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Research paper

Development and optimization of the VISAGE basic prototype tool for forensic age estimation

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ABSTRACT

The VISAGE (VISible Attributes through GEnomics) consortium aims to develop, optimize and validate prototype tools to broaden the use of DNA intelligence methods in forensic routine laboratories. This includes age estimation based on the quantification of DNA methylation at specific CpG sites. Here, we present the VISAGE basic prototype tool for age estimation targeting 32 CpGs from five genes *ELOVL2*, *MIR29B2CHG (herein, MIR29B2C)*, *FHL2*, *TRIM59* and *KLF14*. The assay interrogates these well described age markers by multiplex PCR for bisulfite converted DNA and massively parallel sequencing on a MiSeq FGx instrument. We describe protocol optimizations including tests on five bisulfite conversion kits and an evaluation of the assay's reproducibility and sensitivity with artificially methylated DNA standards. We observed robust quantification of methylation levels with a mean standard deviation of 1.4 % across ratios. Sensitivity tests showed no increase of variability down to 20 ng DNA input into bisulfite conversion with a median difference below 1.6 % between technical replicates.

1. Introduction

Age estimation from biological material can provide essential leads in forensic investigations to find unknown perpetrators of crime typically not identifiable with standard STR-profiling. The investigative value of an unknown person's age is twofold 1) providing intelligence information by itself and 2) making DNA prediction of age-dependent appearance traits (e.g. hair colour, hair loss) more reliable [1]. The analysis of genome-wide DNA methylation profiles from microarray data had revealed that DNA methylation patterns at specific CpG sites are correlated with age [2]. The degree of methylation at such CpGs changes over a person's lifespan and can be used to build age estimation models often referred to as "epigenetic clocks" [3]. Most of the currently available forensic assays are based on technologies such as pyrosequencing (e.g [4].), SNaPshot (e.g [5].), the EpiTyper System (e.g [6].) and more recently also massively parallel sequencing (MPS; e.g [7].). All of these methods require a prior bisulfite conversion (BC) allowing the distinction of methylated versus unmethylated cytosines (represented as thymines after conversion and PCR). However, the harsh chemical treatment during BC leads to DNA degradation [8] and DNA loss in the course of necessary purification steps. This provides challenges arising from the low quantity and quality of DNA obtained from crime scene material. Additionally, most assays were based on singleplex PCR (e.g [9–13].) due to multiplex limitations of used technologies and the challenging primer design for bisulfite converted DNA [14]. The lower complexity of the DNA sequence after conversion leads to an increased occurrence of non-specific primer binding and facilitates dimer formations due to the T and A richness of the sense and antisense strands [15,16]. This results in design constraints that are mostly manageable for singleplex reactions but impede the development of multiplex PCR assays. A possible solution is the restriction of the number of markers to make age estimation through DNA methylation quantification more feasible for forensic applications. Whereas tissue-independent age models use a high number of markers (e.g. 353 markers in [17], 71 markers in [18] or 94 markers in [19]), forensic age estimation models have focused on fewer markers (< 20, e.g. [7,9,10,20,21].) that are highly informative in forensically relevant biological material [22]. However, the simultaneous analysis of

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Table 1
Manufacturer's informations on bisulfite conversion kits.

Short designation	Kit name	Company	DNA input range	Optimum DNA input [ng]	Conversion time	Desulfonation time
MethylEdge	MethylEdge Bisulfite Conversion System	Promega	100 pg -2 µg	200 - 500	1.) 98 °C 8 min 2.) 54 °C 60 min	15 min
Methylamp	Methylamp DNA Modification	Epigentek	> 50 pg	50 - 200	1.) 37 °C 10 min 2.) 65 °C 90 min	8 min
EpiJET	EpiJET Bisulfite Conversion Kit	Thermo Fisher Scientific	50 pg -2 µg	200 - 500	1.) 98 °C 10 min 2.) 60 °C 150 min	20 min
EZ Direct	EZ DNA Methylation-Direct Kit	Zymo Research	> 50 pg	200 - 500	1.) 98 °C 8 min 2.) 64 °C 3.5 h	15 - 20 min
Premium	Premium Bisulfite kit	Diagenode	100 pg -2 µg	200 - 500	1.) 98 °C 8 min 2.) 54 °C 60 min	15 - 20 min

markers in multiplex format is of fundamental importance for the prevention of sample depletion and the implementation of this technology in forensic practice, as shown previously for SNaPshot [23] and MPS [24].

The VISible Attributes through GEnomics (VISAGE) Consortium aims to develop, optimize and, in following studies, forensically validate prototype tools based on MPS to predict externally visible characteristics from the DNA of unknown sample donors. This includes the development of targeted MPS-based tools for genotyping of SNPs to predict appearance and ancestry as well as MPS-based tools for targeted bisulfite sequencing of CpGs for estimating age. Here, we present the development and technical evaluation of the VISAGE basic prototype tool for age estimation (herein BTA). BTA targets 32 age informative CpG sites at five genes, *ELOVL2*, *MIR29B2C* (formerly *C1orf132*), *FHL2*, *TRIM59* and *KLF14,* that were described as strong age predictors for blood samples by Zbieć-Piekarska et al. (2015) [10]. An age prediction model has been developed based on singleplex PCR assays and pyrosequencing. The authors reported a mean absolute deviation (MAD) of 3.9 years in the testing set [10]. The same set of markers was validated in singleplex pyrosequencing assays in a Korean sample set by Cho et al. (2017) who also included *ELOVL2* C_1 and C_2 (Table 2) in their study. They calculated a MAD of 3.3 years after changing CpG positions to those that explain the highest percentage of age-related variance in each marker [13]. Reinforcing the predictive strength of this marker composition, Jung et al. (2019) [23] developed a multiplex SNaPshot assay with a MAD of 3.5 years.

For the BTA, we successfully designed a multiplex PCR for bisulfite converted DNA followed by targeted MPS with the MiSeq FGx. Here, we focus on a detailed description of assay development including the testing of five BC kits and two PCR multiplex kits for protocol optimization. The assay's reproducibility and sensitivity was evaluated using DNA standards of known methylation state, which showed a robust quantification of methylation levels down to 20 ng DNA input.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics commission of the Medical University of Innsbruck (study number 1086/2017) and all volunteers provided written informed consent.

2.2. Experimental design, DNA samples and standards of known methylation state

Assay development was carried out with DNA extracts from blood to ensure that the BTA is optimized for this respective sample type. For final performance assessment, artificially methylated DNA standards were sequenced to control for correct DNA methylation quantification. DNA was extracted from 10 mL whole blood of three sample donors using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany) and quantified by real-time quantitative PCR [25]. These DNA extracts were used in primer optimization, BC kit testing and multiplex PCR optimization. For the assay's performance assessment, the human WGA methylated & non-methylated DNA Set (Zymo Research, Irvine, California, USA) was diluted with 100 µL low TE (10 mM Tris, 0.1 mM EDTA, pH 8) and quantified with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific - TFS, Waltham, MA, USA). Subsequently, these dilutions were adjusted to 20 ng/µL. Fully methylated and non-methylated DNA samples were mixed at different volume proportions to achieve 100 %, 95 %, 90 %, 75 %, 50 %, 25 %, 10 %, 5 % and 0 % methylated DNA standards. All nine methylation ratios were processed in duplicates following the BTA protocol to test reproducibility. This reproducibility study (18 DNA standards) was performed twice, using the Premium Bisulfite kit (Diagenode, Ougrée, Belgium) for run 1 and the EZ DNA Methylation-Direct kit (Zymo Research) for run 2. For sensitivity evaluation, dilution series (200 ng, 100 ng, 50 ng, 20 ng, 10 ng and 1 ng) from five differentially methylated DNA standards (100 %, 75 %, 50 %, 25 % and 0 %) were bisulfite converted in duplicates with the Premium Bisulfite kit. Samples sequenced on the same MiSeq FGx flow cell (Verogen, San Diego, USA) were processed together with one negative control (PCR grade water).

2.3. Bisulfite conversion kit and DNA polymerase testing

Aiming to better understand the effects and performance of bisulfite conversion, five kits from different commercial suppliers (Table 1) were selected based on their presumed amenability for low DNA inputs, as indicated by the manufacturer. Comparative testing was performed following the respective protocols with 200 ng DNA (optimum), 10 ng, 1 ng and 500 pg DNA input. Converted DNA was eluted with 10 µL elution buffer provided by the kits. BC of the five different DNA inputs was carried out in duplicates and eluates were immediately used for amplification. From each BC, 4 µL of converted DNA were used for two singleplex PCRs (308 bp long amplicon of *ELOVL2* gene) to test the performance of the Multiplex PCR Kit (Qiagen) and the ZymoTaq PreMix (Zymo Research). PCRs were performed in 50 µL total volume under the following conditions [25]: initial denaturation at 95 °C for 10 min; 40 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s; final elongation at 72 °C for 10 min. Primer sequences and assay concentrations are listed in Table S1. PCR product yield was quantified fluorometrically using the Qubit dsDNA HS Assay Kit (TFS). The Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, California, USA) was used to control for correct amplicon size and unspecific PCR products (Fig. S1).

2.4. Multiplex PCR

Multiplex PCR primer sequences [9,10] and concentrations are listed in Table 2. Primer positions and amplicon sequences were verified in GRCh38 using Ensemble [26] (www.ensembl.org). The forward primer of *KLF14*designed by Zbieć-Piekarska [10] showed a sequence difference in a C-stretch between GRCh37 and GRCh38. The primer sequence was changed to the bisulfite converted sequence of the newer

Table 2
Genomic locations of target CpGs, multiplex PCR primer sequences and primer concentrations in the final assay.

Gene	Primer sequence (5′-3′)	Strand	Ref.	Concentration [µM]	Amplicon size [bp]	CpG No.	GRCh38
ELOVL2	fwd:AGGGGAGTAGGGTAAGTGAG rev: AAACCCAACTATAAACAAAACCAA	sense	[4]	0.2	267	C_1	Chr6:11044628
						C_2	Chr6:11044631
						C_3[a]	**Chr6:11044634**
						C_4	Chr6:11044640
						C_5	Chr6:11044642
						C_6	Chr6:11044644
						C_7	Chr6:11044647
						C_8	Chr6:11044655
						C_9	Chr6:11044661
MIR29B2C	fwd: GTAAATATATAAGTGGGGGAAGAAGGG rev: TTAATAAAACCAAATTCTAAAACATTC	sense	[25]	0.4	146	C_1	Chr1:207823672
						C_2	Chr1:207823675
						C_3[a]	**Chr1:207823681**
FHL2	fwd:TGTTTTTAGGGTTTTGGGAGTATAG rev: ACACCTCCTAAAACTTCTCCAATCTCC	sense	[25]	0.2	167	C_1	Chr2:105399282
						C_2[a]	**Chr2:105399288**
						C_3	Chr2:105399291
						C_4	Chr2:105399297
						C_5	Chr2:105399300
						C_6	Chr2:105399310
						C_7	Chr2:105399314
						C_8	Chr2:105399316
TRIM59	fwd:TATAGGTGGTTTGGGGGAGAG rev: AAAAAACACTACCCTCCACAACATAAC	sense	[25]	0.2	141	C_1	Chr3:160450172
						C_2	Chr3:160450174
						C_3	Chr3:160450179
						C_4	Chr3:160450184
						C_5	Chr3:160450189
						C_6	Chr3:160450192
						C_7[a]	**Chr3:160450199**
						C_8	Chr3:160450202
KLF14	fwd: GGTTTTAGGTTAAGTTATGTTTAATAGT rev: ACTACTACAACCCAAAAATTCC	sense	[25] [b]	0.4	128	**C_1**[a]	**Chr7:130734355**
						C_2	Chr7:130734357
						C_3	Chr7:130734372
						C_4	Chr7:130734375

[a] Included in final age model of Zbieć-Piekarska et al. (2015) [25].
[b] fwd primer modified to match GRCh38.

assembly from "GGTTTTTAGGTTAAGTTATGTTTAATAGT" to "GGTTTTAGGTTAAGTTATGTTTAATAGT". Notably, both versions showed a similar performance in terms of PCR product yield (data not shown). Additionally, primer sequences were tested *in silico* for the formation of alternative PCR products using Bisearch [16] (default parameters), the formation of primer dimers (AutoDimer [27]) and primer helicity using an in-house developed R script.

For initial annealing temperature optimization, all primers were tested in singleplex gradient PCR using 2 µL eluate from bisulfite conversion of 200 ng DNA with the Premium Bisulfite kit. The multiplex reaction was optimized again by testing annealing temperatures of 55 °C, 57.6 °C and 60° in a gradient PCR and by comparing different denaturation (15 s vs. 30 s), annealing (30 s vs. 60 s) and elongation times (30 s vs. 60 s). Final PCR assays were performed in 25 µL total volume using the Multiplex PCR Kit and the following thermocycler protocol: Initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 10 s, 58 °C for 30 s, 72 °C for 30 s; final elongation at 72 °C for 10 min. In reproducibility and sensitivity studies, 8 µL of the bisulfite converted DNA preparations were used for PCR amplification. PCR products were purified with 1.5X volumes of AMPure XP beads (Beckman Coulter, Brea, California, USA) and quantified using the Qubit dsDNA HS Assay Kit.

2.5. Verification of PCR products

Purified PCR products were analysed using the DNA 1000 or the High Sensitivity DNA Kit (Agilent Technologies) to assess amplicon size and product yield. To verify correct amplification, PCR products from assay optimization and from the final multiplex were typed with Sanger sequencing [28]. Reactions were set up using BigDye Terminator v1.1 Cycle Sequencing Kit (TFS) in 10 µL reaction volumes and 0.3 µM primer (Table 2) with the following cycling protocol: 96 °C for 1 min; 25 cycles of 95 °C for 15 s, 50 °C for 5 s and 60 °C for 4 min. Purification of products was carried out by centrifugation over Sephadex G-100 columns (Amersham, Little Chalfont, UK). Capillary electrophoretic separation was performed on an ABI3500 (TFS) using standard settings. Sequences were analysed with the Sequencer 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA) software.

2.6. Massively parallel sequencing

Libraries were prepared from 50 ng purified PCR products using the KAPA Hyper Prep Kit with KAPA Library Amplification Primer Mix and KAPA SI Adapter Kit Set A + B at 15 µM (all Roche, Basel, Switzerland). Post-ligation and post-amplification clean-ups were performed with 0.8X or 1X AMPure XP beads and eluted in 23 µL or 20 µL low TE, respectively. Libraries were amplified following the manufacturer's protocol using 8 cycles and quantified with the KAPA Library Quantification Complete Kit (Roche) in halved volume. After diluting libraries to 4 nM, samples of reproducibility (N = 19 per flow cell) and sensitivity (N = 21 per flow cell) studies were pooled equimolarly and prepared for sequencing following the MiSeq System Denature and Dilute Libraries Guide, Protocol A. Libraries were diluted to 7 pM and spiked with 2 µL 20 pM PhiX control v3 (Illumina, San Diego, USA). Sequencing was performed on a MiSeq FGx with MiSeq Reagent Kit v2 and 2 × 151 cycles (Verogen).

2.7. MPS data analysis

Fastq files produced by the MiSeq FGx were aligned against a custom reference genome (Table S2) containing only targeted amplicon sequences (+/- 300 bp; GRCh38) using an adapted Burrows-Wheeler

alignment for bisulfite converted DNA sequences – bwa-meth [29]. Samtools [30] was used for BAM file creation, sorting, filtering and indexing. All samples were controlled visually using the Integrative Genomics Viewer (IGV) [31]. To calculate read depth and beta values, total reads for each position of aligned amplicons were extracted using IGV tools using a minimum mapping quality of 30. Beta values were calculated by dividing C reads by the sum of C and T reads. Bisulfite conversion efficiency was taken from all non CpG-Cs per sample and calculated as reversed beta values (T reads divided by the sum of C reads and T reads). Total coverage represents the sum of the number of reads per amplicon and was calculated from one target CpG site per marker (highlighted in Table 2). Normalized read depth was calculated by dividing the read depth at target CpG sites by the total coverage. Statistical analysis was performed with Microsoft Excel and R (https://www.r-project.org/) [32].

3. Results and discussion

3.1. Assay optimization and bisulfite conversion kit testing

Bisulfite conversion is the current method of choice to modify DNA for quantitative methylation analysis. However, it is also known to lead to DNA degradation and loss [8] and therefore, represents a potential bottleneck in DNA methylation analysis. We tested five commercially available BC kits (Table 1) to choose a kit for the BTA protocol. Bisulfite conversion of 200 ng, 10 ng, 1 ng and 500 pg human DNA was performed with each kit and quantified after singleplex PCR (Fig. 1A). Qubit quantification results were used to assess BC kits assuming that high DNA loss and severe DNA degradation during the bisulfite conversion workflow would lead to lower PCR product yield of the 308 bp target sequence of *ELOVL2*. At optimum DNA input (200 ng) all kits showed successful amplification results (Fig. S1). All BC kits tested produced adequate quantification results with 10 ng DNA input, which showed a mean product concentration of 23.0 ng/µl. When lowering the DNA input to 1 ng, all kits but one (Methylamp) achieved a product concentration of more than 1 ng/µl. Highest concentrations at 500 pg DNA input were determined for the EZ Direct (mean = 4.16 ng/µl) and the Premium (mean = 3.02 ng/µl) kit, which were chosen for further testing with the BTA. BC kits have already been evaluated in several studies with regard to DNA yield, fragmentation, specificity and conversion efficiency [33–36]. However, low DNA inputs were only considered by Tierling et al. (2018) [37], which did not include the BC kits picked for BTA optimization. Here, we explored the performance with low DNA inputs to select two kits for further analysis of bisulfite conversion efficiency by targeted MPS.

For optimization of the PCR assay, we evaluated the performance of two PCR kits: the Qiagen Multiplex PCR kit designed for multiplex PCR and the ZymoTaq PreMix optimized for amplification of difficult templates, such as bisulfite converted DNA (ZymoTaq PreMix, Protocol Version 1.0.1). Qubit quantification results after PCR are shown in Fig. 1B. The Qiagen Multiplex kit achieved significantly (Kruskal-Wallis Test: Bonferroni adjusted p-value < 0.0167) higher PCR product yields at 10 ng, 1 ng and 500 pg DNA input compared to the ZymoTaq PreMix and therefore, was used in the final PCR assay.

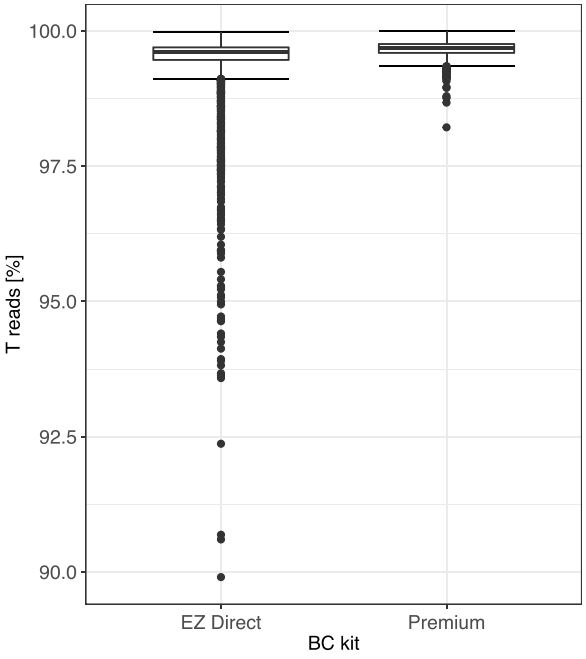

Fig. 2. Bisulfite conversion efficiency was estimated based on the percentage of T reads at non-CpG-C sites. Boxplots show the T reads percentage obtained for non-CpG-Cs of MiSeq FGx Run 1 (Samples: N = 18, non-CpG-Cs per sample: N = 139) using the Premium kit and MiSeq FGx Run 2 (Samples: N = 18, non-CpG-Cs per sample: N = 139) using the EZ Direct kit.

3.2. Bisulfite conversion efficiency

Conversion efficiency of the EZ Direct and the Premium kits was tested in the framework of the reproducibility study (Fig. 2). The overall mean conversion efficiency of the 18 processed differentially methylated DNA standards was high for both kits with more than 99.6 % and 99.4 % conversion for the Premium and the EZ Direct kit, respectively. Bisulfite conversion efficiency of both kits was indicated as > 99.5 % by the manufacturers. Our results appeared less variable within the Premium kit with a minimum mean conversion efficiency of 99.6 % for a single sample whereas the lowest mean conversion efficiency was 98.9 % for the EZ Direct kit. Furthermore, the EZ Direct kit exhibited more outliers with the percentage of T reads in single non CpG-Cs dropping to 89.9 %. Overall, the Premium kit showed more stable conversion rates in combination with a shorter conversion time

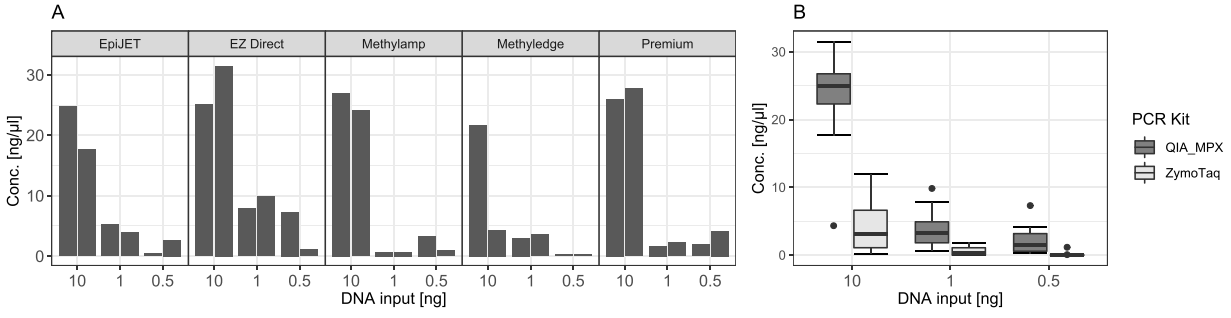

Fig. 1. (**A**) Quantification results for PCR products obtained by using the Qiagen Multiplex Kit after bisulfite conversion of 10 ng, 1 ng and 500 pg DNA with five different bisulfite conversion kits. All reactions were performed in duplicates and quantified using the Qubit fluorometer. (**B**) Quantification results for PCR products obtained by using the Qiagen Multiplex Kit (QIA_MPX) or the ZymoTaq Premix for amplification after bisulfite conversion of the five different kits (N = 10).

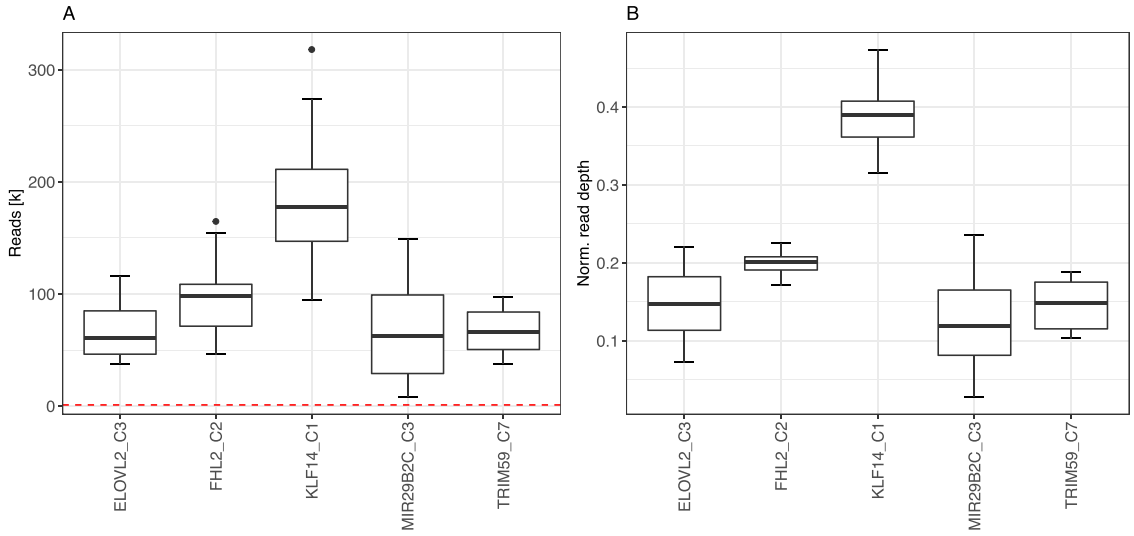

Fig. 3. Boxplots showing (**A**) read depth and (**B**) normalized read depth for all replicates (N = 32) of both MiSeq FGx runs at the five selected target CpG sites.

(1 h vs 3.5 h, Table 1) and was therefore chosen for sensitivity assessment.

3.3. Reproducibility

To test the assay's reproducibility, 200 ng DNA from nine different methylation standards were processed in duplicates with two distinct BC kits on separate MiSeq FGx runs. Both runs yielded high total sequence coverage with a mean of 388,600.9 reads (194,300.4 pairs) per sample for run 1 and 561,369.6 reads (280,684.8 pairs) for run 2. All target positions were covered by more than 5000 reads, exceeding the threshold of 1000 reads (Fig. 3A) that was set following suggestions by Masser et al. (2013) [38] for accurate methylation quantification. Evaluation of the distribution of reads between the five amplicons was performed by normalization of the read depth at one target CpG per marker by the total number of reads (Fig. 3B). *KLF14* yielded higher read depth, which was most likely a result of higher product yields during amplification, as suggested by Bioanalyzer results. At targeted CpG positions, A and G reads were considered as misincorporated bases. To assess variation of methylation levels due to erroneous base calls, the sum of A-reads and G-reads was divided by the total number of reads at the specific target site. Base misincorporation was estimated below 1.5 % (mean = 0.26 %) for all 18 replicates of both runs (Fig. S2), showing that erroneous base calling is expected to introduce only low variation and should not influence final methylation quantification.

The accuracy of age estimation strongly depends on the robust quantification of methylation levels at the targeted CpG positions. Quantification of methylation levels in the two reproducibility runs using different BC kits showed no statistically significant difference (linear regression analysis, Fig. S3); they were, therefore, treated as four replicates in downstream analysis. Plotting observed versus the expected methylation levels at the target CpG sites yielded only little deviation from a linear increase of methylation for *KLF14* and *TRIM59* (Fig. 4). A slightly stronger bias was observed for *FHL2* and *ELOVL2*, while *MIR29B2CHG* was found to overestimate all expected methylation ratios. Measured methylation levels showed a mean standard deviation of 1.4 within ratios, observing 4.3 as the highest standard deviation for the 10 % methylated DNA standard at *MIR29B2CHG*_C3. The difference to the expected methylation level is most likely a result from amplification bias that has been frequently reported for bisulfite converted DNA [39–41]. Moreover, fully methylated DNA standards are not 100 % methylated but defined by the manufacturer as very highly methylated DNA (methylation rates > 95 %), which was corroborated in our study (mean observed methylation of 96.0 % ± 1.9 for nominally fully methylated standards). Furthermore, we found that methylation values within the same amplicon and sample varied on average from 1.4 % for *MIR29B2CHG* with only three target CpG sites up to 3.2 % for *TRIM59* with eight target CpG sites.

3.4. Sensitivity

The optimum DNA input for most of the commercially available BC kits was indicated between 200 and 500 ng DNA by the manufacturers, which is a considerably high amount in the forensic genetic context. Although lower DNA inputs can be used, the tested BC kits showed a strong decrease in amplicon yield from 10 ng to 1 ng human DNA input in the previously described optimization tests (Fig.1). Thus, for the sensitivity study, we analysed a dilution series of five DNA methylation standards (Zymo) from 200 ng to 1 ng. To increase sensitivity, BTA was designed as a multiplex reaction, for which the whole BC eluate can be used in the PCR assay. Target coverage was exceeding the 1000 reads threshold for all DNA inputs and all five markers except for one of the 1 ng replicates at *ELOVL2* target positions (Fig. S4). This indicates that the assay is technically capable of processing low DNA input samples. However, read depth alone is not sufficient to set meaningful low input limits for accurate methylation quantification. To establish such a low limit for initial DNA amount, we considered the increase of absolute differences in measured methylation levels between technical replicates (Fig. 5). We observed constant variation in methylation quantification across dilution steps down to 20 ng DNA input with the highest median difference between duplicates at 50 ng with 1.6 %. When excluding the 10 ng (median = 2.8 %) and 1 ng (median = 9.0 %) samples, the quantified methylation levels were consistent with the results obtained in the reproducibility study (Fig. 4B) showing an average standard deviation of 2.2 % across markers and ratios. Taking into account that the DNA input at PCR level was much lower (approximately 26 % [33] to 45 % [34] DNA loss from optimal input was reported for the Premium kit), the increased variation for 10 ng and 1 ng samples may be explained by stochastic effects. As any quantitative method, DNA methylation analysis is inherently linked to sample representativeness. Therefore, erroneous methylation quantification can be a result of analysing only a small number of DNA molecules that do not reflect the methylation level of the original sample or tissue. Stochasticity in DNA methylation quantification was addressed recently in an *in silico* study by Naue et al. (2018) [42] who calculated that 5 ng of DNA (1392 template molecules) are needed to resolve a 10 % difference in DNA methylation. However, this assumption does not take into account DNA loss during laboratory workflows and technical variation. The

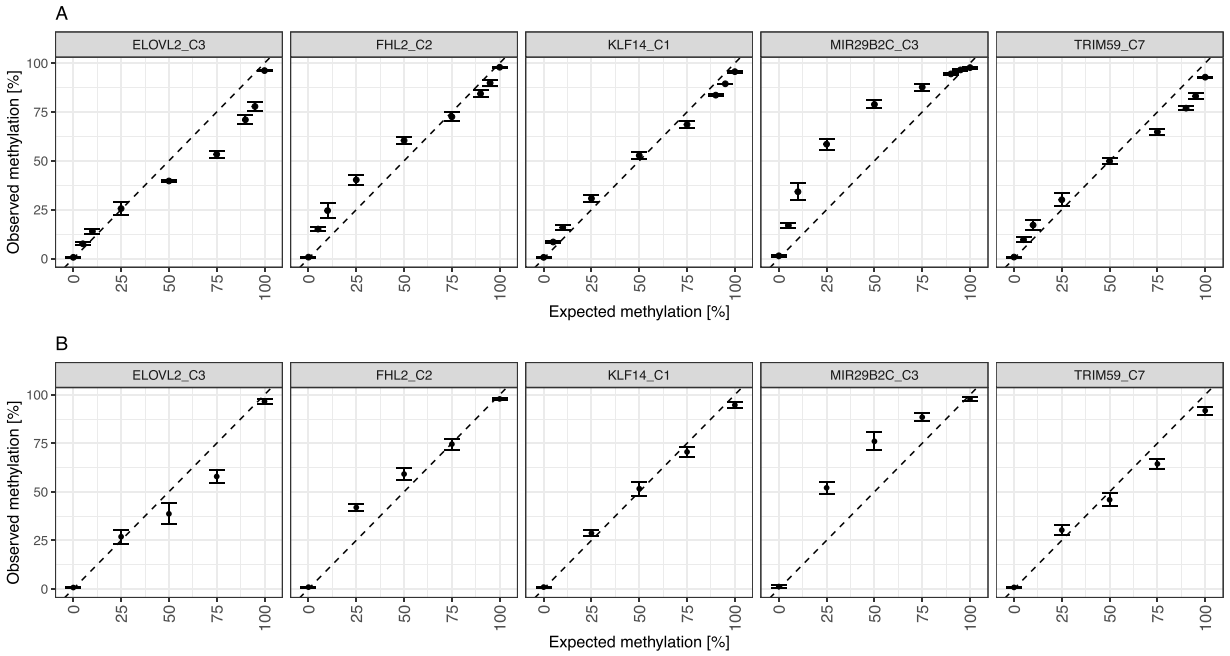

Fig. 4. (**A**) Methylation levels quantified at one target CpG site per marker of the nine DNA methylation standards (N = 4) used to assess reproducibility. (**B**) Methylation levels quantified within the sensitivity study at one target CpG site per marker of five methylation ratios from 200 ng to 20 ng DNA input (N = 8). Error bars represent the standard deviation. Dashed lines depict the line of identity (intercept = 0, slope = 1).

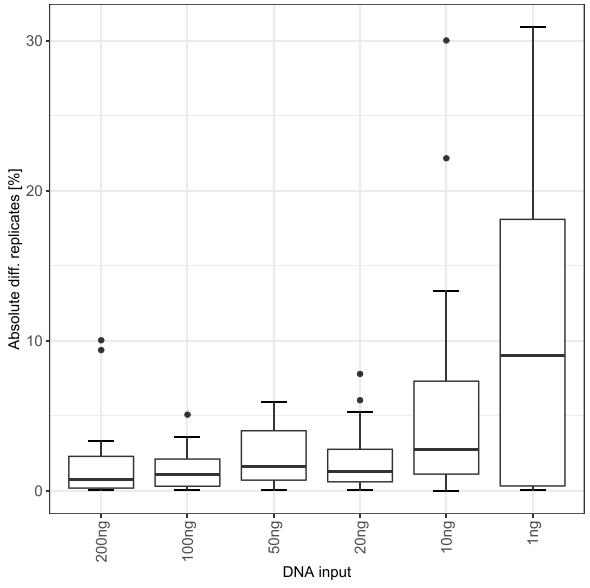

Fig. 5. Boxplots showing the absolute difference in methylation quantification between duplicates. Difference was calculated for one target CpG site per marker and for all five DNA methylated standards (0 %, 25 %, 50 %, 75 % and 100 %; N = 20).

empirically determined methylation values suggested that at least twice of this DNA amount would be needed for reliably telling apart a 10 % difference in methylation levels. Down to 20 ng DNA, none of the duplicates had a difference exceeding 10 % at target CpG sites, while at 10 ng, four target CpGs (*ELOVL2*_C3 at 25 %, *FHL2*_C2 at 50 %, *MIR29B2C*_C3 at 50 % and 25 % methylation rate) showed a greater discrepancy between duplicates (Fig. S5). When examining outliers in IGV, no noticeable differences were observed compared to samples within the expected range.

4. Conclusions

Here, we present the VISAGE basic tool for age estimation from DNA of blood sources based on a bisulfite PCR multiplex simultaneously targeting 32 age-informative CpGs from five genes (*ELOVL2*, *MIR29B2C*, *FHL2*, *TRIM59* and *KLF14*) followed by targeted MPS. Each step of the protocol was optimized in consideration of forensic requirements including a performance test of five BC and two PCR kits. Our results indicated that BC kits are performing differently in terms of DNA recovery of low DNA input samples. However, this performance test was only intended for optimizing the VISAGE protocol as, to the best of our knowledge, a comprehensive sensitivity study of BC kits is missing as of yet. DNA methylation standards were found to be suitable to assess the amplification bias introduced by the method and the reproducibility of DNA methylation quantification. The assay showed robust quantification of DNA methylation levels down to 20 ng DNA input into BC and elevated variability for 10 ng samples whereas measured methylation levels from 1 ng DNA where far from expected values.

This study describes the combination of established DNA methylation markers known to correlate with chronological age into a new multiplex PCR/MPS-based DNA methylation quantification assay and its overall performance with control DNA samples. Further complementing studies including the performance of this tool in other laboratories are underway. The development and validation of a statistical model for age prediction based on the CpG markers and the technology used in the BTA are currently being addressed by the VISAGE Consortium. This includes the generation of data produced with VISAGE prototype tools for age estimation as the method-to-method bias in DNA methylation analysis precludes the use of previously established age-prediction models for this marker set. Moreover, tool developments to include markers for age estimation from DNA of non-blood sources are also ongoing. Such studies will allow the implementation of this VISAGE tool in forensic routine laboratories.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fsigen.2020.102322.

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