Influence of H&E and Papanicolau stains on DNA integrity – systematic review

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INTRODUCTION

The success of the Human Genome Project along with the development of new molecular and bioinformatics methods has allowed a rapid evolution of personalized health care [1]. In that context, molecular pathology plays a key role in providing patient-specific disease signatures allowing to develop targeted and patient-driven therapeutic strategies and to predict therapeutic response, particularly in oncology [1,2]. For that, the presence or absence of mutations is assessed by molecular techniques such as polymerase chain reaction (PCR), Sanger sequencing and next generation sequencing (NGS) [3]. This information complements the morphological analysis obtained by microscopic inspection of tissue sections, commonly from formalin-fixed paraffin-embedded (FFPE) samples, or of cytological specimens (CS)[4]. As cells and other tissue components are invisible, to render them visible under the microscope, it is necessary to perform a previous staining procedure [4]. The routine stain in clinical pathology laboratories for FFPE samples is Hematoxylin and Eosin (H&E), as it allows the visualization of general tissue architecture assisting the evaluation of structural and morphological changes [5]. For cytological specimens, the routine stain is Papanicolau (Pap) [6]. In some cases, FFPE or CS samples are scarce and it is necessary to extract DNA directly from FFPE or CS stained-samples. Furthermore, samples from tumors are usually very heterogeneous containing a variable mixture of neoplastic cells and a variety of normal cells. Molecular analysis of DNA extracted from these mixtures can lead to confusing or false negative results and erroneous conclusions. In order to diminish the interference of normal cells, the tumor area is usually selected and isolated before molecular analysis is performed[7]. This enrichment of neoplastic cells can be performed through manual or automated-assisted microdissection of FFPE, CS or frozen samples [8]. In these cases, it is mandatory to extract nucleic acids directly from stained samples. Although this is a common practice in molecular pathology laboratories, a survey of the literature demonstrates that the reagents used in H&E and Pap stains may induce chemical modifications on DNA. This review collects the available information about the influence of H&E and Pap stains in DNA integrity and explores the mechanisms by which the dyes might interfere with molecular analysis, particularly on PCR-based methods.

RESULTS

H&E stain compromises DNA amplificability (FFPE and CS samples), and removal of hematoxylin is necessary to acquire satisfactory results in PCR-based methods

Stain	Influence on DNA	DNA recovery method	Microdissection (MM - manual, LM-laser assisted)	Samples	References
H&E	Decreased efficiency of PCR of IgH gene (240 to 280 bp products).	Proteinase K and Triton-X	No	FFPE	Diss et al., 1994
	Sucessful PCR amplification of 138 to 239 bp fragments only after destaining.	Chelex 100	MM	FFPE	Medintz et al., 1997
	Sucessfull PCR amplification of 110 to 270 bp fragments after dye removal.	Proteinase K and Chelex 100	No	FFPE	Banaschak et al., 2000
	Inhibition of comparative genomic hybridization (CGH) assay by concentrated samples.	Proteinase K and Tween-20	MM	FFPE	Hirose et al., 2001
	Decreased PCR yield compared to unstained samples.	Lyse-N-Go™	LM	Smears	Sanders et al., 2006
	Lower levels of extracted DNA compared to unstained samples. Sucessfull DNA profilling by PCR.	Phenol-chloroform	No	Smears	Simons & Vintiner, 2011
	Amplification of GAPDH by real-time PCR, microsatellite PCR fragment analyses and Pyrosequencing of KRAS with similar eficiency than that of unstained samples. Microsatellite instability analysis and pyrosequencing of BRAF and KRAS on over 1300 colorectal cancers.	Column-based method	No	FFPE	Morikawa et al., 2012
	Sucessfull STR DNA profiling (Powerplex 16® STR profiles - 100- to 470 bp products) after dye removal	Gradient of non-polar to polar solvent and Column-based method	No	FFPE	Tairis et al., 2018
Hematoxylin	Poor PCR amplification (120 to 215 bp products)	Proteinase K and Tween-20	MM	FFPE	Burton et al., 1998
	No PCR amplification of a 536 bp fragment of the HBB gene (sucessfull after destaining)	Proteinase K	No	smears	Chen et al., 1996
	Sucessfull PCR amplification of a 150 bp fragment of HBB gene	Column-based method	LM	FFPE	Tanji, 2000
	Amplification of GAPDH by real-time PCR, microsatellite PCR fragment analyses and Pyrosequencing of KRAS with similar eficiency than that of unstained samples.	Column-based method	No	FFPE	Morikawa, 2012
	Sucessfull PCR amplification of a 150 bp fragment of HBB gene	Column-based method	LM	FFPE	Tanji et al., 2001
	Lower levels of extracted DNA compared to unstained samples. Diminished PCR efficiency (392 bp fragment of TP53)	Proteinase K	MM	FFPE and frozen	Serth et al., 2000
	Inhibition of PCR amplification that is relieved by dilution of manually dissected samples. Sucessfull PCR amplification in LCM samples (150 bp fragment of HBB gene).	Proteinase K and Tween-20	MM and LM	FFPE and frozen	Ehrig et al., 2001
	Inhibition of PCR amplification of HBB gene (110 bp fragment)	Proteinase K	No	FFPE	Murase et al., 2000
Eosin	Sucessfull PCR amplification of HBB gene (110 bp fragment)	Proteinase K	No	FFPE	Murase et al., 2000
	Amplification of GAPDH by real-time PCR, microsatellite PCR fragment analyses and Pyrosequencing of KRAS with similar eficiency than that of unstained samples.	Column-based method	No	FFPE	Morikawa et al., 2012

Pap stain compromises DNA amplificability, removal of hematoxylin is necessary to acquire satisfactory results in PCR-based methods and DNA degradation might compromise analysis of high molecular weight DNA

Stain	Influence on DNA	DNA recovery method	Microdissection (MM - manual, LM-laser assisted)	Samples	References
Papanicolau	Successful PCR amplification of HBB (268 bp fragment) and HPV L1 (150 bp fragment) genes after destained.	Proteinase K and Tween-20 followed by ethanol precipitation	No	smears	de Lang and Wilander, 2005
	Successful PCR amplification of HBB (110 bp product) and HPV (450 bp product) genes after dye removal.	Proteinase K and Tween-20	No	archived smears	Puranen et al., 1996
	Sucessful PCR amplification of β-actin (317 bp fragment) and TGFβ1 (500 bp fragment) only after phenol-chlorophorm extraction	Phenol-chloroform	No	smears	Gall et al., 1993
	Sucessfull PCR amplification and sequencing of a 188 bp-product of HPV but not of 260 bp.	Guanidinium thiocyanate-silica method	No	archived smears	Smits et al., 1992
	Sucessfull PCR amplification of KRAS (209 bp), SRGAP2 (388 bp) and EGFR (578 and 760 bp). Sucessfull EGFR genotyping.	Column-based method	MM	smears and liquid based cytology	Dejmek et al., 2013
	DNA degradation (mainly <400bp fragments), but sucessfull comparative genomic hybridization, single nucleotide polymorphism and DNA methylation arrays.	Column-based method	No	archived smears	Killian et al., 2010
	Sucessfull comparative genomic hybridization in 2 out 7 samples.	Proteinase K and Tween-20	MM	cytological imprints	Kawauchi et al., 2007
Hematoxylin	Unsucessfull PCR amplification of a 536 bp fragment of HBB gene (sucessfull after destaining)	Proteinase K	No	archived smears	Chen et al., 1996
Aluminum sulfate	Unsucessfull PCR amplification of a 536 bp fragment of HBB gene.	Proteinase K	No	archived smears	Chen et al., 1996
OG-6	Sucessfull PCR amplificationa of a 536 bp product of HBB gene	Proteinase K	No	archived smears	Chen et al., 1996
EA solution	Sucessfull PCR amplificationa of a 536 bp product of HBB gene	Proteinase K	No	archived smears	Chen et al., 1996
Ethylene glycol	Sucessfull PCR amplificationa of a 536 bp product of HBB gene	Proteinase K	No	archived smears	Chen et al., 1996
sodium iodate	Sucessfull PCR amplificationa of a 536 bp product of HBB gene	Proteinase K	No	archived smears	Chen et al., 1996
acetic acid	Sucessfull PCR amplificationa of a 536 bp product of HBB gene	Proteinase K	No	archived smears	Chen et al., 1996

CONCLUSIONS

H&E

- **H&E stain influences the integrity of DNA extracted from both FFPE and cytological samples.**
- **Removal of hematoxylin is necessary to relieve the inhibitory effect of this dye on PCR.**
- **The mechanism by which hematoxylin inhibits PCR might involve several factors: 1. Interference with DNA extraction; 2. Prevention of DNA polymerase attachment ; 3. Rescue of divalent cations.**
- **Proper sample purification and adjustment of PCR conditions are of key importance to achieve satisfactory results in PCR-based methods using H&E-stained samples.**

Papanicolau

- **Papanicolau stain influences the integrity of DNA extracted from cytological samples.**
- **Removal of hematoxylin is necessary to relieve the inhibitory effect of this dye on PCR.**
- **DNA extraction with phenol-chloroform or column-based methods are effective at removing hematoxylin. Alternatively, destaining of cell lysates also results in successful PCR.**
- **Analysis of high molecular weight DNA molecules recovered from Pap-stained samples must be performed with caution and a prior check of DNA integrity must be accomplished.**

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