Comparison and Optimization of DNA Extraction Methods from Cervical Cells Collected in ThinPrep® PreservCyt

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Received: May 2019/ Published: December 2019

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ABSTRACT

Cervical cancer is the ninth leading cancer in women in Portugal. Cervical cancer screening methods have changed since the implementation of the cytology-based test developed by George Papanicolaou until the human papillomavirus-based screening. Nowadays, new biomarkers like deoxyribonucleic acid (DNA) methylation has been proposed as potential triage method of samples testing positive for human papillomavirus

The aim of this study was to optimize and compare three different DNA extraction methods for further methylation analysis. DNA from sixteen cervical samples collected in ThinPrep® PreservCyt was extracted by the phenol-chloroform method, by QIAamp® MinElute™ Media kit (QIAGEN, Hilden) and by Blood DNA Isolation Mini Kit (Norgen Biotek, Ontario). After quantification, DNA was modified by Sodium Bisulfite and amplified by Quantitative Specific Methylation Polymerase Chain Reaction for β-Actin gene. Finally, Cycle threshold (Ct) mean values were evaluated.

The phenol-chloroform method had the higher sample input and, consequently allowed for a higher DNA quantity. Fifteen mL of sample allowed for get 13500ng with the phenol-chloroform method meanwhile 2mL of sample provided 8940ng with QIAamp® MinElute™ Media kit and 927ng with Blood DNA Isolation Mini Kit. However, commercial kits presented higher efficiency and advantages as being less time consuming and easier to perform. No significant differences were observed for amplification Ct values between the three DNA extraction methods neither between samples archived for ten or fourteen months.

Concluding, the three methods provided DNA to amplification but the optimization of commercial kits turned out to be more advantageous.

Key-words: Cervical cancer, HPV, DNA, Extraction, DNA methylation

INTRODUCTION

Cervical cancer (CCx) is the ninth leading cancer in women in Portugal. Each year about 750 cases are newly diagnosed in Portugal and about 340 deaths are due to CCx[1,2].

Persistent Human Papillomavirus (HPV) infection is the main cause of CCx's development. Among the more than 200 types identified, only a limited number of HPV genotypes seem to be implied in CCx carcinogenesis, being classified as high-risk HPV (hrHPV). hrHPV 16 and 18 represent the most carcinogenic genotypes and prevalence studies have shown that 70% of CCx cases are positive for hrHPV 16 or hrHPV[183-5].

The major aim of CCx screening is to identify precursors lesions with potential of progression to cancer and avoid the identification of benign lesions or transient HPV infections[6]. Since the implementation of the Papanicolaou test as a CCx screening test, the incidence and the mortality rate of CCx have decreased substantially, especially in industrialized countries. Nonetheless, cytology-based test presents some limitations as a single screening method. Considering it is highly dependent on the observer expertise, it presents limited sensitivity (50% to 80%)[7,8].

Vaccination programs implementation along with the actual knowledge about HPV infection and CCx development have led to changes in screening methods. Over the last few years, cytology-based test has been replaced by hrHPV test. Several studies have shown that primary hrHPV screening is more sensitive than cytology, although less specific. Its low specificity is mainly due to the transient HPV infections that often occur in young women. This limitation can result in a high number of colposcopy referrals, with subsequent overdiagnosis and decreased cost-effectiveness of the screening[6,8,9]. Thus, triage biomarkers are needed to select women to colposcopy referrals.

Recently, several studies have indicated molecular biomarkers as a strategy to triage women with HPV positive test, specifically molecular biomarkers based on DNA methylation[10,11].

DNA methylation plays an important role in regulation of gene expression, organization of chromatin and genomic imprinting. Commonly, tumor cells display tumor suppressor genes' inactivation as a result of aberrant DNA hypermethylation in respective promoter regions, whereas overall hypomethylation leads to genomic instability[12].

DNA methylation is an early event in carcinogenesis, being proposed as biomarker for early cancer detection. Indeed, methylation biomarkers were suggested to identify precursor lesions with potential to progress to CCx. Hence, several gene panels have been shown to present higher sensitivity and specificity in comparison to conventional cytology. However, independent validations are required prior to implementation of this approach in clinical practice[10-12]. For each sample, DNA methylation analysis requires several steps: 1) DNA extraction, 2) Sodium Bisulfite modification of DNA and 3) Methylation-Specific Polymerase Chain Reaction (MSP).

The general steps of nucleic acids extraction and purification include cell lysis, denaturation of nucleoprotein complexes, inactivation of endogenous nucleases and separation of desired nucleic acid of cell debris. Currently, there are several methods and technologies with different protocols available for DNA isolation[13,14].

Solid-phase nucleic acid purification is the most used method in the commercial extraction kits and it is usually performed using columns. The four principal steps involved in solid-phase extraction are: 1) cell lysis, 2) nucleic acids adsorption, 3) washing and 4) elution. The adsorption of acid nucleic by the solid-phase system depends on the pH and salt content of

the buffer. This process of the extraction is based on hydrogen-binding interaction with a hydrophilic matrix under chaotropic conditions, an ionic exchange under aqueous conditions, affinity and size exclusion mechanisms. Several materials are used in solid-phase extraction method as solid support, for example silica matrices and glass particles[13].

Phenol-chloroform extraction is a liquid-liquid extraction that separates molecules based on their differential solubility in two immiscible liquids. This method is based on the addition of phenol-chloroform to an aqueous solution with previously degraded cells or tissue and mixing the two phases which are then separated by centrifugation. This separation generates an organic inferior phase and an aqueous upper phase. Purified phenol has a higher density than water, therefore, it forms the lower phase and chloroform ensures phase separation. Nucleic acids are soluble in the upper aqueous phase as a result of their polarity. On the other hand, proteins contain various proportions of charged and uncharged domains producing hydrophobic and hydrophilic regions. For that reason, proteins precipitate at the interface between the two phases[15].

The performance of molecular tests requires proper preservation of samples. Recent studies demonstrated that current liquid-based cytology systems for routine cytopathology, including methanol-based solutions like ThinPrep® PreservCyt™, allow cells to be used for genetic screening without affecting performance[16].

This study is part of a project whose aim is to evaluate DNA methylation as potential biomarkers to triage women with samples testing positive for hrHPV. The main goal of the present project was to compare different methods of DNA extraction from cervix cells samples collected into ThinPrep® PreservCyt™ solution. Hence, this study aimed to: 1) compare the quantity of isolated DNA, 2) optimize the protocols of DNA extraction according to the specificities of the samples, 3) compare the efficiency of each method, 4) evaluate if the techniques used for DNA purification affects the sodium bisulfate modification and qMSP and 5) understand the strengths and weaknesses of each DNA extraction method.

MATERIALS AND METHODS

The summary of material and methods are described in the **Supplementary Material**. Briefly:

Samples Collection

Herein, we used the leftovers of the samples obtained by colpocytology collected in ThinpPrep™ PreservCyt™ (HOLOGIC, Toronto) from the CCx Screening Program in the North Region of Portugal provided by Department of Pathology of the Portuguese Oncology Institute of Porto. Eight samples were archived at room temperature for ten months and eight for fourteen months. Samples from sixteen women diagnosed with Negative for Intraepithelial Lesion or Malignancy (NILM) and tested positive for hrHPV were selected for the study (This study was approved by IPO-Porto's Ethics Committee).

DNA Extraction

DNA extraction was performed using the phenol-chloroform method and two kits – QIAamp® MinElute™ Media (QIAGEN, Hilden) and Norgen's Blood DNA Isolation Mini Kit (Norgen Biotek, Ontario). DNA extraction with the commercial kits was performed according to the manufacturers' instructions. The QIAamp® MinElute™ Media Kit is designed for liquid media containing nucleic acids, such as cervical swab transport media. The Norgen's Blood DNA Isolation Mini Kit is designed for the rapid preparation of genomic DNA from whole blood[17,18].

Six samples were used entirely for the purification with phenol-chloroform. For DNA extraction with commercial kits were used the same ten samples for both kits. Two different protocols were performed with each commercial kit. Following extraction, DNA was stored at -20ºC until posterior use.

Phenol-chloroform

Each sample was centrifuged at 3000g for 5 minutes. Cells were ressuspended in 2700µL of cell lysis buffer SE (75mM NaCl e 25mM EDTA), 300µL of 10% SDS and 25µL of proteinase K (20mg/mL) and incubated at 55ºC with agitation at 900rpm until cell complete digestion is achieved. Then 3mL of phenol-chloroform (pH 8) were added to the lysate and it was centrifuged at 3000g for 20 minutes. The aqueous phase was transferred to a new 15mL tube, and 6mL of cold absolute ethanol and 1mL of ammonia acetate at 7.5M were added. After mixing, samples were stored overnight at -20ºC. Subsequently, samples were centrifuged at 3000g for 20 minutes and the pellet was washed with 6mL of ethanol 70% twice. Finally, the air-dried pellets were eluted in 25µL of sterile distilled water.

Blood DNA Isolation Mini Kit – Norgen

To 200µL of sample were added 20µL of Proteinase K (20mg/mL) and 300µL of Lysis Buffer B. After vortexing, samples were incubated at 55ºC for 10 minutes. Then, 110µL of absolute ethanol were added. Samples were transferred to the columns and centrifuged at 6000g for 1 minute. It was added 500µL of Solution WN into the columns and these were centrifuged. Subsequently, the samples were washed twice with 500µL of Wash Solution. Finally, columns were placed in new microtubes and DNA was eluted with 60µL of Elution Buffer B.

Two extractions with different sample quantities were performed. In the first, DNA isolation was performed according to the protocol described above using 200µL of each sample after homogenization. In the second extraction, 2mL of sample were centrifuged and resuspended in 200µL of PBS, 30µL of proteinase K (20mg/mL) were used for lysis and samples were incubated for 30 minutes.

QIAamp® MinElute™ Media

For 250µL of sample, 80µL de Buffer ATL and 20µL of QIAGEN proteinase K were added and the mixture was incubated at 56ºC for 30 minutes with agitation at 900rpm. Subsequently, 250µL of Buffer AL were applied to all samples that were incubated at 70ºC for 15 minutes with agitation at 900rpm. Three hundred (300) µL of absolute ethanol and the lysate was incubated for 5 minutes at room temperature. All samples were transferred into the extension tubes in the columns and the vacuum was switched on in order to the lysate completely drawn through the columns. Subsequently, samples were washed twice, initially with 750µL of Buffer AW2 and then with 750µL of absolute ethanol. After a centrifugation at 20000g for 3 minutes, the columns were transferred to new microtubes and incubated at room temperature for 15 minutes. Finally, nucleic acids were eluted with 60µL of Buffer AVE.

Similarly, to earlier described, two different quantities of samples were tested in parallel: 250µL and 2mL of sample centrifuged and resuspended in 250µL of PBS.

DNA Quantification

Genomic DNA concentrations were measured with the NanoDrop ND-1000 (ThermoScientific, USA) and the Qubit 4 (Life Technologies, USA). Furthermore, for Nanodrop quantification, the A260/A280 ratio was also assessed. Five of the six samples extracted by phenol-chloroform methods were diluted in 50µL of sterile distilled water, due to high concentration and re-quantified.

Sodium Bisulfite Modification

Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, USA) according to the manufacturers' instructions with 150ng and 300ng input of DNA. Modified DNA was eluted with 75µL of sterile distilled water and stored at -80ºC until further use.

Quantitative Methylation-Specific PCR (qMSP)

The modified DNA was used as template and samples were submitted to qMSP for β-Actin amplification. Reactions were performed in 96-wells plates using Applied Biosystems 7500 Real-Time System (Thermo Fisher Scientific, USA). Per each well 2µL of modified DNA, 5µL of XpertFast Probe, 0.4µL of primers and 0.2µL of probe were added. Primers and probe sequences were listed in **Table 1**. Sterile distilled water was added until 10µL of reaction volume was achieved.

Table 1. Probe and β-Actin gene primers sequences

	Sequence
Probe	ACCACCACCCAACACACAATAACAAACACA
Forward Primer	5' TGG TGA TGG AGG AGG TTT AGT AAG T 3'
Reverse Primer	5' ACC AAT AAA ACC TAC TCC TCC CTT AA 3'

The qMSP program consisted of a period of 3 minutes at 95ºC for enzyme activation followed by 45 cycles with 15 seconds at 95ºC for DNA denaturation and 30 seconds at 60ºC for annealing, extension and data acquisition.

All samples were run in duplicated and in each plate 2 negative controls (ddwater) were also included. The modified CpGenome™ Universal Methylated DNA was used as positive control and it was diluted in five serial dilutions by a 5x dilution factor to establish a standard curve to allow methylation quantification and ascertain PCR efficiency.

Statistical Analysis

Statistical analysis was carried out using SPSS (SPSS Inc., an IBM Company, USA) and GraphPad Prism (GraphPad Software, USA).

Non-parametric tests were performed to determine statistical significance in all the comparisons made. In particular, Mann-Whitney U test was used to compare two groups and Kruskal-Wallis test was used in comparison between three or more groups.

The median, minimum and maximum values of the DNA purified quantity and the ratio A260/280 were determined.

Ct Mean obtained for each run was used to compare the different methods and different quantities of DNA used in qMSP as well as the relation between archive time and Ct mean were analyzed.

RESULTS

All purification methods were able to extract measurable quantities of DNA. The total DNA extracted for each method was calculated based on the concentration values given from Qubit 4 (Life Technologies, USA; Table 2). Phenol-chloroform extraction allowed for higher DNA yields, but the sample volume used in this method was also higher. The commercial kits presented better efficiency. Comparing Blood DNA Isolation Mini Kit – Norgen and QIAamp® MinElute™ Media, from a similar volume of sample used for extraction, the QIAamp®'s kit provided a higher DNA amount.

The quality of DNA was assessed by A260/A280 ratios analysis (**Table 3**). The phenol-chloroform method provided DNA with a median A260/A280 ratio value of 1.86, providing DNA with lower protein contamination. Importantly, A260/A280 ratios were less discrepant with phenol-chloroform than the values conferred from the other methods.

Table 2. Median, minimum and maximum values of DNA extracted with the three extraction methods from different initial volume of sample

Methods	Quantity of DNA extracted (ng) [Quantity(ng)/input(mL)]		
	Median	Minimum	Maximum
Phenol-chloroform (n=6) 15 mL	13500.0 [900.0]	181.0 [12.1]	281400.0 [18760.0]
Blood DNA Isolation Mini Kit – Norgen (n=10) 200 µL	140.4 [702.0]	6.6 [33.0]	584.4 [2922.0]
QIAamp® MinElute™ Media (n=10) 250 µL	870.0 [3480.0]	57.2 [228.8]	3696.0 [14784.0]
Blood DNA Isolation Mini Kit – Norgen (n=10) 2 mL	927.0 [463.5]	45.6 [22.8]	3960.0 [1980.0]
QIAamp® MinElute™ Media (n=10) 2mL	8940.0 [4470.0]	84.60 [42.3]	123480.0 [61740.0]

Table 3. Median, minimum and maximum of A260/A280 ratio values obtained from extracted DNA obtained with three extraction methods from different initial volume of sample

Methods	A260/A280 ratio		
	Median	Minimum	Maximum
Phenol-chloroform (n=6) 15 mL	1.86	1.57	1.97
Blood DNA Isolation Mini Kit – Norgen (n=10) 200 µL	1.21	0.93	1.35
QIAamp® MinElute™ Media (n=10) 250 µL	2.42	2.13	3.10
Blood DNA Isolation Mini Kit – Norgen (n=10) 2 mL	1.54	1.12	2.02
QIAamp® MinElute™ Media (n=10) 2mL	2.00	1.90	3.17

After quantification, 150 and 300ng of DNA were modified by Sodium Bisulfite and β-Actin was amplified by qMSP. Ct means were compared to evaluate the efficacy of the different extraction methods (**Figure 1**).

Successful amplification was obtained with 150ng of DNA. DNA from 2mL sample extracted with Blood DNA Isolation Mini Kit – Norgen and DNA from two samples extracted with phenol-chloroform did not amplify. In **Figure 1**, Ct value equals to 40 represent samples without amplification. There were no significant differences between Ct mean for DNA extracted with Blood DNA Isolation Mini Kit – Norgen, QIAamp® MinElute™ Media or phenol-chloroform.

Different quantities of modified DNA input were compared (**Figure 2**). Statistically significant differences were found between Ct mean obtained with 150ng and 300ng of DNA used in qMSP (p=0.0262). The Ct median value achieved with 150ng and 300ng of modified DNA input was 31.50 and 29.38, respectively.

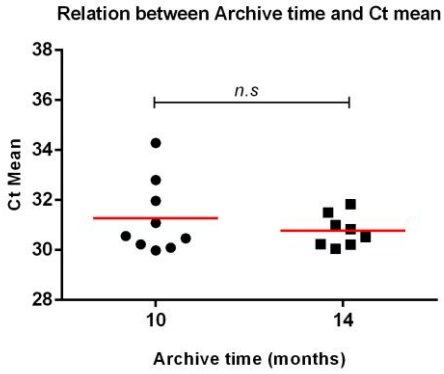

Figure 1 – β-Actin Ct mean of DNA extracted with QIAamp® MinElute™ Media, Blood DNA Isolation Mini Kit – Norgen and phenol-chloroform method. n.s. (not significant)

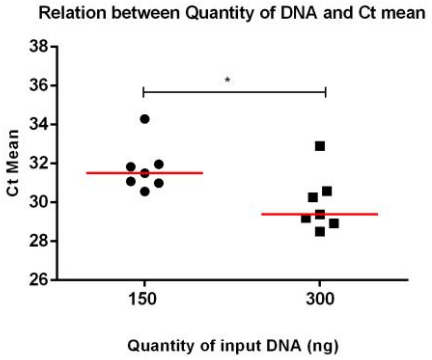

Figure 2 – β-Actin Ct mean for different quantities of modified DNA used for amplification by qMSP. *denote p<0.05

Moreover, in order to understand if the time of preservation in ThinPrep® PreservCyt™ solution might impact on the quality and integrity of DNA and subsequently in the amplification, five samples archived at room temperature since May 2017 and five from September 2017 were used in this study. Ct value was compared between the two periods (**Figure 3**). The median Ct was 30.57 for 10 months and 30.68 for 14 months of

archival. No statistical differences were found between the median Ct in samples with different periods of storage.

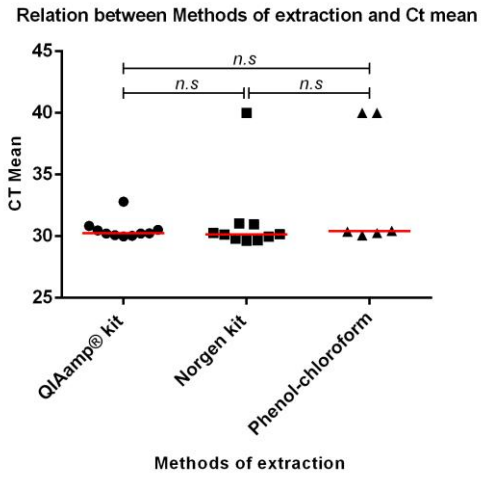

Figure 3 – Effect of archive time in β-Actin Ct mean of samples. n.s. (not significant)

DISCUSSION

CCx remains one of major health problems worldwide. The implementation of effective screening program is essential to decrease cancer incidence and mortality. Despite all the improvements over the years, CCx screening still present limitations[7,8]. Thus, novel biomarkers have been proposed, namely DNA methylation-based biomarkers. To assess these biomarkers, reliable, consistent and accurate results of molecular tests using cytological samples will depend on standardized protocols for maximizing DNA yield and quality. Even though very small quantities of material input can be successfully amplified, the yield of DNA is a major preanalytical factor that determines the success of molecular analysis[19]. This fact entails the need of DNA extraction methods optimization to assure results' reliability. In this study, DNA extraction methods suitable for methylation analysis were optimized and compared.

Phenol-chloroform extraction provided a higher DNA quantity and purity in comparison with commercial kits. In Nanodrop quantifications, A260/280 ratio between 1.8 and 2.0 are usually considered pure DNA. Our results suggested that phenol-chloroform method provides almost pure DNA. The A260/A280 ratio median values of DNA extracted with Blood DNA Isolation Mini Kit – Norgen were below 1.6, showing protein contamination or other contaminants that strongly absorb around 280 nm. The QIAamp® MinElute™ Media Kit besides extracting DNA also extracts RNA. This might explain the obtained A260/A280 ratios above 2.2[19].

Although absolute DNA quantity and purity obtained by conventional extraction was significantly higher, these yields were not reflected in amplification's efficiency since no significant differences were displayed by Ct values. Indeed, since the DNA quantity (not contaminated or contaminated with proteins and RNA) used in amplification was the same for all samples, qMSP did not seem to be negatively affected by those contaminants[20].

Two commercial kits were tested in this project: the QIAamp® MinElute™ Media Kit, optimized to liquid media, and the Norgen's Blood DNA Isolation Mini Kit, designed to DNA extraction from whole blood[17,18]. Overall, our results suggest that Norgen's kit is less efficient than QIAamp®'s kit, although not significantly. This finding might be explained by the fact that Norgen's kit was not specifically designed for the samples used in this study. Therefore, this kit needs additional methodological optimizations.

Although incubation time was increased during these kits' optimization, the total time taken for extractions was significantly lower when compared to phenol-chloroform method. Indeed, this is one of the major advantages for using DNA extraction commercial kits. Importantly, the use of commercial kits decreases the exposure to dangerous chemicals that occurs in conventional DNA extraction method[13]. Furthermore, the usage of

kits prevents the preparation of reagents and decreases the rate of operator errors. Overall, the extraction process with kits that have protocols already optimized is more standardized and thus the results are more reproducible[13].

Since the sample volume recommended by QIAamp® kit manufacturers provided a small quantity of DNA, a larger volume of sample was tested. Although an eight times higher volume was tested, the yields obtained were not directly proportional. This might be explained by an incomplete cell lysis and columns' clogging since the kit is prepared for a low concentration of cells. Thus, higher lysis buffer and proteinase K volumes are required along with longer incubation time to facilitate cells' lysis and ensure that all cells are properly digested.

In order to improve the qMSP performance for the tested samples, different amounts of modified DNA were eluted in the same volume. As expected, samples with more DNA input in sodium bisulfite reaction displayed lower Ct levels in qMSP for reference gene. Therefore, increasing the DNA quantity in qMSP reaction might provide lower Ct value amplification. Nevertheless, excessive amounts of DNA input increase the risk of nonspecific amplification and might inhibit the reaction[20].

Additionally, as demonstrated by other authors, room temperature storage of residual alcohol-based liquid-based preparation cytologic specimens showed no effect in DNA quality, cytomorphology and immunoreactivity during at least one year of storage[21]. Indeed, the samples used in the present study stored for 10 and 14 months in PreservCyt™ solution at room temperature showed no differences in amplification mean Ct. Our results corroborated that this solution preserves DNA for months with quality.

To minimize the external factors affecting the study, extractions with kits were performed with the same samples and similar volumes, samples were homogenized before the needed volume was taken, and purified DNA was eluted in an equal volume of elution buffer.

Notwithstanding, this project presents some limitations. Due to technical problems, the sample number extracted with phenol-chloroform method was smaller than the sample number extracted with commercial kits. Ideally, the phenol-chloroform method should be tested with a smaller volume of sample to get results more comparable. In general, the number of samples tested per protocol could be larger. If these limitations were overcome, results would probably be more consistent for this specific method.

Moreover, as complement to this study, electrophoresis could be performed to assess the DNA integrity. Further protocol optimizations are needed in order to obtain consistent DNA quantities from all samples. Then, the quantity of modified DNA and subsequently DNA amplification needs to be standardized. Finally, genes that will be used to evaluate methylation value as biomarker for women triage when samples are HPV positive are still required to be tested.

In conclusion, the three methods allowed to obtain DNA for β-Actin amplification with qMSP, however all of them had some disadvantages. Since phenol-chloroform is time consuming and requires the usage of toxic reagents, the optimization of DNA extraction kits seemed to be the most suitable methodological approach.

Aknowledgements

This work was supported by a grant from ARS Norte. SS is supported by a PhD fellowship IPO/ESTIMA-1 NORTE-01-0145-FEDER-000027.

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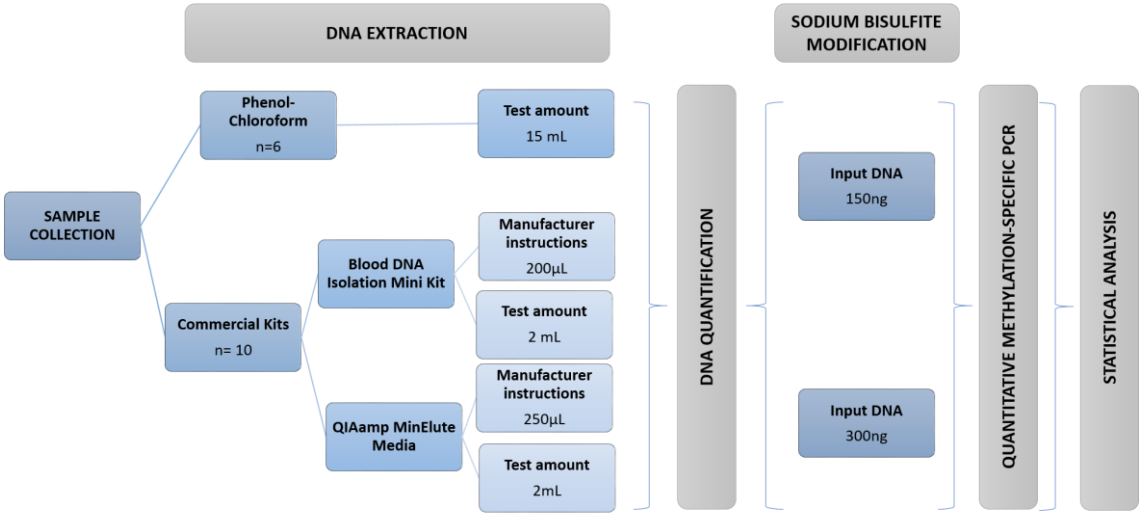
Supplementary material – Materials and Methods



Scheme summarizing of procedures of Materials and Methods section