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Chapter 25
Methylated Cell-Free DNA In Vitro and In Vivo

Tatyana E. Skvortsova, Olga E. Bryzgunova, Alena O. Lebedeva, Viktoria V. Mak, Valentin V. Vlassov, and Pavel P. Laktionov

Abstract We have investigated the stability, circulation and generation of methylated DNA (methDNA) in vivo and in vitro. In serum, the methDNA (free and as nucleoprotein complexes circulating in human blood) was shown to degraded more slowly then unmethylated DNA (unmethDNA). Residual free methDNA circulates in blood for longer than unmethDNA although it is eliminated faster from blood immediately after injection. After injection of human plasma in mice via the caudal vein, human circulating DNA was shown to circulate in blood for a long time, to accumulate in tissues and to diffuse back into the blood. A comparative, quantitative study of RARbeta2 gene methylation in cell-free DNA (cfDNA) and genomic DNA (gDNA) of primary and transformed cells was made using methylation-specific quantitative PCR (MS-qPCR) and pyrosequencing. The MS-qPCR data demonstrate overrepresentation of methDNA sequences in cfDNA of primary cells. Methylation level (ML) of most CpG-sites in cfDNA and gDNA is similar, but certain sites are overmethylated, the extent of their methylation differing between cfDNA and gDNA. Thus, a detailed investigation of methylated cfDNA generation and factors influencing its stability, circulation and elimination from the blood are required for a rational selection of aberrantly methylated tumor markers.

Keywords Circulating DNA · Cell-free DNA · Methylated cirDNA · DNA stability · Pharmacokinetics · Real-time PCR · Pyrosequencing

Abbreviations

cPCR	Conventional PCR
cfDNA	Cell-free DNA
cirDNA	Circulating DNA
gDNA	Genomic DNA

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methDNA	Methylated DNA
ML	Methylation level
MS-qPCR	Methyl-specific quantitative PCR
MSRE-qPCR	Methyl-sensitive quantitative PCR
unmethDNA	Unmethylated DNA

Introduction

Endogenous circulating DNA (cirDNA) has been detected in all biological fluids and has been shown to represent a valuable source of material for medical diagnostics (Fleischhacker and Schmidt 2007). Despite of many publications in this area, the lack of uniformity in presenting and interpreting research data demonstrates that the application of cirDNA as an unambiguous biomarker is, nevertheless, currently overrated. Hence, elucidating the origin, circulation and excretion, function and significance of this nucleic acid is necessary before utilizing cirDNA as a biomarker (van der Vaart and Pretorius 2009).

The activity of DNA-hydrolyzing enzymes in blood and tissues (Cherepanova et al. 2007), the structure of DNA, and DNA to protein interactions in blood are the main factors affecting the circulation of free DNA in blood. Such processes as the generation of cirDNA, its accumulation in tissues (Agrawal et al. 1991; Wilson et al. 2007), and elimination from the organism (Botezatu et al. 2000), obviously influence the concentration of DNA circulating in blood.

Exogenous "naked" DNA rapidly degrades in blood, while DNA composed of natural nucleoprotein complexes (nucleosomes and apoptotic bodies) are capable of long-term circulation in the blood (Wilson et al. 2007) representing the convenient, tumor-specific diagnostic material (Fleischhacker and Schmidt 2007) including aberrantly methylated genes. It should be noted that most CpG pairs in gDNA are methylated, but the features of methDNA in circulation are poorly investigated, although it is known that structure of methDNA is more disposed to Z than to B transition, triplex formation (Hodges-Garcia and Hagerman 1995) and association with specific protein assemblies (Attwood et al. 2002). Interactions of methDNA with specific proteins and the local DNA structure nearby methylated cytosines can interfere with the generation of cfDNA, its circulation and functions.

DNA circulating in the blood of cancer patients originates from both normal and transformed cells (Fleischhacker and Schmidt 2007). Whereas, methylation of cancer suppression gene promoters frequently accompanies cancer transformation and can be readily detected in presence of an excess of unmethDNA with high specificity and sensitivity methDNA are considered as potential oncomarkers Das and Singal 2004. But the potency of methDNA as tumor markers is limited by the restricted presence in the pool of the typical methylation pattern of cirDNA found in tissues (Fleischhacker and Schmidt 2007). Moreover, the data of the recent study of cirDNA in healthy individuals by massively parallel bisulphite sequencing demonstrates that cancer-free cirDNA can contain nearly every conceivable

cytosine-methylation pattern (Korshunova et al. 2008). Thus, the methylation of gDNA as well as methylated cfDNA generation must be also investigated before the practical use of methylated cirDNA in diagnostics.

To understand the contribution of different processes in methylated cirDNA generation, we have investigated the circulation of methDNA and unmethDNA in blood in vivo and methylation of cfDNA and gDNA in vitro.

Material and Methods

Mice. Male mice BALB (7–8 week old, 22.5±3.8 g weight) were purchased from "Genofonds of laboratory animals" (ICG SD RAS, Russia) where they were bred and maintained under standard conditions.

Preparation of unmethylated/methylated DNA (*PCR-products and plasma sample*). The CpG-rich fragments of GSTP1 and RARβ2 genes were produced by PCR using the primers #1, 2 and #3, 4 (Table 25.1), respectively. PCR reaction was performed in the reaction mixture (30 μl) containing 50 ng of gDNA from HUVEC, 300 nM of each primer, *Taq* polymerase buffer (2.0 mM $MgCl_2$), and 1U of *Taq* polymerase (Fermentans, Lithuania). Both PCR-products were purified using non-denaturing 6% PAGE followed-up by electro-elution and aliquots of PCR-products were treated with *Sss*I methylase as recommended by the manufacturers (New England BioLabs).

The blood sample from healthy man was obtained from the Novosibirsk central Clinical Hospital. The plasma fraction was collected under standard conditions (Laktionov et al. 2004).

Stability of methDNA (in vitro *and* in vivo *experiments*). To assess the stability, unmethylated (100 ng)/methylated (24 ng) PCR-products and human plasma (150 pg, containing 70% of the methylated form of the GSTP1 gene) were incubated with 50 μl of mouse serum for different time intervals at 37°C. After incubation, DNA was extracted and quantified by MSRE-qPCR.

40 μl of DNA samples or blood plasma (correspond to 0.6 ng of cirDNA) were injected into mice via the caudal vein using a micro-syringe installed in a Hamilton repeating dispenser (PB600-1). A total of three animals were injected for each experimental point. Unmethylated PCR-product (100 ng/mouse), methylated PCR-product (24 ng/mouse) and a mixture of the two (100+24 ng,) were injected. At different time intervals after injection the blood was taken from the retro-orbital sinus. Blood plasma, serum were prepared as described previously (Laktionov et al. 2004; Fiegl et al. 2005). DNA was extracted and its amount was determined by MSRE-qPCR.

Cultivation of cells and preparation of cell fractions. HeLa (human cervical carcinoma cell line) and HUVEC (human umbilical vein endothelial cells) were cultivated under standard conditions (Morozkin et al. 2004). To study the methylation of cfDNA in different cell types, HeLa and HUVEC were seeded at a density 10^4cells/cm^2. 16 h after seeding, the cells were washed with DMEM and cultivated

Table 25.1 Primers sequences used in this study

N	Genes	Assay	Sequence of primers[a] (5′-3′)	Size, bp	Ann T (°C)	Cycle
1	GSTP1 (X08058, 1149-1312)	cPCR[b]/MSRE-qPCR[c]	F: ggcgccgtgactcagcact	164	65	40
2			R: tggccccatgctgggagct			
3	RARbeta2 (X56849.1, 924-1117)	cPCR	F: atgcgagctgtttgaggact	194	65	40
4			R: ttaccattttccaggcttgc			
5	Independent RARbeta2 (X56849.1, 931-1116)	MS-qPCR[d]	F: ttgtttgaggattgggatg	186	54	40
6			R: taccattttccaaacttactc			
7	Methylated RARbeta2 (X56849.1, 938-1098)		F: aggattgggatgtcgagaacgc	161	64	40
8			R: ctcgaccaatccaaccgaaacg			
9	RARbeta2, external (X56849.1, 858-1128)	Pyro-sequencing	F: ggaagtgagttgtttagaggtt	269	54	40
10			R: caaataatcatttaccattttcca			
11	RARbeta2, internal (X56849.1, 931-1116)		F: ttgtttgaggattgggatgt	186	54	25
12			R: biotin-taccattttccaaacttactc			
13	Sequencing primers		seq1: ttgtttgaggattgggatgt	–		
14			seq2: aggagaatttgggatttttttggga			

[a]All primers were selected using the Primer3 interactive program and synthesized at the Group of Medicinal Chemistry (ICBF SD RAS).
[b]conventional PCR.
[c]methyl-sensitive quantitative PCR.
[d]methyl-specific quantitative PCR.

in fresh growth medium. At different time intervals, growth medium and cells were collected and cfDNA were prepared as described previously (Morozkin et al. 2004).

Analysis of nucleic acids. DNA was isolated using kits for plasma DNA and blood DNA isolation (BioSilica Ltd., Russia). The ML was determined using two different methods: MSRE-qPCR after treatment of the DNA template with the methylation-sensitive endonuclease BstU312I (Fermentas, Lithuania) or MS-qPCR after sodium bisulfite modification by the EZ DNA methylation Kit (Zymo research, USA). Standard templates for any quantitative PCR were obtained by amplification of gDNA with the corresponding set of primers (Table 25.1). Serial dilutions of purified and quantified PCR products were used as standard for DNA concentration. The reactions were performed in the reaction mixture (30 μl) containing 3 μl of template, 1.5 μl of 1,000-fold dilution of SYBR Green I, 300 nM of each corresponding primer, 0.25 mM of each dNTP, *Taq* polymerase buffer (containing 3 mM $MgCl_2$) and 1U of *Taq* polymerase (Fermentans, Lithuania) using the ICycler iQ instrument (BioRad, USA). PCR reactions for each experimental point were conducted in duplicate and repeated at least three times independently to ensure the reproducibility of results. To analyze the degree of methylation in individual CpG-sites by pyrosequencing, biotinylated DNA templates were obtained using two sets of primers. Pyrosequencing was performed using the PSQ 96MA instrument (Biotage AB, USA) according to manufacturer's protocol (PyroGold reagents, Biotage AB, USA). PCR conditions, PCR and sequencing primers are shown in Table 25.1.

Results and Discussion

We have investigated the influence of DNA methylation on DNA stability in blood plasma. It was shown that 93% of unmethylated PCR-product was degraded in a 5 min whereas methylated PCR-product was shown to be more stable: more than 45% remain intact after 60 min of incubation (Fig. 25.1a). The reasons of methDNA stability are not clear: it could be concerned with low efficacy of blood DNA-hydrolysing enzymes or efficient binding with blood proteins as compared to unmethDNA. MethDNA in native complexes circulating in human blood plasma is also more stable in comparison with unmethDNA: after 2 h of incubation with mice serum only 10% of methDNA is degraded (Fig. 25.1b), and its fraction of the total DNA pool increased from 70% up to almost 100% by 50 h (Fig. 25.1c).

Five minutes after injection into mice via the caudal vein 10% of unmethylated (Fig. 25.2a) and only 1% of methylated (Fig. 25.2b) PCR-products were detected in the blood. Starting from this level, the elimination of free DNA from the blood is described by typical biphasic curve: the concentration of unmethylated PCR-product decreased faster (the half-life time is 4.1 min (Fig. 25.2a)) then that of methylated PCR-product (the half-life time is 8.8 min (Fig. 25.2b)). In vitro experiments demonstrated that in mouse plasma, methDNA is at least 10 times more stable than unmethDNA. Fast elimination of methDNA is obviously observed thanks to more efficient accumulation in organs and tissues of methDNA or their complexes

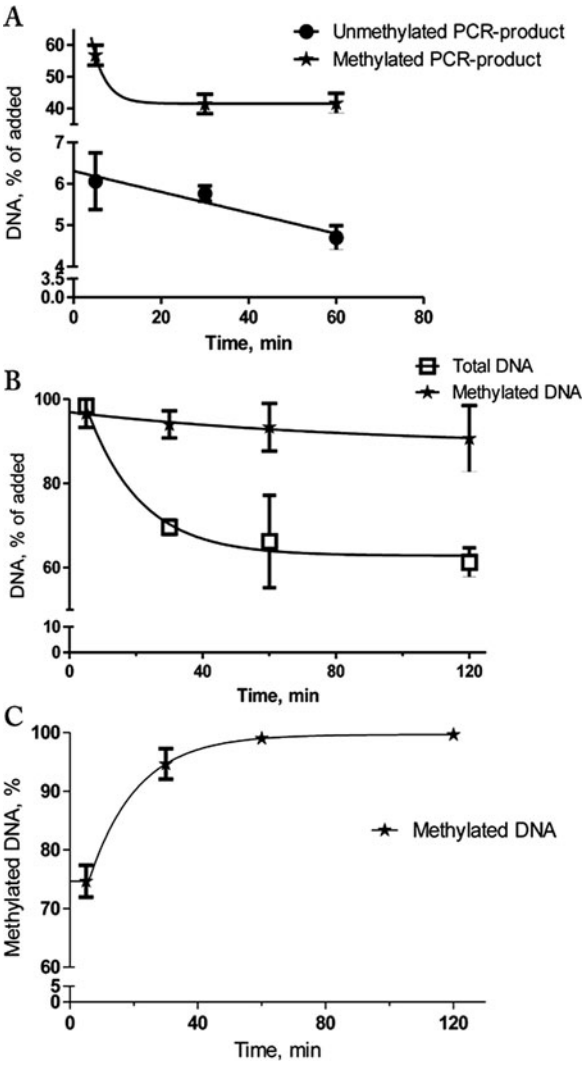

Fig. 25.1 Stability of 164 bp PCR product, product of its methylation with SssI methylase and human plasma cirDNA in mouse serum. One μl of PCR products (**a**) and ten μl of human male plasma (**b**) (70% of methylated GSTP1 in circulating DNA as estimated by real-time PCR) were incubated with 50 μl of mice serum during 5, 30, 60 and 120 min at 37°C. After incubation DNA was isolated and quantified by real-time PCR. Circulating of methylated GSTP1 was estimated from real-time PCR data of Bsh1236I treated and untreated DNA. (**c**) Time dependence of the content of methylated DNA (from human plasma) in mice serum

with blood and tissue biopolymers. The presence of the methDNA in the circulation does not influence the circulation of unmethDNA, and vice versa (Fig. 25.2d).

Five minutes after injection of the human blood plasma only 6% of injected DNA was found to circulate in the mouse bloodstream. The decline of the human DNA concentration during the next 40 min is replaced by an increase up to the 5 min level which is maintained for next 2 h (5.7%) (Fig. 25.2c). These data are apparently, related to a redistribution of cirDNA complexes among the blood and tissues.

The high background of overall methylation found in cirDNA of healthy people by massively parallel bisulphite pyrosequencing raised an additional question regarding the origin of the methylated cirDNA (Korshunova et al. 2008). To reveal

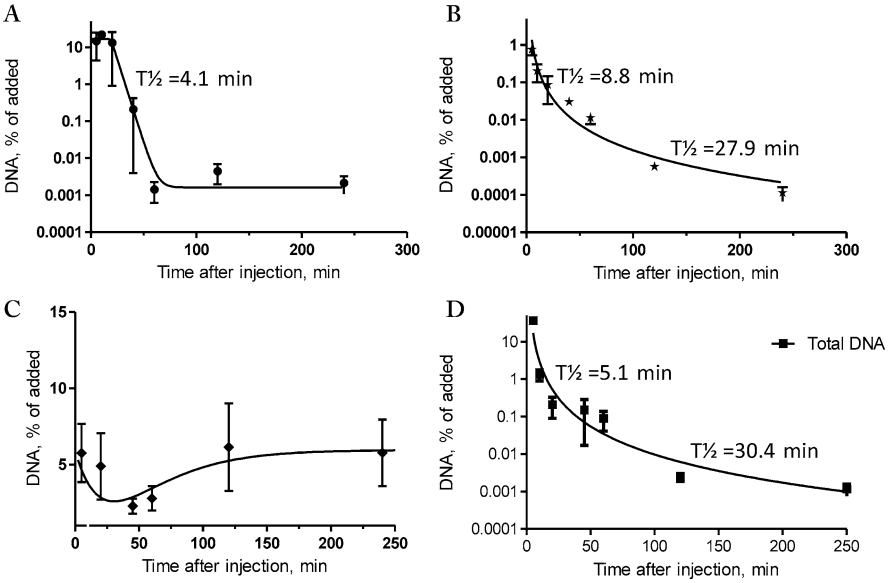

Fig. 25.2 Time dependence of concentration of exogenous DNA injected in mouse blood. PCR (164 bp) product (**a**), its methylated derivative (**b**), 40 μl of human blood plasma (70% of methylated GSTP1 as estimated by real-time PCR) (**c**), both PCR products (unmethylated and methylated) were injected simultaneously (**d**) via the caudal vein. Five, 10, 20, 60, 120, 240 min after injection mice were bled from the retro-orbital sinus, DNA from the plasma was isolated and quantified by real-time PCR

the potential impact of normal cells in this process we made a comparative study of genomic and cfDNA methylation by MS-qPCR and pyrosequencing. RARbeta2 fragment (X56849.1, 924-1117) containing 13 CpG sites was selected for this study. MS-qPCR has a sensitivity of 25 copies and an accuracy of 10%, pyrosequencing has a sensitivity of 2.5% ML and an accuracy of 3.5% for all 13 CpG sites. The docking experiments, when mixtures of different amounts of completely methylated and unmethylated PCR products were tested by MS-qPCR and pyrosequencing, demonstrated a discrepancy of no more than 5% between PCR and pyrosequencing (the data not shown).

The data of MS-qPCR showed that cfDNA and gDNA from HeLa cells have, in general, similar ML (100%) except from 24 h cultivated cells when cfDNA methylation decreased to 50% (Fig. 25.3a). The relative content of the methylated RARbeta2 gene from HUVEC cfDNA is usually higher than in gDNA: there are 1.6% of methylated RARbeta2 in gDNA and 8.9% in the cfDNA. The relative content of the methylated RARbeta2 gene varies during the cultivation of HUVEC, depending on the cells line (cord donor) and increases with cell passage number (Fig. 25.3b and c).

The data of CpG methylation of DNA from HeLa cells obtained by pyrosequencing demonstrated a similar profile for cfDNA and gDNA methylation. The

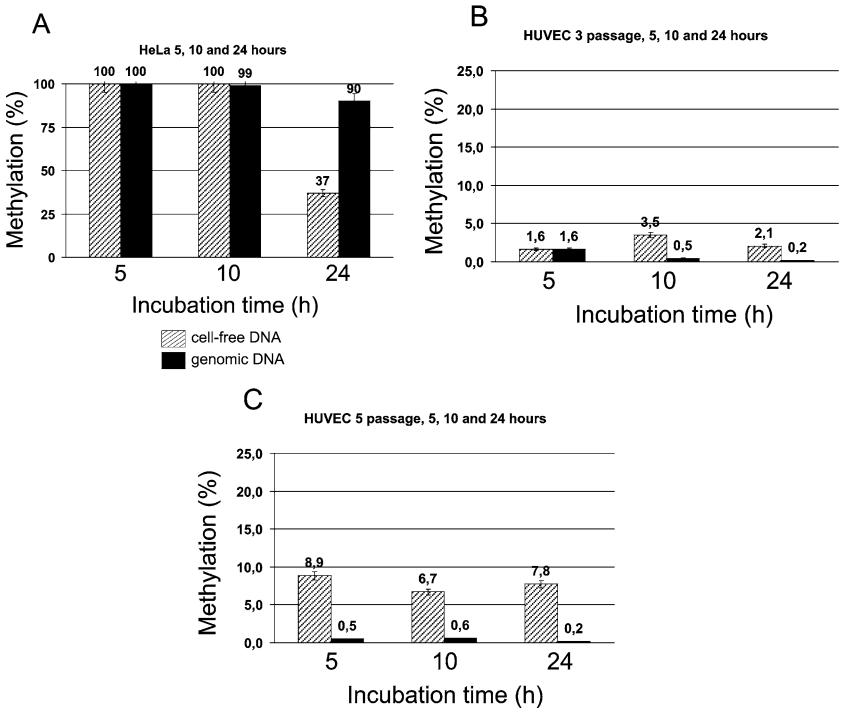

Fig. 25.3 Distribution of methylated RARbeta2 gene in cell-free and genomic DNA of transformed (**a**) and primary (**b** and **c**) cells

low methDNA content from cells cultivated for 24 h determined by MS-qPCR was not confirmed by pyrosequencing (Fig. 25.4). In HUVEC cells the ML of almost all sites were in a range of 3 ± 0.25% both in cfDNA and gDNA. However, the ML of certain CpG-sites depended on the passage number and differs between cfDNA and gDNA (Fig. 25.4).

The methylation data of cfDNA and gDNA in primary cells shows that normal cells can "generate" aberrantly methDNA. Different profiles of cytosine methylation in cfDNA and gDNA raise a question about the processes of the generation of methylated cfDNA. The discrepancy between the pyrosequencing and MS-qPCR data could be concern with a non-classic CpG methylation of cytosines in embryonic stem cells (Lister et al. 2009). Non-CpG methylation can interfere with the estimation of the unmethDNA concentration by MS-qPCR and probably pyrosequencing when primers overlap non-CpG methylated sites (Table 25.2).

The data obtained demonstrates that free methDNA, and particularly methDNA composed of circulating complexes, are more stable, circulates in the bloodstream for a longer time and the continuous circulation led to an overrepresentation of methDNA. Normal cells are involved in the generation of a "background" of aberrantly methylated cfDNA complicating the selection of tumor-specific markers.

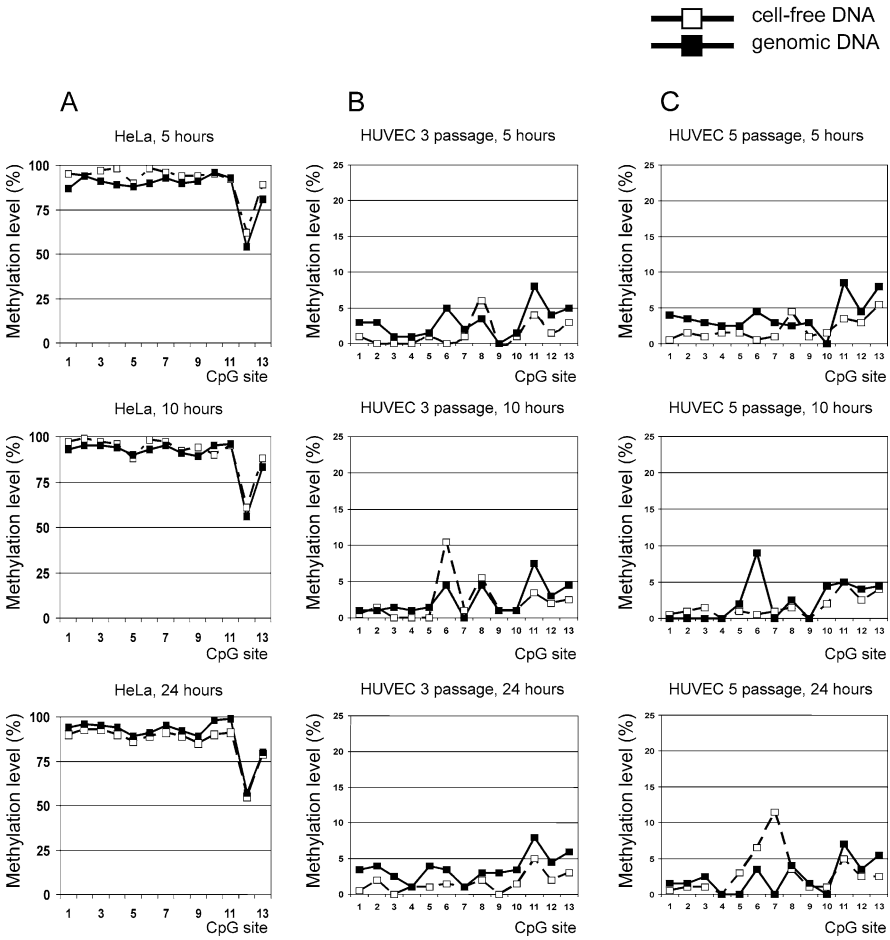

Fig. 25.4 The patterns of methylation in 13 CpG sites in RARbeta2 gene fragment (X56849.1, 931-1117) using cell-free and genomic DNA of transformed (**a**) and primary (**b** and **c**) cells

Table 25.2 Primers sequences for RARbeta2 gene used in this study with marked possible nonCpG methylation position

Destination of primer	Primer sequences prior to chemical conversion (5′-3′)
For first PCR (pyrosequencing) (X56849.1, 858-1128)	F: ggaagtgag**CTG**tt**CAG**aggcc
	R: **CAA**atgatcatttaccattttcca
For second PCR (pyrosequencing) (X56849.1, 931-1116)	F: **CTG**tttgagga**CTG**ggatgc
	R: taccattttc**CAG**gcttgctc
For methylated form RARbeta2 (MS-qPCR) (X56849.1, 938-1098)	F: agga**CTG**ggatg**CCG**agaacgc
	R: ctcggc**CAA**tc**CAGCCG**gggcg

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