New enzymes for DNA technologies

SibEnzyme

INSTRUCTION MANUAL

GlaI-PCR Assay Kit

Catalog No. **K011S, K011L**

Highlights

- The kit is developed for determination of methylation status of human and mammalian DNA fragment with a real-time PCR and for identification of unmethylated RCGY sites within it.
- In case of the only RCGY site in the studied DNA fragment GlaI-PCR assay kit allows to determine a total concentration of DNA molecules and a concentration of DNA molecules with this unmethylated site.
- Primers and a probe for the structure of the analyzed DNA fragment with one (or more) RCGY site must be designed
- GlaI-PCR assay is performed in 3-4 hours and includes 2 stages: DNA hydrolysis and a real-time PCR.
- **NO BISULFITE CONVERSION**

Contents

Web page

http://sibenzyme.com/products/kits

Product Contents

GlaI-PCR Assay Kit		K0011S 200 reactions	K011L 1000 reactions
1	1X TE Buffer	200 µL	1 mL
2	10X SE TMN Buffer	150 µL	750 µL
3	BSA, 10 mg/mL	70 µL	350 µL
4	MD DNA-endonuclease (20 u/µL)	30 µL	90 µL
5	β -mercaptoethanol (200мМ)	12 µL	60 µL
6	10X SE GLAD Buffer	430 µL	2.2 mL
7	dNTP Mix, 10 mM each	90 µL	450 µL
8	SP Taq DNA Polymerase, 5 u/µL	80µL	400 µL
9	Control DNA L-68, 18 ng/µL	10 µL	50 µL
10	Control DNA HeLa, 18 ng/µL	10 µL	50 µL
11	Control mouse DNA, 18 ng/ µL	25 µL	125 µL
12	Control RARB mix (primers + TaqMan probe), 10 µM each	40 µL	200 µL
13	DNA phage λ, 18 ng/µL	10 µL	50 µL
14	DNA Raji, 18 ng/µL	15 µL	70 µl

Storage conditions: -20^{O}C

Note – Expiration date of the reagents in the kit is one year from the date of production. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

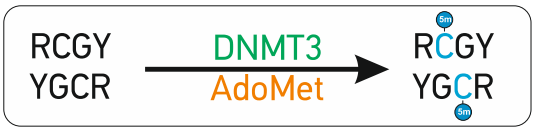
SibEnzyme GlaI-PCR assay kit contains all reagents for analysis except TaqMan Probe and primers. The nucleotide sequences of primers and probe must correspond to the structure of the studied DNA fragment with one or more RCGY sites.

A mixture of primers and probes for GlaI-PCR assay of a human DNA fragment are applied to demonstrate how the method works. The RARB control mixture (primers and fluorescent probe) is provided for analysis of DNA fragment in the 3rd chromosome, in the regulatory region of RARB gene (position 25428290-25428403 in accordance with the genomic Assembly GRCh38/hg38).

Product description

Today an abnormal methylation of regulation regions (promoter and/or first exon) of genes was shown at initial stage of several deceases such as cancer, cardiovascular decease, diabetes and some others.

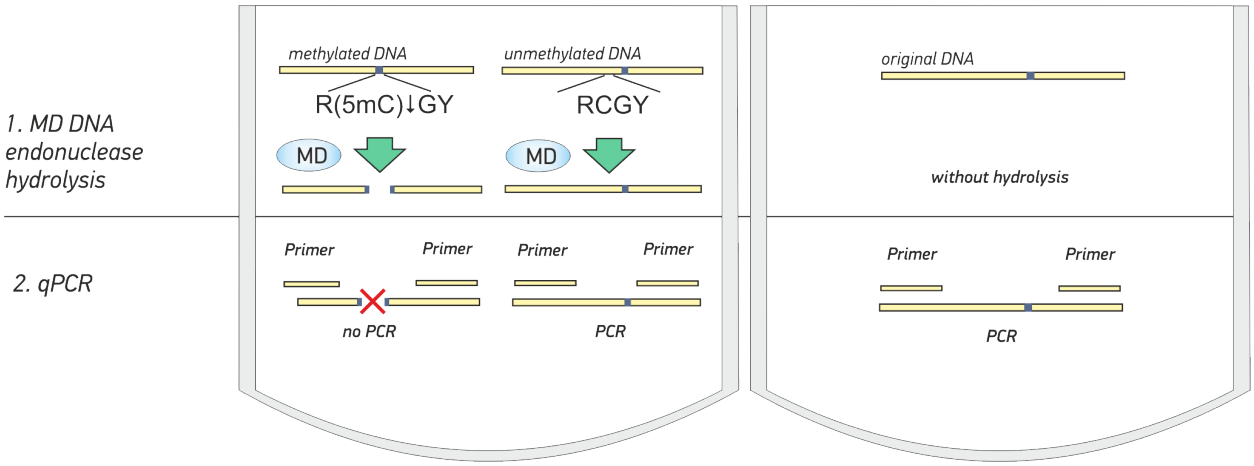
This abnormal *de novo* DNA methylation is performed by DNMT3A and DNMT3B DNA methyltransferases. These enzymes recognize and methylate site 5'-RCGY-3' with formation of 5'-R(5mC)GY-3'/3'YG(5mC)R-5'[1].



Site-specific methyl-directed DNA endonuclease (MD DNA endonuclease), such as GlaI, recognizes exactly this DNA sequence 5'-R(5mC)↑GY-3'/3'YG↑(5mC)R-5' and cleaves it as indicated by symbols ↑, forming blunt ends[2].

GlaI-PCR assay is based on the use of MD DNA endonuclease, such as GlaI, and allows determining the methylation status of the analyzed DNA fragment containing one or more RCGY sites. GlaI-PCR assay is performed in two stages: a) hydrolysis of the analyzed DNA sample by MD DNA-endonuclease, b) real-time PCR with primers bordering the studied DNA fragment [3].

GlaI-PCR



If the R(5mC)GY site is present in the analyzed DNA fragment, MD DNA endonuclease cleaves it and subsequent PCR does not produce the product. At the same time, PCR of the control sample (where there was no hydrolysis by the enzyme) produces a PCR product. The methylation status of the studied DNA fragment is determined comparing the results of PCR of a control sample and DNA after hydrolysis by the enzyme

In case of one RCGY site in the studied DNA fragment, the GlaI -PCR assay method allows determining the concentration of the unmethylated site and calculating its percentage in relation to the total number of DNA molecules with this fragment.

In comparison with other methods for determining DNA methylation, GlaI-PCR assay has a number of advantages:

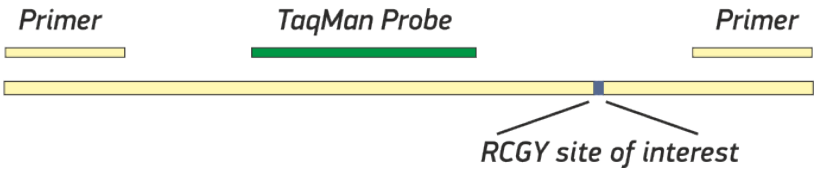
- Simple – 2 easy steps
- Requires only real time PCR-machine
- Quick - only 3-4 hours

Protocol of GlaI-PCR assay

for determination of the percentage of unmethylated site RCGY in DNA preparations.

Preparation of primers and probe

Genomic primers and a fluorescent probe for a DNA fragment containing at least one RCGY site are designed as usual [3].



The PCR Protocol is designed for DNA fragments with GC-percentage no higher than 68-69%.

The study of each sample is performed twice: one PCR from an original DNA and another PCR from hydrolyzed DNA. We recommend carrying out PCR in three repeats (triplets) – totally 6 reactions/2 triplets per sample.
Primers mix (2.4 µl for 1 triplet) includes 2 genomic primers and a fluorescent probe in concentrations of 10 µM each.

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DNA preparation

1. Prepare solutions of N studied DNAs in Milli-Q H_2O or in TE-buffer if you are planning to store DNA solutions. The concentration of the studied DNA, determined spectrophotometrically, should be in the range of 6 ng /µl - 30 ng /µl.

Preparation of DNA standards

2. Prepare 5 test tubes with DNA standards and one control:

Add 26 µl of H_2O to the test tube #1, add 15 µl of H_2O to the test tubes #2, #3, #4, #5 and add 13.5 µl of H_2O to the test tube #6.

Add 4 ml of DNA Raji (the Burkitt lymphoma cell line) at a concentration of 18 ng/ml to the test tube # 1. Gently mix.

Transfer 15 ml of DNA solution from test tube #1 to the test tube #2, gently mix.

Transfer 15 ml of DNA solution from test tube #2 to the test tube #3, gently mix.

Transfer 15 ml of DNA solution from test tube #3 to the test tube #4, gently mix.

Transfer 15 ml of DNA solution from test tube #4 to the test tube #5, gently mix.

Remove 15 µl of the mixture from test tube number 5.

Add 1.5 ml of mouse DNA solution (negative control) at concentration 18 ng/ml to the test tube #6. Gently mix. Shortly centrifuge all tubes.

Stage 1: DNA Hydrolysis

3. Prepare 2 PCR tubes (200 µl) for each sample to be analyzed.

Mix 13.5 µl of H_2O and 1.5 µl of sample DNA in each tube. There will be 2 tubes for each analyzed sample under the number N (sign them" Ne "- experience, and " Nc " - control).

4. Prepare a total reaction mixture for the number of test tubes equal to 2N samples + 6 standards. The reaction mixture is prepared as follows (calculation is provided per one triplet): 12.37 µl of H_2O + 3.15 µl of 10x SE TMN buffer + 0.32 µl of BSA + 0.04 µl of lambda DNA (18ng/µl) + 0.17 µl of β-mercaptoethanol (200mM). After adding each reagent, carefully mix the tube.

5. Add 15.2 ml of the resulting reaction mixture to each of the tubes with numbers Nc and to the tubes with DNA standards ##1-6, carefully mix and shortly centrifuge the tubes.

6. Add (0.19 µl x N) of MD DNA endonuclease to the remaining volume of the reaction mixture. Add 15.2 ml of the resulting reaction mixture to each of the tubes with Ne numbers carefully mix and centrifuge the tube.

The recommended minimum volume of the reaction mixture is calculated for 12 triplets (if the number of triplets in the experiment is less than 12, then the remainder of the reaction mixture is not used).

7. Incubate all test tubes for 40 minutes at 37 °C and shortly centrifuge tubes after incubation.

Stage 2: Real-time PCR

8. Prepare PCR Mix as follows (calculation is provided per one triplet): 20.8 µl H2O + 6.3 µl 10X SE GLAD buffer + 1.2 µl dNTP Mix + 0.6 µl BSA + 2.4 µl primer Mixture for the test DNA fragment + 0.4 µl SP Taq DNA polymerase. After adding each reagent, carefully mix and shortly centrifuge the tube.

9. Add 30.2 µl of the PCR Mix to all the DNA tubes, carefully mix and shortly centrifuge the tube

10. Three times transfer 20 µl of the reaction mixture from each tube to three wells on a PCR plate seal, centrifuge and place into thermocycler.

11. Perform real-time PCR according to the selected amplification profile (with fluorescence detection in FAM channel).

Analysis of results

12. For each sample, two results will be obtained: the concentration of the DNA fragment in the control tube and the concentration of the unmethylated RCGY site in the experiment. The ratio of these values corresponds to the percentage of unmethylated site in the DNA preparation .

Appendix 1: Protocol for determining the proportion of unmethylated site ACGC in the regulation region of RARB gene [3]

The following is a protocol of GlaI-PCR assay for determining the proportion of unmethylated ACGC site in the regulatory region of RARb gene. The experiment is performed using 2 control DNAs: L68 DNA (from the line of diploid cells of the human lung embryo) and HeLa DNA (from the cell line of cervical adenocarcinoma).

DNA preparation

1. Prepare 4 reaction tubes (200 ml) and label them Hc, He, Lc and Le. Mix 13.5 µl of H2O and 1.5 µl of DNA in each test tube (Hc and He - DNA HeLa ; Lc and Le –DNA L68).

Preparation of DNA standards

2. Prepare 5 test tubes with DNA standards and one control:

Add 26 µl of H_2O to the test tube #1, add 15 µl of H_2O to the test tubes #2, #3, #4, #5 and add 13.5 µl of H_2O to the test tube #6.

Add 4 ml of DNA Raji (the Burkitt lymphoma cell line) at a concentration of 18 ng/ml to the test tube # 1. Gently mix.

Transfer 15 ml of DNA solution from test tube #1 to the test tube #2, gently mix.

Transfer 15 ml of DNA solution from test tube #2 to the test tube #3, gently mix.

Transfer 15 ml of DNA solution from test tube #3 to the test tube #4, gently mix.

Transfer 15 ml of DNA solution from test tube #4 to the test tube #5, gently mix.

Remove 15 µl of the mixture from test tube number 5.

Add 1.5 ml of mouse DNA solution (negative control) at concentration 18 ng/ml to the test tube #6. Gently mix. Shortly centrifuge all tubes.

Stage 1: DNA Hydrolysis

3. Prepare a reaction mixture A for 10 test tubes (Hc, Hy, Lc, Ly and 6 standards): 149 µl of H2O + 37.8 µl of 10x SE TMN buffer + 3.8 µl of BSA + 0, 5 µl of lambda DNA (18ng/µl) + 1.9 µl of β-mercaptoethanol (200mM). After adding each reagent, carefully mix a liquid in the tube. Shortly centrifuge the tube.

4. Add 15.2 ml of reaction mixture A to the test tubes Hc, Lc and 6 standards, carefully mix and shortly centrifuge the tubes.

5. Transfer 30.4 µl of the remaining reaction mixture A to a separate tube and add 0.4 µl of MD DNA endonuclease (20 units/µl), mix by pipetting.

6. Add 15.2 ml of the resulting reaction mixture to the test tubes He and Le, carefully mix and shortly centrifuge the tubes.

7. Incubate all test tubes for 40 minutes at 37 °C

8. Prepare a PCR Mixture: 208 µl H2O + 63 µl 10X SE GLAD buffer + 12 µl triphosphate mixture + 6 µl BSA + 24 µl Mixture of" RARb " primers and probe + 4 µl SP Taq DNA polymerase. After adding each subsequent reagent, carefully mix the contents of the test tube, then discard the drops by centrifugation.

9. Add 30 µl of the PCR Mixture to the DNA test tubes , carefully mix the contents of the test tube and drop the drops by centrifugation.

10. Transfer 20 ml of the reaction mixture from each tube to three wells for PCR

3. Prepare 2 PCR tubes (200 µl) for each sample to be analyzed.

Mix 13.5 µl of H_2O and 1.5 µl of sample DNA in each tube. There will be 2 tubes for each analyzed sample under the number N (sign them" Ne "- experience, and " Nc " - control).

4. Prepare a total reaction mixture for the number of test tubes equal to 2N samples + 6 standards. The reaction mixture is prepared as follows (calculation is provided per one triplet): 12.37 µl of H_2O + 3.15 µl of 10x SE TMN buffer + 0.32 µl of BSA + 0.04 µl of lambda DNA (18ng/µl) + 0.17 µl of β-mercaptoethanol (200mM). After adding each reagent, carefully mix the tube.

5. Add 15.2 ml of the resulting reaction mixture to each of the tubes with numbers Nc and to the tubes with DNA standards ##1-6, carefully mix and shortly centrifuge the tubes.

6. Add (0.19 µl x N) of MD DNA endonuclease to the remaining volume of the reaction mixture. Add 15.2 ml of the resulting reaction mixture to each of the tubes with Ne numbers carefully mix and centrifuge the tube.

The recommended minimum volume of the reaction mixture is calculated for 12 triplets (if the number of triplets in the experiment is less than 12, then the remainder of the reaction mixture is not used).

7. Incubate all test tubes for 40 minutes at 37 °C and shortly centrifuge tubes after incubation.

Stage 2: Real-time PCR

8. Prepare a PCR Mixture: 208 µl of H2O + 63 µl of 10X SE GLAD buffer + 12 µl of dNTP mix + 6 µl of BSA + 24 µl of PCR Mix for RARb + 4 µl of SP Taq DNA polymerase. After adding each reagent, carefully mix and shortly centrifuge the tube.

9. Add 30.2 µl of the PCR Mix to all the DNA tubes, carefully mix and shortly centrifuge the tube

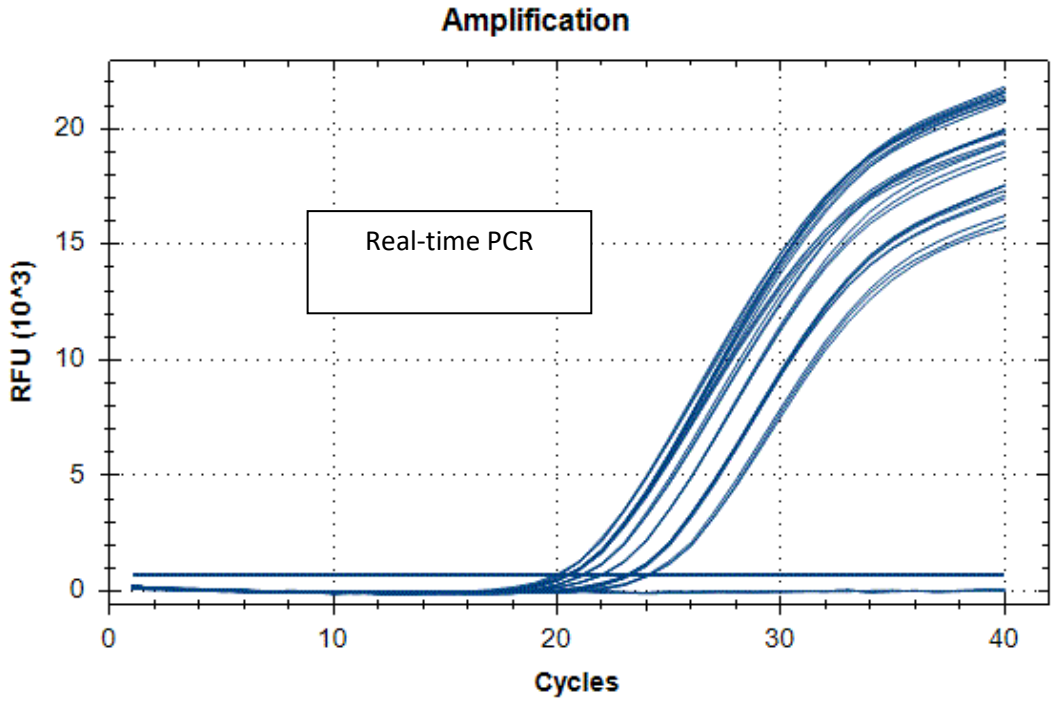
10. Three times transfer 20 µl of the reaction mixture from each tube to three wells on a PCR plate seal, centrifuge and place into thermocycler.

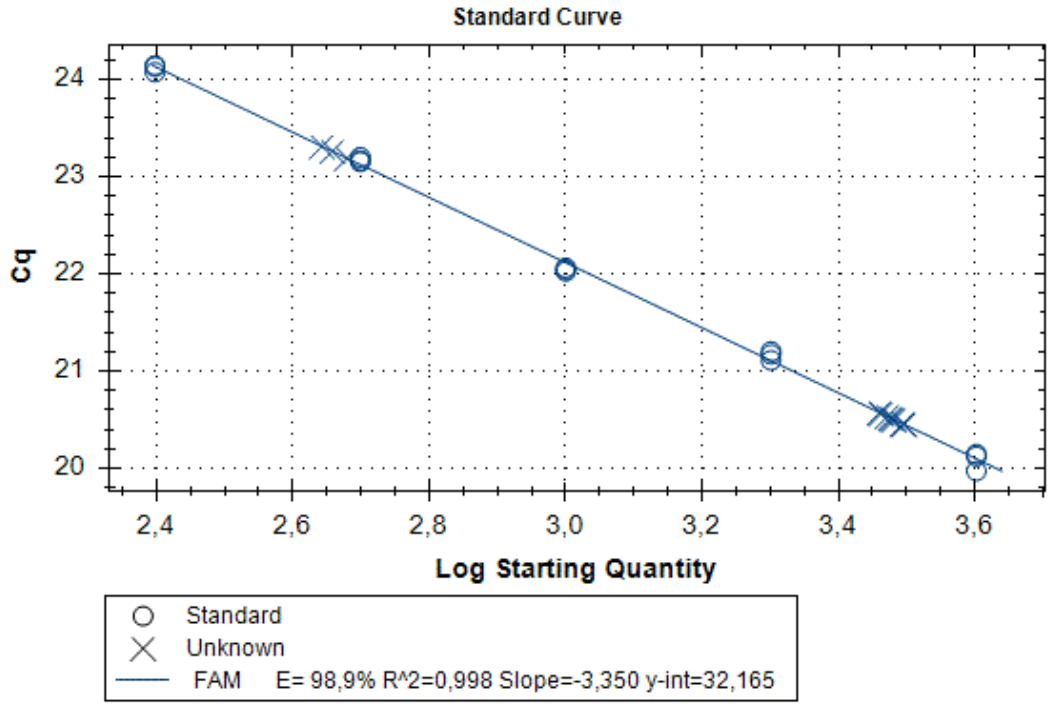
11. Perform real-time PCR according to the selected amplification profile (with fluorescence detection in FAM channel).

Perform a real-time PCR as follows: 3 minutes at 95° C; 5 "blind" cycles: 95° C - 10 seconds; 65° C - 20 seconds; 72°C - 10 seconds; 40 cycles: 95°C — 10 seconds; 65°C — 20 seconds (with detection of a fluorescent signal in the FAM channel); 72°C — 10 seconds. This thermo cycling profile is one of the possible ones and may vary depending on the type of thermal cycler.

The fluorescence growth curves are shown below (Fig.1). Fig.2 demonstrates a graph of Cq dependence on the DNA concentration of standards (-o -) with indication of Cq values for Hela and L-68 DNA (-x -). Fig.3, Fig.4 and Fig.5 show the fluorescence growth curves for DNA HeLa, DNA L68 and the standards DNA, properly.

The results are summarized in Table 1. As can be seen from the data presented in the table, the concentration of the RARB DNA fragment is approximately the same in Lc, Le, and Hc samples and is about 3000 copies at each point of the triplet. At the same time, in He samples, the DNA concentration is about 460 copies, which is 15%. Thus, ACGC site in the RARB gene in DNA L68 is slightly methylated (about 5%), whereas in DNA HeLa it is methylated for 85%.


Amplification
Real-time PCR
RFU (10^3)
Cycles
0
5
10
15
20
0
10
20
30
40


Standard Curve
Cq
Log Starting Quantity
20
21
22
23
24
2,4
2,6
2,8
3,0
3,2
3,4
3,6
Standard
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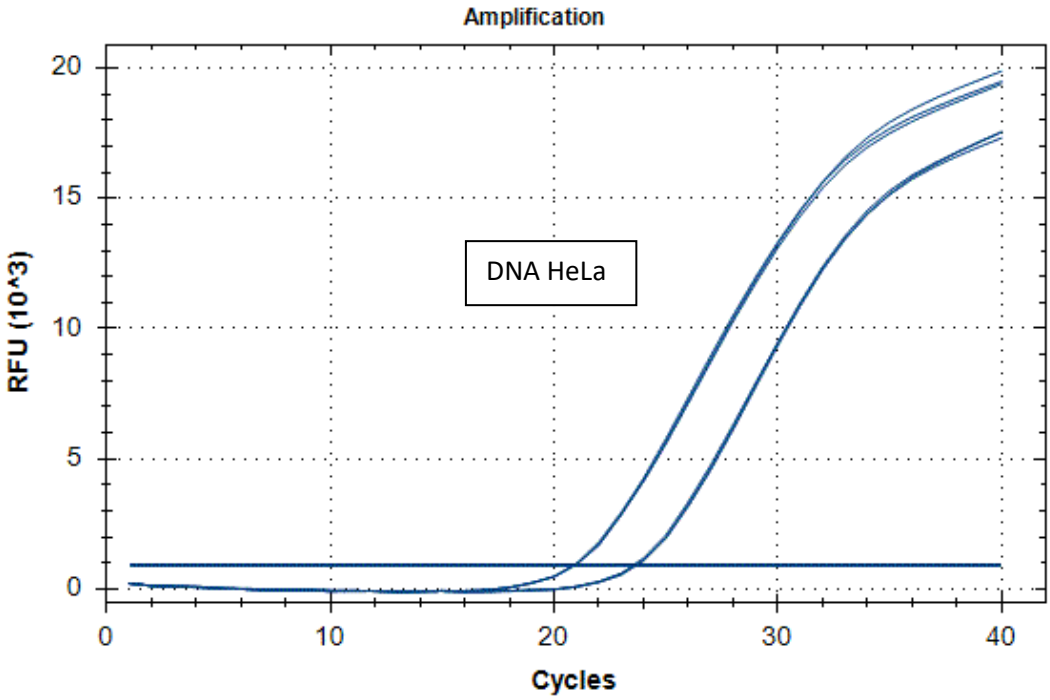
FAM E= 98,9% R^2=0,998 Slope=-3,350 y-int=32,165


Amplification
RFU (10^3)
20
15
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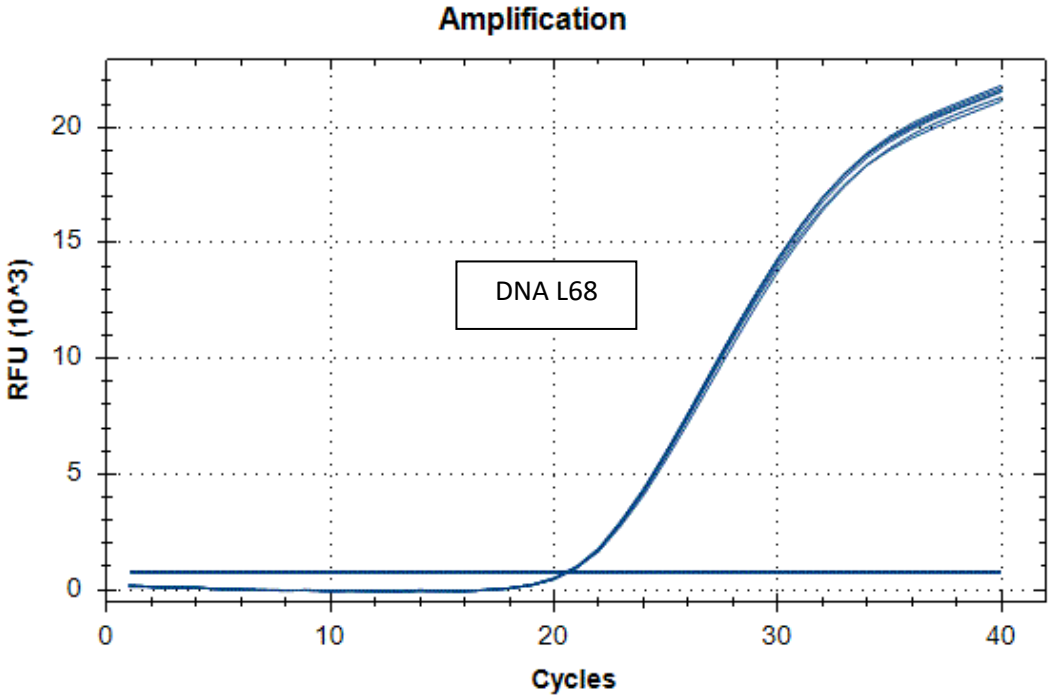
40
Cycles
DNA HeLa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RFU (10^3)
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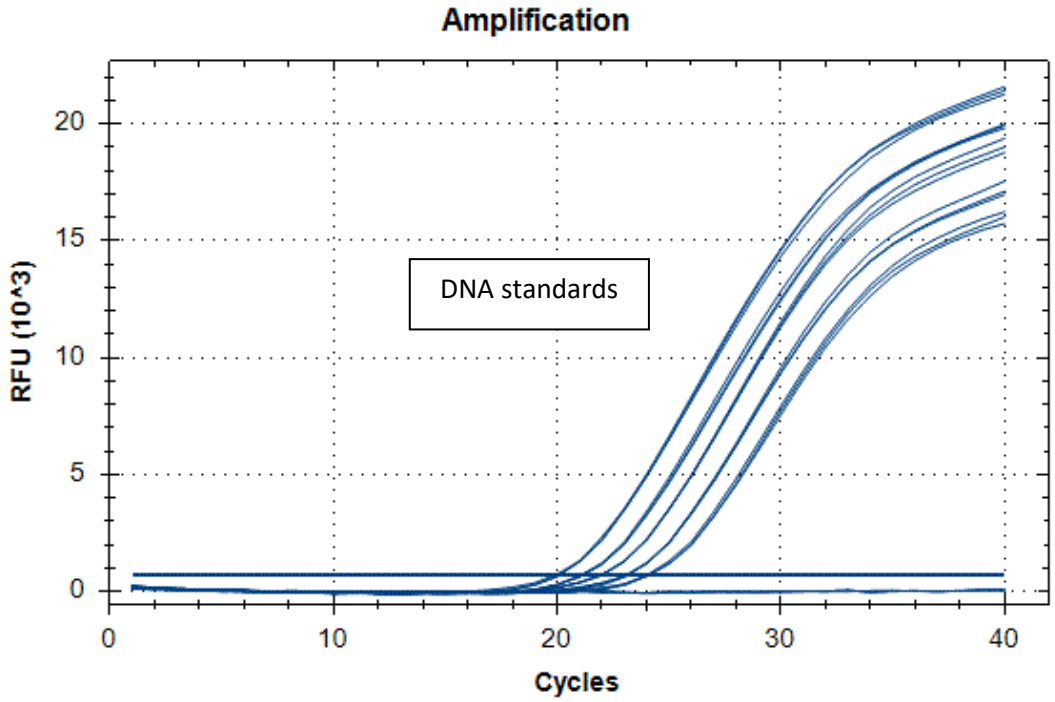
Cycles
DNA L68



References

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2. Tarasova G. V., Nayakshina T. N., Degtyarev S. Kh. Substrate specificity of new methyl-directed DNA endonuclease GlaI. // BMC Molecular Biology 2008, 9:7
3. EV Dubinin, AG Akishev, MA Abdurashitov, SB Oleynikova, VL Sitko, and S Kh Degtyarev. Real time GlaI-PCR assay of regulation regions of human genes HDAC4, RARB and URB1// Research Journal of Pharmaceutical, Biological and Chemical Sciences, vol 7(2), p.p. 667-676 (2016).
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5. Gonchar D.A., Akishev A.G., Degtyarev S.Kh. BlsI- and GlaI-PCR assays – a new method of DNA methylation study // Ovchinnikov bulletin of biotechnology and physical and chemical biology , V.6, No 1, p. 5-12 (2010)

Ordering information

Product description	Catalog No.
GlaI-PCR Assay Kit, 200 rxns	K011S
GlaI-PCR Assay Kit, 1000 rxns	K011L

For individual sale	Catalog No.	Amount(s)
T4 DNA Ligase (200 units/µL)	E319/E320	10000/50000 units
10X SE GLAD Buffer	B013	1 mL
dNTP Mix, 10 mM each	N025	40 µmol
SP Taq DNA Polymerase (5 units/µL)	E333/E334	200/1000 units

Services

Please get in touch with us (info@sibenzyme.com) if you need our assistance with primers design for your R(5mC)GY site of interest.

Primers mix (primers + TaqMan probe) for analysis of your R(5mC)GY site of interest may be synthesized by customer or ordered at SibEnzyme separately.