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From Cell Biology to Tissue Engineering

Comparison of protocols for removal of melanin from genomic DNA to optimize PCR amplification of DNA purified from highly pigmented lesions

Anna Luiza Silva Almeida Vicente[1], Raquel Alves Bianchini[1], Ana Carolina Laus[1], Graziela Macedo[2], Rui Manuel Reis[1,3,4] and Vinicius de Lima Vazquez[1,5]

[1]Molecular Oncology Research Center, [2]Department of Pathology, Barretos Cancer Hospital, Barretos, São Paulo, Brazil, [3]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, [4]ICVS/63B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal and [5]Department of Surgery Melanoma/Sarcoma, Barretos Cancer Hospital, Barretos, São Paulo, Brazil

Summary. Melanin is produced by melanocytes and protects against DNA damage by ultraviolet light. Unfortunately, the melanin protein present in melanoma tumor cells is often co-purified during DNA extraction, and this contamination may inhibit subsequent PCR methods, which directly impacts research applications and the molecular diagnostic tests needed for targeted therapeutics. There are presently no described purification protocols that efficiently remove melanin from genomic DNA. In this study, we compare six different methods for melanin removal from genomic DNA: Agarose Gel Electrophoresis, 1mg Chelex®-100, Chelex®-100 5%, centrifugation, OneStep™ PCR Inhibitor Removal Kit and centrifugation plus OneStep™ PCR Inhibitor Removal Kit. Each comparison was made using 16 formalin-fixed paraffin-embedded (FFPE) and 11 fresh cell line samples. All samples were initially tested using the multiplex PCR reaction for *GAPDH* gene that generates different sized amplified products: 100, 200, 300 and 400 base pairs, which could be inhibited by the addition of exogenous melanin. Six purification protocols were then applied, and all samples that amplified at least one *GAPDH* fragment were sequenced to analyze the presence of the *BRAF* V600E mutation. The efficiencies of amplification decreased for larger sized fragments in all methods. Our comparisons showed that centrifugation combined with the OneStep™ PCR Inhibitor Removal Kit was superior to all other methods for successful *BRAF* sequencing with 100% (100bp), 75% (200bp), 50% (300bp), and 31.3% (400bp) amplification efficiencies for the different amplicon sizes. In conclusion, this genomic DNA extraction method is highly efficient for successful PCR when tumor samples are contaminated with melanin.

Key words: Melanin, PCR inhibitor, Pigmented melanomas, Purification, Polymerase inhibition

Offprint requests to: Vinicius de Lima Vazquez, MD, PhD, , Molecular Oncology Research Center - Barretos Cancer Hospital, Antenor Duarte Villela, 1331, Zip Code: 14784 400, Barretos, São Paulo, Brazil. e-mail: viniciusvazquez@gmail.com

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Introduction

Melanocytes are dendritic cells of the neuroectoderm, and are responsible for the production of the two major types of pigments present in the skin. Activation of wild-type MC1R (melanocortin 1 receptor) promotes the production of darkly pigmented eumelanin, whereas variants with impaired signaling are associated with the production of red/yellow-sulfated pheomelanin. Activation of the MC1R by its endogenous agonists αMSH (alpha-melanocyte-stimulating hormone) and ACTH (melanocortin 2 receptor/adrenocorticotropic hormone) stimulates tyrosinase activity, converting DOPA (dihydroxyphenylalanine) to DOPAquinone (dihydroxyphenylalanine quinone) after the eumelanogenesis and pheomelanogenesis biosynthetic

pigment pathways have diverged. Melanogenesis is completed with the transfer of pigments to the keratinocytes through the melanosomes, thus protecting against DNA damage from ultraviolet (UV) radiation. The genetic damage caused by exposure to the sun and UV radiation leads to abnormal proliferation of the pigmented melanocytes leading to the dark color typical of melanoma.

The discovery that melanomas often have a specific somatic *BRAF* V600 mutation affecting the BRAF/MEK/ERK pathway led to the increasing use of BRAF and MEK therapeutic inhibitors in clinical practice for treatment of advanced metastatic disease in several countries, including North America and Europe (Flaherty et al., 2012). These new drugs are very effective, but before they can be used on patients, it is necessary to determine whether the mutation is present by performing polymerase chain reaction (PCR) tests of genomic DNA (gDNA) derived from a tumor biopsy. A technical challenge for performing PCR testing is that melanin from cancer cells can co-purify with the tumor gDNA inhibiting PCR amplification. It has been demonstrated that melanin is a potent inhibitor of thermostable DNA polymerase that is caused by interactions between the polymerase and melanin proteins. Also, PCR of large amplicons has been found to be more prone to melanin inhibition than shorter PCR tests, due the reduction of the capacity of the polymerase to extend longer synthesized DNA products (Eckhart et al., 2000).

A small number of studies have investigated different ways of removing the contaminating melanin during the DNA extraction process. Purification steps designed to exclude melanin have used NucleoSpin® DNA Clean-Up XS kits (Faber et al., 2013), PowerClean® DNA Clean-Up kits, DNA IQ™ System (Hu et al., 2015) or addition of BSA (bovine serum albumin) (Frouin et al., 2016). However, none of these clean-up methods have demonstrated a high purification efficiency. For example, the BSA assay for *BRAF* V600E analysis of gDNA from formalin-fixed paraffin-embedded (FFPE) samples was only successful for PCR in 61.5% of the samples (Frouin et al., 2016). To our knowledge, there have been no previous systematic studies comparing different methods of removing melanin from gDNA that considered the size of the amplicons and and using gDNA samples from cell lines, fresh tissue or from DNA derived from FFPE.

The aim of this study is to compare six different methods of melanin removal from gDNA (Agarose Gel Electrophoresis, 1mg Chelex®-100, Chelex®-100 5%, centrifugation, OneStep™ PCR Inhibitor Removal Kit and Spin plus OneStep™ PCR Inhibitor Removal Kit), and to evaluate their relative efficiencies for subsequent PCR and sequencing methods. Also, our experimental design allows us to evaluate the impact of different amplicon sizes on assay performance and to determine the efficiency of each method for both freshly collected and paraffin embedded samples.

Materials and methods

Cell culture

For the 11 cultured cell lines (U251, U373, GAMG, SIHA, HCT, NHA, HELA, CASKI, UW497, A375 and FADU), gDNA was extracted using BioPur (Biometrix) following the manufacturer´s recommendations. Concentration and spectrophotometric purity indicators of DNA were measured using NanoDrop 2000 (ThermoFisher) and are summarized in Table 1.

Tissue sample collection

Sixteen FFPE samples from patients with melanoma were retrospectively retrieved from the files of the Department of Pathology at Barretos Cancer Hospital. All the patients were diagnosed between 2000 and 2005 and all cases were re-evaluated by a pathologist who confirmed the diagnosis and identified the tumor region for the molecular analysis. gDNA was obtained from FFPE slides after microdissection using the QIAmp DNA Mini Kit (Qiagen) following the manufacturer´s

Table 1. Concentration and spectrophotometric purity indicators of isolated DNAs from FFPE samples and cultured cell lines measured using NanoDrop 2000 (ThermoFisher).

Sample type	Sample ID	Stock concentration (ng/uL)	A260/280 ratio	A260/230 ratio
FFPE* samples				
	P1	109.7	1.93	1.49
	P2	124.9	1.95	1.51
	P3	420.8	1.88	1.91
	P4	308.7	1.83	1.66
	P5	428.8	1.94	2.24
	P6	377.5	1.87	1.92
	P7	71.6	2.00	1.42
	P8	1572.8	1.87	2.17
	P9	608.2	1.90	1.20
	P10	1124.1	1.92	1.99
	P11	1170	1.91	1.80
	P12	939.7	1.85	1.96
	P13	1172.2	1.85	2.03
	P14	1849.5	1.93	2.16
	P15	798.7	1.78	1.97
	P16	896.1	1.91	2.05
Cultured cell lines				
	L1	1222.9	2.01	2.38
	L2	832.2	1.95	2.48
	L3	1419.1	2.04	2.30
	L4	887.3	2.00	2.22
	L5	614.1	1.83	2.43
	L6	1118.3	2.05	2.40
	L7	636.1	2.03	2.14
	L8	516.9	1.98	2.21
	L9	1178	2.02	2.42
	L10	47.5	2.10	2.00
	L11	28.6	2.00	3.40

*FFPE: formalin-fixed paraffin-embedded.

recommendations. All the samples were metastatic tumors and obtained from patients prior to any radiation or systemic therapy. The biopsies were clinically indicated and not tied to the study. Concentration and spectrophotometric purity indicators of DNA were measured using NanoDrop 2000 (ThermoFisher) and are summarized in Table 1.

Evaluation of the inhibitory effect of melanin on PCR assays

To evaluate the inhibition of melanin in the PCR, in each protocol 1µg of each gDNA and 550 ng (55ng/µL) of commercial melanin (Sigma) were used, according to Hu and collaborators (Hu et al., 2015). Briefly, pure melanin was diluted in ultrapure water at room temperature and vortexed so that known amounts of the exogenous melanin could then be spiked into the gDNA samples being tested to investigate PCR inhibition.

Agarose gel electrophoresis

Gel electrophoresis was used to separate the gDNA from the faster-migrating melanin. All samples were submitted to 1.5% agarose gel electrophoresis for 40 minutes at 90V. After that, gDNA was extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) following the recommendations of the manufacturer.

1 mg Chelex®-100 (Bio-Rad):

One milligram of Chelex-100 was added per sample, mixed and incubated at room temperature for 15 minutes. After centrifugation at 10,000g for 5 minutes, the supernatant containing gDNA was used for PCR reactions (Beadling et al., 2008).

Chelex®-100 5% (Bio-Rad):

An equal volume of Chelex-100 5% (Chelex-100 equilibrated in Qiagen AE buffer) was added per sample and incubated at room temperature for 10 minutes. The mixture was heated to 95°C for 2 minutes, centrifuged at 10,000g for 5 minutes and the supernatant gDNA used for PCR reactions (Beadling et al., 2008).

Centrifugation

This melanin removal protocol was established by our research group. All samples being tested in the study were centrifuged at 20,000g 14°C for 15 minutes so that the contaminating melanin collects in the pellet at the bottom of the tube. The supernatant containing the purified gDNA was used for subsequent PCR reactions.

OneStep™ PCR Inhibitor Removal Kit (Zymo Research)

All samples were purified using the fractionation columns according to the manufacturer's instructions. Before using the column, it was necessary to break the base, remove the cap, and insert it into a collection tube before centrifuging at 8,000g for 3 minutes. The column was then transferred to a 1.5 mL tube, the DNA was added and the samples, which were centrifuged for 8000g for 1 minute.

Centrifugation plus OneStep™ PCR Inhibitor Removal Kit (Zymo Research)

All samples were centrifuged at 20,000g 4°C for 15 minutes and the supernatant gDNA was added in the pre-prepared columns and the samples were centrifuged at 8,000g for 1 minute. This protocol was established by our research group.

PCR conditions

To verify the quality and integrity of the extracted DNA, a multiplex PCR reaction with four pairs of primers for the *GAPDH* gene was performed as described by Van Beers et al. (2006). This multiplex reaction amplifies 4 different fragments of 100 base pairs (bp), 200 bp, 300 bp, and 400 bp, enabling a rapid assessment of DNA quality based on fragment length. Table 2 shows the primers used and the size of the fragments. Conventional PCR multiplex amplification was performed in a volume of 30 µl containing 1.6X PCR buffer, 10 µM deoxyribonucleoside triphosphate (dNTP), 5 µM each of the forward and reverse primers for each fragment, 1 unit of Platinum Taq DNA

Table 2. Primers sequences used for PCR.

Gene	Forward	Reverse	Fragment size
GAPDH	GTTCCAATATGATTCCACCC	CTCCTGGAAGATGGTGATGG	100 bp
GAPDH	AGGTGGAGCGAGGCTAGC	TTTTGCGGTGGAAATGTCCT	200 bp
GAPDH	AGGTGAGACATTCTTGCTGG	TCCACTAACCAGTCAGCGTC	300 bp
GAPDH	ACAGTCCATGCCATCACTGC	GCTTGACAAAGTGGTCGTTG	400 bp
BRAF	TTCATGAAGACCTCACAGTAAAAA	CCACAAAATGGATCCAGACA	107 bp

bp: base pair.

polymerase (Life Technologies), and 100 ng of genomic DNA. The following cycling conditions were used: 94°C for 4 min, 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 7 min, and reactions were performed in Veriti Thermal Cycler (ThermoFisher). A positive control (blood DNA from a healthy donor) and no template control were used in all reactions. Amplification of PCR products was confirmed by 1.5% agarose gel electrophoresis for 60 minutes at 100V.

Analysis of BRAF V600E mutations

The BRAF V600E mutation analysis was performed by PCR followed by direct Sanger sequencing. Briefly, specific primer pairs (Table 2) that flank the target mutated region were amplified by PCR in a reaction containing 10x PCR buffer, 50mM of MgCl2, 10mM dNTP, 10 μM each of forward and reverse primers, 1 unit of Platinum Taq DNA polymerase (Life Technologies), and 50 ng of genomic DNA. The cycling condition was an initial denaturation at 95°C for 15 min, followed by 40 cycles of 95°C denaturation for 45s, 55.5°C for 45s and 72°C elongation for 45s, and 72°C final elongation for 10 min, in Veriti Thermal Cycler (ThermoFisher). Amplification of PCR products was confirmed by gel electrophoresis. Sequencing PCR products was performed using a Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and the ABI PRISM 3500 xL Genetic Analyzer (Applied Biosystems). Figure 1 show a sample considered to be mutated.

Results

We first determined the initial amplification pattern of *GAPDH* of 16 FFPE and 11 cell line gDNA samples by PCR followed by electrophoresis. As expected, all of the four PCR products (100 bp, 200 bp, 300 bp and 400 bp) (Fig. 2A) derived from the DNA samples from the cell lines could be amplified efficiently. For FFPE samples, 16/16 DNA samples amplified the 100 bp PCR product (16/16 FFPE - 100%), 7/16 FFPE DNA samples amplified the 200 bp fragment (43.7%) (three samples had a band with moderate intensity and four had a weak intensity band), only 1/16 samples had weakly stained amplification product for the 300 bp (18.7%) and none of the samples amplified the 400 bp fragment (Fig. 2B). All initial amplification results using the six purification protocols for our subsequent comparisons of efficiencies of melanin removal from gDNA are summarized in Table 3. When 60.0 ng/μL of pure melanin was added in the reaction mixture, PCR was totally inhibited for gDNA from all FFPE and cell line samples (Fig. 2C,D).

Agarose gel electrophoresis

After the contaminating melanin had been removed from the gDNA by agarose gel electrophoresis the efficiency of PCR was evaluated. We observed that there was amplification of 100 bp fragment in 7/16 FFPE samples (43.7%), for the 200 bp in 1/16 samples (6.3%) and for 1/16 samples (6.3%), for 300 bp fragment. No samples amplified the 400 bp fragment (Fig. 3A). We found that in one sample P7, the gel-purified gDNA amplified both 200 bp and 300 bp fragments. Previously this sample had just amplified the 200 bp fragment in the above initial PCR experiment. For cell line gDNAs, 1/11

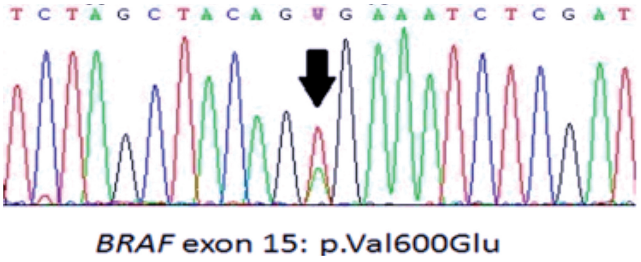

Fig. 1. Example of identification of the V600E mutation (arrow) in a post-purification sample of melanin.

Table 3. Initial amplification of GAPDH and after six protocols for melanin purification, stratified by amplicon size and gDNA origin.

Fragments size	Amplification pre-melanin		Gel Electrophoresis		1 mg Chelex®-100		Chelex-100® 5%		Centrifugation		OneStep™ Kit		Spin + OneStep™ Kit	
	FFPE	Cell Line	FFPE	Cell Line	FFPE	Cell Line	FFPE	Cell Line	FFPE	Cell Line	FFPE	Cell Line	FFPE	Cell Line
100 bp	100.0% (16/16)	100.0% (11/11)	43.8% (7/16)	90.9% (10/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	0.0% (0/11)	100.0% (16/16)	100.0% (11/11)	93.8% (15/16)	100.0% (11/11)	100.0% (16/16)	100.0% (11/11)
200 bp	43.8% (7/16)	100.0% (11/11)	6.3% (1/16)	63.6% (7/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	0.0% (0/11)	12.5% (2/16)	90.9% (10/11)	43.8% (7/16)	100.0% (11/11)	75.0% (12/16)	100.0% (11/11)
300 bp	6.3% (1/16)	100.0% (11/11)	6.3% (1/16)	54.5% (6/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	90.9% (10/11)	12.5% (2/16)	100.0% (11/11)	50.0% (8/16)	100.0% (11/11)
400 bp	0.0% (0/16)	100.0% (11/11)	0.0% (0/16)	27.3% (3/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	0.0% (0/11)	0.0% (0/16)	90.9% (10/11)	6.3% (1/16)	100.0% (11/11)	31.3% (5/16)	100.0% (11/11)

bp: base pair; FFPE: formalin-fixed paraffin-embedded.

(9.1%) did not amplify any fragments, 10/11 amplified the 100 bp fragment (90.9%%), 7/11 (63.6%) amplified the 200 bp fragment, 3 amplified the 300 bp (27.3%) and 3/11 samples (27.3%) amplified the four fragments (Fig. 3B) (Table 3).

1 mg Chelex®-100 and Chelex®-100 5%

Neither of these purification protocols was efficient at amplifying any of the four fragments, for both FFPE and cell line gDNA (data not show) (Table 3).

Centrifugation

After applying our simple centrifugation protocol to the FFPE gDNA the 100 bp fragment was observed in 16/16 FFPE samples (100%) but only 2/16 samples amplified the 200 bp fragment and neither the 300 bp or

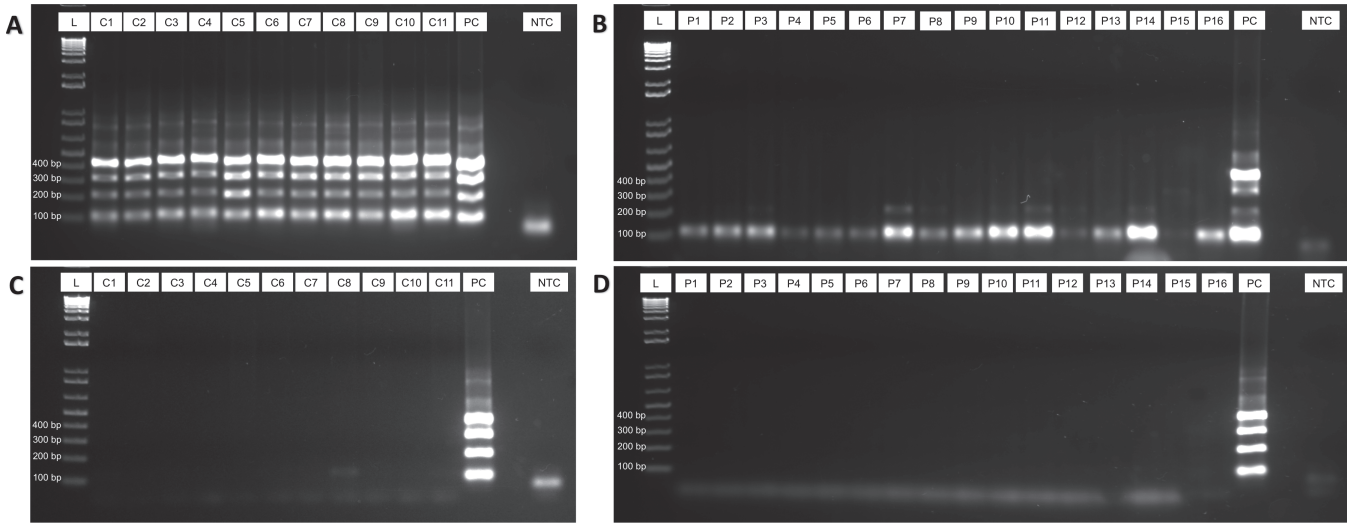

Fig. 2. Initial standard PCR amplification and effects of addition of melanin on PCR. Amplification pattern of GAPDH (100, 200, 300 and 400 bp) for gDNA from cell lines **(A)** and FFPE samples **(B)**. Inhibitory effects of 55 ng/μL of melanin in the PCR for samples of cell lines **(C)** and FFPE **(D)**. L: ladder; P: FFPE samples; C: cell lines; bp: base pair; PC: positive control; NTC: no template control.

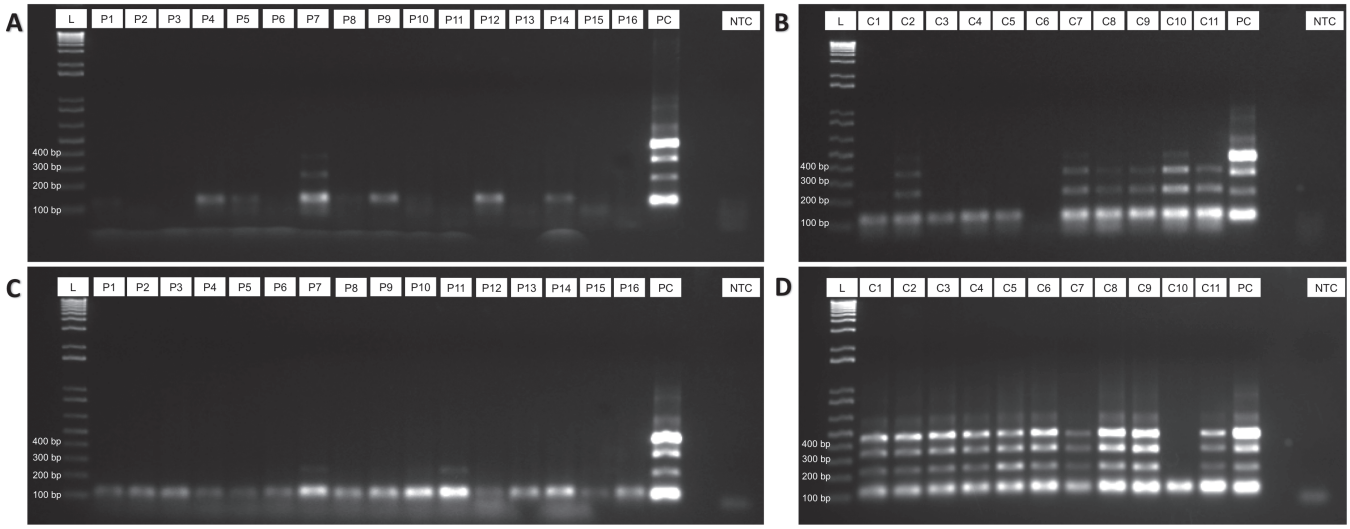

Fig. 3. Protocols for removal of the inhibitory effects of melanin on PCR amplification. The effect of electrophoretic separation of gDNA from melanin and PCR results on purified gDNA from FFPE samples **(A)** and from cell lines **(B)**. Amplification post-purification with the spin protocol for FFPE **(C)** and cell lines **(D)**. L: ladder; P: FFPE samples; C: cell lines; bp: base pair; PC: positive control; NTC: no template control.

400 bp fragment could be amplified (Fig. 3C). In contrast, for cell line gDNA samples 10/11 samples (90.9%) of 200 bp,300 bp, and 400 bp fragments could be amplified. For the smaller 100 bp amplicon, all FFPE and cell line gDNA samples had successful amplification (Fig. 3D) (Table 3).

OneStep™ PCR Inhibitor Removal Kit:

We found that 15/16 (93.8%) of FFPE samples amplified the 100 bp sized amplicon after gDNA purification with OneStep™ PCR Inhibitor Removal kit. For the larger sized 200 bp fragment 7/16 (43.8%) samples had successful amplification, and 2/16 (12.5%) amplified the 300 bp and 1/16 (6.3%) amplified the 400 bp amplicon (Fig. 4A). For the gDNA purified from cell line samples, 11/11 (100%) amplified all four fragments, but in two of them (sample C4 and C11), the 200 bp fragment was weakly amplified (Fig. 4B) (Table 3).

Centrifugation plus OneStep™ PCR Inhibitor Removal Kit

We reasoned that we could maximize the overall efficiency of purification by combining the two protocols with the highest success rates. Our results showed that the combination of centrifugation protocol followed by OneStep™ PCR Inhibitor Removal Kit was efficient in successful amplification of 100 bp fragment in 100% (16/16) of the FFPE samples. Interestingly, there was also an increase of amplification of the 200 bp (12/16 samples - 75%) and the 300 bp fragment (8/16 - 50%) in comparison to our experiments in which we spiked melanin into the initial gDNA templates (Table 3). Furthermore, 5/16 gDNA samples (31.3%) successfully amplified the 400 bp fragment, which was not observed in the previous experiment (Fig. 4C). Also, this protocol was efficient in recovering 100% of cell line samples, maintaining the same amplification pattern prior to the addition of melanin (Fig. 4D) (Table 3).

Finally, all samples in which there was successful amplification post-purification of melanin, regardless of the protocol used, could be successfully used for molecular analyses, such as the identification of the *BRAF* V600E mutation by sequencing (Fig. 1).

Discussion

Melanin inhibits PCR, which is an essential molecular technique for most investigative methods of cancer biology. Co-purification of melanin with gDNA isolated from lesions of patients with melanoma can not only compromise molecular diagnostic testing for the management of patients in need of targeted therapeutics but also limits the use of the tumor gDNA for many research applications. There has been no previous comparison of the impact of different gDNA separation protocols from melanin on the quality of PCR efficiencies gDNA templates from various biological sources with different amplicon sizes.

The gel electrophoresis protocol was the least efficient in maintaining the amplification pattern of the samples pre-addition of melanin, independent of the size of the amplicon and the origin of gDNA. We found that Chelex®-100 was inefficient for melanin purification. For the centifugation and the centifugation plus

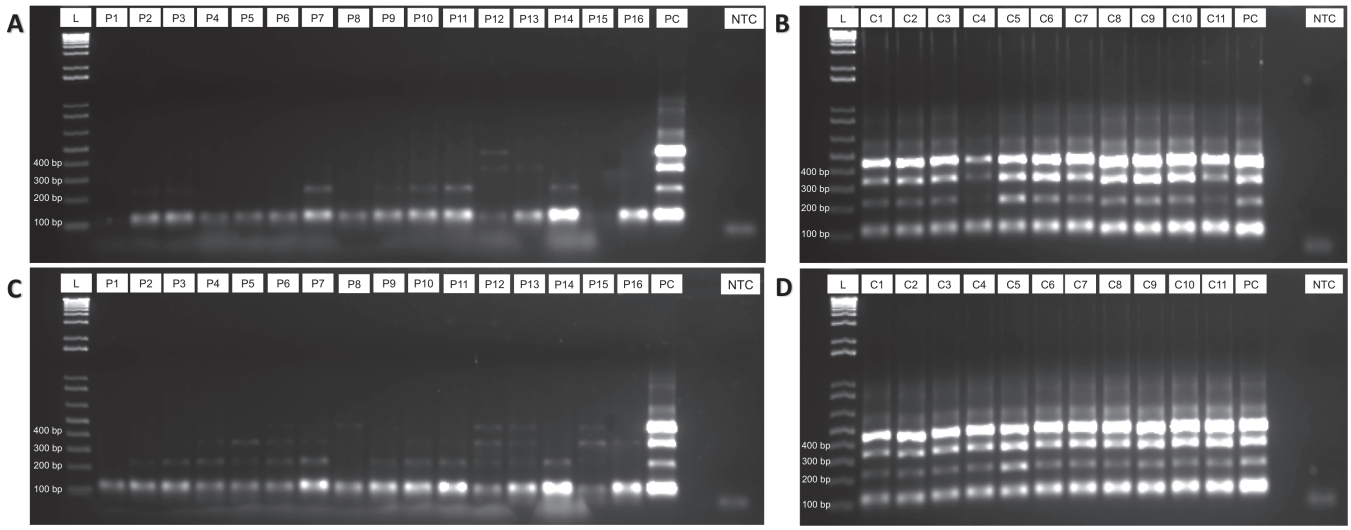

Fig. 4. The two most efficient protocols for removal of the inhibitory effects of melanin on PCR amplification. The efficiency of the OneStep™ PCR Inhibitor Removal Kit to amplify the FFPE **(A)** and cell line samples **(B)**. Combination of the Spin plus OneStep™ PCR Inhibitor Removal Kit purification protocols to recover the amplification pattern of the FFPE **(C)** and cell line samples **(D)**. L: ladder; P: FFPE samples; C: cell lines; bp: base pair; PC: positive control; NTC: no template control.

OneStep™ PCR Inhibitor Removal Kit protocols, we found that the 100 bp fragment for both FFPE-gDNA and cell line-gDNA amplified with the same percentage of success that we obtained before addition of exogenous melanin. However, only one FFPE sample did not amplify this fragment using only OneStep™ PCR Inhibitor Removal Kit. The amplification success rate of the 200, 300 and 400 bp fragments for the cell line-gDNA using purification with centrifugation and centifugation plus OneStep™ PCR Inhibitor Removal Kit was equal to the rate we obtained before addition of exogenous melanin. Interestingly, the combination of centifugation with OneStep™ PCR Removal Kit increases the amplification rate of these fragments in the paraffin samples. Also noteworthy is that the use of our simple centrifugation protocol resulted in amplification at high rates in gDNA cell line samples for the four fragments (Table 3).

In agreement with our results, Hu and colleagues (Hu et al., 2015) showed that the use of Chelex®-100 was inefficient for the removal of melanin from a commercial DNA sample (Hu et al., 2015). However, the two protocols that we used were also briefly described in two publications for the removal of melanin from pigmented samples of patients with melanoma (Beadling et al., 2008; Torres-Cabala et al., 2009). There is no specific information on the percentage of recovery of samples using these methods, as well as a lack of detailed descriptions of the procedures used. It is therefore difficult to draw conclusions regarding the different findings reported in these studies.

Frouin and colleagues (Frouin et al., 2016) showed the efficiency of the BSA and another separation column, the NucleoSpin® Kit (Macherey-Nagel GmbH & Co) to generate conclusive results in the analysis of the *BRAF* V600E mutation in 50 pigmented samples with three levels of melanin contamination, weak, moderate and high. However, none of these samples had very high levels of melanin contamination since without any treatment mutation was detected in 48% of the samples (Frouin et al., 2016). After the BSA treatment the success rate increased to 80%, an increase of 61.5%, and conclusive results were possible in 89.0% of the samples with high melanin levels. They found that the use of the NucleoSpin® Kit maintained the same 48.0% success rate for detecting the presence or absence of the mutation that was found before treatment. However, the use of this Kit was not as efficient for purification at higher levels of melanin contamination. The NucleoSpin® Kit seems to be efficient at retaining larger amounts of contaminating melanin during the clean-up procedure, but it is possible that the column resin interacts with the gDNA in some way so that some conclusive pre-treatment samples were inconclusive after purification.

Our experimental design addressed the possibility of additional inhibitors being introduced by the reagents or processes used for purification. We only used gDNA-FFPE and gDNA-cell line samples that were known to be efficiently amplified by the PCR. We decided to follow the most common scenario, in which the diagnosis routines are performing in FFPE samples, since frozen samples are still rarely available. The culture cells were included for validation in a situation with high quality gDNA.

Then, we added commercial melanin to show that there was no other inhibition in amplification reaction by the different methods of purification. Thus, our findings relate exclusively to the removal of melanin from gDNA and the relative efficiencies of each method. It was then possible to compare the percentage that each method produced and determine whether the pattern of amplification based on the various fragment sizes could be restored when the exogenous melanin was removed from the gDNA. On the other hand, this experimental design made inviable the comparison of the six different protocols when melanin is endogenous in melanoma specimen. However, Frouin and colleagues (Frouin et al., 2016) showed agreement of their results between exogeneous and endogeneous melanin.

Our findings show that the use of the centifugation plus OneStep™ PCR Inhibitor Removal Kit amplified fragments of FFPE samples that had not been previously detected. The samples preserved in paraffin are exposed to fixation and embedded in paraffin resulting in partial degradation of the gDNA. The substances used in this procedure may not be completely removed by gDNA extraction and purification, interfering with subsequent molecular analyses. In our results it is clear that the column of the OneStep™ PCR Inhibitor Removal Kit was also capable of removing some of these contaminants and removal seems to be further improved by sedimentation during centrifugation. Our centrifugation protocol is efficient, simple and low cost, and it can recover approximately 91.0% of the samples for amplicon sizes up to 400 bp, and 100.0% of the 100 bp PCR amplicons. However, for the samples derived from FFPE, we recommend using amplicon sizes up to 100 bp, with a success rate of 100.0%, since we found that when the target amplicon size was 200 bp, the efficiency decreased by 12.5%. The use of agarose gel electrophoresis was also efficient in the separation of melanin and gDNA, especially in the gDNA from cell lines. However, we do not encourage the use of this protocol because it involves extensive manipulation, which may lead to contamination between adjacent samples during electrophoresis. Also, the total concentration of post-purification gDNA was very low.

We chose a multiplex PCR that amplified four fragments of 100 bp, 200 bp, 300 bp, and 400 bp to test the efficiency of different amplicon sizes for the purification gDNA. We found that the amplification of larger fragments was more affected by melanin inhibition than smaller fragments (Eckhart et al., 2000). It is well described that a balance between DNA template and all other components of the PCR reaction, especially the primers, is necessary because PCR

performance is strongly influenced by internal stability, melting temperature, secondary structure or interference with each other (Sint et al., 2012). This template-primer balance is essential when amplifying degraded DNA samples, to avoid the preferential amplification of more efficient primers (Sint et al., 2012). Finally, the idea of using a multiplex PCR was that if a purification protocol were able to restore the amplification of all amplicons, independent of the size, it probably would be efficient in common PCR protocols routinely used in diagnosis laboratories, as in fact we described in our results for *BRAF* evaluation.

In conclusion, we compared six different protocols of purification of contaminating melanin in gDNA samples of different quality with varying sizes of the amplified fragments. Our protocol that combined the separation of melanin from gDNA by centrifugation with subsequent passage through the column of the OneStep™ PCR Inhibitor Removal Kit, recovered 100% of gDNA from cell line and 93.8% of gDNA from FFPE, with amplification of more different sized fragments in comparison to the results obtained before melanin was added to the gDNA. This protocol is feasible and may be used as a routine for research and molecular diagnostic to eliminate the melanin present in heavily pigmented lesions of patients with melanoma.

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Competing financial interest. The author(s) declare no competing financial interests.

Ethical statement. The study was conducted according to the national and institutional ethical policies and it was approved by the Ethic Committee of Barretos Cancer Hospital (Process number: CEP-548/2011). Informed consent was obtained from all participants.

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