routine Practice: Reuse of DNA Extracted from Immunostained Formalin-fixed Paraffin-embedded (FFPE) Slides in Downstream Molecular Analysis of Cancer

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Abstract. *Background/Aim: To date, the conventional formalin-fixed, paraffin-embedded (FFPE) technique is the gold-standard for preserving histomorphology. Once FFPE tissues are stained, slides are routinely archived along with their blocks at biobanks/hospitals. However, the reuse of fixed and stained biospecimens as DNA source is not a common routine practice worldwide and, thus, indicates the need of studies to investigate the feasibility of extracting DNA from already immunohistochemistry (IHC) FFPE-stained slides and its possible reuse in subsequent downstream molecular analyses. Materials and Methods: FFPE IHC slides from colorectal cancer (CRC) patients were prepared and stored in the CEGMR Biobank. The workflow consists of digitalization of IHC stained slide's image, removing the slide cover-slip, crude dissection and DNA extraction. Following DNA quality assessment, mutation analysis of CTNNB1 and methylation profile of CDH1 were performed. Results: High-quality DNA was obtained allowing 60% concordance between CDH1 methylation and membranous E-cadherin expression pattern. Clean CTNNB1 DNA chromatograms with evenly-spaced peaks were observed. Conclusion: This study is a proof of concept to recycle and reuse DNA from IHC stained slides with suitable concentration and integrity for further downstream molecular applications. These findings will enhance the pathologists' knowledge, attitudes and practices (KAP) towards the use of these biospecimens and support the implementation of this approach in clinical pathology practice. Therefore, the scientific community will benefit from the largest comprehensive database of human fully annotated FFPE biospecimens already available at their disposal in order to demystify the complexity and the heterogeneity of many challenging diseases and foster the transition towards precision medicine.*

Since the completion of the Human Genome Project (HGP) in 2003, scientists have had increased access to extremely valuable genomic data to investigate genetic factors, gene–environment interactions and their potential effects on human health and welfare. Together with new breakthroughs in cutting-edge technologies, the HGP has initiated a new medical research era marked by huge milestones showing evidence of genetic, genomic and/or epigenomic causes for many diseases and disorders (1-3). Consequently, high expectations have been expressed for more accurate and individualized healthcare (1, 4-6). Despite these promising

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developments of high-throughput technologies, many diseases (*e.g.*, cancer) are still threatening human welfare mainly because of their polygenic, complex and yet poorly understood molecular processes. Furthermore, new challenges related to biospecimen quality and annotation, large-scale experiment planning, big data sets management and several ethical, legal and social issues have arisen in order to ensure a safer application of precision medicine to the public (7-10). In this context, modern biobanks, as collection and storage of biospecimens for diagnostic, treatment and biomedical research purposes, have become a core platform and an essential discipline to support both genomic medicine progress and clinical discoveries (11-13). To date, such biological repositories are the driving force that feeds the scientific community with fully annotated biospecimens, the main fuel of high-quality research resource and personalized healthcare (14-16). Nowadays, the availability of high-quantity and -quality biological samples with associated clinicopathological follow-up data is considered a crucial resource to set up large-scale studies and develop more effective tools and drugs that enhance the clinical service offered to patients (17, 18). The need for large sets of fully annotated and high-quality biospecimens has led to a noticeable and worldwide surge of biorepositories in terms of number, size, procedures and management (19, 20). With patient consent, any biospecimen leftover after any clinical examination is a valuable resource for translational studies of many complex diseases and should be handled and stored according to the best Standards Operating Procedures (SOPs) (5, 21-23). In this context and in diagnostic pathology units, formalin has been routinely used as a fixative for many decades to maintain the structural integrity of a specimen. As a consequence, huge collections of freshly collected biospecimens are stored as formalin-fixed, paraffin-embedded (FFPE) blocks for further use in diagnosis, prognosis and prediction of diseases or in translational research in order to customize and improve the healthcare services offered to patients (24). To date, the conventional FFPE technique has been considered as the gold standard for preserving histomorphology, while cryopreservation of tissue is the gold standard for biomolecule preservation (25). From the FFPE block, a pathological slide is made and used for diagnostic purposes using Hematoxylin & Eosin (H&E) staining or for prognostic prediction and therapeutic planning using immunohistochemistry (IHC) techniques to target different proteins and evaluate their expression patterns (26-28). Once these tissues are stained by either procedure, the slides are usually not properly archived or ignored while the FFPE blocks are stored for long periods at hospitals.

Although, it is widely thought that tissue fixation, processing, embedding through parafinization, staining steps, storage methods and microdissection of these tissues may compromise the integrity of biomolecules (DNA, RNA and proteins), many studies demonstrated that these analytes, contained within these FFPE samples, are still useful and informative (28, 29).

In this context and in order to overcome the shortage of high-quality and fully-annotated biological samples, it is necessary to be able to recover high-quality and pure nucleic acids from these biospecimens fixed in slides even after staining with either H&E or IHC methods. In fact, using FFPE microsections fixed on already stained IHC slides to extract DNA for subsequent molecular analysis is not a common practice worldwide and indicates the need for further investigation in this direction. Therefore, the current study explores a new approach to extract DNA from already stained FFPE slides for use in studying cancer molecular biology. The objective of this study is to recover pure DNA with acceptable quantity and integrity from already stained specimens and assess its use in subsequent downstream molecular analyses and profiling (at both genetic and epigenetic levels). Once confirmed, this approach will increase the value of samples and expand their use in medical and translational research applications in the genomic medicine era marked by a shortage in high quality and fully annotated biospecimens. This methodology may be translated into clinical and translational research applications for molecular profiling and biomarkers' analysis of several diseases (30). It will also make the reuse of enormous number of FFPE stained slides a new golden alternative that will improve biospecimens' management best practices and feed the large longitudinal biobanking-based studies with huge cohorts of biospecimens and controls (24, 31, 32).

This proof of concept study entails IHC image digitalization, cover-slip removal, tissue collection, DNA extraction and quality assessment. Afterwards, recycled DNA was tested in downstream molecular analysis applications for cancer diagnostics.

Materials and Methods

FFPE slides and IHC. FFPE blocks from informed consent patients, diagnosed with invasive colorectal cancer (CRC) between 2001 and 2009, were retrieved from the archives of the Department of Pathology at King Abdulaziz University (KAU), Jeddah, Saudi Arabia. FFPE slides were produced and used for IHC analysis of several markers in the Center of Excellence in Genomic Medicine Research (CEGMR), KAU. Manual, indirect IHC protocol is a routine analysis in the CEGMR that is performed according to manufacturer's recommendations and as described by our group elsewhere (33-35). Stained FFPE IHC slides were thereafter digitalized and archived at room temperature in the CEGMR Biobank, King Abdulaziz University. This study was approved by the CEGMR Research Bioethics Committee, King Abdulaziz University.

Cover-slip removal from stained FFPE IHC slides. All IHC slides' images should be digitalized before cover-slip removal and DNA extraction in order to archive crucial information related to the

Table I. *CDH1 primers and probe details.*

Forward primer (5'-3')	Reverse primer (5'-3')	Probe oligo sequence (5'-3')
AGGGTTATCGCGTTTATGCG	TTCACCTACCGACCACAA	6FAM-ACTAACGACCCGCCCACCCFA

Table II. *Recycled DNA concentration and quality as shown by spectrophotometry.*

Sample ID	Concentration (ng/μl)	A260	A280	260/280	260/230	Sample type	Factor
S06-3081	493.9	9.878	5.074	1.95	1.29	DNA	50
S06-1569	697	13.941	6.92	2.01	1.45	DNA	50
S07-1543	872.6	17.453	8.76	1.99	1.56	DNA	50
S07-1450-D	770.2	15.404	7.743	1.99	1.23	DNA	50
S07-2421	396.5	7.929	3.972	2	1.17	DNA	50

biospecimen morphology and proteins'/markers' expression patterns. In order to obtain sufficient starting material, 3 to 5 stained FFPE IHC slides from the same FFPE block (same patient) were pooled and plunged in xylene-filled jars until the cover-slips were released. The incubation period was from 5 to 7 days at room temperature. In total, stained FFPE slides from 62 patients were used (Figure 1).

Sample collection and DNA extraction. After cover-clip removal, stained microsections from used FFPE slides were gently and crudely dissected manually using a sterile and DNA-free scalpel. Samples from the same patients were pooled in a 2-ml microcentrifuge tube and stored at –20˚C. DNA extraction from the IHC stained tissues was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA) according to manufacturer's guidelines (36). While the lysis step of this kit helps to release DNA and prevent possible inhibitory effects induced by formalin cross-linking of nucleic acids, the QIAamp spin columns allow washing and purification of samples followed by a final elution step in a new sterile tube to collect purified DNA (Figure 1).

Purity and concentration of the recycled DNA were assessed using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) according to manufacturer's recommendations. DNA integrity was confirmed by gel electrophoresis.

Target gene mutation analysis (CTNNB1 gene). Recycled DNA was subsequently used as a template to perform a PCR (*CTNNB1* primers: F: 5'-GCTGATTTGATGGAGTTGG- 3'; R: 5'-CTCTTA CCAGCTACTTGTTC-3') followed by gel electrophoresis for the target gene *CTNNB1*. Sanger sequencing was performed on the purified gel band (QIAquick Gel Extraction Kit; Qiagen) of 19 recycled DNAs to assess the *CTNNB1* sequence identity as an indicator of DNA integrity.

CDH1 promoter methylation analysis. The methylation profile of the *CDH1* promoter region through the determination of CpG Island Methylator Phenotype status was performed using the MethyLight (Qiagen) assay as previously described (37). DNA from 62 CRC patients was subjected to bisulfite conversion using the Epitect Biosulfite Conversion kit (Qiagen). The MethyLight assay for *CDH1* methylation was performed using StepOne real time-polymerase chain reaction (RT-PCR) (Applied Biosystem ABI, Foster City, CA, USA) with primers and probe details reported earlier (38) and summarized in Table I. The RT-PCR running program conditions were as follows: initial denaturation at 95˚C for 10 min followed by 45 cycles of denaturation at 95˚C for 10 s, annealing at 60˚C for 1 min. Samples exhibiting percentage of methylated ratio (PMR) cut-off ≥10 compared to the reference unmethylated gene were considered as having a hypermethylated *CDH1* promoter.

Results and Discussion

Retrieval and quality of DNA from stained IHC FFPE slides - Proof of concept. To achieve cover-clip removal, stained and digitalized IHC FFPE slides were incubated for 5 to 7 days in xylene. Following DNA extraction from crude microdissection tissues, DNA quality assessment by spectrophotometry showed peaks at 260 nm wavelength and satisfactory DNA concentrations, integrity and quality in most of the samples (Table II, Figure 2). Therefore, DNA with satisfactory quality can be retrieved from immunostained FFPE slides. Due to its properties to dissolve paraffin, xylene helps in cover-slip removal from stained IHC FFPE slides, while preserving the quality of the DNA (39).

Mutation analysis in CRC patients. Although DNA was successfully recovered from IHC FFPE slides, additional downstream applications are required to assess its usefulness in molecular applications as target gene mutation analysis. Since our biospecimens came from CRC patients, the *CTNNB1* gene was selected for mutation analysis given the strong evidence of correlations between the proliferative

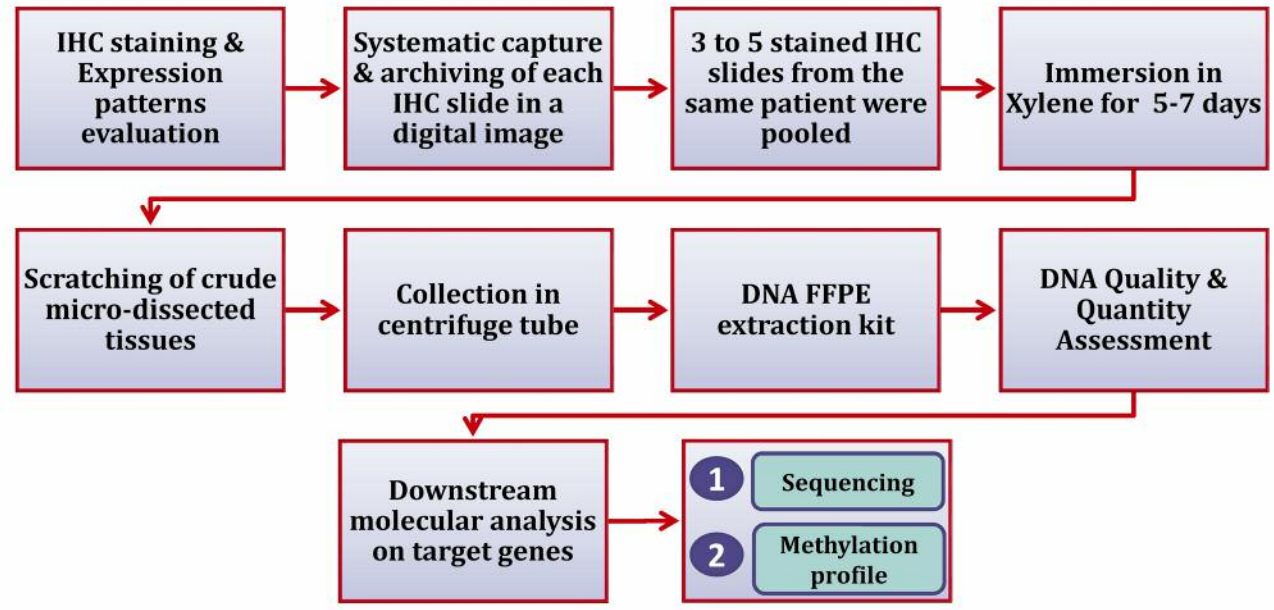

Figure 1. *Study workflow. IHC, Immunohisochemistry, FFPE, formalin-fixed, paraffin-embedded.*

ability of colorectal carcinoma cells and the genetic alterations of *CTNNB1* (40-42). Although the reported mutation frequencies were variable among populations and regions, it was worthwhile to assess the presence of possible mutations in this interesting prognostic biomarker of CRC using our reused DNA as a template.

A PCR using specific primers was performed to amplify the *CTNNB1* sequence. The PCR product size was assessed by gel electrophoresis. The size of the target gene recovered was around 190 bp, as shown by the clear and uniform gel bands in Figure 3. However, having the expected gel electrophoresis band does not reveal possible artifacts in the *CTNNB1* sequence. In fact, tissue fixation and embedding with paraffin or other fixatives are thought to affect DNA quality (36, 43). Therefore, the *CTNNB1* gel bands were purified and sequenced. The Sanger sequencing results showed very clean electropherograms with evenly-spaced peaks each with only one color represented (Figure 4). The sequencing results were blasted against the NCBI database for *CTNNB1* sequence identity. A very high identity rate of 97% was obtained by blasting the sequence to the NCBI database (http://blast.ncbi.nlm.nih.gov/ Blast.cgi), thus confirming the integrity of the crude microdissected DNA for this target gene. In all our sequenced samples, no variations in the CTNNB1 coding sequence were reported. This result is a further piece of evidence supporting a good quality and integrity of the recycled DNA. In addition to PCR and sequence analysis, the possibility to reuse recycled DNA was extended to include additional downstream molecular analyses, *e.g.*, the methylation profile.

CDH1 promoter methylation profile. DNA methylation of multiple promoters is used as a biomarker for early detection and as a tool for monitoring patients with CRC. The identification of methylation patterns can be considered for early detection or prognostic biomarkers playing a critical role in cancer prevention and patient monitoring purposes (44).

In our laboratory, DNA from FFPE slides was used to assess CDH1 cytoplasmic and membranous protein expression by IHC staining. Since promoter methylation of *CDH1* was demonstrated in CRC patients by various investigators and suggested as an early prognosticator for this carcinoma (45-47), the MethyLight assay of the *CDH1* promoter region was performed using the reused DNA from these stained IHC FFPE slides as a template. Data analysis of the amplification plots showed that 45% (28 out of 62) of *CDH1* promoter was hypermethylated in our cohort of CRC patients (Table III), which is consistent with previous methylation incidence rates (48). Although this methylated profile did not correlate significantly with any clinicopathological feature, it appears to occur at an early phase of colon carcinogenesis, as suggested by Xu *et al.* (49). The concordance between *CDH1* promoter methylation and its protein expression performed by IHC on the original CRC slides before DNA reuse was assessed using 55 samples matched with their E-Cadherin protein expression.

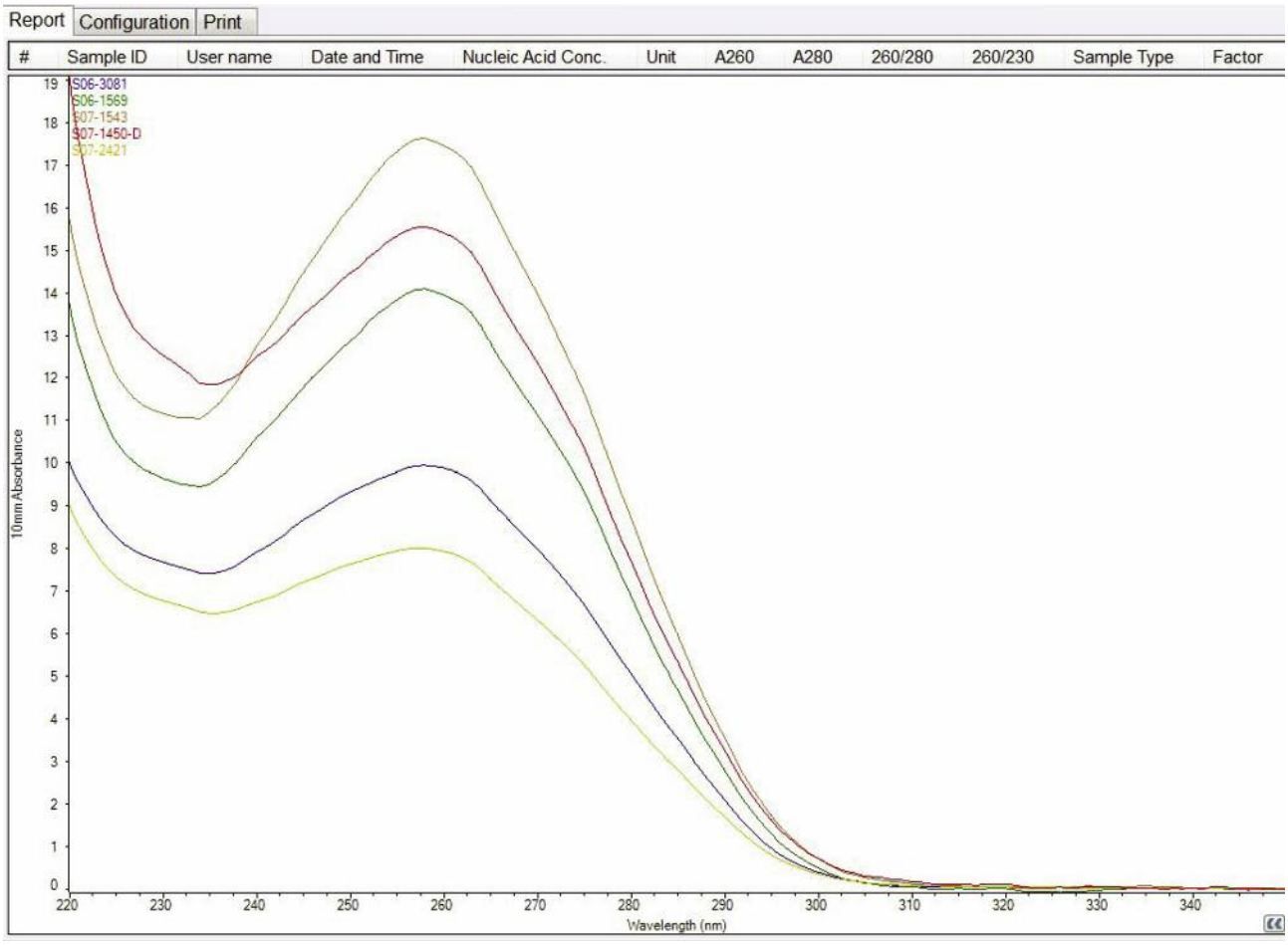

Figure 2. *Reused DNA peaks as shown by NanoDrop.*

Interestingly, a 60% of concordance rate between E-cadherin promoter methylation and both membranous and cytoplasmic protein expression was recorded (Table IV). These findings are again in agreement with a previous study focusing on *CDH1* methylation in CRC (48). Taken together, both the *CDH1* methylation profile and its concordance with the E-Cadherin protein expression are consistent with previous studies in methylation and provide further evidence that the reused DNA extracted from already immunostained IHC FFPE slides can be reused for methylation profiling.

This study indicated that this new approach, to extract the DNA from IHC FFPE slides in hospitals and pathology departments worldwide, is feasible and useful for further downstream molecular analyses. The reused DNA had enough quantity and quality to perform non-exclusively mutation analysis and methylation profile.

In Europe alone, it is estimated that 25 to 30 million biospecimens taken by surgery or biopsy are collected in hospitals every year (50). Most of these samples are archived after being fixed mainly with formalin. These processed tissue specimens are, as described by Dr. Giorgio Stanta, "a giant virtual bio-banking system" (50) that offers the largest comprehensive database of human biospecimens linked to their full clinicopathological follow-up records immediately available to the scientific community. These valuable biospecimens are a huge asset for translational medicine to

Table III. *Methylation frequency of CDH1 promoter gene in CRC tissues (n=62).*

	Methylated	Non-methyalated
No. of CRC cases	28	34
Methylation state (%)	45	55

Table IV. *Concordance between CDH1 methylation and membranous protein expression in CRC patients.*

	Methylation state		
	0 (Non-methylated)	1 (Methylated)	Total
Protein by IHC			
0 (No expression)	7	10	17
1 (positive expression)	23	15	38
Total	30	25	55

IHC, Immunohistochemistry.

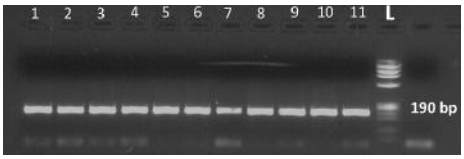

Figure 3. *Gel electrophoresis of CTNNB1 gene (190 bp) from DNA retrieved from already immunostained FFPE IHC slides. L, Gel ladder as a control; lanes 1 to 11, numbers' of assessed samples; IHC, immunohisochemistry, FFPE, formalin-fixed, paraffin-embedded.*

demystify the complexity and the heterogeneity of many challenging diseases, such as cancer, validate several biomarkers and execute effective longitudinal cohort-based studies. Therefore, our suggested approach will offer the possibility to benefit from these large cohorts of biospecimens by extracting high-quality DNA. We have shown that this DNA extracted from IHC FFPE slides can be used to perform downstream molecular applications on target genes, including PCR, mutation analysis, sequencing and methylation profiling. This cost-effective approach is also very useful given the latest developments in the clinical practice in which minute biopsy materials will not be sufficient to perform additional molecular analyses and/or translational research. We do believe that the implementation of this new approach based on IHC image digitalization, archiving of IHC PPFE slides and subsequent downstream molecular reuse in the pathology departments' routine practice will first enhance their staffs' knowledge, attitudes and practices (KAP) towards the value of these biospecimens. Then, these reused samples will significantly support our efforts to alleviate the burden of complex and rare genetic diseases (50, 51) and improve, therefore, the quality of patients' healthcare.

Conclusion

This study provided a proof of concept of a promising workflow allowing successful extraction of DNA from IHC FFPE slides that will be of primary importance for education, translational medicine, as well as for biobanking, through improvement of best practices in biospecimens management. Results demonstrated that used FFPE slides, routinely underused/wasted at pathology departments, can be reused to extract DNA with satisfactory quality and are useful for several downstream molecular applications on target genes, including PCR, mutation analysis, Sanger sequencing (*CTNNB1*) (β-catenin) and methylation profile of *CDH1* (E-cadherin). The suggested protocol will help demystifying cancer clonal heterogeneity where IHC digital image will serve as a guide for both crude and/or laser microdissection of interested clones in order to interrogate cancer subclones.

However, further studies are required to validate and refine this approach mainly at the cover-slip removal step duration using different chemicals and/or incubations conditions while maintaining the integrity of the extracted biomolecules that will be useful for further molecular applications in medical and/or forensic applications. Once validated using a larger cohort of samples, this new evidence-based approach will provide opportunities to access a huge number of biospecimens (used slides) with full clinical information, which is a valuable asset towards studying personalized care at the molecular level.

It should also be considered to set up a suitable post-diagnostic archiving procedure in Pathology Departments and molecular diagnostics laboratories for used FFPE slides to optimize their use in further molecular diagnostic or translational medicine research. However, additional awareness, education, training and quality assessment are required for suitable implementation of this procedure in the daily clinical pathology practice. This study may also serve to provide insight to improve recovery of nucleic acids from bioevidence in investigative genetics and forensic sciences where degraded or limited quantity samples are often encountered.

Conflicts of Interest

All Authors declare the absence of any institutional or commercial affiliations that might pose a conflict of interest regarding the publication of this manuscript.

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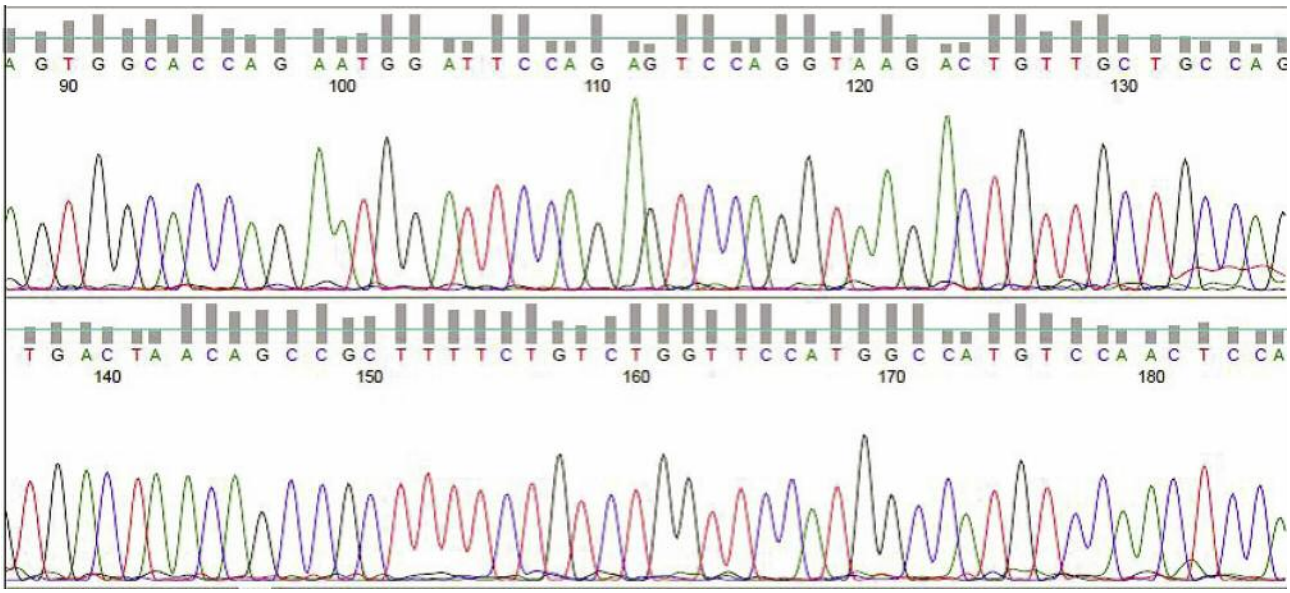

Figure 4. *CTNNB1 DNA Sanger sequencing electropherogram with evenly-spaced peaks represent different nucleotides each fluorescently labeled for automatic detection by sequencing. Each color represents one nucleotide as following: purines are guanine (black) and adenine (green) with their complementary pyrimidines that are cytosine (blue) and thymine (red), respectively.*

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