Optimizing conditions for *MGMT* promoter methylation status analysis in glioblastoma FFPE samples

Original Article

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Abstract:

The methylation status of the *MGMT* promoter represents the valuable prognostic and predictive marker in glioblastoma (GBM) patients undergoing treatment with alkylating agents such as Temozolomide. Although Formalin-Fixed and Paraffin-Embedded Tissue (FFPE) signifies the most commonly used source for tissue-based molecular testing, its use in Methylation-Specific Polymerase Chain Reaction (MSP) analysis manifests certain limitations due to low DNA integrity. Our study aimed to identify the optimal *MGMT* promoter MSP reaction conditions concerning the utilization of bisulfite-converted FFPE-derived template DNA. Several optimizing reactions were conducted and subjected to ImageJ software analysis. As a result, 4U of HotStarTaq and 125 ng of template DNA were specified as necessary for successful MSP reactions. The confirmation of optimization success was obtained through comparison of semi-quantitative values of DNA methylation levels between reference Fresh Frozen tissue and corresponding FFPE sample obtained from the same GBM patient.

Key words:
glioblastoma, FFPE, methylation, *MGMT*, MSP, optimization, Taq polymerase

Apstract:

Optimizacija uslova MSP reakcije za promotorni region *MGMT* gena kod FFPE uzoraka glioblastoma

Status metilacije promotornog regiona *MGMT* gena predstavlja jedan od najvažnijih prognostičkih i prediktivnih markera pacijenata obolelih od glioblastoma (GBM) podvrgnutih lečenju alikirajućim agensima poput Temozolomida (TMZ). Iako uzorci tkiva fiksiranih u formalinu i ukalupljenih u parafinske blokove (FFPE) predstavljaju najuobičajeniji izvor DNK materijala prilikom molekularno-bioloških analiza, njihova upotreba pri metodi lančane reakcije polimeraze specifične za metilaciju (MSP) ispoljava određena ograničenja usled niskog integriteta DNK. Cilj ovog rada predstavljalo je definisanje optimalnih uslova MSP reakcije za *MGMT* promotorni region prilikom korišćenja bisulfitno-konvertovane DNK izolovane iz FFPE uzoraka kao matrice za MSP reakciju. Izvedeno je nekoliko reakcija optimizacije, a dobijeni rezultati su obrađeni u ImageJ programu. Kao rezultat, HotStarTaq polimeraza u količini od 4U i DNK matrica u količini od 125 ng izdvojene su kao neophodne za uspešnu MSP reakciju. Potvrda uspešnosti reakcija optimizacije dobijena je upoređivanjem semi-kvantitativnih vrednosti nivoa metilacije DNK između referentnog sveže-zamrznutog uzorka (FF) i odgovarajućeg FFPE uzorka poreklom od istog GBM pacijenta.

Ključne reči:
glioblastomi, FFPE, metilacija, *MGMT*, MSP, optimizacija, Taq polimeraza

Introduction

Glioblastoma (GBM) refers to the highly infiltrative type of gliomas, malignant brain tumors. Owing to great morphological and genetic heterogeneity, it is characterized by extremely low five-year survival rates – only 5%, and an annual incidence of 5.26 per 100,000 people (Soomro et al., 2017). Although it is widely accepted that gliomas originate from normal glial cells, increasing evidence supports the role of various cell types such as glial or neural precursors and stem cells as their possible origin (Chen et al., 2017). Recent findings

in the molecular biology of glioma have led to the novel, improved system of classification which was established by the World Health Organization (WHO) in 2016 (Louis et al., 2016). Namely, the shortcomings of histopathology based classification were exceeded through the incorporation of molecular diagnostic criteria - testing for Isocitrate Dehydrogenase (*IDH*) mutation, chromosome 1p/19q deletion, and histone mutations. In 2010, The Cancer Genome Atlas (TCGA) classification of GBMs had identified 4 subtypes of glioblastomas based upon gene expression profiles and genomic clustering – proneural, neural, classical, and mesenchymal subtypes (Verhaak et al., 2010). The methylation of the Cytosine-phosphate-Guanine (CpG) islands in the promoter region of O⁶-methyl guanine-DNA methyltransferase gene (*MGMT*) represents one of the most common epigenetic alterations in GBM. It is present in both primary and secondary GBM in 42% and 79% patients respectively (Soomro et al., 2017). The MGMT enzyme is an excision repair enzyme that removes alkyl adducts from the O^6-position of the guanine. Thus, it protects normal cells from carcinogens by repairing double-strand breaks and base mispairing which leads to apoptosis and cell death. During that process it is being irreversibly deactivated, which is why MGMT is referred to as "suicidal" enzyme (Thon et al., 2013; Soomro et al., 2017). Regarding the MGMT role of counteracting the activity of alkylating agents, its inactivation through epigenetic silencing was recognized as an important and clinically relevant factor in GBM patients undergoing treatment with alkylating agents such as Temozolomide (TMZ). The positive methylation status of the *MGMT* promoter represents a strong and independent predictive factor of favorable survival in GBM. The median survival was significantly longer in patients with a methylated *MGMT* promoter (21.7 months) in comparison with the patients lacking methylation (12.7 months). Additionally, a high frequency of *MGMT* promoter methylation was documented in long-term GBM survivors undergoing TMZ treatment (Thon et al., 2011; Thon et al., 2013). Given the fact that methylated cytosine in CpG islands exhibits the same base-pairing interactions as unmethylated cytosine, the evaluation of methylation status using conventional hybridization-based methods, i.e. microarrays and PCR, is not suitable (Holmes et al., 2014). To address that, Frommer et al. have designed a protocol that utilizes bisulfite–induced modification of genomic DNA resulting in the conversion of unmethylated cytosine to uracil, while 5-methylcytosines remain intact (Frommer et al., 1992). Such bisulfite-converted DNA could be subsequently analyzed via PCR in order to acquire desired epigenetic information. With the numerous commercially available kits and several technological advances, current bisulfite-treatment protocols are more convenient and user friendly in comparison with the original 16 hours protocol (Holmes et al., 2014). However, the success of bisulfite-conversion may vary considerably depending on the quality of DNA samples and the choice of tissue samples (Tournier et al., 2012). There are strong suggestions for avoiding Formalin-Fixed and Paraffin-Embedded Tissues (FFPE) samples for bisulfite conversion and following Methylation-Specific Polymerase Chain Reaction (MSP). These are supported by evidence that the use of FFPE induces non-reproducible bisulfite conversion leading to unreliable and inconsistent results for methylation levels (Tournier et al., 2012). In contrast with FFPE, the use of Fresh Frozen tissue (FF) brings reproducible and satisfactory results, owing to the process of cryo-preservation which provides the adequate DNA preservation. Given that the FFPE samples consist of degraded DNA generally less than 300 bp, the main challenge with managing FFPE samples is to provide an efficient cell lysis which releases DNA of sufficient quality and quantity for further analysis (Holmes et al., 2014). What makes it even more difficult, formalin-fixation induces the formation of DNA-protein crosslinks, which often could not be completely removed by common lysis protocols. Nevertheless, as FFPE samples are widely available, this type of tissue sample is the most commonly used source for tissue-based molecular testing (Dietrich et al., 2013). Alongside the low pricing of long term storage, FFPE is often the only available material for retrospective studies and the most important material for standard routine diagnostics in the era of personalized medicine (Dietrich et al., 2013). Therefore, several commercially available kits for FFPE DNA isolation and bisulfite conversion were developed to ensure results as reliable as possible (Holmes et al., 2014; de Ruijter et al., 2015; Ludgate et al., 2017; Kint et al., 2018). This study aimed to define optimal PCR conditions for evaluation of *MGMT* promoter methylation status in the FFPE sample obtained from GBM patient, considering the recommendations from the previous studies (Dietrich et al., 2013). Also, to investigate the validity of the results, they were compared with *MGMT* promoter methylation status obtained from the FF sample of the same GBM patient.

Material and methods

Patient and tumor specimens

Both FF and FFPE tissue tumor specimens were collected from the GBM patient (male, 63 years old) operated on at the Neurosurgery Clinic (The

Clinical Centre of Niš, Serbia) in 2013. The patient underwent total resection of the tumor and had a Karnofsky score of ≥80%. The diagnosis of glioblastoma WHO grade IV was confirmed by an expert neuropathologist (N.V and M.K). The written informed consent of study participation was obtained from the GBM patient. The Ethics Committee of the Faculty of Medicine, Niš, Serbia, approved the informed consent form and study protocol (01-2113-10).

DNA isolation and bisulfite conversion

Extraction of genomic DNA was performed using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) from 25 mg of FF sample and QiAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany, Catalogue No. 56404) from 8 freshly cut sections with a thickness of 10 μm from FFPE sample. A total of 2 μg of genomic DNA was modified by sodium bisulfite using EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany) for the FF DNA sample and Epitect Plus FFPE bisulfite kit (Qiagen, Hilden, Germany, Catalogue No. 59144) for FFPE DNA sample. BioSpec–nano UV–Vis Spectrophotometer

Table 1. Primer sequences and amplicon size

Gene	Primer Sequence (5'-3')	Amplicon Size (bp)
MGMT unmethylated (U)	F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT R: AACTCCACACTCTTCCAAAAACAAAACA	93
MGMT methylated (M)	F: TTTCGACGTTCGTAGGTTTTCGC R: GCACTCTTCCGAAAACGAAACG	81

(Shimadzu, Kyoto, Japan) was utilized for the determination of quantity and quality of isolated DNA and bisulfite converted samples. Isolated DNA samples were inspected for degradation and visualized by running the DNA samples on 2% agarose gel.

Methylation-Specific Polymerase Chain Reaction (MSP)

All of the MSP reactions were carried out in a total volume of 20 μL containing 0.2 μM dNTP mix, 1 × PCR buffer with 1.5 mM $MgCl_2$ (Qiagen, Hilden, Germany) and 10 pM of appropriate forward and reverse primer (**Tab. 1**).

Amplification reactions were performed in a Mastercycler Gradient (Eppendorf) using the following program: 95 °C for 15 min, then 35 cycles of 95 °C for 50 s, 59 °C for 50 s and 72 °C for 50 s, and a final extension at 72 °C for 10 min.

MSP optimization reactions with four different amounts of bisulfite-converted template DNA (31.25 ng, 62.5 ng, 125 ng, and 250 ng) in combination with 1U and 4U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany, Catalogue No. 203203) were conducted according to suggestions from the previous study (Dietrich et al., 2013). Following the determination of template DNA optimal concentration, another set of optimization reactions was designed regarding the investigation of the optimal concentration of HotStarTaq polymerase for successful *MGMT* MSP reactions using FFPE DNA isolates. Therefore, MSP reactions with 1U, 2U, and 4U of HotStarTaq polymerase and unmethylated set of *MGMT* primers were conducted simultaneously with MSP reaction including template DNA obtained from the FF sample and 1U of HotStarTaq polymerase. For comparison of methylation level evaluation between FFPE and FF DNA isolates, another set of MSP reactions was carried out using both sets of *MGMT* primers and FFPE and FF isolates as DNA templates, followed by agarose gel electrophoresis. MSP reactions using FFPE-derived bisulfite-converted template DNA were conducted in duplicate.

Analysis of Methylation Data

After each optimization, MSP gel images were subjected to ImageJ software analysis (National Institute of Health, Bethesda, MD, USA) with the aim of measuring the fluorescence intensity of methylated (M) and unmethylated (U) MSP bands. M/U intensity ratio values represent the common approach of semi-quantitative evaluation of the *MGMT* promoter methylation level (Christians et al., 2012).

Results

FF and FFPE DNA isolates

Concentrations and purity of genomic DNA isolates and bisulfite-converted DNA samples are presented in **Tab. 2**.

Table 2. Quantity and quality of isolated DNA and bisulfite converted samples (BioSpec–nano UV–Vis Spectrophotometer)

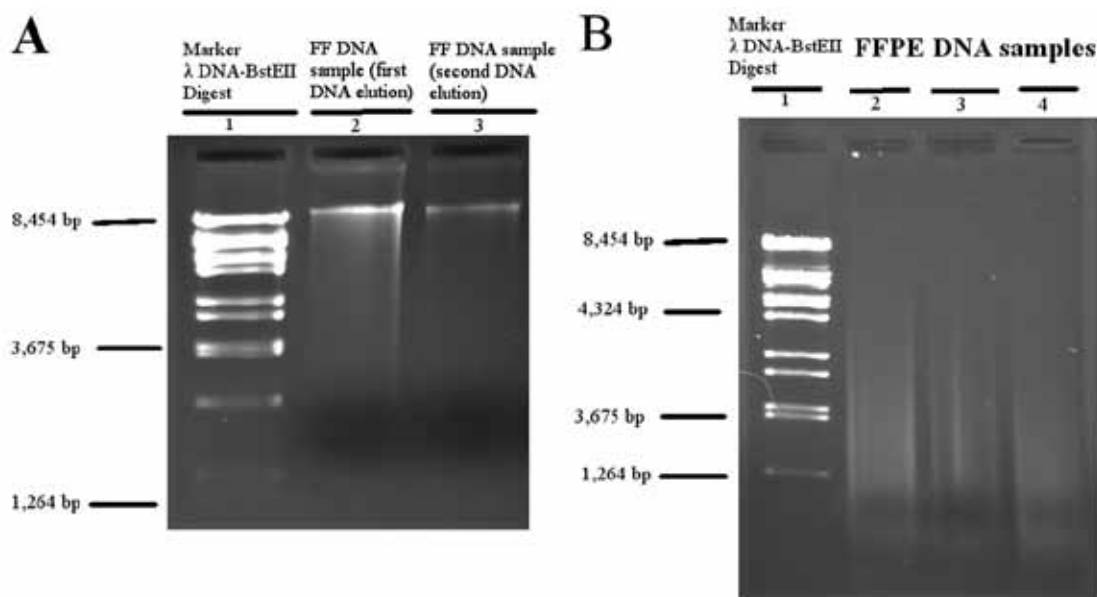|	Total yield of DNA (ng)	OD 260/280	Yield of bisulfite-converted DNA per single conversion (ng)
FFPE DNA isolate (from 8 sections with a thickness of 10 μm)	4,537.5	1.94	2,265.8
FF DNA isolate (50 mg of tissue)	35,209.2	1.87	2,094.4

Fig. 1. Agarose gel electrophoresis images of isolated DNA samples (~400ng) **A**) High-quality DNA isolates obtained from FF samples (4000-9000 bp fragments). Lane 1 – marker λ DNA-Bst EII (New England BioLabs® Inc. #N3014S), Lane 2- FF DNA sample (first DNA elution), Lane 3- FF DNA sample (second DNA elution) **B**) Low-quality FFPE DNA samples with highly degraded DNA fragments: Lane 1 – marker λ DNA-Bst EII, Lanes 2,3,4 - FFPE DNA samples.

Total yield of isolated DNA from FFPE sample was 4,537.5 ng and 35,209.2 ng from FF sample, with OD 260/280 values of 1.94 and 1.87, respectively. Recorded yield of bisulfite-converted DNA per single conversion was 2,265.8 ng for FFPE and 2,094.4 ng for FF DNA sample.

Agarose gel images representing the integrity of isolated DNA are shown in **Fig. 1**.

Agarose gel images of FF DNA samples displayed that most of the DNA fragments have migrated conjointly with the largest fragments of the DNA ladder marker (~9000 bp) thereby forming a noticeable band. In contrast, highly degraded FFPE DNA samples were presented on agarose gels as characteristic large smears originating from DNA fragments of various sizes, mostly shorter than 1,264 bp.

MGMT MSP template DNA concentration optimization in FFPE samples

Agarose gel image of MSP optimization of optimal template DNA quantity is presented in **Fig. 2**.

With the clear difference in fluorescence intensity of both methylated and unmethylated *MGMT* MSP products for 1U and 4U of HotStarTaq polymerase, the most appropriate M and U MSP products were recorded for 125 ng quantity of template DNA and 4U of HotStarTaq polymerase.
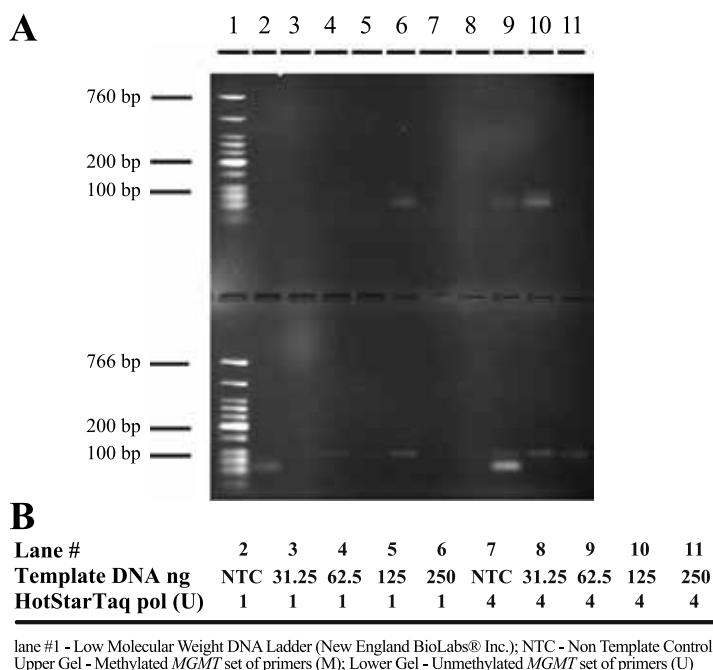

Lane #	2	3	4	5	6	7	8	9	10	11
Template DNA ng	NTC	31.25	62.5	125	250	NTC	31.25	62.5	125	250
HotStarTaq pol (U)	1	1	1	1	1	4	4	4	4	4

lane #1 - Low Molecular Weight DNA Ladder (New England BioLabs® Inc.); NTC - Non Template Control;
Upper Gel - Methylated *MGMT* set of primers (M); Lower Gel - Unmethylated *MGMT* set of primers (U)

Fig. 2. MSP optimization for FFPE DNA isolates **A**) Agarose gel image **B**) legend

Lane #	2	3	4	5	6
Sample type	NTC	FF	FFPE	FFPE	FFPE
Template DNA (ng)	/	125	125	125	125
HotStarTaq pol (U)	1	1	1	2	4

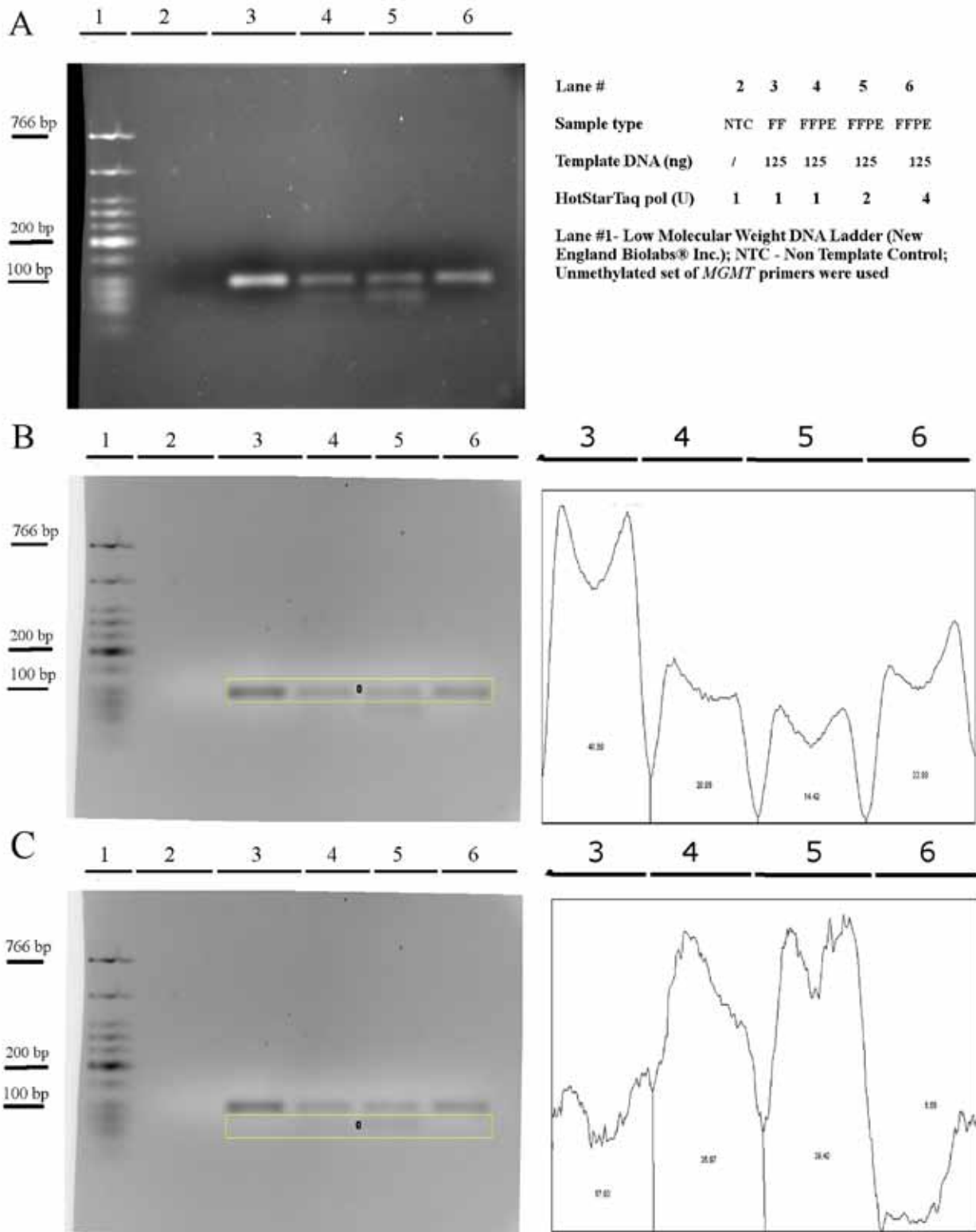Lane #1- Low Molecular Weight DNA Ladder (New England Biolabs® Inc.); NTC - Non Template Control; Unmethylated set of *MGMT* primers were used

Fig. 3. Results of MSP optimization regarding defining the optimal amount of HotStarTaq polymerase. **A**) Original agarose gel image with a legend on the right, presenting FFPE unmethylated *MGMT* promoter MSP products with 1U, 2U and 4U of HotStarTaq polymerase alongside with FF unmethylated *MGMT* MSP with 1U of HotStarTaq polymerase product as a positive control. **B**) ImageJ software processed image with fluorescence intensity analysis of specific unmethylated *MGMT* MSP product bands, with corresponding chart representing their relative values. **C**) ImageJ fluorescence intensity analysis of primer-dimer presence in MSP reactions.

Table 3. Fluorescence intensity values of 3 different concentrations of HotStarTaq polymerase obtained using ImageJ software analysis

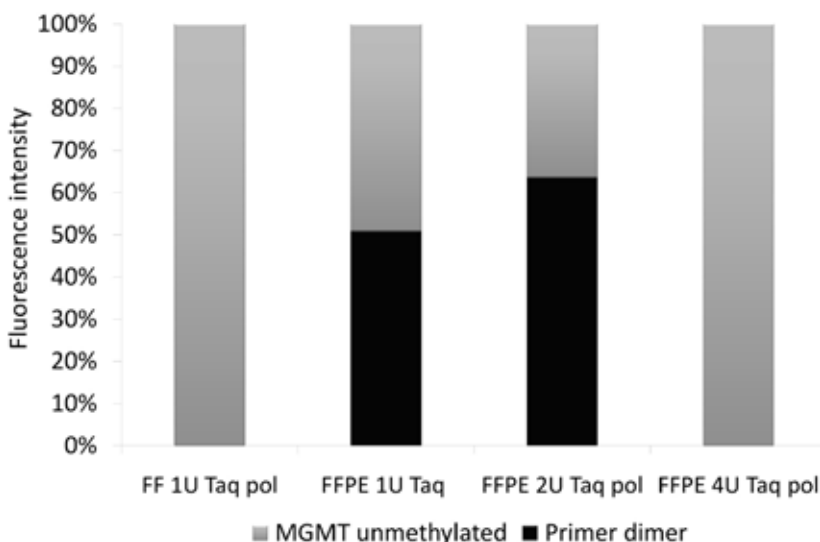| Total amount of HotStarTaq polymerase units in MSP reaction	1U	2U	4U
Fluorescence intensity of unmethylated MGMT MPS product compared to FF refference (%)	48.27	34.67	57.43
Fluorescence intensity of primer-dimers compared to FF refference (%)	200.60	219.85	37.16
Product / primer-dimer intensity ratio compared to FF refference (%)	49.10	36.30	113.12

Fig. 4. *MGMT* product / primer-dimer fluorescence intensity ratios detected for 125 ng of template DNA and 3 different concentrations of HotStarTaq polymerase in FFPE and reference FF DNA bisulfite-converted samples

MGMT **MSP HotStarTaq polymerase concentration optimization in FFPE samples**

Regarding further determination of the optimal amount of Hot Start Taq polymerase, agarose gel image was acquired following MSP optimization and processed with ImageJ software analysis (**Fig. 3**).

Fluorescence intensity values acquired using the ImageJ software have confirmed the 4U of HotStarTaq polymerase as an optimal amount for successful MSP *MGMT* reaction in FFPE samples. Among tested concentrations, the highest fluorescence intensity value of unmethylated MSP products was recorded for 4U of HotStarTaq polymerase. It was equivalent to 57,43% of the recorded fluorescence intensity value of the reference FF DNA *MGMT* MSP product. Furthermore, the fluorescence intensity value of primer-dimers was the lowest for the 4U concentration, being even lower than the one recorded for the reference FF DNA template MSP - 37.16% of the reference value. Finally, only for the 4U HotStarTaq polymerase concentration

was documented that the *MGMT* product/primer-dimer fluorescence intensity ratio exceeds 100% of the reference value (113.12 %), suggesting optimal reaction conditions (**Tab. 3** and **Fig. 4**).

MGMT **methylation assesment in FFPE and FF sample**

Figure 5 (A) shows the agarose gel image of *MGMT* MSP reactions which included bisulfite-converted template DNA of the same patient originating from both FFPE and FF samples, alongside with randomly chosen FFPE sample from another GBM patient. M/U ratio values of FF and FFPE samples were acquired via ImageJ software analysis **Fig. 5** (B).

As a result of ImageJ software analysis, both the FFPE and FF samples were assessed as strongly methylated semi-quantitative category of *MGMT* promoter methylation (M/U ratio <1). The observed difference in M/U ratio values was 8.47% (**Tab. 4**).

Discussion

Results from our study have confirmed the great difference in DNA fragmentation level between FF and FFPE DNA isolates (**Fig. 1**). In contrast with the FF sample, the FFPE sample from our study was in great portion consisted of DNA fragments shorter than ~1,000 bp, which were forming a smear and lacking a distinguishable band on the agarose gel. This observation is consistent with previously described FFPE DNA isolate properties originating from the tissue preparation and fixation process. Namely, it was shown that mechanical stress resulting from cross-linking during the fixation process, the concentration of formalin, pH, and salt, the temperature, and tissue type have a great impact on the quality of the FFPE-derived DNA (Ludgate et al., 2017). Among such factors, the deparaffinization was emphasized as the crucial process which reduces the quality of the isolated DNA (Sengüven et al.,
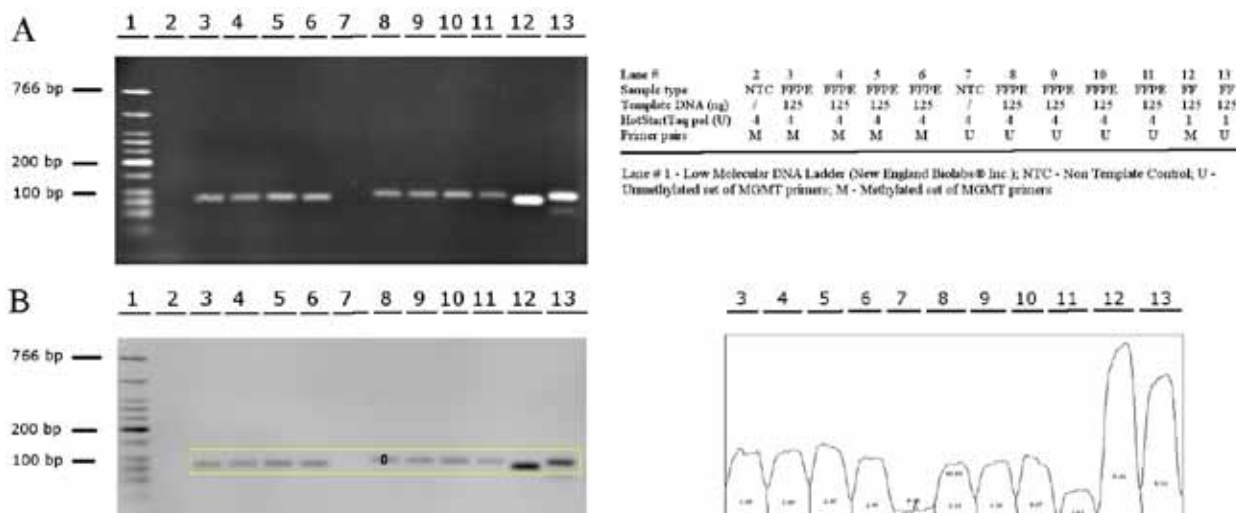
Fig. 5. Differences in *MGMT* promoter methylation assessment between FF and FFPE samples: **A**) Agarose
gel image presenting methylated and unmethylated *MGMT* promoter MSP products with the corresponding
legend on the right side. Lanes 3 and 4 - methylated *MGMT* promoter MSP products involving FFPE samples
of the previously listed GBM patient as template DNA; Lanes 8 and 9 - unmethylated *MGMT* promoter MSP
products involving FFPE samples of the previously listed GBM patient as template DNA; Lanes 12 and 13
- Reference FF sample *MGMT* promoter MSP products (methylated and unmethylated); Lanes 5,6,10,11
- *MGMT* promoter MSP products (methylated and unmethylated) assessed with FFPE bisulfite-converted
sample of different GBM patient. **B**) Fluorescence intensity levels of methylated (M) and unmethylated (U)
MSP bands obtained using ImageJ software.

2014). Given the fact that bisulfite conversion further
degrades DNA, the challenges which arise in DNA
methylation analysis are no surprising (Patterson
et al., 2011). As a consequence, MSP reaction
conditions concerning methylation analysis may
significantly differ in case of using the FFPE instead
of the FF bisulfite-converted sample as template
DNA. Considering the significance of FFPE tissues
as a valuable DNA source for *MGMT* methylation
analysis in GBM patients, as well as for clinical
and cancer research in general, our main goal was
to reveal the optimal *MGMT* promoter methylation
MSP reaction conditions which could bring the
results as valid as those involving FF-derived DNA.

The most significant MSP reaction parameters
– *Taq* polymerase and template DNA concentrations
were selected considering the findings of improved
PCR performance in FFPE tissue samples presented
by Dietrich et al. (2013). Primarily, our study tested
4 concentrations of template DNA (31.25 ng, 62.5
ng, 125 ng, and 250 ng) and 2 concentrations
of HotStarTaq polymerase (1U and 4U) for
the capability of alleviating PCR inhibition and
amplifying methylated *MGMT* promoter (81 bp)
and unmethylated *MGMT* promoter (93 bp) MSP
products. In concordance with previous findings,
this optimization reaction revealed the 4U of
HotStarTaq polymerase, alongside with 125 ng of
template DNA as optimal concentrations for both
methylated and unmethylated MSP products. As

seen in **Fig. 2**, the gradual increase of template DNA
amount enhances the MSP product intensity, both
in 1U and 4U reaction subgroups. This overcoming
of PCR inhibition occurs through increasing the
probability of the presence of template molecules
of proper length and integrity. As a result, higher
template concentrations and 1U concentration
of HotStarTaq polymerase emerged as one of
the possible solutions for optimal amplification
conditions. Furthermore, a 4-fold increase of
the HotStarTaq polymerase clearly showed the
successful amplification in replicates containing low
amounts of template DNA. The listed observations
were suggesting the optimal amount of 125 ng and
4U of HotStarTaq polymerase in both methylated
and unmethylated *MGMT* products. However,
the presence of additional primer-dimer bands
on agarose gel could lead to misinterpretation of
methylation level analysis.

To address that issue and further elucidate
optimal MSP conditions, another optimization
reaction was conducted. Given the relatively short
length of the *MGMT* promoter MSP products, this
optimization introduced the 2U concentration of
HotStarTaq polymerase as a possible solution for
economizing enzyme consumption. For that purpose,
optimization reaction was performed including 3
concentrations of HotStarTaq polymerase (1U, 2U,
and 4U), 125 ng amount of template DNA, and
unmethylated *MGMT* promoter primer set (**Fig.**

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3). FF DNA bisulfite-converted sample served as a control MSP reaction. Despite successful amplification of the specific product in 1U and 2U replicates, an ImageJ software analysis showed the significant presence of primer-dimer bands in 1U and 2U, but not in 4U replicate, confirming the 4U concentration of HotStarTaq polymerase as the optimal one (**Fig. 4**).

Since the original study concerning *MGMT* promoter methylation in GBM patients, there were several common approaches of methylation status assessment (Esteller et a., 2000). In order of improving the qualitative assessment results obtained by the end-point PCR method, the semi-quantitative approach which utilizes additional ImageJ software analysis of gel images was proposed (Dietrich et al., 2013). Thus, through the measuring of fluorescence intensity ratios of methylated and unmethylated *MGMT* promoter product bands, patients could be sorted into three groups – unmethylated (M/U ratio =0), with weak promoter methylation (M/U ratio between 0 and 1) and strongly methylated (M/U ratio >1). Although this approach is widely replaced by qPCR methods of methylation status assessment, for this study it presents a valuable method for validation of optimizing MSP reaction results. By comparing M/U ratio values with those of FF samples, FFPE *MGMT* promoter MSP reaction conditions could be tested.

For that purpose, the final set of *MGMT* promoter MSP reactions in our study have included both reference FF and FFPE DNA isolates, performed in duplicate with previously defined PCR conditions (4U HotStartTaq polymerase and 125 ng of template DNA). Indeed, M/U ratios obtained for corresponding FF and FFPE samples in our study were very similar, differing only in 8.47 percent (**Tab. 4**). In that manner both of the samples were assessed as strongly methylated (**Tab. 4**), although the absolute intensity values were almost 3-fold different (**Fig. 5B**). This observation suggests that the optimizing conditions for *MGMT* promoter MSP using FFPE-derived DNA samples presented in this study were properly defined.

Conclusion

The results acquired in our study were in line with previous findings concerning the use of FFPE tissue in MSP reactions. Thus, gel electrophoresis analysis of the FF and FFPE DNA isolates confirmed the high level of degradation in FFPE isolates. Based on several optimizing reactions, 4U HotStarTaq polymerase and 125 ng of template DNA could be singled out with high certainty as suitable concentrations for successful MSP reaction concerning methylated and unmethylated *MGMT* promoter amplification

products and FFPE-derived template DNA. Although the above-mentioned PCR conditions refer to the amplification of specific *MGMT* promoter PCR products using the end-point PCR method, they could also be taken into consideration while performing MS-qPCR reactions. However, it should be noted that due to the small sample size and a relatively small number of measurements, the presented results were not additionally supported by some form of statistical analysis.

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