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GLAD-PCR assay of selected R(5mC)GY sites in URB1 and CEPBD genes in human genome.

Alexandr G Akishev[1,2], Murat A Abdurashitov[1,2]*, Viktoriya L Sitko[1,2], Nina A Netesova[2], Irina F Radaeva[2], Elena A Nechaeva[2], and Sergey Kh Degtyarev[1,2]

[1]SibEnzyme Ltd, Novosibirsk, Russia.
[2]State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk region, Russia.

ABSTRACT

The optimal conditions of GLAD-PCR assay of A(5mC)GT site in URB1 gene in human DNA have been determined as follows: a hybrid primer with 4 additional bases,0.33 units of SP-Taq DNA polymerase in 20 µl of reaction mixture, concentration of $MgCl_2$ is 3 mM, concentration of DNA ligase is 10 U/µl. GLAD-PCR assay of tandem of GCGC sites in CEBPD gene was carried out in these optimal conditions and confirmed a complete methylation of tandem of GCGC sites in DNA from malignant cell lines Raji, U-937 and Jurkat. We believe these conditions may be also used for GLAD-PCR assay of other R(5mC)GY sites in the mammalian genomes. Distinct and clear curves of the fluorescence accumulation in case of methylated sites and an absence of fluorescence signal in case of non-methylated sites show a big potential of GLAD-PCR assay in DNA methylation analysis.
Keywords: DNA methylation, real time PCR, DNA endonucleases, ligation-mediated PCR.

Corresponding author

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INTRODUCTION

*De novo*DNA methylation of RCGY sites in human and mammalians genomes is one of the main mechanisms of epigenetic regulation of genes activity. Enzymes DNMT3a and DNMT3b catalyze modification of RCGY site with formation of 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' sequence. Earlier we discovered a new methyl-directed site-specific DNA endonuclease GlaI [1]. GlaI recognizes and cleaves DNA sequence 5'-R(5mC)^GY-3'/3'-YG^(5mC)R-5' as indicated by caret signs [2]. Based on this unique substrate specificity of GlaI we developed a new method of GLAD-PCR assay which allows to determine methylation of RCGY site of interest in human genomic DNA [3]. GLAD-PCR assay includes three steps: (1) GlaI cleavage of R(5mC)GY sites in DNA, (2)ligation of the obtained DNA fragments to the oligonucleotide adapter and (3)PCR with TaqMan probe and primer, which are complementary to target DNA fragment, and a so-called hybrid primer, which is complementary to the adapter. A proposed method was used to determine methylation of RCGY sites in regulation region of ELMO1 and ESR1 genes in DNA preparation from healthy and cancer tissues [4]. Recently we studied methylation of ACGT site in URB1 gene and showed a complete methylation of this site in human leukocyte DNA [5]. This result corresponds to the literature data and allows us to carry out an optimization of conditions of GLAD-PCR assay.

In this work we have carried out an optimization of GLAD-PCR assay of A(5mC)GT site inURB1 gene. The obtained optimal conditions of GLAD-PCR assay have been used to study methylation of tandem of GCGC sites in regulation region of CEBPD tumor suppressor gene.

MATERIALS AND METHODS

Materials

Normal saline (JSC "Krasfarma", Krasnoyarsk), trypsin solution (Trypsin-Versen) (LLC "Biolot", St. Petersburg), phenol (JSC "Reagent", Novosibirsk), chloroform (JSC "Reagent", Novosibirsk), isoamyl alcohol (JSC "Reagent", Novosibirsk), RNase A (OOO "Samson-Med", St. Petersburg), SDS ("Helicon", Moscow). Phosphamidites (Biosset, Novosibirsk, Russia). Buffer A (1M EDTA, 1M NaCl, 1M Tris-HCl, pH 8.0, at 25 °C), buffer B (0,1 M Tris-HCl, pH 7,5 at 25 °C; 0,1 M NaCl and 30 mM $MgCl_2$), 4M NaCl, 3M potassium-acetate buffer (pH 7.0 at 25 °C), proteinase K, BSA, λDNA, 10x TE buffer (100 mM Tris HCl pH 7,0 at 25 °C; 10 mM EDTA), 10x TMN buffer (100 mM Tris-HCl pH 7.9 at 25 °C, 50 mM $MgCl_2$, 250 mM NaCl), 50 mM $MgCl_2$, dNTP mix (concentration 10 mM each), 10x GLAD-Mg buffer (500 mM Tris-SO_4 pH 9.0 at 25°C, 300 mM KCl, 100 mM $[NH_4]_2SO_4$), methyl-directed site-specific endonuclease GlaI, T4 DNA ligase and SP-Taq DNA polymerase – produced by SibEnzyme Ltd. (Novosibirsk, Russia).

Purification of DNA from blood samples and from cell lines.

Participating blood donors voluntarily joined this study with the written informed consent to have their biologic specimens to be analyzed, and the work had been approved by the Ethical Committee of the Seversk Biophysical Research Centre, consistent with the WMA Declaration of Helsinki.

Purification of leukocyte DNA was performed as described earlier [5].Cell lines L-68, HeLa, Jurkat, Raji and U937 from the “Collection of microorganisms” department of State Research Center of Virology and Biotechnology “Vector” (Koltsovo, Novosibirsk region, Russia) were used in a work and grown as indicated earlier [6]. A pellet of approximately 10 million cells of each cell line after washing with buffer B was frozen at -20 °C. A frozen pellet was dissolved in1.35 ml of buffer A with addition of 75 µl 10% SDS and 20 µl proteinase K (20 mg/ml) and a further treatment was carried out similar to a purification of leukocyte DNA (see above).

Preparation of DNA solutions

Concentration of the genomic DNA preparations was adjusted to 9 ng/µl (approximately 3000 copies of haploid genome in 1 µl) in 1x TE buffer. DNA standards(15, 30, 60, 150, 300, 600, 1500 copies/µl)were prepared from a leukocyte DNA by dilution in TE buffer withλDNA (70 ng/µl).

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DNA hydrolysis with GlaI

1 µl of DNA standard was added to 14 µl H_2O and 15 µl obtained DNA solution was incubated in 21,5 µl reaction mixture containing TNM buffer with addition of 0.1 µg/µl BSA, 2 % of DMSO and GlaI at concentration 0.07 units/µl. Reaction mixture was incubated at 30 °C for 30 minutes followed by thermoinactivation at temperature 65 °C for 10 minutes and cooling to 4 °C.

Ligation

Reaction mixture for DNA ligation (30 µl) was obtained from products of GlaI digestion (21,5 µl) and adapter, ATP and DNA ligase. Adapter was obtained by incubation of oligonucleotide A1 5'-CCTGCTCTTTCATCG-3'and oligonucleotide A2 5'-CGATGAAAGAGCAGGp-3' (10 µM of each) in 50 µl at 65°C for 5 minutes and subsequent cooling to 10°C. Reaction mixture for DNA ligation contains adapter (0.5 µM), ATP (0.5 mM) and DNA ligase (10 units/µl) and is incubated at 25 °C for 15 minutes.

Structure of primers and TaqMan probes

DNA-synthesizer ASM800 (Biosset, Russia) was used for synthesis of oligonucleotide primers and TaqMan probes. Oligonucleotides were purified by reversed-phase chromatography on a PLRP-S 300A ("Polymer Laboratories Ltd", UK) with subsequent gel filtration on "Sephadex G-25 DNA-grade" (Amersham-Pharmacia, USA). Determination of the copies number in DNA preparations was performed by comparative real time PCR of a target DNA sample and DNA standards. Structures of the synthesized oligonucleotides are presented in Table 1.

Table 1: The nucleotide structures of the designed oligonucleotides

Synthetic oligonucleotide	Description	Structure
URB1F	genomic primer	5' GCGAAGGATGTCCCCGACAC 3'
URB1Z	probe	5' FAM-CTGCTGCCACAGTGACCTGCCCA-BHQ1 3'
URB1H-3	hybrid primer with 3 additional bases	5' CGCCTGCTCTTTCATCGGTG 3'
URB1H-4	hybrid primer with 4 additional bases	5' CGCCTGCTCTTTCATCGGTGC 3'
URB1H-5	hybrid primer with 5 additional bases	5' CGCCTGCTCTTTCATCGGTGCC 3'
URB1H-6	hybrid primer with 6 additional bases	5' CGCCTGCTCTTTCATCGGTGCCG 3'
CEBPDF	genomic primer	5' CGAGGAGGTTCCAAGCCCACAAAC 3'
CEBPDZ	probe	5' FAM-CACTCCCTCTGCCAGCACTCCA-BHQ1 3'
CEBPDH-4	hybrid primer with 4 additional bases	5' CGCCTGCTCTTTCATCGGCTG 3'

PCR

PCR for each DNA sample was performed in 20 µl of reaction mixture in triplets. Original 60 µl of reaction mixture contains 30 µl of ligation products plus 30 µl of PCR reaction mixture. The PCR reaction mixture includes GLAD-Mg buffer with 0.2 mM of each dNTP, 0.4 µM of each primer and TaqMan probe, BSA 0.2 µg/µl. Concentrations of $MgCl_2$, SP-Taq DNA polymerase and DNA ligase are varied in course of optimization as described below. DNA standards were prepared from leukocyte DNA at concentration 5, 10, 20, 50, 100, 200, 500 and 1000 copies of haploid genome in 20 µl PCR. Real-time PCR was performed in the "CFX-96 Thermal Cycler Amplifier" (Bio-Rad, USA). Initiation was performed during 3 min at 95 °C, followed by 45 cycles of amplification: the DNA melting - 95 °C, 10 sec; annealing - 63 °C for 20 sec with detection of fluorescence in the FAM channel; elongation - 72 °C for 5 sec. Data analysis and processing was carried out using the "Bio-Rad CFX Manager V. 2.1" software.

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RESULTS AND DISCUSSION

DNA structure of the amplified regions of URB1 and CEBPD genes

GLAD-PCR assay is one of the ligation-mediated PCR(LM-PCR) variants [7]. It is based on the addition of short synthetic adapter oligonucleotide duplex to the DNA fragments obtained after GlaI cleavage. In the further amplification, "hybrid" primeris used which is composed from two parts - the larger part is complementary to the sequence of the added adapter, and the remaining part contains only a few bases which correspond to the adjoined genomic sequence at GlaI cleavage site. The second primer and the fluorescent probe completely correspond to the genomic sequence of the studied DNA region.

The coordinates and nucleotide structures of the amplified regions in thereference human genome assembly GRCh38/hg38 are shown on Figure 1.In case of the amplified region of CEBPD gene there are two possible cleavage sites for GlaI in the close vicinity. The adjacent five nucleotides at 5' side for both sites are identical thus allowing to use the same hybrid primer for both sites. Thus, in this case GLAD-PCR will reflect methylation of any from these two GCGC sites.

URB1
chromosome 21, positions 32334235–32334294
GCGAAGGATGTCCCCGACACTCGGCTGCTGCCACAGTGACCTGCCCAGGCTCCGGCAC^GT

CEBPD
chromosome 8, positions 47738426-47738510
CGAGGAGGTTCCAAGCCCACAAACAGGAAGAAGAGAAGGCCCTGGAGTGCTGGCAGAGGGAGTGTCATTCCCAGCAGC^GCAGC^GC

Figure 1: Structures of amplified regions in URB1 and CEBPD genes. The annealing sites for genomic primer and probe are underlined with single and double lines, correspondingly. RCGY sites are shown in grey. Cleavage position of GlaI (if CG pair is methylated) are shown by caret signs.

The influence of hybrid primer length on GLAD-PCR assay efficiency

The length and composition of the additional part of hybrid primer may have a strong influence on the DNA polymerization process, so we have tested several variants of hybrid primers at the first step of optimization for URB1 gene.

5 U/µl of DNA ligase were used at sample preparation step. The PCR mix contained 3 mM Mg^{2+}and0,67 U SP-Taq polymerase (other components concentrations of the mix are given in "Materials and Methods" section).Initiation was performed during 3 min at 95 °C, followed by 45 cycles of amplification: melting at 95°Cfor 10 sec, annealingfor 20 sec, elongation at 72°C for 5 sec. The annealing temperatures were 55, 61.7, 62.6 и 63°C depending on the length of hybrid primer (from 3 to 6 additional bases, correspondingly).

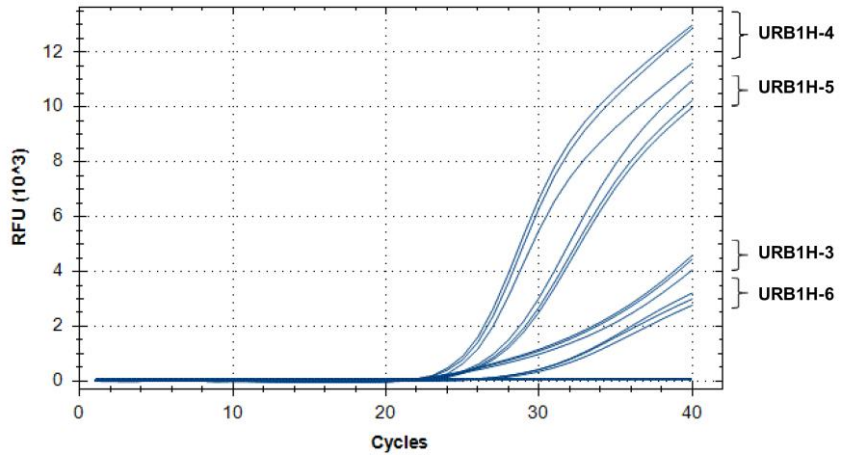

Figure 2: Curves of fluorescence accumulation depending on the length of hybrid primer.

Figure 2 presents the PCR curves for all four reactions with different hybrid primers. According to the data of Figure 2 the maximum of fluorescence signal was observed in the reactions with URB1H-4 hybrid primer. In the case of 5 additional nucleotides (hybrid primer URB1H-5) the rate of fluorescence accumulation is lower. Hybrid primers URB1H-3 and URB1H-6 do not provide the necessary efficiency in PCR. Probably, the usage of hybrid primer with additional three nucleotides (URB1H-3) leads to amplification of many non-target GlaI fragments which have 5'-CAC-3'/3'-GTG-5' on the both ends. This non-specific reaction results in a strong decrease in amplification of the target DNA region. In the case of hybrid primer with additional six nucleotides (URB1H-6) the PCR efficiency is significantly lower, probably, due to GC-rich structure of the primer end which allows binding to non-specific regions of genomic DNA.

Thus, URB1H-4 is the most appropriate primer for the usage in the further experiments where we evaluated an influence of different concentration of SP-Taq DNA polymerase, $MgCl_2$and DNA ligase on efficiency of PCR.

Dependence of GLAD-PCR assay from concentration of SP-Taq DNA polymerase

First, we analyzed an influence of different concentrationof SP-Taq DNA polymerase on PCR.10 U/µl of DNA ligase was used at DNA ligation step. The PCR mixture was supplemented with 3 mM $MgCl_2$ (final concentration). The selected URB1H-4 hybrid primer was used in the reaction. The annealing temperature in PCR was 63°C, whereas other PCR conditions were not changed. Figure 3 shows the obtained real-time PCR curves using different concentrations of the target DNA (DNA standards 5, 10, 20, 50, 100, 200 and 500 copies of haploid genome).

Though the Cq values for all reactions are near the same (excepting the case of the lowest polymerase amount), the form of curves is most proper in the case of 0.33 U of SP-Taq DNA polymerase in 20 µl of reaction mixture. We can see well pronounced plateau zone and the highest fluorescence accumulation is achieved. This is evidence of higher efficiency and correctness of targeted amplification using this enzyme concentration.

The influence of Mg^{2+}concentration on GLAD-PCR assay

It's well known that concentration of magnesium ions influences on the success of PCR [8]. Four different concentrations of $MgCl_2$in GLAD-PCR have been tested. Oligonucleotide adapter was ligated using 10 U/µl of DNA ligase. 0.33 U of SP-Taq DNA polymerase was added toeach20 µl ofPCR mixes. All other conditions were the same as in previous experiment. Figure 4 shows the obtained results using standard curves for different DNA concentrations.

In this case we estimated the amount of the successful low-copy (5-20 haploid genomes) reactions. As a rule PCRs of only several copies of target DNA molecules are usually not reproducible due to irregular distribution of molecules in the initial solutions and to some other factors [9]. According to the data presented in Figure 4 concentration 3.0 mM $MgCl_2$provides the most successful low-copy reactions and this value (3.0 mM) was chosen for the next step.

The influence of DNA ligase concentration on GLAD-PCR assay

Figure 5 demonstrates the results of GLAD PCR assay of 5-500 copies of DNA molecules depending on concentration of DNA ligase. The success of GLAD-PCR assay depends on complete ligation of oligonucleotide adapter to GlaI fragments. It should be mentioned that GLAD-PCR assay was designed to perform all procedures in one tube, so the final PCR mix contains DNA ligase which is not a standard reagent for PCR. It is not clear if the presence of this protein may affect the polymerization reaction. Figure 5 shows the results of PCRs depending on DNA ligase concentrations.

The best results of the amount of detected low-copy PCRs were obtained at 10 U/µl concentration of DNA ligase. Thus, the optimal conditions for GLAD PCR assay of A(5mC)GT site in URB1 gene may be recommended as follows: hybrid primer with 4 additional bases, 0,33 units of SP-Taq DNA polymerase in 20 µl of reaction mixture, concentration of $MgCl_2$ is 3 mM, concentration of DNA ligase is 10 U/µl.

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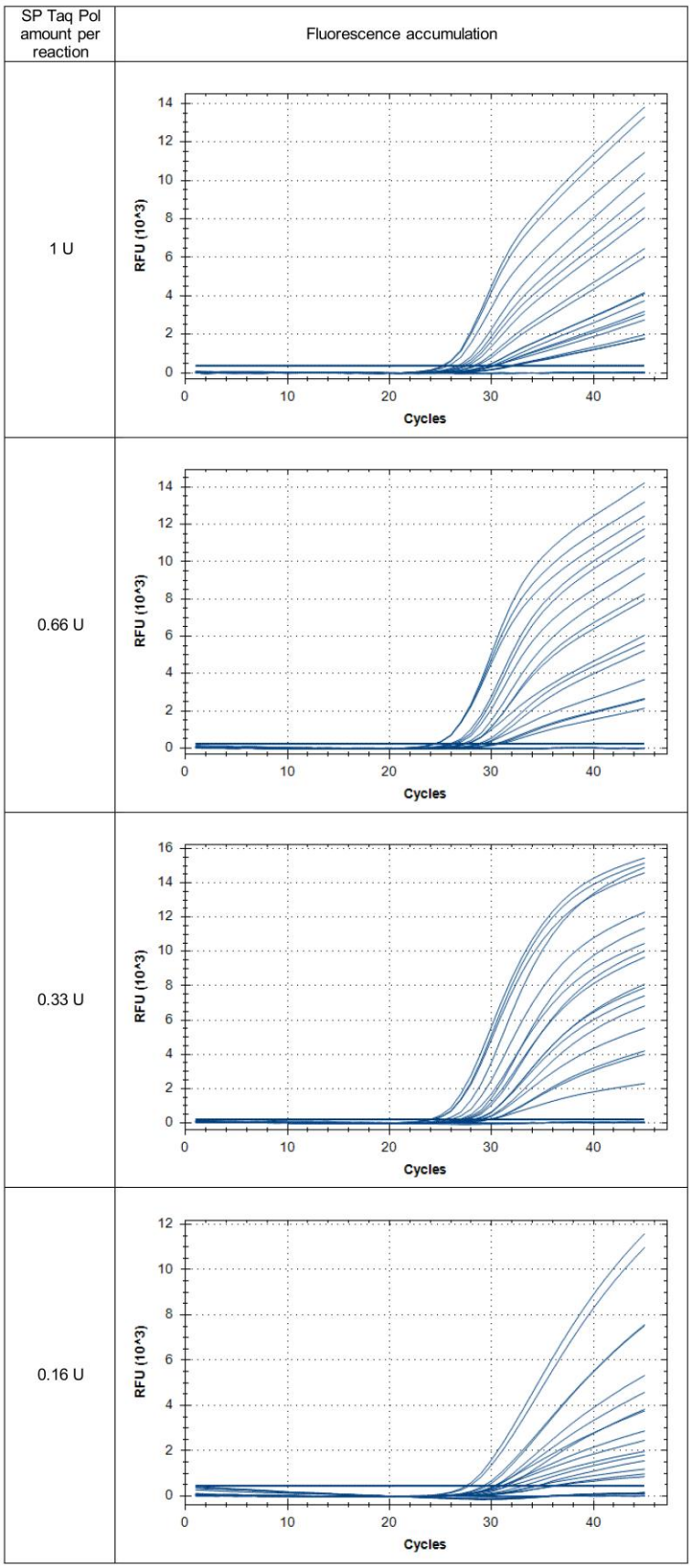

Figure 3: Curves of fluorescence accumulation depending on the DNA polymerase amount and target DNA copy number.

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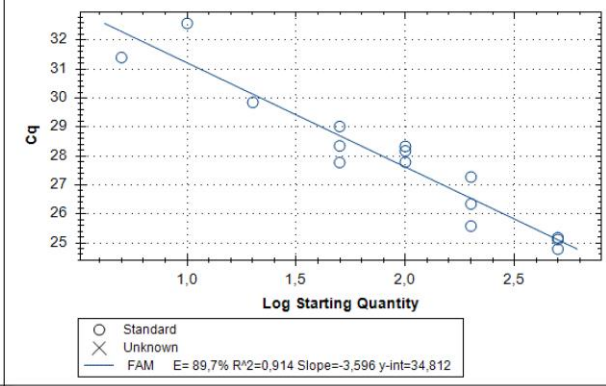
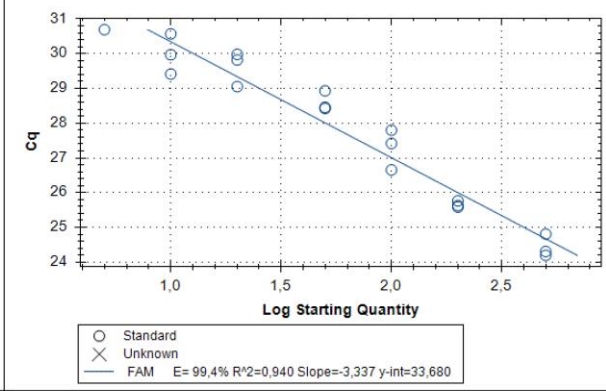
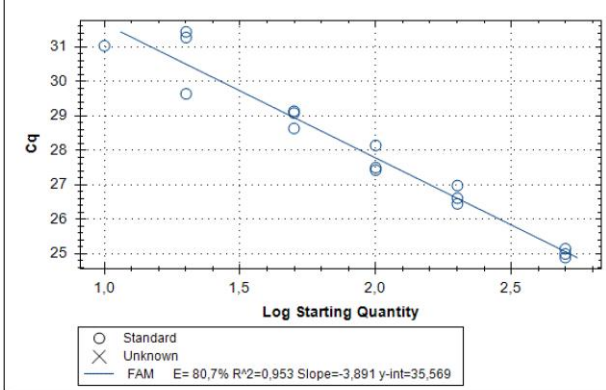
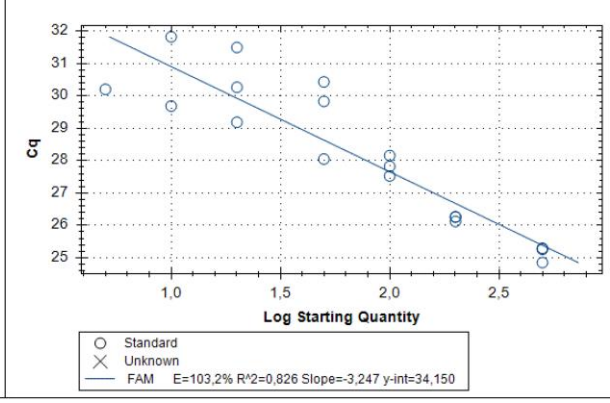
$MgCl_2$ concentration in the PCR mixture	PCR standard curve
2.5 mM	
3.0 mM	
3.5 mM	
4.0 mM	

Figure 4: Standard curves at different $MgCl_2$ concentrations for the used DNA standards (5-500 genomic copies).

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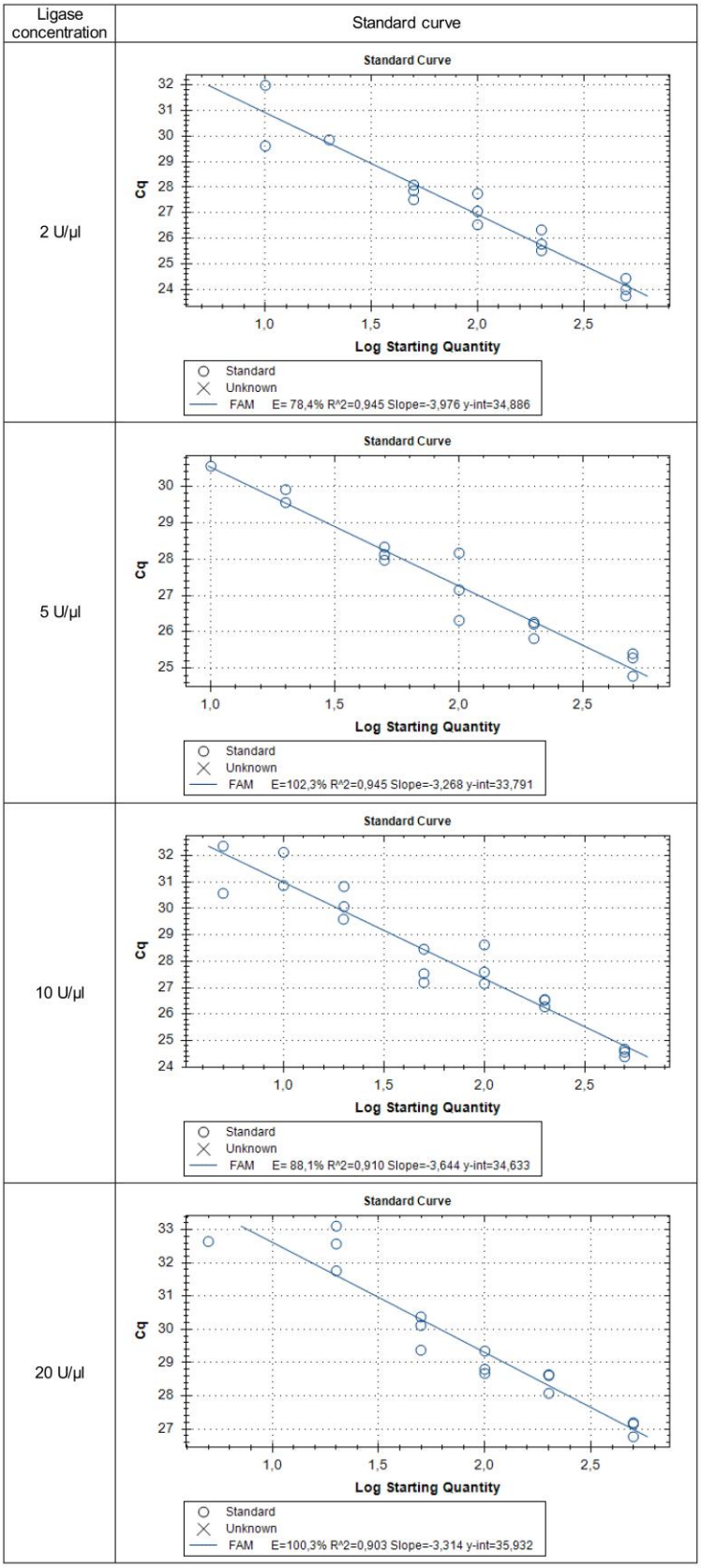

Figure 5: Standard curves at different DNA ligase concentrations for the used DNA standards (5-500 genomic copies).

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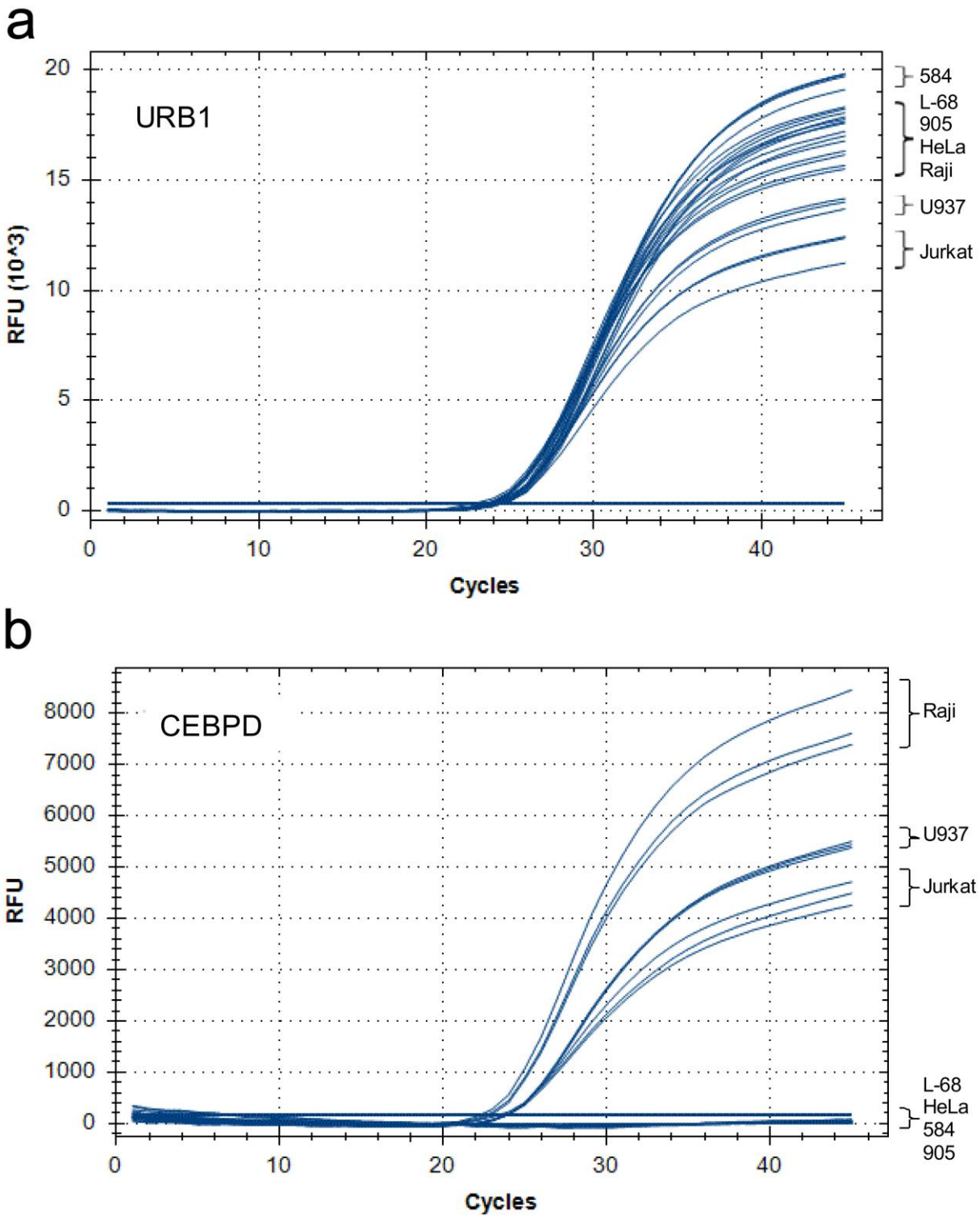

Figure 6: Curves of fluorescence accumulation obtained using 1000 copies of genomic DNAs from different cell lines and leukocytes samples 584 and 905. RCGY sites in URB1 (a) and CEBPD (b) genes were targets for GLAD-PCR assay.

GLAD-PCR assay of selected R(5mC)GY sites in URB1 and CEBPD genes in DNA preparations from leukocytes and human cell lines

In the determined optimal conditions we have carried out GLAD-PCR assay of A(5mC)GT site in URB1 gene in DNA preparations from malignant cell lines HeLa, U937, Raji, Jurkat, non-malignant cell line L-68 and leukocytes from healthy donors (samples 584 and 905). Figure 6a demonstrates the obtained curves of fluorescence accumulation for each template DNA at concentration ~1000 copies of haploid genomes in reaction mixture. According to these results ACGT site in the URB1 gene is methylated in all studied DNAs. Methylation of ACGT site in the URB1 gene in all studied DNAs is known from RRBS data presented by ENCODE/HudsonAlpha project (https://www.encodeproject.org) [10]. In case of leukocyte DNA a complete methylation of this site is shown in our previous paper [5].

Based on the determined optimal conditions of GLAD-PCR assay for A(5mC)GT site in URB1 gene we performed GLAD-PCR assay of tandem of GCGC sites located in the regulatory region of CEBPD gene. CEBPD is one of the well-known tumor suppressor genes which is often hypermethylated in cancer. Earlier we showed a complete methylation of this tandem of sites in some malignant cell lines [11]. The GC content of the amplified CEBPD region (61.1%) is lower than the studied region of URB1 gene (68.2%). A structure of the CEBPD tandem sites in the amplified region is given on Figure 1.GLAD-PCR assay of both GCGC site may be done with one hybrid primer because 5'-regions of these sites are the same.

The results of GLAD-PCR assay of tandem of GCGC sites in CEBPD gene in different DNA preparations are presented in Figure 6b. Data in Figure 6b show a high methylation of at least one of GCGC site from a studied tandem in DNA from malignant cell linesRaji, U-937 and Jurkat and an absence of methylation of GCGC sites in DNA from L-68 and HeLa cell lines and leukocyte DNA. A direct sequencing of the studied CEBPD region of HeLa and L-68 genomic DNAs has shown an absence of any nucleotide substitution compare to reference genome sequence (data not shown). So, GlaI does not cleave this tandem sites due to non-methylated cytosine residues at these positions in HeLa and L-68 genomes.

These results correspond to the data we obtained earlier, when, based on results of BlsI-PCR assay, we showed a complete methylation of tandem of GCGC sites in CEBPD regulatory region in DNA from malignant cell lines and an absence of these sites methylation in healthy DNA [11].

Thus, in this work we have determined the optimal conditions of GLAD-PCR assay of A(5mC)GT site in URB1 gene. GLAD-PCR assay of tandem of GCGC sites in CEBPD gene was carried out in these optimal conditions and confirmed a complete methylation of tandem of GCGC sites in DNA from malignant cell lines Raji, U-937 and Jurkat. We believe these conditions may be also used for GLAD-PCR assay of other R(5mC)GY sites in the mammalian genomes. Distinct and clear curves of the fluorescence accumulation in case of methylated sites and an absence of fluorescence signal in case of non-methylated sites show a big potential of GLAD-PCR assay in DNA methylation analysis.

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