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Short communication

Determination of the DNA methylation level of the marbled crayfish: An increase in sample throughput by an optimised sample preparation

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Abstract

Using a previously described capillary electrophoretic method with laser-induced fluorescence detection the genomic methylation level can be determined exactly. We present a sample preparation that eliminates the surplus of fluorescence marker used for coupling resulting in an increase of sample throughput from 75 to 250 analyses per week. The sensitivity of the method was also increased, which allows the determination of methylation levels under 1%. With these changes in sample preparation a methylation level of $1.64 \pm 0.03\%$ in hepatopancreas DNA of the recently discovered marbled crayfish could be determined.

Keywords: DNA methylation; 5-Methylcytosine; Epigenomics; CE-LIF; Marbled crayfish

1. Introduction

The epigenetic code, i.e. the methylation of cytosines, various histone modifications and DNA-binding proteins, is, unlike the genetic code, always being rewritten and erased. Epigenetic mistakes are involved not only in mutations in the genotype, but also in the development of abnormalities, cancer and other diseases. As a result of increasing interest in epigenetic questions, the analytical method for determining the genomic methylation level and other DNA modifications, developed by us in 2002 [1] and shown in Fig. 1, has come to be used by several research groups [2–13]. Because of the continuously increasing need of a reproducible and rapid method to determine the methylation level, we present here, on the basis of the analysis of a crayfish-DNA, a clear improvement of the previously published method as regards sample throughput and sensitivity. The samples analysed are hepatopancreas DNA from the marbled crayfish, which is capable of unisexual reproduction [14,15] and was discovered first in the mid-1990s in the German aquarium trade.

2. Materials and methods

2.1. Reagents

4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl ethylene-diamine (BODIPY) was purchased from MoBiTec (Göttingen, Germany), spleen phosphodiesterase (SPD) from Calbiochem (La Yolla, USA), Lambda-DNA from New England Biolabs (Beverly, USA) and micrococcal nuclease (MN) and calf-thymus DNA (CT-DNA) were obtained from Sigma (Steinheim, Germany). *Hpa*II restriction endonuclease, *Hpa*II methylase, *S*-adenosylmethionine, NEBuffer1 and NEBuffer for *Hpa*II methylase were purchased from New England Biolabs (Beverly, USA). The Lambda *Eco*RI/*Hin*d III marker was from MolekularBiologisches & Biochemisches Labor Dr. Bartling GmbH (Bielefeld, Germany). *N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethane sulphonic acid (HEPES), 1-ethyl-3-(3′-*N*,*N*′-dimethylaminopropyl)-carbodiimid (EDC) and paraffin oil were purchased from Fluka (Steinheim, Germany). The synthesis of the reference standard 2′-deoxy-5-methylcytidine-3′-monophosphate (5m-dCMP) was published earlier [6]. All other chemicals used were of highest purity and were purchased from Merck (Darmstadt, Germany). Water was purified via an ultrapure laboratory water

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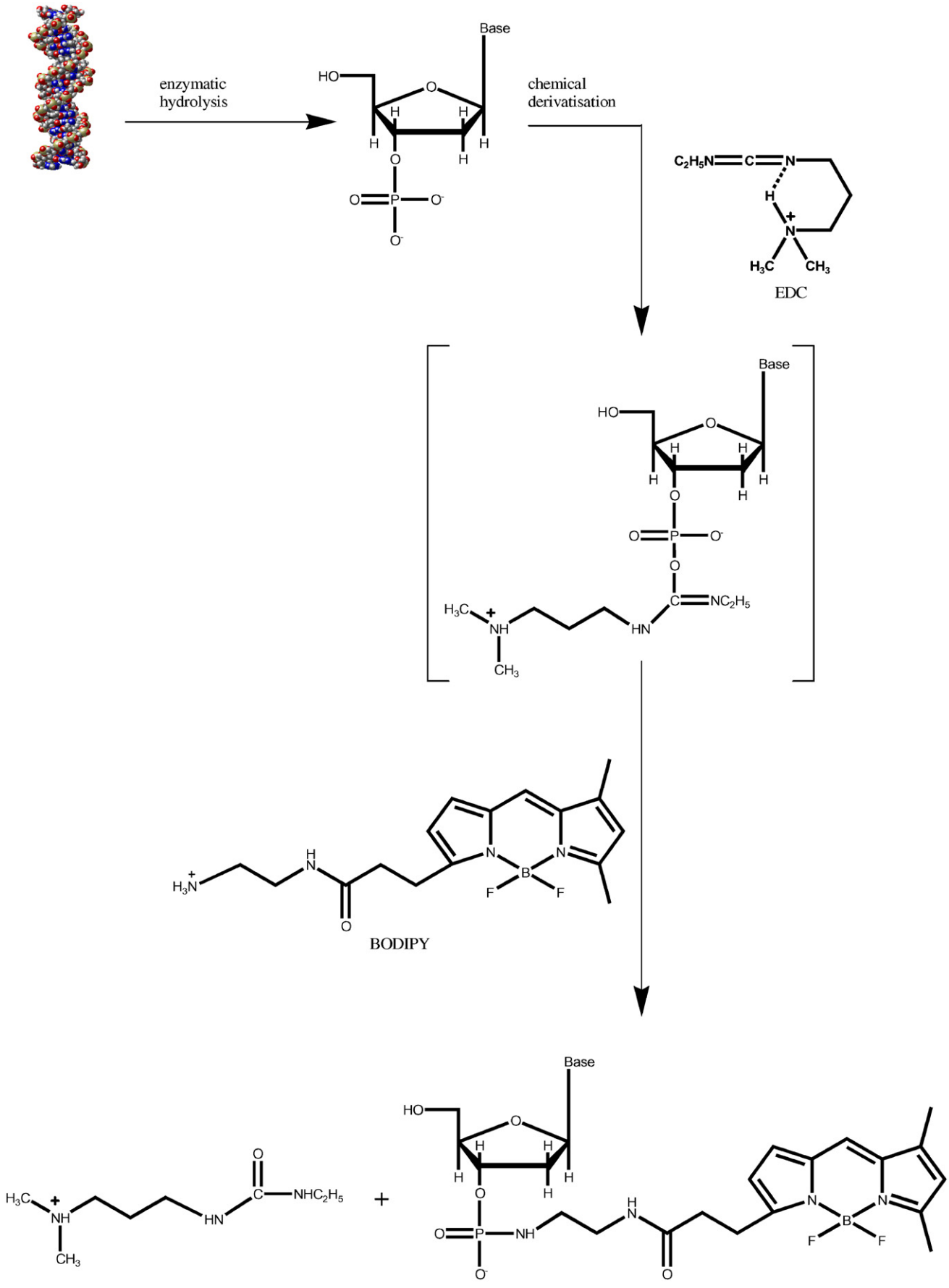

Fig. 1. Outline of the analytical method to determine the methylation level with CE-LIF after carbodiimide-assisted derivatisation.

system (Milli-Q synthesis A10) from Millipore (Schwalbach, Germany), and the water quality was 18.2 MΩ cm with TOC 5–10 ppb.

2.2. Capillary electrophoresis

Capillary electrophoresis was carried out on a PACE^{TM} MDQ system with a laser-induced-fluorescence detector (argon-ion laser with λ_{em} = 488 nm) from Beckman Coulter (Munich, Germany). The fused-silica capillary used was purchased from CS-Chromatography-Service (Langerwehe, Germany) and had a total length of 50 cm (with the detection window at 40 cm) and an inner diameter of 50 μm. For improving the long-term stability of the capillary, it was treated by the heat-curing technique described by Baeuml and Welsch [16], and to avoid high injection fluctuations, about 2 mm of the polyimide coating was burned away at the capillary inlet. That prevents gradual build-up of welled and frayed polyimide coating in front of the capillary inlet [16].

The separations were achieved with an electrolyte consisting of 95 mM SDS in a solution of 90% (v/v) sodium phosphate buffer (20 mM, pH 9.0) and 10% (v/v) MeOH as organic modifier (5s-sample injection at 0.5 p.s.i. at 20 °C and an applied voltage of 18 kV). The cathode was the outlet in all runs. For conditioning, the capillary was rinsed with 1 M NaOH (15 min), 1 M HCl (15 min), 1 M NaOH (15 min), water (5 min) and electrolyte (10 min). Before each run it was rinsed with 200 mM sodium dodecyl sulphate (1 min), 1 M NaOH (1.5 min), water (1 min) and finally with electrolyte (2 min).

2.3. Hydrolysis and derivatisation of genomic DNA samples

Method A: 1 μg DNA was diluted in water (5 μL) and hydrolyzed by incubation for 3 h at 37 °C with 4.2 μL of an enzyme mixture (MN (150 mU/μL)/SPD (12.5 mU/μL) and 0.8 μL buffer (100 mM $CaCl_2$ in 250 mM HEPES, pH 6.0)). To the hydrolysate were added 1.8 M EDC (15 μL; in 50 mM HEPES, pH 6.4), 27 mM BODIPY FL EDA (15 μL; in 50 mM HEPES, pH 6.4) and 15 μL HEPES (50 mM, pH 6.4), and the sample was incubated in the dark for 21 h at 25 °C. The derivatisation solution was diluted 1:200 in ultrasonically degassed purified water before injection.

Method B: 10 μg DNA was hydrolysed as described in method A. To the hydrolysate were added 1.8 M EDC (20 μL; in 50 mM HEPES, pH 6.4), 27 mM BODIPY FL EDA (20 μL; in 50 mM HEPES buffer, pH 6.4), and 50 mM HEPES (20 μL, pH 6.4), and the sample was incubated in the dark for 21 h at 25 °C. The derivatisation solution was diluted 1:200 in ultrasonically degassed purified water before injection.

2.4. Reduction of BODIPY and salt content

Fifty-five microliter of the derivatised sample were transfered into a 15 mL cap and diluted with 425 μL water. To the solution was slowly added 52.5 mM sodium tetraphenylborate (550 μL; in 1 mM sodium phosphate buffer, pH 6.0). After mixing, 11 mL methylene chloride were added to the solution, mixed again and centrifuged for 4 min at 3000 rpm. The aqueous phase was isolated and analysed by CE.

3. Results and discussion

One problem of the previous analytical method [1–3] is that only BODIPY satisfies all necessary criteria for use as a fluorescence marker in the determination of the methylation level. The criteria are (i) laser-induced fluorescence excitation above 400 nm to reduce interference by native fluorescent matrix compounds, (ii) a primary amine group to facilitate the formation of phosphoramidates with nucleotides, (iii) a pure (without isomers) commercially available fluorescence marker and (iv) no further functional groups in the molecule to avoid any oligomerisation. In our opinion, these essential criteria are fulfilled only by the BODIPY we used, which can be excited at 488 nm with an argon-ion laser.

Unfortunately, light, temperature and oxygen cause this fluorescence marker to form, even on brief storage, decomposition products that are also fluorophores and emit photons after excitation at 488 nm. These side-products of the derivatisation reaction lead to a problem with DNA samples with low methylation level or when there are small quantities of DNA (≤1 μg) because the increased decomposition or side-products prevent examination of the 5m-dCMP signal after only one or two measurements of a single sample aliquot. However, to determine small changes in the methylation level with precision, up to ten repetitions of an analysis are necessary. Because of the poor sample stability described, the autosampler cannot be used optimally, with the result that only 75 analyses (each 40 min; including rinsing steps) can be done per week (with a 10 h working day and 5 days per week).

First attempts to determine the methylation level of the marbled crayfish, which appears to be particularly suitable for research on epigenetics because of its parthenogenetic mode of reproduction and high adaptability to a wide range of experimental conditions [14,15], revealed very low genomic methylation. The analysis of these samples was thus complicated by the instability of the fluorescence marker, as described above. Fig. 2 shows the determination of the methylation level of a 1 μg hepatopancreas DNA sample from marbled crayfish by the analytical method described earlier [1–3] after only 100 min in the autosampler. Whereas paraffin oil covering [3,17] allows 12–15 analyses in series with 10 μg DNA samples with concentration of 5m-dCMP higher than 2%, no improvement was obtained with the 1 μg DNA samples from the marbled crayfish (data not shown). Only the first measurement of each aliquot was analysable. In the ensuing electropherogram, a co-eluting decomposition signal prevents the data analysis of the methylation level.

To avoid the interfering decomposition signals of the fluorescence marker, a solid-phase extraction (SPE) was used to separate the derivatisation products from the surplus of BODIPY, but this sample preparation led to more decomposition products in the sample, for which reason a precipitation of the positively charged BODIPY and the degradation product of EDC (at a pH 6.0) with sodium tetraphenyl borate was developed (see *Reduc-*

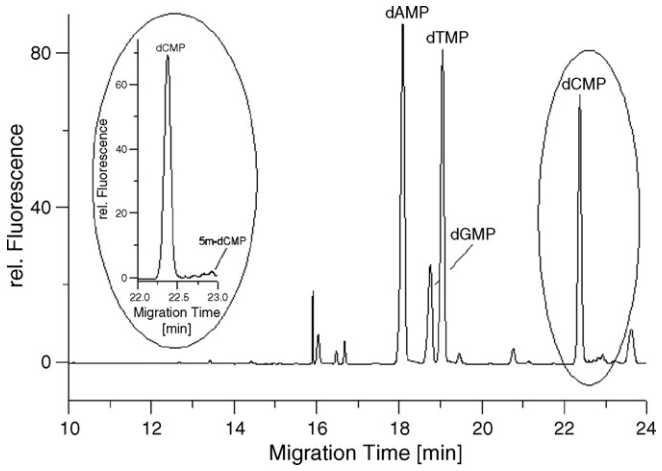

Fig. 2. Analysis of the 1 μg hepato-pancreas DNA of the marbled crayfish after 100 min of storage at RT in the autosampler. The sample was 100-fold diluted.

tion of Bodipy and salt content). With this method the positively charged fluorescence marker and degradation product of EDC (for structure see Fig. 1, [18]) are complexed by the tetraphenyl borate anion and removed from solution. To prevent the inclusion of the derivatised nucleotides in the voluminous precipitate, in a following step the tetraphenyl borate complex was diluted in methylene chloride. In this solvent the derivatised nucleotides are almost insoluble. After centrifugation the fluorescence-labelled nucleotides are in the aqueous phase, which is used without further preparation for injection. With this sample preparation, the surplus BODIPY and the degradation product of EDC were almost quantitatively (98%) eliminated. Fig. 3a and b show the electropherograms (68 min instead of 30 min data acquisition) of a CT-DNA (10,000-fold diluted) without the new sample preparation (Fig. 3a) and with the described elimination of BODIPY and the degradation product of EDC (Fig. 3b). The latter cannot proved in this way because of the lack of fluorophoric groups, but CZE analysis with UV detection of these samples shows the reduction of the degradation product of EDC (data not shown).

For a precise determination of cytosine methylation levels in genomic DNA the calculation of the fluorescence quantum yield factor (QYF) is necessary. Therefore, we calibrate the method with a complex, yet well defined standard, the bacteriophage lambda DNA that had been methylated in vitro with the bacterial methylase M. *Hpa*II [2,6]. Methylated and unmethylated lambda DNA was then hydrolysed, derivatised, and after the elimination of the surplus of BODIPY, analysed (methylated and unmethylated lambda DNAs were each analysed 25 (1 μg DNA) and 30 (10 μg DNA) times) by capillary electrophoresis. The nucleotide-specific median correction factors thus determined for 10 μg DNA obtained by this procedure were: dAMP 0.68 ± 0.001, dGMP 1.76 ± 0.001, dTMP 0.98 ± 0.001, dCMP 1.08 ± 0.006 and 5m-dCMP 0.91 ± 0.04. For 1 μg DNA the correction factors were: dAMP 0.67 ± 0.001, dGMP 1.62 ± 0.006, dTMP 0.92 ± 0.001, dCMP 1.26 ± 0.003 and 5m-dCMP 1.00 ± 0.05. The ranges are the standard error of the mean (methylated and unmethylated lambda DNA) with

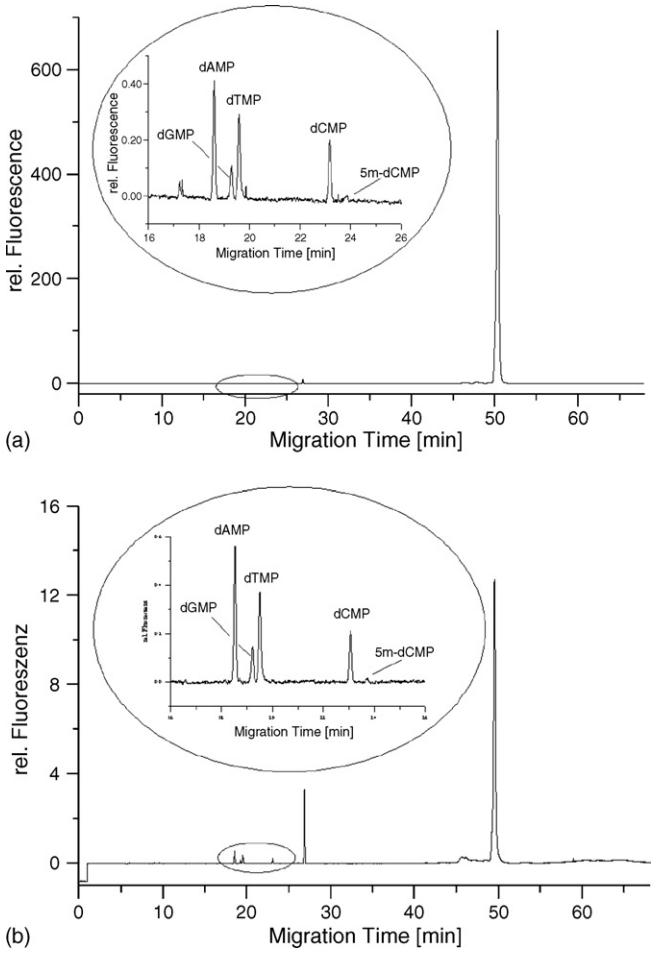

Fig. 3. Sixty-eight minute electropherograms of a CT-DNA with the previously used sample preparation (a, [1–12]) and with the described elimination of BODIPY and the degradation product of EDC (b). The enlarged part of the electropherograms shows the signals of the derivatised nucleotides. To demonstrate the reduction of BODIPY, a high dilution was used, which prevented the determination of 5m-dCMP (only dAMP, dGMP, dTMP and dCMP are observed).

the exception of 5m-dCMP (only methylated lambda DNA), for which only the standard deviation could be determined.

To determine the reproducibility of the sample preparation, 10 μg aliquots from an identical source (CT-DNA) were hydrolysed and derivatised in four parallel, independent reactions. The surplus of BODIPY and the degradation product of EDC in the nucleotide mixture from each reaction were then eliminated by the sample preparation described above and analysed several times (n = 15–20) by capillary electrophoresis (in an 80-run sequence), and the cytosine methylation levels were determined with the new correction factors. This revealed a very high degree of reproducibility between individual sample preparation steps as well as between individual measurements (methylation level of the four aliquots: 6.27 ± 0.16, 6.25 ± 0.10, 6.45 ± 0.18 and 6.42 ± 0.19%).

The storage stability of the prepared samples was tested in that part of a sequence in which the multiple analyses (n = 18) of 1 μg methylated lambda DNA were carried out between 16 and 33 h of storage in the rack of the autosampler. The methyla-

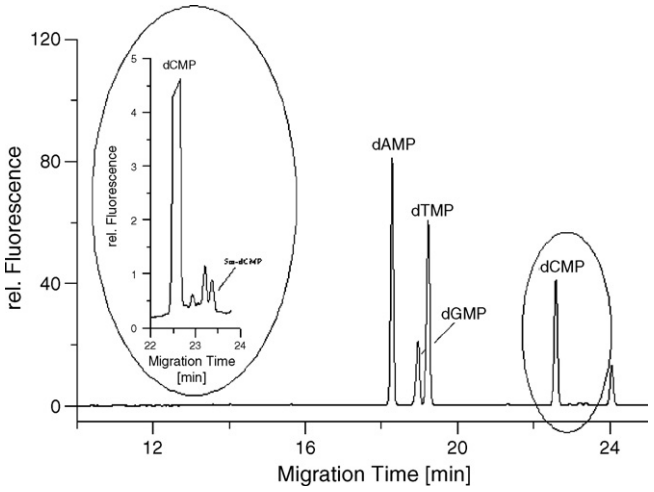

Fig. 4. Analysis with the newly developed sample preparation of 1 μg hepatopancreas DNA of a marbled crayfish which was stored for 44 h at RT before determination. The sample was 100-fold diluted.

tion level determined was 2.75 ± 0.12 (theoretical value: 2.7%), demonstrating a clear increase in the stability of the derivatised sample by the more extensive sample preparation.

Without the interfering decomposition products, very small DNA samples (≤1 μg) with a low methylation level, such as DNA from marbled crayfish, can now be determined exactly. Fig. 4 shows an electropherogram of the analysis of 1 μg hepatopancreas DNA from a marbled crayfish; the derivatised sample was stored for 44 h at RT before analysis to demonstrate again the sample stability. This is the first time that the methylation level of this animal has been analysed. The methylation level in this tissue was determined with a sequence of 18 runs as 1.64 ± 0.03%. A 200-fold dilution is necessary unless the sample is prepared as described here (see Fig. 2). With this new sample preparation only a 10- to 100-fold dilution is needed before analysis. This leads to an increase in sensitivity of the CE-LIF method.

4. Conclusion

The rapid and inexpensive sample preparation presented here permits an increase in sample throughput and allows the accurate determination of low methylation levels. In addition, the analysis of DNA in small quantities (≤1 μg) is simplified because of the lack of decomposition products and allows for the first time the determination of the methylation level of hepatopancreas DNA of the recently discovered marbled crayfish.

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