Biol. Chem., Vol. 381, pp. 269 – 272, March 2000 · Copyright © by Walter de Gruyter · Berlin · New York

Short Communication

Biotin-Avidin Microplate Assay for the Quantitative Analysis of Enzymatic Methylation of DNA by DNA Methyltransferases

Markus Roth and Albert Jeltsch*

Institut für Biochemie, Fachbereich 8,
Justus-Liebig-Universität, Heinrich-Buff-Ring 58,
D-35392 Gießen, Germany

* Corresponding author

An assay is described to measure methylation of biotinylated oligonucleotide substrates by DNA methyltransferases using [methyl-^{3}H]-AdoMet. After the methylation reaction the oligonucleotides are immobilized on an avidin-coated microplate. The incorporation of [^{3}H] into the DNA is quenched by addition of unlabeled AdoMet to the binding buffer. Unreacted AdoMet and enzyme are removed by washing. To release the radioactivity incorporated into the DNA, the wells are incubated with a non-specific endonuclease and the radioactivity determined by liquid scintillation counting. As an example, we have studied methylation of DNA by the *Eco*RV DNA methyltransferase. The reaction progress curves measured with this assay are linear with respect to time. Methylation rates linearly increase with enzyme concentration. The rates are comparable to results obtained with the same enzyme using a different assay. The biotin-avidin assay is inexpensive, convenient, quantitative, fast and well suited to process many samples in parallel. The accuracy of the assay is high, allowing to reproduce results within ± 10%. The assay is very sensitive as demonstrated by the detection of incorporation of 0.8 fmol methyl groups into the DNA. Under the experimental conditions, this corresponds to methylation of only 0.03% of all target sites of the substrate. Using this assay, the DNA methylation activity of some M.*Eco*RV variants could be detected that was not visible by other *in vitro* methylation assays.

Key words: DNA methyltransferase / DNA modification / *Eco*RV / Enzyme assay / Protein-DNA interaction.

Methylation of DNA occurs in most eukaryotes and prokaryotes and is essential in mammalia (reviews: Noyer-Weidner and Trautner, 1993; Adams, 1995; Cheng, 1995a, b; Ahmad and Rao, 1996a; Siegfried and Cedar, 1997; Schluckebier *et al*., 1998). It is involved in the control of DNA-replication, post-replicative repair and regulation of transcription. DNA nucleobases can be methylated at the N^6 position of adenine, N^4 position of cytosine or C^5 position of cytosine. The modification is introduced post-replicative by DNA methyltransferases (MTases) which use S-adenosylmethionine (AdoMet) as a donor for an activated methyl group. Despite being developed over several years, MTase assays still are notoriously difficult. Usually radioactively labeled AdoMet is employed which carries a methyl group labeled either with [^{14}C] or [^{3}H]. To detect low amounts of methylation, [methyl-^{3}H]-AdoMet is preferable, because it is available at much higher specific activity (2 – 3 TBq/mmol) than [methyl-^{14}C]-AdoMet (2 GBq/mmol). After the methylation reaction, the labeled DNA must be separated from unincorporated AdoMet and the incorporation of [^{3}H] into the DNA measured. This is usually achieved by spotting the methylated DNA onto a DE-cellulose filter sheet followed by washing with buffer, ethanol and ether, drying and counting of the dried filter plates (Rubin and Modrich, 1977). This assay has been used very often (see Reich and Mashhoon, 1991; Mi and Roberts, 1992; Dryden *et al*., 1993; Willcock *et al*., 1994; Kossykh *et al*., 1995; Marzabal *et al*., 1995; Ahmad and Rao, 1996b for some examples). Other assays are based on coupling of the DNA to cellulose (Hübscher *et al*., 1985) or on thin-layer chromatography (Jeltsch *et al*., 1998). However, with these assays the background level of radioactivity often obscures low degrees of DNA methylation. The background is caused by AdoMet that adsorbs to the filter sheets or co-precipitates with the MTase on the filter. Moreover, since [^{3}H] is a very weak β-emitter, counting of radioactivity that is bound to a filter plate leads to a strong reduction in counting yield and accuracy.

Here, we present an alternative method to measure modification of DNA by DNA MTases: biotinylated oligonucleotide substrates are methylated using [methyl-^{3}H]-AdoMet. To separate methylated DNA form unreacted AdoMet, the DNA is bound to an avidin-coated microplate. Incorporation of [^{3}H] into the DNA is quenched by the presence of an excess of unlabeled AdoMet in the binding buffer. Subsequently, unreacted AdoMet is removed by washing. The immobilized DNA is digested with a non-specific endonuclease to release the radioactivity from the microplate. Finally, the amount of methyl groups transferred to the DNA is quantified by liquid scintillation counting of the solution obtained after nucleolytic digestion.

To demonstrate the feasibility of the new assay, DNA methylation experiments with the *Eco*RV DNA MTase were carried out. This enzyme recognizes GATATC sequences and modifies the first adenine residues within (Bougueleret *et al*., 1984; Nwosu *et al*., 1988). Figure 1A displays a time course of the methylation of 0.5 μM RVL19 DNA with 0.9 μM M.*Eco*RV, showing that the biotin-avidin MTase assay is linear with respect to time. Linear regression of the first 4 data points yields a rate of incorporation of methyl groups into the DNA of 5.5 fmol min^{-1}. Three independent experiments yielded deviations below ± 10% for each individual data point and the slopes derived from the data. As expected, no incorporation of radioactivity was observed using RVL19MM which contains a G^mATATC/G^mATATC (^mA: 6-methyladenine) recognition site, because this site is already methylated at both target adenines (Figure 1B). The experiments with RVL19MM DNA allow to estimate the background of the method to be 44 ± 9 cpm (mean and standard deviation taken from 8 independent experiments) which is close to the background signal of the counter. To find out how the observed rates depend on the enzyme concentration, methylation kinetics were car-

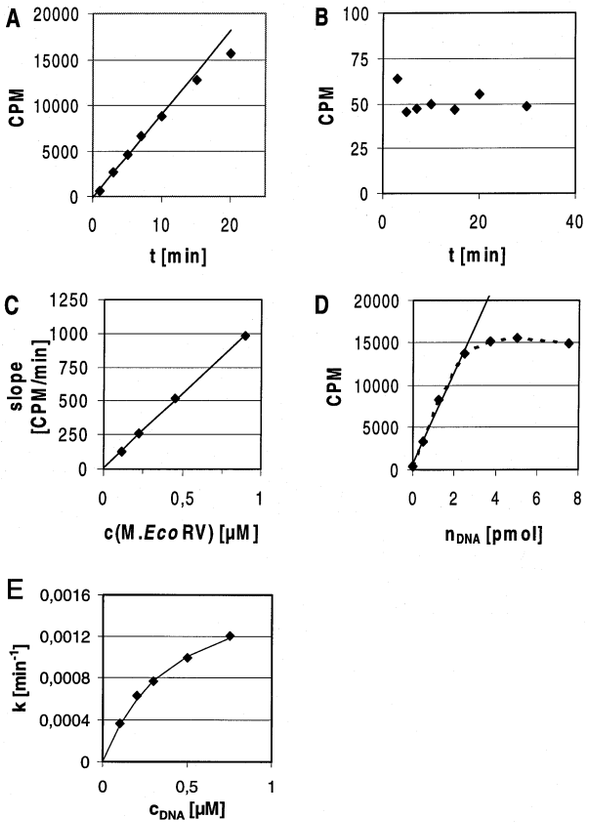

Fig. 1 Examples of Results Obtained with the Biotin-Avidin Methylation Assay.

(A) Time course of methylation of RVL19. HPLC-purified RVL19 [d(CGCGGCCGATATCCCGGGC)/d(Bt-TTTTTTGCCCGGGATATCGGCCGCG)] was purchased from Interactiva (Ulm, Germany). To coat microplates (E.I.A./R.I.A. Plate, flat bottom, high binding, Cat. No. 9018, Costar Corp., Cambridge, MA, USA) with avidin, 1 μg avidin (Sigma) dissolved in 100 μl of 100 mM $NaHCO_3$ (pH 9.6) was dispensed into each well and incubated overnight at 4 °C. The wells were washed five times with 200 μl PBST (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 0.05% v/v Tween 50, pH 7.2). Coated plates can be stored several days at 4 °C. His_6-tagged M.*Eco*RV was purified as described (Jeltsch *et al*., 1998, 1999a). Methylation reactions were carried out using 0.5 μM RVL19 oligonucleotide and 0.9 μM M.*Eco*RV in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 50 ng/μl BSA in the presence of 0.76 μM labeled [methyl-^{3}H]-AdoMet (2.68 TBq/mmol, NEN) at ambient temperature. To measure the time course of DNA methylation, aliquots comprising 10 μl were removed from the reaction mixture and pipetted into the wells of a microplate that contains 10 μl of 10 mM unlabeled AdoMet (Sigma) in 10 mM H_2SO_4 to quench the incorporation of [^{3}H] into the DNA. PBST supplemented with 500 mM NaCl and 1 mM EDTA was added to a total volume of 50 μl and the mixture incubated for 30 min to allow binding of the oligonucleotides to the microplate. The wells were washed five times with 200 μl PBST supplemented with 500 mM NaCl to remove the unreacted AdoMet and the enzyme. High salt binding and washing buffer was used to prevent binding of the MTase to the DNA. Complete removal of the MTase is important, because unreacted AdoMet could bind to the protein and thereby be retained. Subsequently, the DNA was degraded using 0.7 μg (700 U) *Serratia marcescens* nuclease in 100 μl of 50 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$ for 30 min at ambient temperature. The released radioactivity was analyzed by liquid scintillation counting of the reaction mixture after adding 2 ml Liquid Scintillator Quicksafe A (Zinsser Analytic). In this work a laboratory preparation of the *Serratia marcescens* nuclease was used. The enzyme is commercially available (Benzonase™, Merck, Darmstadt, Germany). As an alternative other non-specific endonucleases like DNaseI might be used. To avoid degradation, labeled and unlabeled AdoMet was stored in small aliquots in 10 mM H_2SO_4 at –20 °C and thawed only once.

(B) Time course of methylation of RVL19MM. HPLC-purified RVL19MM [d(CGCGGCCG^{m}ATATCCCGGGC)/d(Bt-TTTTTTGCCCGGG^{m}ATATCGGCCGCG)] was purchased from Primm srl (Milano). The methylation reaction was carried out under the same conditions as described in (A). Note that no incorporation of radioactivity is observed, because RVL19MM contains a G^mATATC/G^mATATC site that is already methylated at both target adenines.

(C) Dependence of the apparent rate of methylation of RVL19 on the enzyme concentration. The time course of methylation of 0.5 μM RVL19 was determined in the presence of 0.1–0.9 μM M.*Eco*RV as described in (A). Linear regression of the initial parts of the reaction progress curves yielded the apparent rates of DNA methylation at each enzyme concentration [cpm/min] that are shown in the Figure.

(D) Binding capacity of one well of the microplate for oligonucleotides. RVL19 (0.5 μM) was methylated using 0.9 μM M.*Eco*RV as described above. Different amounts of the reaction mixture were applied to the wells of a microplate and the samples washed, eluted as described above and the retained radioactivity analyzed by liquid scintillation counting.

(E) Michaelis-Menten analysis of the rates of DNA methylation of RVL19M [d(CGCGGCCGATATCCCGGGC)/d(Bt-TTTTTTGCCCGGG^{m}ATATCGGCCGCG)] at different DNA concentrations. Methylation reactions were carried out as described in (A). In all experiments samples containing 5 pmol DNA were removed and analyzed as described. The line shows a least-square fit of the data to the Michaelis-Menten equation (K_m = 0.43 μM, k_{cat} = 0.11 h^{-1}).

ried out with 0.5 μM RVL19 using different concentrations of M.*Eco*RV and analyzed by linear regression of the initial part of the reaction progress curves. As shown in Figure 1C, the rates depend linearly on the enzyme concentration as expected from the theory of enzymatic catalysis.

To measure the capacity of the coated microplates for binding biotinylated oligonucleotides, RVL19 DNA was methylated for 20 min under the same conditions as in Figure 1A and increasing amounts of the reaction mixture were applied to the wells of the microplate. As shown in Figure 1D, the amount of retained radioactivity increases linearly up to a load of 2.5 pmol DNA. This behavior is indicative of a complete binding of the DNA to the microplate. On the basis of the level of radioactivity observed under saturation with oligonucleotide, the capacity of one well can be estimated to be about 2.7 pmol biotinylated oligonucleotide. To ensure complete release of the bound DNA by the nuclease treatment, RVL19 was methylated as above and 5 pmol DNA bound to each well of the microplate. The wells were incubated with the nuclease for various times between 5 and 60 min. Complete release of the radioactivity was observed even after 5 min of nuclease treatment (data not shown) demonstrating that the nuclease step is very efficient.

To compare the rates of DNA methylation determined with this assay with results obtained using a different assay, we have carried out a Michaelis-Menten analysis of the methylation of RVL19M containing one hemimethylated GATATC/G^{m}ATATC site by M.*Eco*RV. As shown in Figure 1E, a K_m value of 0.43 μM and a k_{cat} of 0.11 h^{-1} was determined. Considering the K_m value for AdoMet (12 μM) (Jeltsch *et al*., 1998) and the maximum binding capacity of the microplate, the apparent k_{cat} determined in this experiment at c_{AdoMet} = 0.76 μM corresponds to a k_{cat} of 3.8 h^{-1} under saturation with AdoMet. Using [methyl-^{14}C]-labeled AdoMet and a thin layer chromatography assay we determined the k_{cat} and K_m values of the methylation of an asymmetric 20mer [d(GATCGTAGATATCGCATCGA)/d(TCGATGCGATATCTACGATC)] to be 3.9 h^{-1} and 0.39 μM (Jeltsch *et al*., 1998). This comparison confirms that the rates determined with the biotin-avidin microplate assay are reliable.

As the biotin-avidin assay has a very low background of radioactivity (Figure 1B), it should allow to demonstrate DNA methylation even at low levels. As a test case, we investigated the methylation of DNA by two M.*Eco*RV mutants that display a reduced but nevertheless detectable activity in *E. coli* cells (K16A and Y258A) (Roth *et al*., 1998). Despite of being active *in vivo*, catalytic activity could not be detected with the purified proteins *in vitro* so far (Roth *et al*., 1998). Using the same enzyme preparation as in the previous study we reinvestigated these variants with the new assay. As shown in Table 1, both of the variants were able to incorporate significant amounts of radioactivity into the DNA. After background correction the observed cpm values correspond to 0.8 fmol methyl groups incorporated into the DNA in the case of K16A and 1.7 fmol in the case of Y258A. This is a very high sensitivity, as demonstrated by the observation that DNA methylation by these variants could not be detected with several other methods (Roth *et al*., 1998 and unpublished observations). Given the capacity of the microplate for oligonucleotides under our experimental conditions (2.7 pmol), the amounts of methylation correspond to a modification of 0.03% and 0.06% of the substrate DNA in the case of K16A and Y258A, respectively. These results demonstrate that the biotin-avidin assay is well suited to detect DNA methylation at very low levels. It should be noted that the sensitivity of the biotin-avidin MTase assay is high with respect to the total amount of methyl groups transferred as well as with respect to the level of methylation of the substrate.

Table 1 Methylation of RVL20asym by M.*Eco*RV and M.*Eco*RV Mutants[1].

Mutant	cpm	Standard deviation	Number of experiments
M.*Eco*RV	35600	5100	4
K16A	170	12	4
Y258A	327	19	4
No enzyme	29	10	8

[1] Cloning, purification and characterization of GST-tagged M.*Eco*RV variants has been described earlier (Friedrich *et al*., 1998; Roth *et al*., 1998; Jeltsch *et al*., 1996b). Methylation reactions were carried out using 0.1 μM asymL20 [d(GATCGTAGATATCGCATCGA)/d(Bt-TTTTTTCGATGCGATATCTACGATC)] in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 50 ng/μl BSA in the presence of 0.76 μM labeled [methyl-^{3}H]-AdoMet at ambient temperature for 16 hours. Enzyme concentrations were 180 nM (M.*Eco*RV), 94 nM (Y258A) and 20 nM (K16A). 100 μl of the reaction mixture were transferred to the microplate. Subsequently, the samples were processed as described in the legend to Figure 1A.

The new MTase assay described here has several advantages:

i) It allows detection of the radioactivity incorporated into DNA in solution by liquid scintillation counting. This is important, because [^{3}H] is a very weak β-emitter that can only be detected in solution with high efficiency.

ii) Separation of substrates and products is achieved by an affinity purification step. Therefore, the unreacted AdoMet is removed very efficiently regardless of the buffer composition and enzyme concentration resulting in a very low background of radioactivity. This is particularly important if enzymes (or enzyme variants) with very low catalytic activity are studied, because a higher background may obscure low degrees of DNA-methylation.

iii) The method has a very high sensitivity as demonstrated by detection of incorporation of 0.8 fmol of methyl groups into the DNA as well as by the ability to measure methylation of the DNA at a level of 0.03%.

iv) The assay is very accurate as illustrated by our finding that reaction rates were reproducible with standard deviations of less than 10%.

v) The assay is inexpensive, convenient, fast and, due to the usage of the microplate format, many samples can be processed in parallel. Therefore, the assay is well suited for automation and robotic applications.

Acknowledgements

Expert technical assistance of H. Büngen is gratefully acknowledged. Thanks are due to I. Franke for providing a preparation of the *Serratia marcescens* endonuclease and A. Pingoud for stimulating discussions and support. This work has been supported by the Deutsche Forschungsgemeinschaft (JE 252/2-3) and the European Community (BIO4-CT98-0328).

References

Adams, R.L.P. (1995). Eukaryotic DNA methyltransferases – structure and function. Bioessays *17*, 139–145.

Ahmad, I., and Rao, D.N. (1996a). Chemistry and biology of DNA methyltransferases. Crit. Rev. Biochem. Mol. Biol. *31*, 361–380.

Ahmad, I., and Rao, D.N. (1996b). Functional analysis of conserved motifs in *Eco*P15I DNA methyltransferase. J. Mol. Biol. *259*, 229–240.

Bougueleret, L., Schwarzstein, M., Tsugita, A., and Zabeau, M. (1984). Characterization of the genes coding for the *Eco*RV restriction and modification system of *Escherichia coli*. Nucleic Acids Res. *12*, 3659–3677.

Cheng, X. (1995a). DNA modification by methyltransferases. Curr. Opin. Struct. Biol. *5*, 4–10.

Cheng, X. (1995b). Structure and function of DNA methyltransferases. Annu. Rev. Biophys. Biomol. Struct. *24*, 293–318.

Dryden, D.T.F., Cooper, L.P., and Murray, N.E. (1993). Purification and characterization of the methyltransferase from the Type I restriction and modification system of *Escherichia coli* K12. J. Biol. Chem. *268*, 13228–13236.

Friedrich, T., Roth, M., Helm-Kruse, S., and Jeltsch, A. (1998). Functional mapping of the *Eco*RV DNA methyltransferase by random mutagenesis and screening for catalytically inactive mutants. Biol. Chem. *379*, 475–480.

Hübscher, U., Pedrali-Noy, G., Knust-Kron, B., Doerfler, W., and Spadari, S. (1985). DNA methyltransferases: activity minigel analysis and determination with DNA covalently bound to a solid matrix. Anal. Biochem. *150*, 442–448.

Jeltsch, A., Friedrich, T., and Roth, M. (1998). Kinetics of methylation and binding of DNA by the *Eco*RV adenine-N^6 methyltransferase. J. Mol. Biol. *275*, 747–758.

Jeltsch, A., Christ, F., Fatemi, M., and Roth, M. (1999a). On the substrate specificity of DNA methyltransferases: adenine-N^6 DNA methyltransferases also modify cytosine residues at position N^4. J. Biol. Chem. *274*, 19538–19544.

Jeltsch, A., Roth, M., and Friedrich, T. (1999b). Mutational analysis of the methylation of target base flipping by the *Eco*RV adenine-N^6 DNA methyltransferase. J. Mol. Biol. *285*, 1121–1130.

Kossykh, V.G., Schlagman, S.L., and Hattman, S. (1995). Phage T4 DNA [N^6-adenine] methyltransferase. J. Biol. Chem. *270*, 14389–14393.

Marzabal, S., DuBois, S., Thielking, V., Cano, A., Eritja, R., and Guschlbauer, W. (1995). Dam methylase from *Escherichia coli*: kinetic studies using modified DNA oligomers: hemimethylated substrates. Nucleic Acids Res. *23*, 3648–3655.

Mi, S., and Roberts, R.J. (1992). How M.*Msp*I and M.*Hpa*II decide which base to methylate. Nucleic Acids Res. *20*, 4811–4816.

Noyer-Weidner, M., and Trautner, T.A. (1993). Methylation of DNA in prokaryotes. In: DNA Methylation: Molecular Biology and Significance, J.P. Jost and H.P. Saluz, eds. (Basel, Switzerland: Birkhäuser Verlag), pp. 40–108.

Nwosu, V.U., Connolly, B.A., Halford, S.E., and Garnett, J. (1988). The cloning, purification and characterization of the *Eco*RV modification methylase. Nucleic Acids Res. *16*, 3705–3720.

Reich, N.O., and Mashhoon, N. (1991). Kinetic mechanism of the *Eco*RI DNA methyltransferase. Biochemistry *30*, 2933–2939.

Roth, M., Helm-Kruse, S., Friedrich, T., and Jeltsch, A. (1998). Functional roles of conserved amino acid residues in DNA methyltransferases investigated by site-directed mutagenesis of the *Eco*RV adenine-N^6-methyltransferase. J. Biol. Chem. *273*, 17333–17342.

Rubin, R.A., and Modrich, P. (1977). *Eco*RI methylase physical and catalytic properties of the homogeneous enzyme. J. Biol. Chem. *252*, 7265–7272.

Schluckebier, G., Labahn, J., Granzin, J., and Saenger, W. (1998). M.*Taq*I: Possible catalysis via cation-pi interactions in N-specific DNA methyltransferases. Biol. Chem. *379*, 389–400.

Siegfried, Z., and Cedar, H. (1997). DNA methylation: a molecular lock. Curr. Biol. *7*, R 305–307.

Willcock, D.F., Dryden, D.T.F., and Murray, N.E. (1994). A mutational analysis of the two motifs common to adenine methyltransferases. EMBO J. *13*, 3902–3908.

Received October 26,1999; accepted December 21,1999