

Efficacy of bisulfite modification and DNA recovery using commercial kits from samples of genomic and circulating DNA

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Abstract: This study had evaluated three commercially available bisulfite modification kits with regard to the procedural DNA loss and the efficacy of cytosine conversion to uracil. The efficacy of DNA conversion was estimated by pyrosequencing of eight CpG sites of unmethylated and fully enzymatically methylated templates of RAR β gene promoter region and cirDNAs of healthy donors and prostate cancer patients. The procedural DNA loss was calculated by methyl-independent qPCR for ubiquitously distributed L1RE1 retrotransposable elements. Our data demonstrates that all commercial kits displayed similar conversion efficacy of the unmethylated cytosines close to 99% independently of DNA concentration. However, when low DNA concentrations were used, the observed basic level of genomic DNA methylation increased to 11% depending on the position of CpG site. Qiagen and Chemicon kits recovered no more than 20% starting material at a high DNA input (500 ng/probe) and only 2.7–5.8% for low DNA input (10 ng/probe). EZ DNA Methylation-Gold Kit from Zymo Research provided the highest recovery regardless of the initial DNA input with average rates of no less than 86%. These results suggest that the EZ DNA Methylation-Gold Kit is the most appropriate tool for bisulfite modification of cirDNA when assaying DNA in low amounts.

Keywords: DNA Methylation, Bisulfite Conversion, Pyrosequencing

1. Introduction

To detect methylated sequences even in the presence of excess amounts of unmethylated templates, the majority of technologies rely on sodium bisulfite modification of the input material, which converts the epigenetic DNA differences into genetic ones. In particular, unmethylated cytosine nucleotides are converted to thymidines (by uracil), whereas methylated cytosines (5mC) still pair as cytosines. Since subsequent analyses, like methylation-specific PCR (MSP) or sequencing, rely on the differentiation between methylated and unmethylated cytosine residues, the incorrect performance of the procedure greatly influence the further results. When analyzing minute amounts of DNA like cirDNA in a cancer

diagnostic setting, it is of paramount importance to avoid any DNA loss during the several manipulation steps, especially involving bisulfite modification itself or the accompanying DNA recovery [1]. Efficacy of DNA conversion and recovery also interferes with data of pyrosequencing or Massive Parallel Sequencing data.

The efficacy of bisulfite modification protocols is extremely important in relation to the endogenous cell-free DNAs circulating in the blood (cirDNA), which represents a valuable source of material for non-invasive cancer diagnosis [2, 3]. Indeed, concentrations of cirDNA in the plasma of healthy/cancer persons vary between 10 ng/ml and hundreds of nanograms per milliliter [3, 4]; cirDNA are strongly fragmented and cirDNA from tumor cells circulate in excess of cirDNA from the normal cells [3]. Aberrantly methylated DNAs are among the most sensitive

and specific cirDNA cancer markers not only because they are frequently found in cancer but also because bisulfate modification provides detection of aberrantly methylated targets in the large excess of unmethylated one [5]. Thus, bisulfite DNA conversion eventually determines the sensitivity and specificity of subsequent cirDNA analysis.

This study has evaluated the efficacy of three commercially available bisulfite modification kits for treatment of cirDNA with regard to procedural DNA loss and the efficacy of cytosine conversion to uracil.

2. Materials and Methods

2.1. Sample Collection and Treatment

Blood samples of 70 healthy donors (19-63 years old) and 20 previously untreated prostate cancer patients (pT2-3pN0-xpM0-x, 45-78 years old) were obtained from the Central Clinical Hospital and Municipal Clinical Hospital no. 1, Novosibirsk, Russia. The experimental protocol was approved by the Ethics Committees of the Hospitals, and informed consent was obtained from all patients involved in the study. Venous blood was collected and plasma was separated as earlier described [6].

To obtain the preparations for analysis, individual samples of the healthy blood plasma were pooled (1-ml sample from each donor) to obtain the specimen of 70 ml; analogously, 20 individual 1-ml samples of prostate cancer patients were pooled to give the specimen of 20 ml. CirDNAs were isolated using the Blood Plasma DNA Isolation Kit (BioSilica Ltd., Novosibirsk, Russia) [7] according to the manufacturer's instructions and eluted into nuclease-free water. Genomic DNA (by definition [8], unmethylated in the region of RARbeta2 gene promoter) was isolated from leukocytes of healthy donors using Genomic DNA Isolation Kit (BioSilica Ltd., Novosibirsk, Russia). Fully methylated genomic DNA was obtained from Millipore (Billerica, MA, United States). DNA concentrations and purity were determined by OD_{260}/OD_{280}

(Genesys 10 UV, Thermo Electronics, United States).

2.2. DNA Modification

The genomic (10 or 500 ng) and cirDNAs from patients (80 ng) were modified by treatment with sodium bisulfite using the following kits; EpiTect Bisulfite Kit (Qiagen, Hilden, Germany; Catalog no. 59104), EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, United States; Catalog no. D5006), and CpGenome Fast DNA Modification Kit (Chemicon, Temecula, CA, United States; Catalog no. S7824) as recommended by manufactures. Eluted DNAs were brought to equal volumes (40 μ l). All experiments were repeated twice and in duplicates for each experimental point.

2.3. Quantification of DNA before and after Modification

Genomic and cirDNA were quantified using methyl-independent quantitative real-time PCR (Q-PCR) for detection of the reverse chain of LINE1 elements. Q-PCR was performed in an ICycler iQ5 (Bio-Rad, Hercules, CA, United States). Oligonucleotides were synthesized at the Laboratory of Medical Chemistry, Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia. Sequences of all primers and PCR conditions are listed in Table 1. The PCR mixture (30 μ l) contained 5 μ l of DNA template, 1.5 μ l of 1000-fold diluted SYBR Green I, 10 nM fluorescein, 300 nM of each primer, 0.25 mM of each dNTP, 1 U of *Taq* polymerase (Fermentas, Vilnius, Lithuania), and *Taq* polymerase buffer (65 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.05% Tween 20, and 3.5 mM $MgCl_2$). The PCR products amplified from bisulfite, treated/untreated DNA were used as standards for DNA quantification before and after conversion respectively. The accuracy (variation coefficient) of real-time PCR was 7% and sensitivity, 230 copies of PCR product per reaction (equal to 0.5 pg of genomic DNA), the efficacy 98,3-99,2%.

Table 1. Primers and PCR conditions.

Target sequence	Primer sequences (5'-3')	PCR conditions	Product size, bp
LINE1, LINE1 retrotransposable element 1(AL162574.14, 162925-163131)	F: ttttgaataggtgtgtgt	180 s - 95°C, (25 s - 95°C, 15 s - 60°C, 40 s - 72°C) x 40	207
	R: acttacactccacacaata		
RARbeta2, retinoic acid receptor, beta, isoform 2 [Homo sapiens], wild-type (X56849.1, 924-1117)	F: atgcgagctgtttgaggact	3 min - 95°C, (25 s - 95°C, 15 s - 64.5°C, 40 s - 72°C) x 40,	192
	R: ttaccattttccaggcttgc	5min - 72°C	
RARbeta2, retinoic acid receptor, beta, isoform 2 [Homo sapiens], external (X56849.1, 858 -1128)	F: ggaagtgagttgttagaggtt	3 min - 95°C, (20 s - 95°C, 10 s - 54°C, 30 s - 72°C) x 40,	269
	R: caaataatcatttaccatttcca		

Target sequence	Primer sequences (5'-3')	PCR conditions	Product size, bp
RARbeta2, retinoic acid receptor, beta, isoform 2 [Homo sapiens], internal (X56849.1, 930-1116)	F: ttgtttgaggattgggatgt	5 min - 72°C	186
	R: biotin-taccattttccaaacttactc	3 min - 95°C, (20 s - 95°C, 10 s - 54°C, 30 s - 72°C) x 20, 5 min - 72°C	
RARbeta2, sequencing primer (X56849.1, 930-950)	ttgtttgaggattgggatgt	*	68

*Pyrosequencing was performed as described in the text.

2.4. Estimation of Pyrosequencing Accuracy and Sensitivity

Solutions with a known ratio of unmethylated to completely methylated PCR products were used for estimation of the accuracy and sensitivity of pyrosequencing platform. To obtain unmethylated PCR products, 50 ng genomic DNA from HUVEC cells was amplified using RARbeta2 wild-type primers (Table 1, N3 and N4) in a conventional PCR. The PCR mixture (30 µl) contained 5 µl of DNA template, *Taq* polymerase buffer (65 mM Tris-HCl pH 8.8, 16 mM (NH₄)₂SO₄, 0.05% Tween-20, and 2 mM MgCl₂), 0.25 mM of each dNTP, 1 U of *Taq* polymerase (Fermentas, Vilnius, Lithuania), and 300 nM of each primer. The PCR conditions are given in Table 1. The PCR products were purified by non-denaturing 6% PAGE and subsequent electroelution. Part of PCR product was treated with *SssI* methylase (New England BioLabs, Ipswich, MA, United States) as recommended by the manufacturers and purified using a PCR Product Isolation Kit (BioSilica Ltd., Novosibirsk, Russia). Both PCR products were quantified by OD₂₆₀ (Genesys 10 UV, Thermo Electronics, United States).

Completely methylated and completely unmethylated PCR products were combined to obtain a panel of methylation standards contained 0, 2.5, 5, 10, 50, and 100% of methylated DNA with a final DNA concentration of 1 pg/µl. The degree of methylation of the standards was determined by pyrosequencing as described below. Methylated standards were modified with an EZ DNA Methylation-Gold Kit according to the manufacturer's protocol (Zymo Research). DNA (5 µl) was amplified with biotinylated RARbeta2 internal primers in a conventional PCR as described above (PCR conditions are given in Table 1) for subsequent pyrosequencing immediately after bisulfite modification. Single-stranded biotinylated DNA were isolated using streptavidin coated beads on a Pyrosequencing workstation (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing was performed in a PSQ 96MA (Pyrosequencing AB, Sweden) device using a PyroGold SQA reagent kit (Pyrosequencing AB, Sweden) and 0.45 µM sequencing primer (Table 1) according to the manufacturer's protocol.

2.5. Pyrosequencing of Genomic and Circulating DNAs

CirDNA was extracted from the samples and bisulfite-modified as described above. DNA was amplified in two steps by nested PCR: 5 µl of DNA per reaction were amplified at the first PCR step with external primers and 1 µl of the obtained PCR products was reamplified at the second step with internal primers. The composition of PCR mixture was similar to that described above for conventional PCR. Pyrosequencing was done as earlier described. The primers and PCR conditions are listed in Table 1. The genomic DNA from leukocytes of healthy donors or universal methylated genomic DNA obtained from Millipore (10 and 500 ng) was amplified in two-step PCR and pyrosequenced as described for cirDNA. Pyrosequencing was done in duplicates for each experimental point.

3. Results

The bisulfite-based methods have been successfully used for methylation analysis of DNA from various sources [9-14]. These methods are based on the selective deamination of cytosine to uracil by treatment with sodium bisulfite and subsequent PCR-based assays (MS-PCR, Q-MS-PCR, bisulfite sequencing, COBRA, microarrays and pyrosequencing). During PCR amplification of bisulfite-modified DNA, original cytosines appear as thymine, while 5-methylcytosines (5mC) appear as cytosine. The ideal bisulfite DNA conversion protocol must provide a complete conversion of cytosine residues to uracil, whereas all 5mC should remain intact. In addition, the loss of DNA during the modification reaction due to non-specific degradation and at the re-isolation steps should be kept as small as possible.

The aberrantly methylated DNAs released into the circulating blood by tumor cells are expected to be convenient markers for non-invasive tumor diagnostics [5]. However, fragmentation of cirDNA along with minute amounts of tumor-specific aberrantly methylated DNA circulating in the excess of unmethylated "normal" cell-free DNA demands very efficient protocols of bisulfite conversion minimizing the false DNA conversion and any DNA loss.

To evaluate the DNA loss after chemical conversion, DNA was quantified using methylation independent Q-PCR for detection of LINE1 elements. To obtain a sufficient number of identical samples of cirDNA from healthy donors and cancer patients, separate samples from different donors were combined to obtain “healthy” and “cancer” blood plasma. Pooling of separate samples was

required for accurate comparison of the data of subsequent tests. To estimate the efficacy of bisulfite treatment, we first characterized the accuracy and reproducibility provided by the pyrosequencing platform in question, and then assessed the methylation level of each cytosine in the examined RARbeta2 region (Fig. 1) after DNA conversion with using 3 kits.

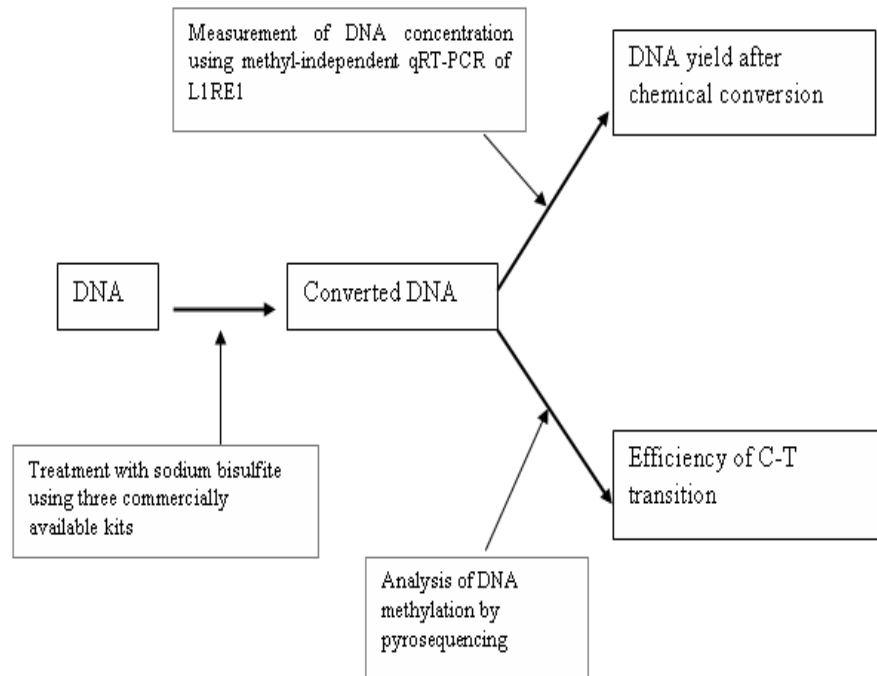


Figure 1. Scheme of experiments. To evaluate DNA loss after chemical conversion, DNA was quantified using methylation-independent qPCR for detection of LINE1 elements. To estimate the efficacy of bisulfite treatment, first, the accuracy and reproducibility of the pyrosequencing platform was characterized and then the methylation level of each cytosine in the studied RARbeta2 region.

3.1. Accuracy of Evaluation of the Degree of Methylation Using Pyrosequencing Platform

Pyrosequencing was recently adapted to study DNA methylation and, thus, become a useful tool for determination of the global DNA methylation content of a sample [15-17]. For this purpose, pyrosequencing is ideal epigenotype-mapping tool, realized as Pyrosequencing workstation (Pyrosequencing AB, Uppsala, Sweden), which is able to quantitatively analyze up to 10 CpG sites in a single sequencing run of 80 nucleotides [18].

The method of bisulfite modification is based on modification of cytosine (in contrast to 5 methyl cytosine) with bisulfate with subsequent deamination of cytosine but not 5mC. It was shown that reaction efficacy depends on time, temperature and bisulfite concentration [4], and hypo- and over-modification can occur, demanding accurate selection of the conditions of modification/deamination. As is shown earlier [19], pyrosequencing-based DNA analysis determines methylation with an accuracy of 3–5%. To validate the accuracy and sensitivity of our pyrosequencing assay, we used the panel of methylation standards that contained 0,

2.5, 5, 10, 50, and 100% of methylated DNA. The average methylation of eight studied CpG sites of RARbeta2 (Fig. 2) gene was determined using one sequencing primer (Table 1). The average mean, minimum, and maximum levels of methylation (expressed as a percentage) were then determined for each DNA mixture. As is shown in Table 2, there was a slight overestimation of methylation levels in the mixtures containing a very low rate of methylated DNA (0, 2.5, 5, and 10% mixtures) and a very clear underestimation of methylation level in the mixture containing 100% of methylated DNA. Nevertheless, the correlation between the methylation level detected in pyrosequencing assay and the rate of methylated DNA is linear. The mean of Pearson coefficient is 0.999. Pyrosequencing showed a mean accuracy rate of 96% (range, 92% for the mixture containing 100% methylated DNA to 97.6% for the mixture containing 2.5% methylated DNA; Table 2). In addition, to evaluate the variation of pyrosequencing assay, three independent sequencing runs were performed using the panel of methylation standards. The degree of methylation at individual CpG sites showed a high reproducibility with a mean variation of 1.5% (range, 0.0 to 6.1 for individual CpG sites).

Table 2. Degree of methylation of individual cytosines determined by pyrosequencing of the mixtures with a known content of unmethylated and methylated PCR products.

Content of methylated PCR-product, %	Mean* degree of methylation, %, and (SD) for individual CpG sites (1–8)							
	1	2	3	4	5	6	7	8
100	95.4 (1.8)	82 (0.7)	95.4 (1.1)	95 (1.4)	98.8 (1.6)	96.4 (0.9)	95.2 (1.3)	93.8 (1.6)
50	50.4 (3.4)	47.2 (3.7)	49.8 (2.5)	48.2 (2.9)	55.6 (4.1)	56.2 (6.1)	52.2 (4.7)	53.8 (5.4)
10	12.6 (0.5)	13 (1.9)	11.2 (0.8)	10.6 (0.5)	15 (1.2)	15.2 (1.6)	12.8 (0.8)	15.8 (2.2)
5	7 (0.0)	6.8 (0.8)	7 (0.0)	7 (0.7)	8.6 (0.5)	8 (0.0)	7.2 (0.4)	8.4 (1.5)
2.5	4.4 (0.9)	4.4 (0.9)	3.4 (0.5)	2.8 (1.6)	5 (0.0)	5.6 (1.3)	4 (0.0)	6.2 (1.6)
0	2.6 (0.5)	2.6 (0.9)	2.6 (0.5)	2.2 (0.8)	3.2 (0.4)	2.6 (0.5)	2.4 (1.8)	4.2 (0.8)

* The mean was calculated for three independent runs.

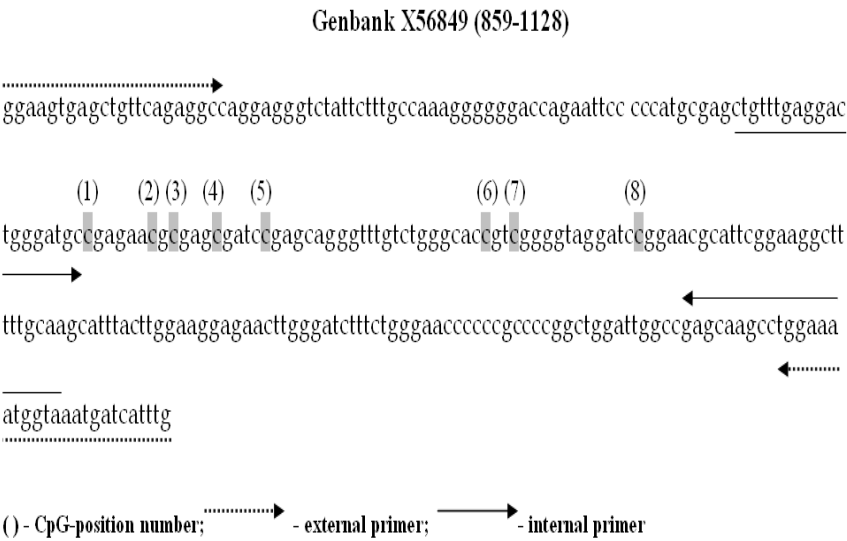


Figure. 2. Location of the primers used for PCR and pyrosequencing of the promoter region of RARbeta2 gene. Potentially methylated sites are marked grey.

3.2. Efficacy of DNA Conversion after Modification with Different Commercial Kits

The genomic DNA from leukocytes of healthy donors unmethylated in the analyzed region of RARbeta2 gene and universally methylated genomic DNA obtained from Millipore, along with the cirDNA isolated from the plasma of healthy donors and prostate cancer patients were treated with different kits to evaluate the degree of individual CpG cytosine methylation by pyrosequencing. Comparison of

the average methylation of all CpG sites demonstrates that all kits give a similar level of DNA conversion/over-conversion (Table 3). The levels of individual cytosine methylation in unmethylated and methylated DNAs along with in the cirDNA from healthy donors and prostate cancer patients determined by different kits varied insignificantly. The same DNA preparations were used in all experiments; thus, the non-coincidence of the data of individual CpG methylation is obviously related to the compositions of kits and the treatment protocols.

Table 3. Degree of methylation (%) of individual cytosines in the CpG dinucleotides of *RARBeta2* gene (*X56849.1*, 951-1007).

Commercial kit	DNA	DNA, ng	Mean* degree of methylation, %, and (SD) for individual CpG sites (1–8)								Mean (SD), %
			1	2	3	4	5	6	7	8	All eight sites
Qiagen	Methylated genomic	10	99 (1.4)	99 (0.7)	96 (0.7)	99 (1.4)	100 (0.0)	100 (0.0)	83 (2.8)	100 (0.0)	97 (0.9)
		500	96 (1.4)	97 (1.4)	96 (1.4)	96 (1.4)	99 (0.0)	100 (0.0)	84 (2.1)	100 (0.0)	96 (1)
	Unmethylated genomic	10	5 (0.7)	8 (1.4)	5 (0.7)	4 (2.1)	10 (0.7)	17 (1.4)	4 (2.8)	18 (4.2)	9 (1.8)
		500	6 (0.7)	6 (0.0)	7 (1.4)	6 (0.7)	9 (0.0)	15 (0.7)	8 (1.4)	12 (0.7)	7 (0.7)
	cirDNA from plasma of healthy controls	80	6 (1.4)	6 (0.7)	7 (1.4)	8 (2.1)	8 (0.7)	11 (0.7)	7 (2.8)	10 (2.8)	8 (1.6)
	cirDNA from plasma of prostate cancer patients	80	22 (1.4)	22 (1.4)	21 (1.4)	21 (0.7)	24 (0.7)	20 (2.1)	10 (2.8)	19 (1.4)	20 (1.5)
	Methylated genomic	10	94 (3.5)	95 (0.7)	89 (7.1)	90 (6.4)	95 (3.5)	93 (4.9)	74 (7.1)	90 (7.1)	90 (5)
		500	96 (0.7)	97 (0.7)	96 (0.7)	96 (0.7)	99 (0.7)	99 (0.7)	77 (3.5)	100 (0.0)	95 (1)
	Unmethylated genomic	10	11 (2.8)	5 (0.7)	11 (2.8)	6 (1.4)	8 (0.7)	7 (0.7)	6 (2.8)	5 (1.4)	7 (1.7)
		500	4 (0.7)	5 (1.4)	6 (1.4)	5 (0.0)	7 (2.1)	9 (0.7)	5 (1.4)	8 (0.7)	6 (1.1)
Zymo Research	cirDNA from plasma of healthy controls	80	5 (2.8)	5 (1.4)	5 (2.8)	4 (1.4)	6 (0.7)	8 (1.4)	5 (2.8)	7 (1.4)	6 (1.8)
	cirDNA from plasma of prostate cancer patients	80	20 (2.8)	16 (0.7)	29 (7.1)	30 (3.5)	26 (4.9)	14 (4.9)	7 (3.5)	12 (6.4)	19 (4.2)
	Methylated genomic	10	99 (0.7)	99 (0.7)	99 (0.7)	99 (0.7)	100 (0.7)	100 (9.9)	74 (5.7)	100 (1.4)	96 (2.6)
		500	98 (0.7)	98 (0.7)	97 (0.7)	98 (0.7)	100 (0.7)	99 (5.7)	72 (2.8)	97 (2.1)	95 (1.8)
	Unmethylated genomic	10	7 (1.4)	9 (2.1)	7 (1.4)	7 (2.8)	7 (1.4)	5 (2.8)	7 (4.2)	5 (2.1)	7 (2.3)
		500	8 (0.0)	8 (0.7)	9 (0.0)	8 (0.7)	5 (0.7)	6 (1.4)	3 (2.8)	5 (1.4)	7 (1)
Chemicon	cirDNA from plasma of healthy controls	80	4 (1.4)	4 (0.7)	5 (1.4)	5 (2.8)	3 (1.4)	7 (2.8)	4 (3.5)	4 (2.8)	5 (2.1)
	cirDNA from plasma of prostate cancer patients	80	23 (2.8)	24 (1.4)	27 (1.4)	27 (2.8)	30 (0.7)	16 (9.9)	9 (5.7)	15 (2.1)	21 (3.4)

Pyrosequencing of methylated DNA demonstrates that the methylated cytosines are stable to bisulfite conversion by all commercial protocols. The degree of methylation of individual cytosines contained in the genomic DNA and cirDNA from healthy donors amounts to 4–11% for the Zymo Research kit, 6–17% for the Qiagen kit, and 3–9% for the Chemicon kit. Pyrosequencing of the unmethylated PCR product treated with Zymo Research kit demonstrated up to 4% methylation of individual cytosines. Thus, an incomplete cytosine conversion is only partially responsible for an increased methylation of cytosines in the DNA from healthy donors, which obviously have low but detectable level of basic methylation [13]. The reagents from Chemicon and Zymo Research provide the least level

of incomplete cytosine conversion.

3.3. DNA Recovery after Modification with Different Commercial Kits

A minute amount of free DNA circulating in the blood [3], fragmentation of cirDNA [3], and the presentation of different DNA sequences in the pool of cirDNA nonequivalent to genomic DNA [3, 13, 21] demand an appropriate method for measuring the cirDNA concentration [3]. As much as 80 ng of cirDNA was used to provide a credible data in the tested range of cirDNA concentrations after bisulfate conversion, as medium DNA amount extracted from 1 ml of prostate cancer patients plasma.

We have earlier demonstrated that qPCR of LINE1 elements, uniformly distributed in the human genome, is an adequate method for estimating the genomic or cirDNA at minute concentrations [20]. To evaluate the DNA yield after modification with different commercial kits, the qPCR was elaborated for L1RE1, a LINE1 retrotransposable element, with a methyl-independent primer to the reverse strand that lacked cytosines in the primer recognition site [20]. Since the bisulfite conversion of the internal region that contains cytosines interferes with the melting temperature of PCR products, two PCR products were prepared for PCR calibration: one from untreated DNA and the other from bisulfite-treated DNA (untrPCR product and bstrPCR product, respectively). To measure the concentration of native DNA, untrPCR product was used for calibration, and bstrPCR product was used as a calibration standard to measure the concentration of bisulfite-treated DNA. The discrepancy between the calibration plots constructed with untrPCR and bstrPCR products were undetectable at the concentrations exceeding 50 pg/probe but reached 40% when 0.5 fg of the PCR products were compared by qPCR. Since all the tested kits demonstrate similar level of cytosine conversion (Table 3), such a strategy for PCR calibration for measuring of DNA concentration appears to be reasonable.

The efficacy and reproducibility of DNA recovery were evaluated from the data on the yield of DNA \pm SD (Table 4). Processing of 500 ng of unmethylated or methylated genomic DNA with EpiTect Bisulfite Kit (Qiagen) and CpGenome Fast DNA Modification Kit (Chemicon) yielded a similar quantity of DNA, namely, about 20% of

the starting amount. Recovery of 10 ng with these kits is less efficient, especially for methylated DNA. EZ DNA Methylation-Gold Kit (Zymo Research) demonstrated more reasonable data for both types and quantities of DNA.

Processing of 80 ng of the cirDNA from blood plasma of healthy controls and prostate cancer patients with CpGenome Fast DNA Modification Kit (Chemicon) resulted in better DNA yield than EpiTect Bisulfite Kit (Qiagen). Conversion of the cirDNAs by EZ DNA Methylation-Gold Kit (Zymo Research) demonstrated the highest possible DNA yield of 100% (Table 4) demonstrating in this way absence of usually occurred DNA degradation during bisulfite treatment and DNA loss during isolation [1].

Convergence (%) of the data obtained by the kits (Table 4) was estimated as a min/max variance of the mean data for different DNA recovery from solutions with different concentrations. The recovery of unmethylated DNA (500 ng, marked grey in Table 4) was selected as a reference and considered as zero. Data convergence reflects uniformity in the recovery of DNA of different sizes, structures, and compositions. EZ DNA Methylation-Gold Kit (Zymo Research) provided the most uniform yield of unmethylated, methylated genomic and circulating DNAs from the blood plasma of prostate cancer patients and healthy donors (Table 4). EpiTect Bisulfite Kit (Qiagen) and CpGenome Fast DNA Modification Kit (Chemicon) demonstrated a lower yield, especially at low DNA concentrations, and a significant variation of convergence ($-71/+10$ and $-87/+95$, respectively).

Table 4. Recovery of DNA after treatment with different commercial kits.

DNA	Initial amount	Qiagen	Zymo Research	Chemicon
		Mean, % (SD)	Mean, % (SD)	Mean, % (SD)
Methylated	10 ng	5.8 (0.05)	92 (8)	2.7 (0.2)
	500 ng	22 (4)	68 (3)	19 (7)
Unmethylated	10 ng	16 (4)	81 (37)	14 (2)
	500 ng	20 (4)	75 (9)	21 (7)
Plasma cirDNA from healthy controls	80 ng	16 (2)	100 (5)	19 (7)
Plasma cirDNA from prostate cancer patients	80 ng	11 (1)	100 (5)	41 (13)
Mean (SD)		15 (3)	86 (16)	19 (7)
Convergence, % (from/to)		-71/+10	-9/+33	-87/+95

In sum, EZ DNA Methylation-Gold Kit from Zymo Research provided the highest yield of DNA after bisulfite treatment and isolation; the mean yield of DNA was no less than 86%. In addition, the efficacy of DNA recovery after treatment with Zymo Research kit does not depend on starting DNA concentration. This is in contrast to other kits tested, which provided no more than 20% yield for high DNA concentrations and only 2.7–5.8% for low DNA concentrations. A low concentration of cirDNA in the blood and, correspondingly, low amounts of these DNAs available for analytical procedures along with their fragmentation as well as size and compositional diversity of DNA fragments demand the most efficient protocol of DNA bisulfite treatment. A high recovery rate of DNA after treatment with EZ DNA Methylation-Gold Kit demonstrates that this kit is the most appropriate for cirDNA treatment. Thus, the treatment of cirDNA from both healthy donors and cancer patients with EZ DNA Methylation-Gold Kit provides almost quantitative DNA recovery after treatment and confirms that this protocol can be recommended for treatment of cirDNA.

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Competing Interests

The authors do not receive any reimbursements, fees, funding, or salary from any organization that may in any way gain or lose financially from the publication of this manuscript, nor hold any stocks or shares from such an organization. The authors do not hold any patents relating to the theme of the manuscript nor have any relation with organizations that holds or have applied for such patents. The authors also do not have financial or non-financial competing interests.

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