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Detection and Quantitation of the Activity of DNA Methyltransferases Using a Biotin/Avidin Microplate Assay

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Summary

The biotin–avidin microplate assay is a sensitive method to measure methylation of biotinylated oligonucleotide substrates by DNA methyltransferases (MTases). The methylation reaction is carried out in solution using [methyl-^{3}H]-AdoMet. Afterwards, the oligonucleotides are immobilized on an avidin-coated microplate, where the incorporation of [^{3}H]-labeled methyl groups into the DNA is stopped by addition of unlabeled AdoMet to the binding buffer. Separation of radioactively labeled DNA from unreacted AdoMet and enzyme is performed by washing steps. Subsequently, the radioactivity incorporated into the DNA is released by a nucleolytic digestion of the DNA. By liquid scintillation counting, the amount of DNA methylation can be determined. Advantages of the microplate assay are its high sensitivity which allows the detection of low amounts of DNA methylation, the efficient separation of reaction components resulting in a low background of radioactivity and a high accuracy (±10%) and reliability. Furthermore, the assay is very convenient, fast and well suited for automation.

Key Words: DNA modification; DNA methyltransferase; biotin–avidin interaction; enzyme assay.

1. Introduction

DNA methylation is an important and essential modification of DNA observed in most prokaryotes and eukaryotes that has many biological functions. In prokaryotes, DNA methylation is used to coordinate DNA replication and cell cycle, to direct post-replicative mismatch repair and to distinguish between self and nonself DNA. In eukaryotes, it is involved in processes

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like gene regulation, maintenance of genome integrity, regulation of development and protection of genome against selfish DNA *(*for review *see* **refs.** ***1–5****)*. Erroneous DNA methylation causes different diseases including cancer ***(6****,****7)***. DNA methyltransferases (MTases) catalyze the transfer of a methyl group to the N^6 position of adenine, N^4 position of cytosine or C^5 position of cytosine by using S-adenosyl-L-methionine (AdoMet) as a donor for an activated methyl group *(*for review *see* **refs.** ***1*** and ***8****)*. Because of its diverse biological functions, methods for analysis of this process are very important and deserve continuous refinement. Several assay systems have been developed to study the activity of DNA MTases *(*for review *see* **refs.** ***9*** and ***10****)* including digestion of the DNA by methylation-sensitive restriction enzymes ***(11–13)***, bisulfite modification for detection of 5-methylcytosine ***(14****,****15)*** or the separation and quantitative determination of modified nucleosides by high pressure liquid chromatography (HPLC) ***(16–18)***. Another class of methylation assays relies on the usage of AdoMet that carries a radioactive label on its methyl group that is transferred to the DNA by the MTase. These assays require the separation of methylated DNA and unused cofactor which can be achieved by (i) spotting the reaction mix onto a DE-cellulose filter sheet ***(19)***, (ii) coupling of DNA to cellulose ***(20)***, or (iii) thin layer chromatography ***(21)***. Some years ago, we have introduced a biotin–avidin microplate format for this purpose ***(22)***. This assay is a sensitive in vitro method to assay the activity of DNA MTases that allows measuring low amounts of DNA methylation in a fast, accurate way.

In the microplate assay described in this chapter (*see* **Fig. 1**), biotinylated oligonucleotide substrates are methylated using [methyl-^{3}H]-AdoMet. During the reaction, the radioactive label is transferred from the coenzyme to the DNA. After the methylation reaction, the methylated DNA must be separated from unreacted AdoMet and coenzyme bound to the enzyme. For this separation, the DNA is immobilized on the surface of an avidin-coated microplate. During this step, incorporation of [^{3}H] into the DNA is quenched by the addition of an excess of unlabeled AdoMet in the binding buffer. In the next step,

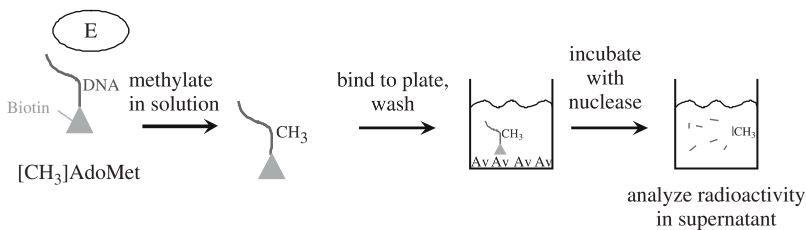

Fig. 1. Schematic drawing of the biotin/avidin microplate DNA methylation assay described in this chapter.

the free AdoMet and enzyme-bound AdoMet are removed by washing. In the end, the bound DNA is digested with a nonspecific nuclease to release the incorporated radioactivity. After the digestion, the radioactivity in the solution is measured by liquid scintillation counting to quantify the amount of methyl groups transferred to the DNA by DNA MTases.

This protocol has advantages in comparison to published protocols. First, the background of radioactivity is low, because of the efficient removal of unreacted AdoMet. Second, the detection of [^{3}H] by liquid scintillation counting is possible with high efficiency. Third, results can be accurately reproduced with small deviations of ±10%. Taken these points together, this assay is very well suited to detect methylation of DNA at a level of smaller than 0.1% of total methylation of the DNA. The assay provides quantitative data of high accuracy and reproducibility. Furthermore, it enables the usage of the microplate format to process many samples in parallel in a fast and inexpensive way. Therefore, the method is prone for automation and high-throughput approaches.

2. Materials

1. Microplates (e.g., E.I.A./R.I.A. plate, flat bottom, high binding, cat. no 9018, Costar Corp., Cambridge, MA, USA; or comparable product from any other manufacturer); store coated plates at 4°C.
2. Avidin (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany); store at 4°C.
3. Unlabeled AdoMet (Sigma); 10 mM solution in 10 mM H_2SO_4; aliquot; store at –20°C.
4. 100 mM $NaHCO_3$ pH 9.6.
5. Phosphate Buffered Saline Tween-20 (PBST): 140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 0.05% Tween 50, pH 7.2
6. Multichannel pipette.
7. Biotinylated oligonucleotide substrates; anneal and prepare a stock solution that is stored at –20°C.
8. Reaction buffer for the methylation reaction, composition depends on the enzyme studied.
9. [methyl-^{3}H]-AdoMet (3.22 TBq/mmol, NEN), store at –20°C in small aliquots.
10. Nonspecific endonuclease, for example, S*erratia marcescens* nuclease in 50 mM Tris/HCl pH 8, 5 mM $MgCl_2$; the enzyme is commercially available as Benzonase from Merck (Merck Biosciences GmbH, Bad Soden, Germany), DNaseI might be used as well.
11. HCl, 0.05 M.
12. Liquid Scintillator Solution Rotizint® Eco Plus (Carl Roth GmbH + Co. KG, Karlsruhe, Germany); Irritant.
13. Liquid scintillation counter and counter vials

3. Methods

1. Preparation: Coat the microplates with avidin; dissolve 1 μg avidin in 100 μl of 100 mM $NaHCO_3$, pH 9.6 (*see* **Note 1**), pipette 100 μl per well and incubate overnight at 4°C. Coated plates can be stored for 2 weeks at 4°C.
2. Before use, wash the wells five times with 200 μl PBST to remove unbound avidin (*see* **Note 2**).
3. Prepare the binding buffer consisting of 5 μl 10 mM unlabeled AdoMet in 35 μl PBST supplemented with 500 mM NaCl (*see* **Note 3**) and distribute it in each well of the microplate to quench the incorporation of [^{3}H] into the DNA after the methylation reaction.
4. Methylation reactions are carried out in 10–50 μl in a reaction tube. Typically, 0.1–10 μM biotinylated oligonucleotide and 1 nM–10 μM enzyme is used in a buffer adapted to the enzyme to be studied, for example, 100 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), pH 8.0, 50 mM NaCl, 1 mM EDTA (Ethylenediamine tetraacetic acid), 0.5 mM DTT (Dithiothreitol), 200 ng/μl bovine serum albumin in the presence of 0.75 μM labeled [methyl-^{3}H]-AdoMet (3.22 TBq/mmol, NEN) (*see* **Note 4**). Methylation reactions can be carried out at different temperatures. The reaction can be started by addition of enzyme, DNA or coenzyme. To measure a time course of methylation (*see* **Note 5**), remove aliquots of 1–5 μl from the reaction mixture at each time point and pipette them into the wells of the microplate that contain the binding buffer with an excess of unlabeled AdoMet (*see* **Note 3**) to quench the incorporation of [^{3}H] into the DNA. After the last time point, incubate the plate for 30 min at room temperature to allow binding of the oligonucleotide substrates to the avidin on the microplate.
5. Wash the wells five times with 200 μl PBST supplemented with 500 mM NaCl to remove the unreacted AdoMet and the enzyme (*see* **Notes 2** and **6**).
6. Digest the immobilized DNA by adding 0.7 μg *S. marcescens* nuclease (*see* **Note 7**) in 100 μl 50 mM Tris/HCl pH 8.0, 0.5 mM $MgCl_2$ per well and shake the microplate for 30 min at room temperature. As an alternative, release of the DNA could be achieved by adding 100 μl 0.05 M HCl per well (which disrupts the avidin–biotin interactions) and shaking the microplate for 30 min at room temperature (*see* **Note 8**).
7. After digestion, the whole solution is transferred from the wells into a scintillation vessel. Each sample is mixed with 2 ml Liquid Scintillator Solution and subjected to liquid scintillation counting to quantify the amount of methyl groups transferred to the DNA (*see* **Note 9**).

4. Notes

1. Wrong pH leads to a lower binding efficiency of avidin to the surface of the microplate.
2. Avoid scratching the bottom of the plate with the pipette tips. Tilting of the plate facilitates complete removal of avidin or washing buffer.
3. Prepare the binding buffer that contains an excess of unlabeled AdoMet to quench the incorporation of [^{3}H] into the DNA directly before using. Unlabeled AdoMet

should be stored in small aliquots in 10 mM H_2SO_4 and thawed only once to avoid degradation. Use high-salt buffer to prevent binding of the MTase to the DNA after stopping the reaction.

4. Labeled AdoMet should be stored at –20°C, aliquoted and thawed only once.
5. Carry out each measurement at least in duplicates.
6. Complete removal of the MTase and unreacted labeled AdoMet is very important for a low background of radioactivity. After each washing step, no buffer should be left in the wells.
7. The purification of *S. marcescens* nuclease was performed similarly as described by Friedhoff et al. ***(23)***: The His_6-Nuclease fusion protein was expressed in TGE900 *Escherichia coli* cells. Protein overexpression was induced at a cell density of 0.5–0.6 at OD 600 nm by changing the temperature from 28 to 42°C, and the cells were grown for an additional 2 h. All following steps were carried out at 4°C. The cells were harvested by centrifugation (15 min and 3000 g) and washed with STE buffer [10 mM Tris/HCL (pH 8.0), 0.1 mM EDTA, 0.1 mM NaCl]. The cell pellet was resuspended in buffer A (10 mM Tris/HCl, pH 8.2), and the cells were disrupted by ultrasound. Cell debris were removed by centrifugation (1 h at 15,000 g), and the pellet was resuspended in buffer B (6 M urea, 10 mM Tris/HCl, pH 8.2, 10 mM imidazole) and kept overnight at 4°C on a shaker. After a new centrifugation (1 h at 15,000 g), the supernatant was applied onto a Ni-NTA column (Qiagen GmbH, Hilden, Germany) equilibrated with buffer B. The column was washed with 150 ml buffer B, eluted with buffer B containing 200 mM imidazole and collected in fractions. Fractions containing the nuclease were pooled and dialyzed overnight against 10 mM Tris/HCl, pH 8.2. The concentration of the nuclease was determined using an extinction coefficient of $\varepsilon_{280\ nm}$ = 44,620 M/cm. The enzyme was stored in small aliquots at –80°C. After thawing, keep the nuclease at 4°C. For checking the activity of the nuclease and estimation how much enzyme is required for full digestion perform a methylation assay as described and digest the biotinylated oligonucleotides using different amounts of nuclease. The enzyme is commercially available as Benzonase from Merck.
8. Release of DNA by HCl was only checked in assays using MTases with good activity and was found to be equally efficient as enzymatic digestion. However, the influence of the HCl on the efficiency of Liquid Scintillation counting should be tested.
9. Mix the solution obtained after digestion and Liquid Scintillator Solution by vortexing or inverting the scintillation vials before counting.

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