New enzymes for DNA technologies

SibEnzyme

INSTRUCTION MANUAL

GLAD-PCR Assay Kit

Catalog No. **K009S, K009L, K010/1, K010/3, K010/5, K010/32**

Highlights

- Study of DNA methylation in human and mammalians
- Determination of presence and quantity of R(5mC)GY site in the point of interest in analyzed DNA by comparison with the calibration line
- Assay is based on the primers and TaqMan probe designed by a researcher
- All reagents for control experiments and formation of the calibration line are included
- Less than 4 hours for assay including DNA hydrolysis, adapter ligation and PCR
- **NO BISULFITE CONVERSION**

Contents

Web page

http://sibenzyme.com/products/kits

Ver 1.2

Product Contents

GLAD PCR Assay Kit		K009S 200 reactions	K009L 1000 reactions	K010/1*, K010/3, K010/5, K010/32
1	1X TE Buffer	200 µL	1 mL	1 mL
2	10X SE TMN Buffer	160 µL	800 µL	160 µL
3	DMSO	80 µL	400 µL	80 µL
4	BSA, 10 mg/mL	70 µL	350 µL	70 µL
5	MD endonuclease (20 u/µL)	10 µL	35 µL	10 µL
6	Universal adapter, double-stranded (10 µM)	110 µL	550 µL	110 µL
7	ATP, 10 mM	110 µL	550 µL	110 µL
8	T4 DNA Ligase (200 u/µL)	100 µL	450 µL	100 µL
9	10X SE GLAD Buffer	430 µL	2.2 mL	430 µL
10	$MgCl_2$, 50 mM	120 µL	600 µL	120 µL
11	dNTP Mix, 10 mM each	90 µL	450 µL	90 µL
12	SP Taq DNA Polymerase, 5 u/µL	40µL	200 µL	40 µL
13	Control DNA Raji, 18 ng/µL	10 µL	50 µL	10 µL
14	Control DNA HeLa, 18 ng/µL	10 µL	50 µL	10 µL
15	Control DNA λ, 18 ng/ µL	25 µL	125 µL	25 µL
16	Control URB1 mix (primers + TaqMan probe), 10 µM each	40 µL	40 µL	40 µL
17	Control CEBPD mix (primers + TaqMan probe), 10 µM each	15 µL	15 µL	15 µL
18	Hybrid primers*, (20 µM)	—	—	N vials, 170 µL each

Storage conditions: -20°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 GLAD-PCR Assay kit contains all reagents for analysis of any R(5mC)GY site in human or mammalian genomes except TaqMan Probe and primers. The sequences of these oligonucleotides should correspond to the DNA region nearby the analyzed R(5mC)GY site (see page 4 for protocol).

GLAD-PCR Assay of two R(5mC)GY sites in human genome are used as an example of the method application. Mixes of TaqMan Probes and primers for analysis of only these two R(5mC)GY sites are included:

1. "Control URB1 mix (primers + TaqMan probe)" (p.16) is provided for analysis of A(5mC)GT site in regulation region of URB1 gene. Position of this site (according to a human genome assembly GRCh38/hg38) is 32334291-32334294 in chromosome 21.
2. "Control CEBPD mix (primers + TaqMan probe)" (p.17) is provided for analysis of G(5mC)GC site in regulation region of CEBPD gene. Position of this site (according to a human genome assembly GRCh38/hg38) is 47738502-47738505 in chromosome 8.

* You can choose 1, 3, 5 hybrid primers or order full range of 32 hybrid primers from the list in Appendix 3

Product description

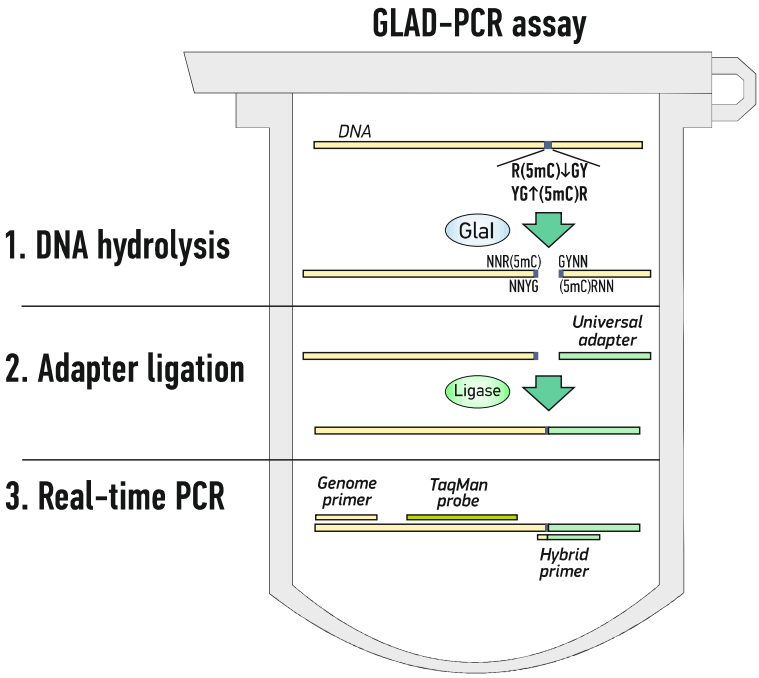
Today an abnormal methylation of regulation regions (promoter and/or first exon) of genes was shown at initial stage of several deceases such as cancer, cardiovascular decease, diabetes and some others.

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].

The site-specific methyl-directed DNA endonuclease (MD endonuclease), included in the set, 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].

GLAD-PCR assay is based on application of the MD endonuclease such as GlaI [3] and includes three steps:

1. Hydrolysis of studied DNA with MD endonuclease
2. Ligation with the universal adapter
3. Subsequent real-time PCR with Taqman probe.
   One primer is designed for DNA region of interest, structure of another primer is based on an adapter sequence.



PCR product is produced only if R(5mC)GY site is present in the point of interest in studied DNA.

GLAD PCR assay has been developed to determine minimal quantities of 5'-R(5mC)^GY-3'/3'YG^(5mC)R-5' sites in a presence of unmethylated DNA. Such DNA preparations are typical for clinical samples of blood and tissues. GLAD PCR assay allows to determine methylation of RCGY site of interest in human and mammalian genomes.

GLAD-PCR assay is performed in one tube, takes about 3-4 hours and determines even several copies of R(5mC)GY site of interest.

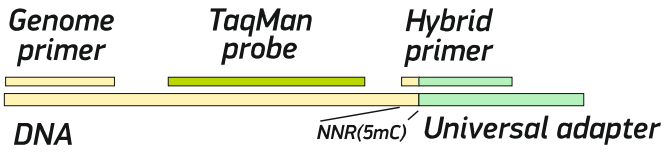
In comparison with other methylation detection methods GLAD-PCR has strong advantages:

- Simple – 3 easy steps
- Requires only real time PCR-machine
- Quick - only 3-4 hours
- Sensitive – detects even several copies of selected R(5mC)GY site

Protocol

Preparation of primers and probe

Genomic primer and TaqMan probe located near 5'-R(5mC)^GY-3' site of interest are designed as usual [4].



Hybrid primer is a DNA sequence 5'-CCTGCTCTTTCATCGGYNN-3 ', wherein 5'-end of 15-nucleotide primer corresponds to universal adapter and tetranucleotide part at the 3'-end (underlined) is complementary to the genomic sequence at MD endonuclease hydrolysis point. This structure implies the existence of 32 variants of hybrid primers* corresponding to different possible terminal sequences after MD endonuclease hydrolysis of all possible variants of NNR(5mC)^GY sequence.

We recommend to carry out GLAD-PCR assay of each DNA sample in triplet.

Primers mix (2.5 µL for one triplet) consists of genomic primer, hybrid primer and TaqMan probe in 10 µM concentration of each.

DNA preparation

1. Prepare the solutions of N studied DNAs in Milli-Q H_2O or in TE buffer if you are planning to store DNA solutions.

Stage 1: DNA hydrolysis

2. Use 200 µL PCR-microtubes for DNA hydrolysis, mix 14 µL H_2O and 1 µL of each studied DNAs in N tubes.

3. Prepare DNA Hydrolysis Mix for N tubes:
(3.6 µL·N H_2O + 2.2 µL·N 10X SE TMN Buffer + 0.5 µL·N DMSO + 0.2 µL·N BSA); Vortex, Briefly Centrifuge (VBC). Add 0.1 µL·N of MD endonuclease (mix by pipette tip carefully, without bubbles). We recommend use N=12 or more.

4. Add 6.5 µL of DNA Hydrolysis Mix to each of N tubes with DNA solutions (mix by pipette tip carefully, without bubbles). Incubate 30 min at 37 °C, discard droplets of condensate by centrifugation.

Stage 2: Universal adapter ligation

5. Prepare DNA Ligation Mix for N tubes:
(4.6 µL·N H_2O + 1.6 µL·N Adapter + 1.6 µL·N ATP + 1.2 µL·N T4 DNA Ligase); (VBC).

6. Add 8.5 µL of DNA Ligation Mix to each tube with digested DNA (p.4); (VBC).

7. Incubate 15 min at 25 °C.

Stage 3: Real-time PCR

8. Prepare PCR Mix for studied R(5mC)GY site:
(19 µL·N H_2O + 6.3 µL·N 10X SE GLAD Buffer + 1.6 µL·N $MgCl_2$ + 1.3 µL·N dNTP Mix + 0.6 µL·N BSA + 2.5 µL·N the selected primers + TaqMan probe mix + 0.4 µL·N SP Taq DNA pol) (VBC after added reagents).

9. Add 30 µL of PCR Mix to each tube after DNA ligation (p.7) (VBC).

10. Withdraw 20 µL of the reaction mixture 3 times (triplet) from each tubes and place them in PCR plate (totally Nx3 samples, maximum 96), seal, centrifuge, place into thermocycler.

* See Appendix 3 for a list of hybrid primers

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

Appendix 1: Protocol of the A(5mC)GT site determination in regulation region of URB1 gene and G(5mC)GC site in regulation region of CEBPD gene [4]

Below we provide a protocol of GLAD-PCR assay for 6 triplets of control Lambda DNA, HeLa DNA and Raji DNA:

1. Mark 6 PCR-microtubes (200 µL) with letters A,B,C,D,E,F; mix 14 µL H_2O and 1 µL DNA in each tube (A, B - Lambda DNA; C, D - HeLa DNA; E, F - Raji DNA).

2. Prepare DNA Hydrolysis Mix:
22.9 µL H_2O + 13.5 µL 10X SE TMN Buffer + 2.7 µL DMSO + 1.3 µL BSA; Vortex, Briefly Centrifuge (VBC).
Add 0.6 µL of MD endonuclease (mix by pipette tip carefully, without bubbles).

3. Add 6.5 µL of DNA Hydrolysis Mix to each tube (mix by pipette tip carefully, without bubbles).

4. Incubate 30 min at 37 °C, discard droplets of condensate by centrifugation.

5. Prepare DNA Ligation Mix:
27.4 µL H_2O + 9.6 µL Adapter + 9.6 µL ATP + 7.2 µL T4 DNA Ligase; (VBC).

6. Add 8.5 µL of DNA Ligation Mix to each tube with digested DNA (p.4); (VBC).

7. Incubate 15 min at 25 °C.

8. Prepare PCR Mix for URB1 for 3 triplets:
57 µL H_2O + 19 µL 10X SE GLAD Buffer + 4.8 µL $MgCl_2$ + 3.9 µL dNTP mix + 1.9 µL BSA + 7.5 µL URB1 mix + 0.6 µL SP Taq DNA pol; (VBC).

9. Prepare PCR Mix for CEBPD for 3 triplets:
57 µL H_2O + 19 µL 10X SE GLAD Buffer + 4.8 µL $MgCl_2$ + 3.9 µL dNTP + 1.9 µL BSA + 7.5 µL CEBPD mix + 0.6 µL SP Taq DNA pol; (VBC).

10. Add 30 µL of PCR Mix for URB1 to tube A, to tube C and to tube E; (VBC).

11. Add 30 µL of PCR Mix for CEBPD to tube B, to tube D and to tube F; (VBC).

12. Withdraw 20 µL of the reaction mixture 3 times (triplet) from each tube and place them in PCR plate (totally 18 samples), SEAL, CENTRIFUGE, PLACE INTO THERMOCYCLER.

13. Perform the real-time PCR: in case of CFX-96 Thermal Cycler Amplifier" (Bio-Rad, USA) the amplification profile is as follows: 3 min at 95 °C; 5 “blind” cycles: 95 °C for 10 sec; 63 °C for 20 sec; 72 °C for 5 sec; 40 cycles: 95 °C for 10 sec; 63 °C for 20 sec (with fluorescence detection in FAM channel); 72 °C for 5 sec. Amplification profile may differ for another PCR amplification system.

Expected results of control experiment

Region \ DNA	Raji DNA	HeLa DNA	Lambda DNA (negative control)
URB1	(+) Methylated	(+) Methylated	(-)
CEBPD	(+) Methylated	(-) Unmethylated	(-)

Appendix 2: Protocol of the calibration line formation for determination of the number of DNA copies carrying A(5mC)GT site in regulation region of URB1 gene [4]

Below we provide a protocol of GLAD-PCR assay for determination of the number of DNA copies carrying A(5mC)GT site in regulation region of URB1 gene. One tube with control Lambda DNA and 5 tubes with dilutions of Raji DNA have been used to obtain the calibration line.

1. Mark 6 PCR-microtubes (200 µL) with numbers 1-6; add 28 µL H_2O to the vial 1, add 15 µL H_2O to vials 2, 3, 4, 5 and add 14 µL H_2O to the vial 6. Add 2 µL of Raji DNA to the vial 1, mix carefully. Withdraw 15 µL of DNA solution from vial 1 and add this aliquot to vial 2, mix carefully. Withdraw 15 µL of DNA solution from vial 2 and add this aliquot to vial 3, mix carefully. Withdraw 15 µL of DNA solution from vial 3 and add this aliquot to vial 4, mix carefully. Withdraw 15 µL of DNA solution from vial 4 and add this aliquot to vial 5, mix carefully. Withdraw 15 µL of DNA solution from vial 5 and discard this aliquot. Add 1 µL of Lambda DNA to the vial 6, mix carefully, briefly centrifuge.

2. Prepare DNA Hydrolysis Mix:
22.9 µL H_2O + 13.5 µL 10X SE TMN Buffer + 2.7 µL DMSO + 1.3 µL BSA; after each addition mix carefully, briefly centrifuge. Add 0.6 µL of MD endonuclease, mix by the pipette tip carefully, without bubbles, briefly centrifuge.

3. Add 6.5 µL of DNA Hydrolysis Mix to each tube, mix by pipette tip carefully, without bubbles, briefly centrifuge.

4. Incubate 30 min at 37 °C, discard droplets of condensate by centrifugation.

5. Prepare DNA Ligation Mix:
27.4 µL H_2O + 9.6 µL Adapter + 9.6 µL ATP + 7.2 µL T4 DNA Ligase; after each addition mix carefully, briefly centrifuge.

6. Add 8.5 µL DNA Ligation Mix to each tube with digested DNA (p.4); mix carefully, briefly centrifuge.

7. Incubate 15 min at 25 °C.

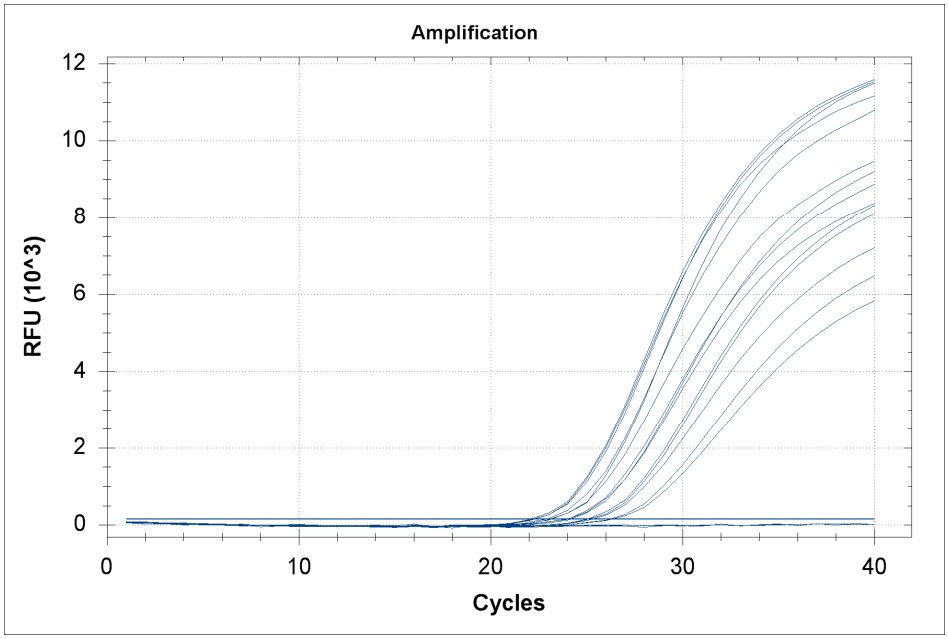
8. Prepare PCR Mix for A(5mC)GT site in regulation region of URB1 gene:
114 µL H_2O + 38 µL 10X SE GLAD Buffer + 9.6 µL $MgCl_2$ + 7.8 µL dNTP mix + 3.8 µL BSA + 15 µL URB1 mix + 1,2 µL SP Taq DNA pol; after each addition mix carefully, briefly centrifuge.

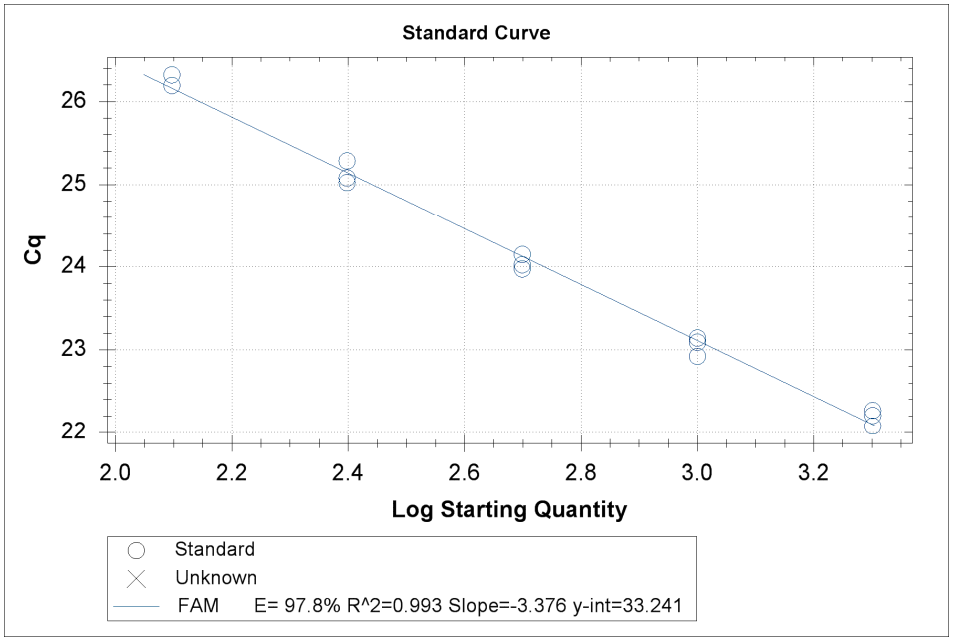
9. Add 30 µL of PCR Mix for URB1 to tubes 1, 2, 3, 4, 5 and 6. Mix carefully, briefly centrifuge.

10. Withdraw 20 µL of the reaction mixture 3 times (triplet) from each tube and place them in PCR plate (totally 18 samples), SEAL, CENTRIFUGE, PLACE INTO THERMOCYCLER.

11. Perform the real-time PCR: in case of CFX-96 Thermal Cycler Amplifier" (Bio-Rad, USA) the amplification profile is as follows: 3 min at 95 °C; 5 “blind” cycles: 95 °C for 10 sec; 63 °C for 20 sec; 72 °C for 5 sec; 40 cycles: 95 °C for 10 sec; 63 °C for 20 sec (with fluorescence detection in FAM channel); 72 °C for 5 sec. Amplification profile may differ for another PCR amplification system.

Expected results of experiment





Appendix 3: Hybrid primers sequences

HP1:	5' CCTGCTCTTTCATCGGCAA 3'	(19)	HP17	5' CCTGCTCTTTCATCGGTAA 3'	(19)
HP2:	5' CCTGCTCTTTCATCGGCAC 3'	(19)	HP18	5' CCTGCTCTTTCATCGGTAC 3'	(19)
HP3:	5' CCTGCTCTTTCATCGGCAG 3'	(19)	HP19	5' CCTGCTCTTTCATCGGTAG 3'	(19)
HP4:	5' CCTGCTCTTTCATCGGCAT 3'	(19)	HP20	5' CCTGCTCTTTCATCGGTAT 3'	(19)
HP5:	5' CCTGCTCTTTCATCGGCCA 3'	(19)	HP21	5' CCTGCTCTTTCATCGGTCA 3'	(19)
HP6:	5' CCTGCTCTTTCATCGGCCC 3'	(19)	HP22	5' CCTGCTCTTTCATCGGTCC 3'	(19)
HP7:	5' CCTGCTCTTTCATCGGCCG 3'	(19)	HP23	5' CCTGCTCTTTCATCGGTCG 3'	(19)
HP8:	5' CCTGCTCTTTCATCGGCCT 3'	(19)	HP24	5' CCTGCTCTTTCATCGGTCT 3'	(19)
HP9:	5' CCTGCTCTTTCATCGGCGA 3'	(19)	HP25	5' CCTGCTCTTTCATCGGTGA 3'	(19)
HP10	5' CCTGCTCTTTCATCGGCGC 3'	(19)	HP26	5' CCTGCTCTTTCATCGGTGC 3'	(19)
HP11	5' CCTGCTCTTTCATCGGCGG 3'	(19)	HP27	5' CCTGCTCTTTCATCGGTGG 3'	(19)
HP12	5' CCTGCTCTTTCATCGGCGT 3'	(19)	HP28	5' CCTGCTCTTTCATCGGTGT 3'	(19)
HP13	5' CCTGCTCTTTCATCGGCTA 3'	(19)	HP29	5' CCTGCTCTTTCATCGGTTA 3'	(19)
HP14	5' CCTGCTCTTTCATCGGCTC 3'	(19)	HP30	5' CCTGCTCTTTCATCGGTTC 3'	(19)
HP15	5' CCTGCTCTTTCATCGGCTG 3'	(19)	HP31	5' CCTGCTCTTTCATCGGTTG 3'	(19)
HP16	5' CCTGCTCTTTCATCGGCTT 3'	(19)	HP32	5' CCTGCTCTTTCATCGGTTT 3'	(19)

References

1. Handa, V., and Jeltsch A. “Profound sequence preference of Dnmt3a and Dnmt3b mammalian DNA methyltransferases shape the human epigenome” // 2005, J. Mol. Biol. 348, 1103-1112

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. Kuznetsov V.V., Abdurashitov M.A., Akishev A.G., Degtyarev S.Kh. “Method of determining nucleotide sequence Pu(5mC)GPy at predetermined position of continuous DNA” // Patent RU 2525710

4. A.G. Akishev et al. “GLAD-PCR assay of selected R(5mC)GY sites in URB1 and CEPBD genes in human genome” // Res J Pharm Biol Chem Sci, 8(1): pp.465-475, 2017

Ordering information

Product description	Catalog No.
GLAD PCR Assay Kit, 200 rxns	K009S
GLAD PCR Assay Kit, 1000 rxns	K009L
GLAD PCR Assay Kit with hybrid primers, 200 rxns, 1 hybrid primer	K010/1
GLAD PCR Assay Kit with hybrid primers, 200 rxns, 3 hybrid primers	K010/3
GLAD PCR Assay Kit with hybrid primers, 200 rxns, 5 hybrid primers	K010/5
GLAD PCR Assay Kit with hybrid primers, 200 rxns, 32 hybrid primers	K010/32

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.