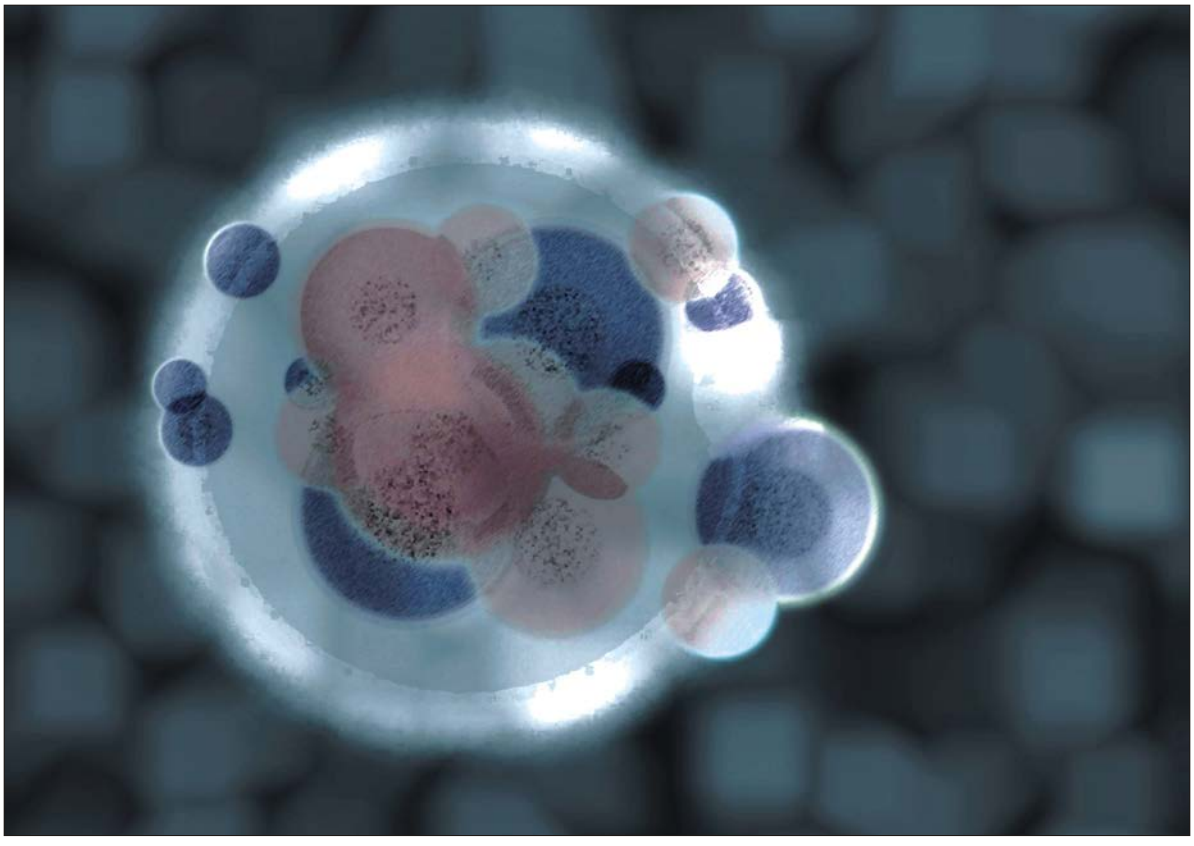

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 10 (3): 261-266, 2010
ISSN 1727-3048

Comparison of the Three Methods for DNA Extraction from Paraffin-Embedded Tissues

[1]M.H. Mirmomeni, [1]S. Sajjadi Majd, [2]S. Sisakhtnezhad and [1]F. Doranegard
[1]Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran
[2]Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

Abstract: The aim of this study was to compare three different techniques for optimizing DNA extraction from Paraffin-Embedded Tissues (PETs) in order to generate DNA suitable for PCR. Paraffin-embedded tissues are common resources for molecular genetic studies but the extraction of high quality nucleic acid from them is problematic. DNA extraction methods used include modified phenol-chloroform protocol, salting out method using ammonium acetate and commercial kit (QIA amp DNA Mini Kit (50)-QIAGEN). DNA was extracted using these methods and qualified using spectrophotometer analysis, electrophoresis and PCR amplification. The tissue specimens used for detection of p53 and Nat2 genes consist of liver tissues with hepatocellular carcinoma (HCC) and stomach tissues which showed stomach adenocarcinoma. DNA was extracted using the aforementioned methods. Subsequently, PCR amplification for p53 and Nat2 genes was performed and 196 and 783 bp fragments were yielded for p53 and Nat2 genes, respectively. Finally, comparison of the electrophoresis pattern in all three methods indicated that no significant differences were detected and all three methods showed equally efficient results.

Key words: DNA extraction, paraffin-embedded tissue, PCR

INTRODUCTION

Tissue removed from the human body for diagnostic purposes can be fixed, paraffin wax embedded and stored for several years. These specimens are valuable sources of material for research (Pinto and Villa, 1998; Sjoholm *et al*., 2005; Chan *et al*., 2001; Gillio-Tos *et al*., 2007; Gilbert *et al*., 2007). Many different methods and technologies are available for the isolation of genomic DNA. Most of the techniques used for DNA extraction from paraffin- embedded tissue include three steps: deparaffinization, digestion and purification. In general, all methods involve dissolvation of the wax in xylen and ethanol, lysis of the cells, followed by the removal of debris and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved by digestion with proteinase K, followed by salting-out, organic extraction, or binding of the DNA to a solid phase support (either anion-exchange or silica technology). DNA is usually recovered by precipitation using ethanol or isopropanol. The choice of a method depends on many factors: the required quantity and molecular weight of the DNA, the purity required for downstream applications and the time and expanse (Poinar and Stankiewikz, 1999).

A general method of DNA extraction from paraffin wax embedded tissues, uses multiple washes with a protease usually followed by phenol-chloroform-isoamylalcohol treatment for DNA purification. The last step is important to reduce protein contamination that could interfere with the PCR assay (Pinto and Villa, 1998; Noguera *et al*., 2001). This method is proved to be very efficient, however, it is laborious, time consuming and requires several centrifugation and wash steps which increase the risk of sample contamination. However, the present study has proposed modifications in this method.

DNA extraction from these specimens is suitable for PCR amplification (An and Fleming, 1991; Poljak *et al*., 2000; Vollzenandt *et al*., 1993) and many studies on PCR-based gene analysis using tissues stored as paraffin blocks have been published (Rivero *et al*., 2006; Coates *et al*., 1991; An and Fleming, 1991; Hein, 2006). The salting-out DNA extraction procedure did not completely remove or decrease the presence of inhibitors to PCR in a considerable number of samples (Noguera *et al*., 2001). The aim of this study was to compare three different techniques in order to optimize DNA extraction from paraffin-embedded tissues with the aim of generating DNA suitable for PCR. These methods include phenol-chloroform (Vollzenandt *et al*., 1993), salting out (Rivero *et al*., 2006) and commercial kit for DNA extraction. For each extraction technique, analysis was performed on some 10 μm sections of liver (hepatocellular carcinoma) and stomach (adenocarcinoma) tumor (An and Fleming, 1991; Liu *et al*., 2002; Hein, 2006), which was taken from paraffin-embedded tissues.

Corresponding Author: Mohammad Hossein Mirmomeni, Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

MATERIALS AND METHODS

Most DNA extraction methods consist of three stages including deparaffinization, protein digestion and DNA purification.

Tissue specimens: Paraffin embedded tissue blocks from HCC patients and patients affected stomach adenocarcinoma related to 1998 to 2007 were gathered from Kermanshah hospitals (Taleghani and Emam Reza Hospitals) and their pathological diagnostic were confirmed by pathologist.

Deparaffinization method: Ten micrometer thick sections of each block of PETs were collected in a 1.5 mL micro tube and were dewaxed by adding 1 mL xylene (Merk) and incubating for 30 min at 55°C each, then micro tubes were centrifuged at 8000 rpm for 10 min and supernatant was discarded until the paraffin was completely removed. This stage was repeated three times. The pellet was washed by applying a series of ethanol (Merk) (absolute, 90 and 70% of ethanol), shaking and centrifugation at 12000 rpm for 10 min, each time.

Digestion method: For digestion, 500 μL DNA extraction buffer (1 M NaCl (Merk); 1 M Tris-HCL (Merk), pH 8; 0.5 M EDTA (Merk), PH 8; 10% Sodium dodecyl sulfate) and 40 μL of Proteinase K (Cinnagen) (10 mg mL^{-1}) were added to the sample, micro tubes were briefly vortexed and were incubated at 55-56°C overnight with gentle agitation every 4 h. When protein digestion was deemed adequate, proteinase K was inactivated by heat at 85°C for 15 min (Chen *et al*., 2000; Fan and Gulley, 2001). Then, deparaffinized and digested samples were submitted to one of the following DNA extraction methods.

DNA extraction methods: In this study for preparing suitable DNA, three different techniques were used for optimizing the DNA extraction method from paraffin-embedded tissues.

Modified phenol-chloroform DNA extraction method: After proteinase K inactivation, 0.5 mL of saturated phenol (Fluka) pH 8 was added and micro tubes were gently shaken by hand for 5 min and centrifuged for 5 min at 8000 rpm. Supernatant was collected in a new tube and was then mixed with 220 μL phenol and 220 μL chloroform/isoamylalcohol (Merk) (24:1), followed by centrifugation (5 min at 8000 rpm) to obtain the aqueous phase. At the next step, 440 μL chloroform/isoamylalcohol (24:1) was added and centrifuged for 5 min at 8000 rpm. Supernatant was transferred to the new tube and sodium acetate (Merk) (3 M, pH 5.2) (0.1 supernatant volume) and 100% ethanol were added (3 times of supernatant volume) for an overnight DNA precipitation at -20°C or 60 min at -80°C. The precipitated DNA was collected by centrifugation for 30 min at 4°C, washed with 70% ethanol, air dried at room temperature and re-suspended in 30-50 μL of double distilled water or TE buffer (Rivero *et al*., 2006).

Salting-out extraction method: After proteinase K inactivation, 400 μL ammonium acetate solution (final concentration 3.5 M) was added to the micro tubes. Micro-tubes were then vortexed for 20 sec at high speed, incubated on ice for 30 min and centrifuged at 12000 rpm for 30 min supernatant was transferred to another tube, 600 μL isopropanol was added and centrifuged at 14000 rpm for 10 min and kept in -200°C for 2 h. After that, Supernatant was discarded and DNA pellet was washed in 70% ethanol and centrifuged at 14000 rpm for 1 min. Supernatant was discarded and pellet was dried in 37°C. Subsequently, DNA was dissolve in 30-50 μL buffer (Rivero *et al*., 2006).

Commercial DNA isolation kit: The extraction steps were performed according to the user guide of commercial DNA isolation kit (QIAGEN). After dewaxed-tissues were digested with 180 μL of ALT buffer and 35 μL proteinase K and incubated overnight at 56°C. After digestion, 200 μL of AL buffer was added and incubated at 70°C for 10 min, followed by mixing with 200 μL of 100% ethanol. The solution was transferred in to a spin column, centrifuged for 1 min at 8000 rpm and washed with 500 μL AW1 and 500 μL AW2 buffers. DNA was eluted with 200 μL of AE buffer preheated to 70°C and further incubated at 70°C for 5 min before collection by centrifugation (Aplenc *et al*., 2002).

Polymerase Chain Reaction (PCR): The DNA obtained was used for amplification of a 196 bp fragment of p53 gene and a 783 bp fragment of Nat2 gene. The PCR was carried out in total volume of 25 μL, consisting of 10X PCR buffer(Cinnagen), 3 mM dNTP (Cinnagen), 4 mM $MgCl_2$ (Cinnagen), 25 Pmoles of each primer and one units of Taq DNA polymerase (Cinnagen). The primers for p53 gene PCR were F (GW-XI-1C): 5` CTTGCCACAGGTCTCCCC 3` and R (GW-XI-1D): 5` GCCACTTGCCACCCTGCACA 3` (Liu *et al*., 2002); those for the Nat2 gene PCR were F: 5` AGTTGGGCTT AGAGGCTATTT 3` and R: 5` ATCACCCAACTCACTAA TTATCAA 3`. Amplification conditions were 30 sec at 94°C, 30 sec at 60°C (p53 PCR) or 60 sec at 72°C (nate2 gene) and 30 sec at 72°C for 35 cycles.

Statistical analysis: In this study, data analysis was performed using MINI-TAB statistical analysis software version14.0. Data obtained from three different DNA extraction methods for all HCC and SAC were analyzed

and variance analysis was used to verify possible differences among the amount of DNA obtained from the different extraction methods. Differences were considered statistically significant when the probability (P) was less than 0.05 (Rivero *et al*., 2006).

RESULTS

DNA was extracted using three methods including phenol-chloroform method, salting-out method and commercial Kit method. The comparison of efficiency of the DNA extraction and purification was carried out by measuring the DNA concentration and suitability of PCR. The yield and purity of DNA for each method was estimated by measuring the A260 and A260/A280 absorbance ratio, respectively (Table 1). Results show that, all three extraction methods produced significant yields of DNA (above 3 μg), which are adequate to the PCR reaction.

The quality and length of extracted DNA was compared with agarose gel electrophoresis. Figure 1 shows the electrophoretic pattern of DNA recovered by the three extraction methods. The electrophoretic pattern was identical for all methods used. The DNA was not homogenously stained since, in all methods, slightly stronger intensity at the bottom of DNA smear was observed, suggesting that low molecular weight DNA was predominant.

The DNA concentration was calculated from the A260 for DNA samples and the purity of DNA was assessed by calculating A260/A280 ratio for protein impurities.

At the next step, successful DNA extraction was assessed by PCR amplification of p53 and Nat2 genes. Electrophortic patterns indicated 196 and 783 bp fragments amplified with two pair primers for p53 and Nat2 genes, respectively. PCR amplified products were analyzed by electrophoresis on 1.7% agarose gel and visualized by ethidium bromide staining and UV transilluminator (Fig. 2a, b).

Finally, data obtained from the yielding of DNA, was submitted to a variance analysis to verify possible differences among from the different extraction methods.

Table 1: Purity (P) and yield of DNA (Y) in different extraction methods used

Method	Phenol-chloroform		Salting-out		Kit	
Specimen	P	$Y_{(\mu g)}$	P	$Y_{(\mu g)}$	P	$Y_{(\mu g)}$
HCC1	1.77	23.00	1.73	8.30	1.67	12.50
HCC2	1.79	17.15	1.72	23.50	1.74	13.50
HCC3	1.86	14.55	1.66	16.00	1.77	11.00
SAC1	1.74	15.50	1.97	19.85	1.85	12.55
SAC2	1.70	42.00	1.89	31.50	1.83	11.50
SAC3	1.77	35.50	1.76	17.90	1.94	14.50

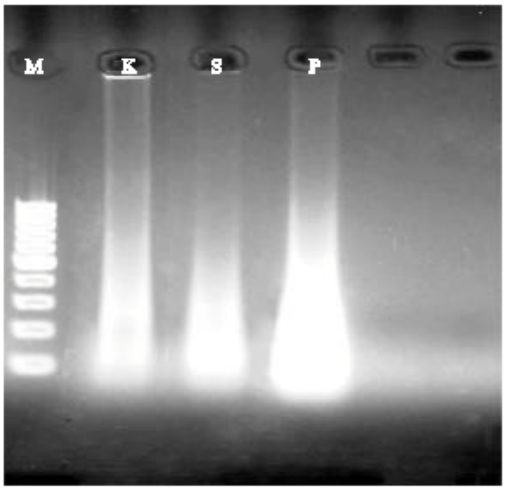

Fig. 1: Electrophoresis pattern of DNA obtained by each extraction method. K, P, S represent DNA extracted from kit, phenol-chloroform, salting-out methods, respectively and M is 100 bp marker

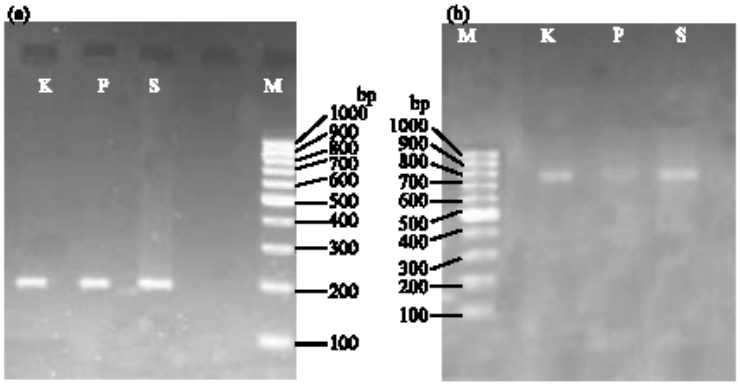

Fig. 2: Amplified DNA fragments of HCC and SAC. (a) 196 bp amplicons of HCC and (b) 783 bp amplicons of SAC with DNA extracted from Kit (K), Phenol-chloroform (P) and salting-out (S) methods

Differences were considered statistically significant when the probability(p) was less than 0.05. Results show that, variance analysis did not show statistically significant differences between the amounts of DNA for each extraction method studied ($p>0.05$).

DISCUSSION

Paraffin wax-embedded tissues are valuable sources of DNA for analysis. Although paraffin-embedded tissues are commonly regarded as presenting incurred during the fixation and embedding processes, it has been demonstrated that DNA obtained from PETs are suitable for use in PCR.

The study of relationship between the allelic polymorphism of Nat2 gene encode xenobiotic metabolizing N-acetyl transferase 2 enzyme (Agundez, 2008; Ambrosone *et al*., 2008) and the risk of various cancers is one of the most important present investigations (Walraven *et al*., 2008). In the other hand, the p53 tumor suppressor gene is the most frequently mutated gene in several human cancer (Lu *et al*., 2001; Diller *et al*., 1990; Kern *et al*., 1992; Rezaii *et al*., 2008) and mutation in codon 249 of p53 gene has been identified as a hot spot mutation for HCC (Llovet and Beaugrand, 2003; Garrcia *et al*., 2000). Thus, we decided to study these genes in population of Iran west and DNA extraction with high quality as the primary step is necessary for attaining these goals.

Routinely used methods extracting DNA from formalin- fixed tissues consist of many steps, including deparaffinization in xylene, washing in a descendent series of ethanol, protein digestion and DNA purification. Several methods and technologies are available for DNA extraction from PETs, but the traditionally and most frequently used method to separate protein from DNA is the phenol-chloroform method (Noguera *et al*., 2001; Fan and Gulley, 2001; Cao *et al*., 2003; Sepp *et al*., 1994; Coura *et al*., 2005). The basis of this separation is the difference in solubility of the nucleic acids, proteins and lipids in these organic solvents. The cells are lysed using a detergent and then mixed with phenol, chloroform and isoamylalcohol. The advantage of this method is that high-purity DNA can be obtained; however, this method has some disadvantages for being laborious, time-consuming and a cumbersome technique. Furthermore, the procedure uses toxic compounds and may not give reproducible yields. DNA using this method may contain residual phenol and/or chloroform, which can inhibit enzyme reactions in downstream applications and therefore may not be sufficiently pure for sensitive downstream applications such as PCR. The process also generates toxic waste that must be disposed at with care and in accordance with hazardous waste guidelines. In addition, this technique is almost impossible to automate, making it unsuitable for high-throughput applications (Noguera *et al*., 2001; Satiroglu-Tufan *et al*., 2004; Cao *et al*., 2003).

Another conventional method is the salting-out method which uses high concentration of salt solutions such as potassium acetate or ammonium acetate. Proteins and other contaminants precipitate from the crud cell lysate. After centrifugation, precipitants are discarded and DNA is recovered by alcohol precipitation. Removal of proteins and other contaminants using this method may be inefficient and RNase treatment, dialysis and/or repeated alcohol precipitation are often necessary before the DNA can be used in downstream applications. DNA yield and purity are highly variable using this method (Noguera *et al*., 2001).

Ternary method frequently used for DNA extraction is the Kit method. Kit technology avoids the use of toxic substances and can be used for different throughput requirements as well as for different scales of purification but it is expensive (Noguera *et al*., 2001; Cao *et al*., 2003).

Present results showed no significant differences between the amounts of DNA obtained from each of the extraction methods studied. However, the ammonium acetate DNA extraction method described is more convenient and less toxic than the phenol-chloroform method. Moreover, when analyzing PCR amplification, it is observed that the salting-out extraction method described is as efficient as the phenol-chloroform and the commercially available DNA isolation kit method (Noguera *et al*., 2001).

The electrophoretic pattern observed corroborates the theory of poor preservation and high degradation of DNA extracted from fixed tissues, considering that most DNA obtained was of low molecular weight (Santos *et al*., 2009). Thus, Nat2 gene 783 bp products were more difficult to amplify despite the extraction methods used.

The results indicated that although the phenol-chloroform method is effective for extracting high quality DNA, the toxic solutions used and its lengthy procedure reduces its benefits. Moreover, although using commercial kits has widely expanded recently, their high cost limits their use.

In conclusion, salting out procedure is safer, simpler, more effective and cheaper than the other methods described. The salting out method does not use many organic solvent and the entire extraction procedure uses only three eppendorf tubes for each sample, negating the need for the transfer of the supernatants between tubes, lowering the use of consumables in the laboratory and

reducing the possibility of contamination. However, amplification of DNA, particularly 783 bp amplicons, with salting out method is very hard indicating that DNA from PETs is variably damaged during processing and most extracted DNA are low molecular weight.

REFERENCES

Agundez, J.A., 2008. Polymorphisms of human N-acetyltransferases and cancer risk. Curr. Drug Metab., 9: 520-531.

Ambrosone, C.B., S. Kropp, J. Yang, S. Yao, P.G. Shields and J. Chang-Claude, 2008. Cigarette smoking, N-*Acetyltransferase* 2 genotypes and breast cancer risk: Pooled analysis and meta-analysis. Cancer Epidemiol. Biomarkers Prev., 17: 15-26.

An, S.F. and K.A. Fleming, 1991. Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues. J. Clin. Pathol., 44: 924-927.

Aplenc, R., C. Orudjev, J. Swoyer, B. Manke and T. Rebbeck, 2002. Differential bone marrow aspirates DNA yields from commercial extraction kits. Leukemia, 16: 1865-1866.

Cao, W., M. Hashibe, J.Y. Rao, H. Morgenstern and Z.F. Zhang, 2003. Comparison of methods for DNA extraction from paraffin-embedded tissues and buccal cells. Cancer Detection Prevention, 27: 397-404.

Chan, P.K.S., D.P.C. Chan, K.F. To, M.Y. Yu, J.L.K. Cheung and A.F. Cheng, 2001. Evaluation of extraction methods from paraffin wax embedded tissues for PCR amplification of human and viral DNA. J. Clin. Pathol., 54: 401-403.

Chen, W.N., C.J. Oon and M.C. Moh, 2000. Detection of hepatitis B virus surface antigen mutants in paraffin-embedded hepatocellular carcinoma tissues. Virus Genes, 20: 263-267.

Coates, P.J., A.J. d'Ardenne, G. Khan, H.O. Kangro and G. Slavin, 1991. Simplified procedures for applying the polymerase chain reaction to routinely fixed paraffin wax sections. J. Clin. Pathol., 44: 115-118.

Coura, R., J.C. Prolla, L. Meurer and P. Ashton-Prolla, 2005. An alternative protocol for DNA extraction from formalin fixed and paraffin wax embedded tissue. J. Clin. Pathol., 58: 894-895.

Diller, L., J. Kassel, C.E. Nelson, M.A. Gryka and G. Litwak *et al*., 1990. P53 functions as a cell cycle control protein in osteosarcomas. Mol. Cell. Biol., 10: 5772-5781.

Fan, H. and L.M. Gulley, 2001. DNA extraction from paraffin-embedded tissues. Met. Mol. Med., 49: 1-4.

Garrcia, C.A., A. Ahmadian, B. Gharizadeh, J. Lundeberg, M. Ronaghi and P. Nyren, 2000. Mutation detection by pyrosequencing: Sequencing of exons 5-8 of the p53 tumor suppressor gene. Gene, 253: 249-257.

Gilbert, M.T.P., T. Haselkorn, M. Bunce, J.J. Sanchez and S.B. Lucas *et al*., 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues-which methods are useful when?. PLoS ONE, 2: e537-e537.

Gillio-Tos, A., L. de Marco, V. Fiano, F. Garcia-Bragado, R. Dikshit, P. Boffetta and F. Merletti, 2007. Efficient DNA extraction from 25-year-old paraffin-embedded tissues: Study of 365 samples. Pathology, 39: 345-348.

Hein, D.W., 2006. N-acetyltransferase 2 genetic polymorphism: Effects of carcinogen and haplotype on urinary bladder cancer risk. Oncogene, 25: 1649-1658.

Kern, S.E., J.A. Pietenpol, S. Thiagalingam, A. Seymour, K.W. Kinzler and B. Vogelstein, 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science, 256: 827-830.

Liu, H., Y. Wang, Q. Zhou, S.Y. Gui and X. Li, 2002. The point mutation of p53 gene exon7 in hepatocellular carcinoma from Ahui Province, a non HCC prevalent area in China. World J. Gastroenterol., 8: 480-482.

Llovet, J.M. and M. Beaugrand, 2003. Hepatocellular carcinoma: Present status and future prospects. J. Hepatol., 38: S136-S149.

Lu, X., G. Magrane, C. Yin, D.N. Louis, J. Gray and T.V. Dyke, 2001. Selective inactivation of p53 facilitates mouse epithelial tumor progression without chromosomal instability. Mol. Cell. Biol., 21: 6017-6030.

Noguera, N.I., C.E. Tallano, I.M. Bragos and A.C. Milani, 2001. Modified salting-out method for DNA isolation from newborn cord blood nucleated cells. J. Clin. Lab. Anal., 14: 280-283.

Pinto, A.P. and L.L. Villa, 1998. A spin cartridge system for DNA extraction from paraffin wax embedded tissues. Mol. Pathol., 51: 48-49.

Poinar, H.N. and B.A. Stankiewikz, 1999. Protein preservation and DNA retrieval from ancient tissues. Proc. Natl. Acad. Sci. USA., 96: 8426-8431.

Poljak, M., K. Seme and N. Gale, 2000. Rapid extraction of DNA from archival clinical specimens: Our experiences. Eur. J. Physiol., 439: 42-44.

Rezaii, J., H. Ashegh, M. Hasibi, M. Behzadi, F. Khodadadi and M. Heidarali, 2008. The investigation of p53 and Ki-67 gene mutations in relation with helicobacter pylori infection in patients with gastric cancer. J. Med. Sci., 8: 564-568.

Rivero, E.R.C., A.C. Neves, M.G. Silva-Valenzuela, S.O.M. Sousa and F.D. Nunes, 2006. Simple salting-out method for DNA extraction from formalin-fixed, paraffin embedded tissues. Pathol. Res. Practice, 202: 523-529.

Santos, S., D. Sa, E. Bastos, H. Guedes-Pinto, I. Gut, F. Gartner and R. Chaves, 2009. An efficient protocol for genomic DNA extraction from formalin-fixed paraffin-embedded tissues. Res. Vet. Sci., 86: 421-426.

Satiroglu-Tufan, N.L., F. Bir and E. Duzcan, 2004. Rapid and effective DNA amplification by polymerase chain reaction directly from paraffin-embedded tissue. Aegean Pathol. J., 1: 33-38.

Sepp, R., I. Szabo, H. Uda and H. Sakamoto, 1994. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. J. Clin. Pathol., 47: 318-323.

Sjoholm, M.I.L., G. Hoffmann, S. Lindgren, J. Dillner and J. Carlson, 2005. Comparison of archival plasma and formalin-fixed paraffin-embedded tissue for genotyping in hepatocellular carcinoma. Cancer Epidemiol. Biomarkers Prevention, 14: 251-255.

Vollzenandt, M., A.P. Dicker, R. Fanin, D. Banerjee, A. Albino and J.R. Bertino, 1993. Polymerase chain reaction analysis of DNA from paraffin-embedded tissue. Methods Mol. Biol., 15: 80-88.

Walraven, J.M., Y. Zang, J.O. Trent and D.W. Hein, 2008. Structure/function evaluations of single nucleotide polymorphisms in human N-acetyltransferase 2. Curr. Drug Metab., 9: 471-486.