# GLAD PCR analysis of aberrant DNA methylation in cancer

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GlaI hydrolysis and Ligation Adapter Dependent PCR (GLAD PCR) is the novel method to determine R(5mC)GY sites produced by methylation with DNMT3A and DNMT3B. GLAD PCR analysis is performed in one tube and includes 3 steps: DNA hydrolysis with site-specific methyl-directed DNA endonuclease GlaI, universal adapter ligation and 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. GLAD PCR analysis has been used to study an aberrant methylation of regulatory regions of RARB, EGFR, HS3ST2, TWIST1 and some other genes in malignant cell lines. GLAD PCR analysis allows detecting several copies of methylated DNA and may be used in routine laboratory and clinical practice.

## Introduction

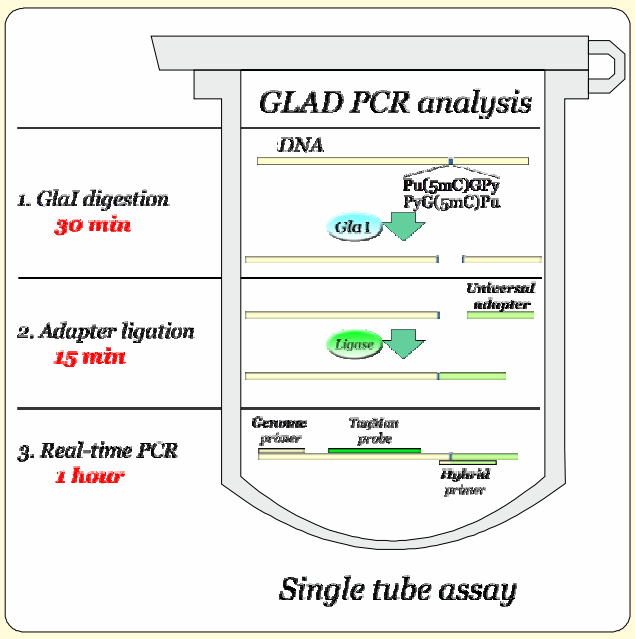
*De novo* DNA methylation in mammals is performed by **DNMT3A** and **DNMT3B** DNA methyltransferases.

PuCGPy / PyGCPu —DNMT / AdoMet→ Pu(5mC)GPy / PyG(5mC)Pu

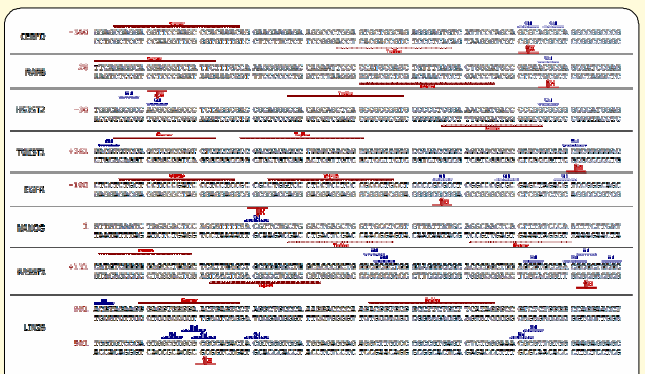
Recently we have discovered and characterized a new DNA-endonuclease **GlaI** [1]. GlaI belongs to the **novel** type of site-specific **methyl-directed** DNA-endonucleases which hydrolyze **only methylated DNA**. GlaI recognizes DNA sequence **Pu(5mC)GPy**.

Pu(5mC)GPy / PyG(5mC)Pu —GlaI→ Pu(5mC)↓ G Py / Py G ↑(5mC)Pu

## Method




## Studied genes




1 — transcription start. H — position of hybrid primer.

## Studied DNA

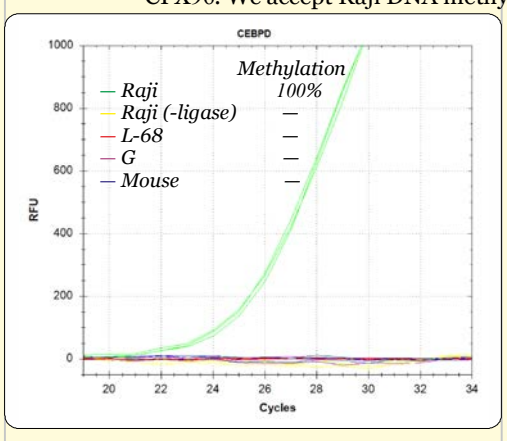
***Malignant cell lines***
***Raji*** — Burkitt's lymphoma,
***Jurkat*** — acute T-cell leukemia,
***U-937*** — histiocytic lymphoma,
***HeLa*** — cervix adenocarcinoma,

***Control***
***L-68*** — fibroblast cell line,
***G*** — human peripheral blood DNA,
***Mouse*** — A/He mouse DNA, negative control

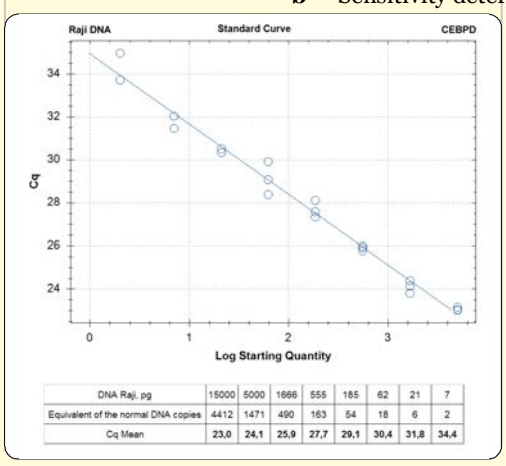
## GLAD PCR analysis of aberrant methylation in regulatory regions of tumor suppressor genes

**Fig. 1.** GLAD PCR analysis of DNA methylation in regulatory region of **CEBPD** tumor suppressor gene. RCGY site is in positions -270..-267 from transcription start.

**a** — Amplification chart of GLAD PCR assay of 15 ng DNA per reaction using Bio-Rad CFX96. We accept Raji DNA methylation to be 100% according to [2,3].

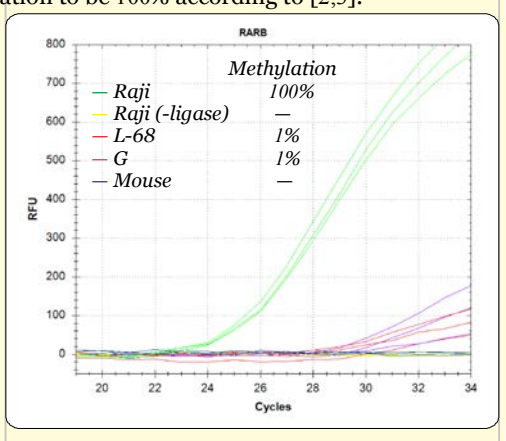


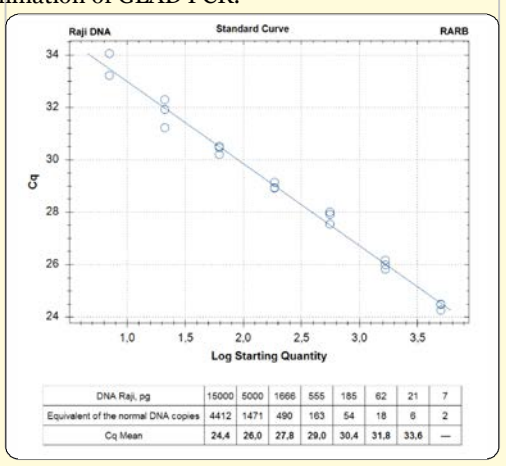

**b** — Sensitivity determination of GLAD PCR.




| DNA Raji, pg | 15000 | 5000 | 1666 | 555 | 185 | 62 | 21 | 7 |
|---|---|---|---|---|---|---|---|---|
| Equivalent of the normal DNA copies | 4412 | 1471 | 490 | 163 | 54 | 18 | 6 | 2 |
| Cq Mean | 23,0 | 24,1 | 25,9 | 27,7 | 29,1 | 30,4 | 31,8 | 34,4 |

**Fig. 2.** GLAD PCR analysis of DNA methylation in regulatory region of **RARB** tumor suppressor gene. RCGY site is in positions 112..115 from transcription start.




| DNA Raji, pg | 15000 | 5000 | 1666 | 555 | 185 | 62 | 21 | 7 |
|---|---|---|---|---|---|---|---|---|
| Equivalent of the normal DNA copies | 4412 | 1471 | 490 | 163 | 54 | 18 | 6 | 2 |
| Cq Mean | 24,4 | 26,0 | 27,8 | 29,0 | 30,4 | 31,8 | 33,6 | — |

It is well known that an abnormal methylation of certain DNA regions, mostly promoter and first exon of tumor suppressor genes, is a serious sign of cell malignancy. Differences in methylation of CpG island of these regions has been observed for many cancer cell lines and earlier we have developed PCR assay to discriminate malignant cell lines based on different pattern of CpG islands methylation [3].

Here we have shown that a number and positions of methylated RCGY sites in regulatory regions of tumor suppressor genes vary in different malignant cell lines. It allows to consider a characterization of cancer cell lines based on the results of GLAD PCR assay of the selected RCGY sites located in regulation region of tumor suppressor genes.

Cancer lines typing based on GLAD PCR assay of regulatory regions takes into account an epigenetic issue for differentiation of these cell lines and may be used for a rapid epigenetic characterization of malignant cell lines.

**Table 1.** Epigenetic typing of human cancer cell lines. GLAD PCR assay of 15 ng DNA per reaction. We accept Raji DNA methylation to be 100% for all tumor suppressor genes.

| Gene \ DNA | Raji | Jurkat | U-937 | Hela | L-68 | G | Mouse |
|---|---|---|---|---|---|---|---|
| RASSF1A | 100 | 55 | — | — | — | — | — |
| CEBPD | 100 | 1,3 | 1,0 | — | — | — | — |
| TWIST1 | 100 | 1,0 | 0,5 | — | — | — | — |
| EGFR | 100 | 1,0 | 1,2 | — | — | — | — |
| LIN28 | 100 | 90 | 100 | 90 | — | — | — |
| RARB | 100 | 60 | 90 | 100 | 1,0 | 1,0 | — |
| HS3ST2 | 100 | 12 | 13 | 55 | 0,5 | 0,5 | — |
| NANOG* | 1,1 | 0,4 | 0,4 | 1,1 | 55 | 100 | — |

* — gene regulator of cell differentiation

Methylation, % | 0 | <1,5 | 1,5-50 | 50-100 |

**RASSF1A** — Ras association (RalGDS/AF-6) domain family member 1
**CEBPD** — CCAAT/enhancer binding protein (C/EBP), delta
**TWIST1** — twist basic helix-loop-helix transcription factor 1
**EGFR** — epidermal growth factor receptor
**LIN28** — RNA-binding protein LIN-28
**RARB** — retinoic acid receptor, beta
**HS3ST2** — heparan sulfate (glucosamine) 3-O-sulfotransferase 2
**NANOG** — homeobox protein NANOG

## Reference

**1.** BMC Mol Biol. 2008 Jan 15;9:7. doi: 10.1186/1471-2199-9-7.
**2.** http://science.sibenzyme.com/article12_article_53_1.phtml
**3.** http://science.sibenzyme.com/article8_article_58_1.phtml

**Patent is pending**

## Conclusions

- A new method of GLAD PCR assay has been developed to study DNA methylation. Method includes GlaI hydrolysis of studied DNA, the universal adapter ligation and subsequent real-time PCR of the studied RCGY site. Method is performed in one tube, takes about four hours and allows to determine several copies of methylated DNA.
- GLAD PCR assay has been applied to study aberrant methylation of selected RCGY sites in regulatory region of tumor suppressor genes. GLAD PCR assay has revealed different patterns of RCGY sites methylation in four malignant cell lines. All studied RCGY sites are highly methylated in Raji cells and unmethylated in control fibroblast line.
- GLAD PCR assay may be used for determination of methylation status of particular RCGY sites and for a rapid epigenetic characterization of malignant cells.

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