The EpiTect Methyl qPCR Assay as novel age estimation method in forensic biology

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Abstract

Human aging is associated with epigenetic modification of the genome. DNA methylation at cytosine is apparently the best characteristic among this modification which occurs through mammalian life time. Such methylation changes at regulatory region can provide insights to track contributor age for criminal investigation.

The EpiTect Methyl II PCR system (QIAGEN) was used to compare methylation levels of CpG islands of the promoter regions of 4 age related genes (*NPTX2*, *KCNQ1DN*, *GRIA2* and *TRIM58*). However more genes were investigated, only these were the success ones. This technique is based on the detection of remaining input genome after digestion with a methylation-sensitive restriction enzyme. This study examined 80 female DNA samples of various ages (18-91 years) obtained from blood, using primers designed to flank the studied gene loci. The data obtained from DNA methylation quantification showed successful discrimination among volunteered ages. Overall, the difference between predicted and real age was about 11 years and absolute mean differences (AMD) was only 7.2 years error. We suggest the EpiTect system can be used as fast and simple innovative tool in future forensic age estimation.

1 Introduction

Human aging is multifactorial process and associated with epigenetic modification at specific genome sites. Genome-wide analysis shows that global hypomethylation is typically associated with aging [1-4]. Using epigenetic signature differences to classify and distinguish age level is a current topic area of forensic interest. DNA methylation on cytosines is the best characterised among the epigenetic modifications which occurs through mammalian life time. Blood samples have been collected from female volunteers aged 18-91 years. The aim of the study was to find a correlation between methylation level and human age by using EpiTect System - a rapid and simple methodology originally intended for cancer epigenetics research, but here applied to

forensic aging research where it can operate with low concentrations of template DNA and without the need for bisulphite chemical conversion.

Methylation changes at specific gene regulatory regions can provide insights to define contributor age for criminal[5]. Researchers have found that 60–70% of promoter regions of the human genome overlap with CpG islands [6-10]. These islands are regions of DNA (not less than 200 bp) with a high frequency (>50%) of CpG sites [7, 11, 12]. CpG Islands located in promoter regions of specific genes of interest which are known to show altered methylation levels as a function of age were analysed using the EpiTect Methyl qPCR Assay.

The methylation analysis technique used in our study relies on the detection of the remaining input genome after digestion with methylation-sensitive restriction enzymes. Following cleavage, intact DNA is quantified by real-time PCR. Subsequently, by comparing this with assay results from mock digest (no enzyme added), the relative fractions of methylated or unmethylated digestion can determine by using the ΔCt method, and the result displayed as percentage methylated (M) and unmethylated (uM). The promoter region of genes which are highly expressed are mostly unmethylated, regardless of the high GC content [10]. Existing technologies to quantify DNA methylation pattern include: combined bisulphite/restriction analysis (COBRA) [13], methylation-specific PCR[14], bisulphite sequencing [15], high resolution melting (HRM) analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [16], next-generation sequencing [17], Pyrosequencing®[18] and fluorescence-based real-time MethyLight PCR[19]. The workflows commonly start with an initial stage of sodium bisulphite DNA conversion. This step is critical for the analysis because any technical mistakes made here profoundly alter apparent methylation level and cannot be corrected without repeating the treatment: however, additional DNA may not be available for this in the forensic setting. Thus, despite their single-base resolution of the methylome, these methods are not free from drawbacks such as protocol optimisation difficulties, the laborious nature of processing many samples, as well as the requirement for special instruments and equipment.

2 Materials and Methods

To identify gene promoter methylation, EpiTect® Methyl qPCR Array (SA Bioscience, Qiagen, Hilden, Germany) was used according to the manufacturer's instructions.

Biological samples were collected after ethical approved: a consent form was discussed and signed by the volunteers. Blood swabs were taken using ForensiX tubes by the investigator, its advantages detailed in our previous study [20]. To decrease the biological parameter diversity, only females were recruited (80 volunteers), their ethnic background (Kurdish) and age range (18-91 years) as phenotype information were recorded independently.

2.1 Restriction Digestion

After measurement of DNA concentration by qPCR quantitative method, the extracted DNA was measured for purity purposes by Nanodrop-1000 UV-vis spectrophotometer. Only those samples which passed both above criteria were exposed to digestion performed by using EpiTect Methyl II DNA Restriction Kit (SA Bioscience, cat#: 335452). The final volume of components was mixed thoroughly and centrifuged briefly in a micro centrifuge tube. Four reaction digestions were carried out: no-enzyme (Mo), Methylation Sensitive enzyme (Ms), Methylation dependent enzyme (Md) and Methylation sensitive and dependent enzymes (Msd) as showed in **Table 1** .

Table 1 Restriction Digestion Step: Reaction digestions set up for each gene and each sample. Four tubes were labelled as (Mo, Ms, Md and Msd). Sensitive A or dependent B enzymes were added to Ms, Md and Msd tubes respectively, but ddH2O was added to Mo instead of enzyme.

Component	M_o	M_s	M_d	M_{sd}
Reaction Mix	28 µl	28 µl	28 µl	28 µl
Methylation sensitive Enzyme A	--	1 µl	--	1 µl
Methylation- dependent Enzyme B	--	--	1 µl	1 µl
RNase-/DNase-free water	2 µl	1 µl	1 µl	--
Final volume	30 µl	30 µl	30 µl	30 µl

The components were mixed gently by pipette and briefly spun down. The mixture was incubated at 37 ºC overnight, followed by heating for 20 minutes at 65 ºC to inactivate the enzymes.

2.2 PCR Set up

Individual reactions were prepared for each of the four digestions (Mo, Ms, Md and Msd) in a tube according to Table 2.

Table 2

PCR Set Up: The table shows the PCR set up preparation with amplifications performed in a 96-well plate.

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
PCR primer mix	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Mo digest	5.0 µl	-	-	-
Ms digest	-	5.0 µl	-	-
M_d digest	-	-	5.0 µl	-
M_{sd} digest	-	-	-	5.0 µl
RNase/ DNase free water	6.5 µl	6.5 µl	6.5 µl	6.5 µl
Final volume	25 µl	25 µl	25 µl	25µl

2.3 QPCR instrument setup to Quantify DNA Methylation Analysis

The EpiTect Methyl II PCR Arrays which does not require bisulphite conversion can be run on any real-time PCR instrument with instrument-specific SYBR Green Master Mix acquired from SABiosciences. As a consequence of this, RT2SYBR Green ROX™ qPCR Mastermix (SA, Bioscience, cat# 330520) was selected in this experiment to be used with our Stratagene Mx3005P real time PCR instrument (Agilent Technologies, CA, USA). SYBR® Green (with dissociation curve) protocol was used thermal profile set up with following condition as shown in **Error! Reference source not found.**.

Table 3

Real time PCR programme: The table shows the PCR condition to quantify genomic DNA for methylation level study.

Temperature	Time	Number of cycles
95°C	10 min	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min	40 cycles

For dissociation curve, segment three was added to the programme: 95 ºC for 1 min and one cycle, then annealing at 55 ºC for 30 seconds and another denaturation at 95 ºC for the same period and one cycle.

3 Discussion of results

The EpiTect system was originally used in detection of breast cancer [21] and brain cancer [22]. Therefore, this is the first study to use it to study DNA methylation for forensic purposes.

Human DNA of various ages (18-91 years) obtained from blood, using pre designed primers in the kit that flank a studied genes loci. The results of the 80 samples (each repeated twice) were displayed as percentage of unmethylated and methylated fraction. This shows that DNA methylation successfully discriminated volunteered age.

It is known that DNA methylation has a crucial role in normal cell differentiation and development [23, 24]. Generally the promoter regions are hypomethylated and CGIs lack DNA methylation in the gene [25, 26]. However a small percentage are hypermethylated as well [24]. Based on the assumption that epigenetic changes may drive the heterogeneity of the cell, the promoter methylations of 13 candidate genes (*BCAS4, KCNQ1DN, GRIA2, TRIM58, EDARADD, TOM1L1, SOGA1, NPTX2, FGF7, ZC3H12D, ASPA, PDE4C* and *ITGA2B*) and details are summarized in Table 4).

Previous studies have showed that these genes can be used as forensic marker for either age discriminate or tissue identification [27-29]

Table 4

Screening of 13 candidate genes using EpiTect Methyl qPCR Array: The table shows description, symbol, location and PCR product size of all 13 studied genes which screened by using EpiTect Methyl system.

No.	Gene description	Symbol	UniGen no.	NCBI ID	Catalog #	CpG island location	PCR size
1-	KCNQ1 downstream neighbour	KCNQ1DN	Hs. 127821	55539	EPHS102112-1A	Chr11: 2890388-2891337	157bp
2-	Breast carcinoma amplified seq4	BCAS4	Hs. 708239	55653	EPHS109350-1A	Chr20: 49411401-49412142	254bp
3-	Glutamate receptor	GRIA2	Hs.32763	2891	EPHS111325-1A	Chr4:158141404-158141836	189bp
4-	Tripartite motif containing 58	TRIM58	Hs.269151	25893	EPHS101433-1A	Chr1:248020330-248021552	262bp
5-	EDAR-associated death domain	EDARADD	Hs.352224	128178	EPHS101394-1A	Chr1: 236558459 - 236559336	196bp
6-	Aspartoacylase	ASPA	Hs.171142	443	EPHS105621-1A	Chr17: 3375006 - 3375237	215bp
7-	Target of myb1	TOM1L1	Hs.153504	1040	EPHS106189-1A	Chr17: 52977866 - 52978307	147BP
8-	Phosphodiesterase 4C	PDE4C	Hs.132584	5143	EPHS107250-1A	Chr19: 18335072 - 18337375	217bp
9-	KIAA0889	SOGA1	Hs.460807	140710	EPHS109245-1A	Chr20: 35491001 - 35492805	257BP
10-	Neuronal pentraxin II	NPTX2	Hs.3281	4885	EPHS113305-1A	Chr7: 98245805 - 98247759	174bp
11-	Integrin, alpha 2b	ITGA2B	Hs.411312	3674	No products	Chr17:42476290-42477054	765bp
12-	Fibroblast growth factor 7	FGF7	Hs.567268	2252	No products	Chr:1549549862-49820528	64148bp
13-	Zinc finger CCCH-type	ZC3H12D	Hs.632618	340152	No products	Chr6: 149447630..149485012	37382bp

However, in our study only 4 of 13 genes were showed significant different methylation levels according to age Table 5.

Table 5

Location of four Interest Genes: The table shows bioinformatics ad location of four genes which correlated with age and passed EpiTect system criteria. In addition to both SEC and DEC which is sensitive and dependent enzyme control respectively.

Gene Symbol	Catalogue Number	CpG island Position	UniGene number	NCBI ID	PCR product size
GRIA2	EPHS111325-1A	Ch4:158141404-158141836	Hs.32763	2891	189
KCNQ1DN	EPHS102112-1A	Chr11:2890388-2891337	Hs.127821	55539	157
NBTX2	EPHS113305-1A	Chr7:98245805-98247759	Hs.3281	4885	174
TRIM58	EPHS101433-1A	Chr1:248020330-248021252	Hs.269151	25893	262
EP-SEC	EPHS115450-1A	Chr0:0-0			159
EP-DEC	EPHS115451-1A	Chr0:0-0			220

The level of methylation percentage was used to test for association between examined genes (*NPTX2, GRIA2, TRIM58* and *KCNQ1DN*) and age. Correlation values (R-square) for methylation at these genes were 0.452, 0.501, 0.808 and 0.550 respectively. The Pearson correlation (p-value) was significant in all cases (p-value < o.o5).

Meanwhile the assay results for *EDARADD, FGF7, ZC3H12D, TOM1L1, SOGA1* and *BCAS4* genes did not show any correlation between age and methylation level (data not shown) and no significant differences were found (p-value >0.05). Regarding the analysis of the CGIs for *ASPA, PDE4C* and *ITGA2B* genes, a suitable assay was not available from SA-Bioscience.

When information about these three genes was sent to the bioinformatics department of QIAGEN, response was that it would not be possible to create an assay for *ASPA, PDE4C* and *ITGA2B*, "*because there were not enough restriction sites in the provided sequences or within 100 bp on either side of them in the genomic context, so no primer design is feasible"* (Pers. Comm., from QIAGEN [30]. Blood DNA was used in this study, but more experiments need for other tissue types to investigate the discrimination power among different ages. Other biological fluids like saliva might give similar result as it is popularly thought that buccal epithelial

cells are the main source of DNA in saliva, but one study shows that up to 74% of the DNA in saliva comes from white blood cells [31]. Furthermore, all oral samples are not equal, because DNA quality from the same amount of saliva can be very variable and can explain the differing methylation extent of same age donors [31] . Saliva can be enriched in epithelial and blood cells, especially when the donor has gingivitis (i.e. teeth gum diseases) and resulting bleeding. Epigenetics is interaction between genetics and environment, so external factor such lifestyle, diet, pregnancy and even natural disasters, have an effect on methylation patterns [32]. It could be argued that the EpiTect technique is a favourable route because it assesses multiple CpG sites simultaneously rather than single CpG sites. Most biological changes of DNA methylation are known to occur at multiple CpG sites simultaneously [10, 33, 34]. This regional analysis may better represent the methylation status of a CpG island than a specific analysis of single sites.

Generally the human genome is hypomethylated through aging. It has been observed that the new-born DNA had 494,595 more methylated CpG (mCpGs) dinucleotide than did the centenarian DNA (16,280,495 vs. 16,775,090 on the Watson and Crick strands, respectively [3]. But CGI methylation levels increase in promoter region in most cases, as these entire four target regions were hypermethylated with aging as the scatter plots between each gene and age shown in Fig. 1.

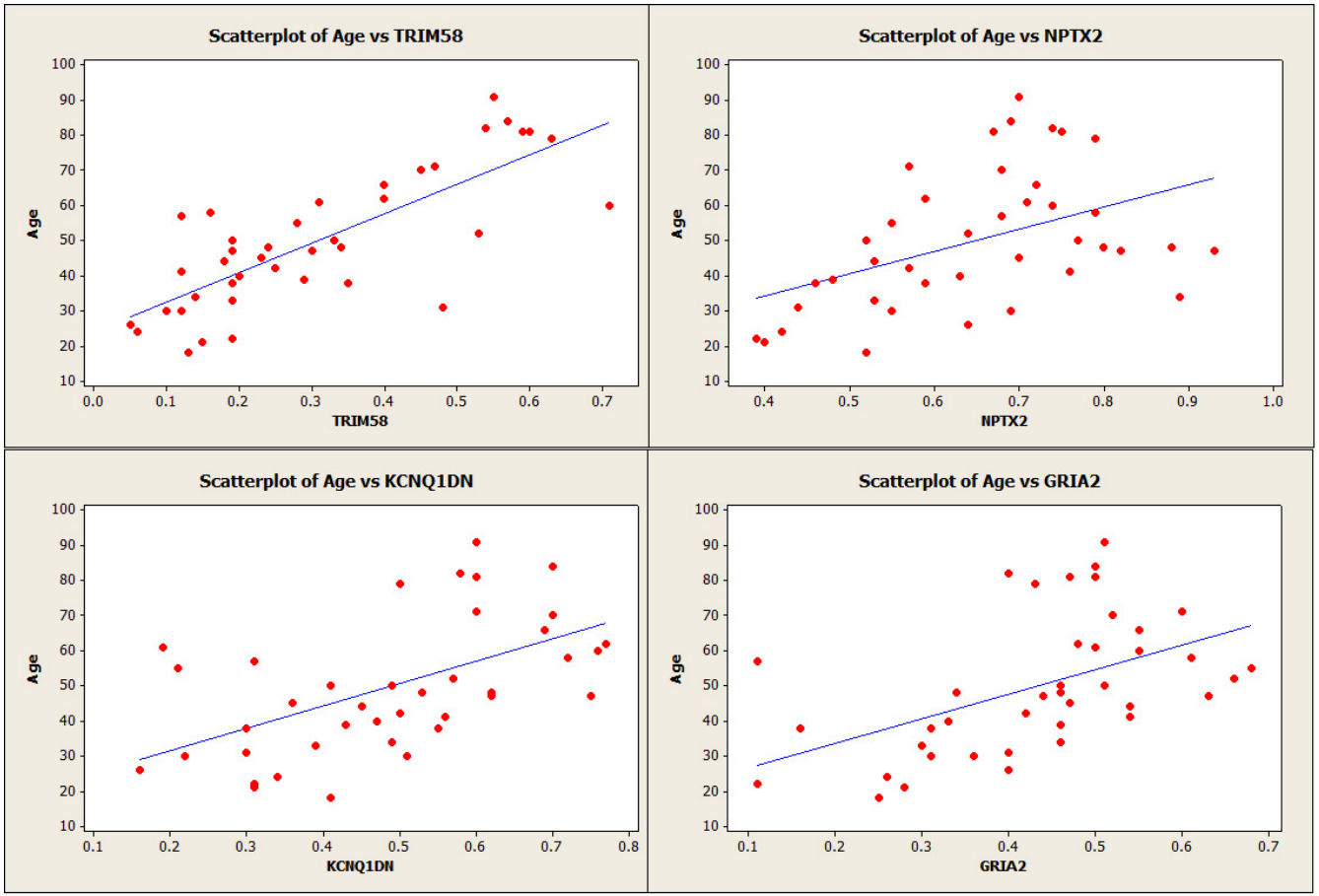

Fig. 1. Prediction of age by methylation level: The figure shows scatterplot of Correlation between methylation level of (*NPTX2, KCNQ1DN, GRIA2* and *TRIM58*) genes and age.

Statistical methods to predict age from methylation data were performed and multivariate linear regression found the "standard error" value from the analysis report. The result of R value, R squared value, adjusted R squared value and "standard error of estimate" is 0.848, 0.720, 0.687, and 10.80734, respectively. The equation model that built in previous step was applied to build a linear regression to predict the real age for the female blood samples as shown in Fig. 2. Overall the calculation differences between predicted and real age was about 11 years and the absolute mean deviation (AMD) only in error by 7.2 years. Of course there will be some difference between the two values, and the standard error value is usually larger than the average absolute difference value.

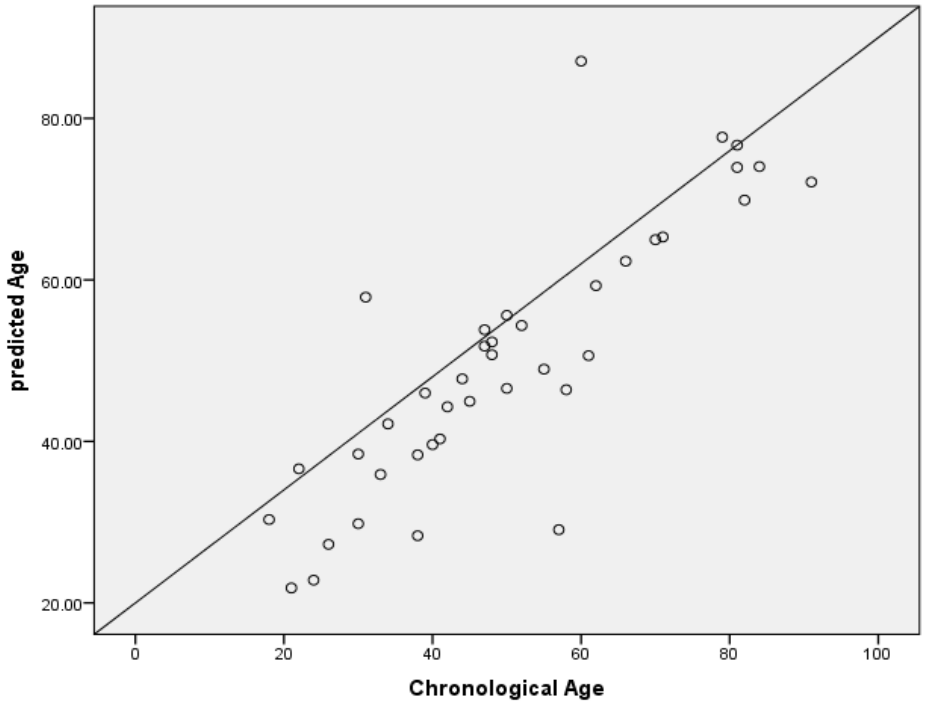

Fig. 2. Predicted age versus chronological age: The figure shows a scatter plot of predicted age against chronological age of blood samples with regression line.

The results of this study show that the EpiTect system can be used to estimate the age of the donor through DNA methylation levels with only 11 year error. Despite the usefulness of this figure for forensic purposes, some recent studies have presented an estimation of age with only 5-7 years error [5, 35]. However, our result is still better than any other age estimation approach such as the correlation with of telomere length or other approaches. This is a relatively small sample size; caution must be applied, as the findings might not be transferable to other gender (men) or females from different population. The present findings seem to be consistent with Koach *et. al*. (2011) who have shown that the methylation level of

CpG sites in *NPTX2, KCNQ1DN, GRIA2* and *TRIM58* promoters strongly correlate with age with 11 error year [29]. What is surprising in our study is that, the analysis was done without DNA conversation with bisulphite sodium.

Therefore, EpiTect Methyl qPCR system is interesting alternative, as only qPCR instrument need and available in any laboratory. It is a complete system, from sample isolation to result. More biological, quicker and cheaper are other advantages of this system which is complementary to bisulphite methods as well.

In comparison to other method, only 20ng DNA per gene was required for methylation analysis. In contrast Cygenia (http://www.cygenia.com/) for instance, needs at least 150 ng DNA or 1mL of blood for methylation diagnostic using pyrosequencing. ZymoResearch (http://www.zymoresearch.com/) is another bioscience company for methylation pattern analysis service, but 0.5-1 µg DNA is required. Furthermore they charge about £ 75 (€ 95) / £ 90 ($150) per sample, compare this to the EpiTect Assay which only costs £10-15 per sample as shown in Table 6.

Table 6

EpiTect system VS other Techniques: The table compares EpiTect with pyrosequencing and Illumina next generation sequencing.

Technique	**EpiTect Methyl System**	**Pyrosequencing**	**Next Generation Sequencing**
Product	SA bioscience	Cygenia	ZymoResearch
Amount require	20ng/ Gene	150 ng	0.5-1 µg
Quality Absorbance: 260/280 ratio	high > 1.7)	high > 1.7)	high > 1.7)
Instrumentation	Only qPCR	Pyromark	Illumina
Bisulphite Sodium Treatment	No	Yes	Yes
Cost	£ 10-15	£75	£90
Time (hours)	12	35	35

3.1 Principle of the commercial EpiTect system

The EpiTect Methyl II qPCR system (SA Bioscience, Qiagen, Hilden, Germany) is based on the quantitative detection of remaining input DNA within a sample population after treatment with methyl-sensitive and methyl-dependent enzymes[36, 37]. Primers are designed by an optimized computer algorithm to ensure that the amplicon contains

cutting sites for both digestion types and are specifically designed for analysing the DNA methylation status of CpG islands. SYBR Green-based real time PCR detection is employed after digestion. To make the system work, each genomic DNA is subjected to four separate digestion treatments according to the manufacturer`s protocol. The first condition is Mock digest (Mo) which means no enzymes were added and the product of this digestion represents the total amount of input DNA for real time PCR detection. The second condition is a methylation-sensitive digest (Ms) which cleaves unmethylated DNA and real time PCR therefore hypermethylated DNA. The methylation-dependent digest (Md) is the third condition, which digests methylated DNA and the qPCR detects the remaining unmethylated DNA. The final condition is for the double digest (Msd), both enzymes were added, and all DNA molecules (both methylated and unmethylated) are digested. This reaction measures the background fraction of input DNA refractory (R) to enzyme digestion and double digestion (Mdd). The relative amounts of DNA species from the targeted regions are assessed a comparative ΔCT calculation using an automated Excel-based data analysis template provided by the manufacturer. As little as 2µg genomic DNA can be used to profile the methylation status of target genes and methylated DNA can be reliably detected in heterogeneous samples [38]. This technology yields data comparable in quality to bisulphite Sanger sequencing and Illumina® assays, without the need for the bisulphite conversion step [25]. The basic principles of the EpiTect Methyl II PCR System along with performance, verification, and application data to demonstrate its robust potential for methylation profiling in various biological systems and for the screening of DNA methylation biomarkers[25, 38].

3.2 Data analysis

After quantification programme has completed, the row C_T values were obtained according to manufacturer`s instruction of the used real-time PCR instrument. Standard Microsoft Excel-based template automatically performed all threshold cycle difference (ΔC_T) based calculations from the raw value of the (C_T) to determine the methylation level of specific gene[22]. The C_T values of both (sensitive and dependent) digests with the mock digestion values will be analyse with a free software program which it calculates and reports the methylated and unmethylated proportion of the DNA. This free software is available online and can be downloaded at: (http://www.sabiosciences.com/dna_methylation_data_analysis.php).

The EpiTect® Methyl II PCR Array System is accurate and reliable technology for DNA methylation studies of several genes simultaneously[25]. In this fast and an innovative technology, predesigned and ready-to-use primers were provided to detect reliable screening of high frequency of CpG sites termed CpG islands (CGIs) at promoter region which is generally lack DNA methylation in the gene [25, 26].

3.3 Statistical analysis for the EpiTect assay

Statistical analyses were performed for all data using Excel 2010 (Microsoft Corporation), SPSS and Minitab v16 (Minitab Inc.). First simple linear regression (LR) for each group of females ranging in age from 18 to 91 years was used to analyse the relationship (Correlation R^2) between the methylation percentages of each single gene. The result was considered significant at Statistical Significance (i. e. P-values) below 0.05 (p-value < 0.05).Standardized regression coefficients (β) were used to compare the effect size of particular gene. In the next step, multivariate linear regression (MLR) was applied, allowing simultaneous analysis of all the tested genes. In the second step, a linear regression prediction model between predicted and real age was developed based on the methylation data obtained for the same samples.

3.4 Validation of enzyme digestion efficiency

A success of the EpiTect Methyl II PCR system depends on efficient DNA digestion by methylation specific restriction enzyme (MSRE) and methylated dependent restriction enzyme (MDRE). Each assay includes specific enzyme control methylation-sensitive enzyme control (SEC) and methylation-dependent enzyme control (DEC) for monitoring the cutting efficiencies of these enzymes and to ensure that the result is reliable and reproducible [25]. After the C_T values are pasted into the Microsoft® Excel® data analysis spreadsheet, a "Pass" or "Fail" result is returned for the SEC and DEC controls Fig. 3. Each of SEC and DEC specific primers are included in each EpiTect assay and are available separately as primer assay (Cat.#.EPHS115450-1A and EPHS115451-1A), respectively.

Two control DNA molecules (one completely methylated and other unmethylated) are spiked in buffer of EpiTect Methyl II DNA restriction kit Cat. #. (335452), each control DNA has

specific enzyme-sensitive target regions flanked by unique primer regions. The results were accepted when both SEC and DEC assays passed the quality control. Thus for SEC, the C_T values of Ms and Mo digests were pasted in to excel data analysis template. If the C_T values difference was equal or greater than four (ΔC_T [M_s-M_o] > 4), the software showed "Pass" in the analysis for. Likewise the difference between C_T values of Md and Mo digests $_{should}$ be (ΔC_T [M_d-M_o] > 4) for DEC assay. These results confirm that more than 93.6% of the control DNA molecules were cleaved, so the enzyme was active and digested input DNA samples efficiently.

1	Samples		1		2	
2	Symbol	Catalog Number	UM	M	UM	M
3	NPTX2	EPHS113305-1A	35.22%	64.78%	54.04%	45.96%
4	KCNQ1DN	EPHS102112-1A	44.78%	55.22%	11.12%	88.88%
5	GRIA2	EPHS111325-1A	25.10%	74.90%	42.00%	58.00%
6	TRIM58	EPHS101433-1A	56.91%	43.09%	12.10%	87.90%
7	SEC	EPHS115450-1A	Pass		Pass	
8	DEC	EPHS115451-1A	Pass		Pass	

Instructions | Raw Data | QC Data Report | **Results** | Calculations

Fig. 3. SEC and DEC control: The figure shows the result of the Microsoft excels data analysis and how both controls (SEC and DEC) passed.

An analytical window (W) in the Microsoft Excel data analysis template represents the CT value differences between double digest and mock digests as in Fig. 4. **Error! Reference source not found.**

To have reliable result, (W) should be greater than 3 (ΔC_T [M_{sd}-M_o] > 3) which is mean more than 87.5% of input DNA was digested. In contrast if (W) was less than three (W < 3) this means that refractory DNA percentage (R) is greater than 12.5 percent (R > 12.5%). Thus data quality control report (QC Report) worksheet in the excel software is reported as a "Failure" Fig. 4.

	A	B	C	D	E	F
1	Samples		1		2	
2	Gene Symbol	Catalog Number	W	R	W	R
3	BCAS4-1	EPHS109350-1A	2.74	Failure	-4.45	Failure
4	KCNQ1DN	EPHS102112-1A	-4.81	Failure	-0.81	Failure
5	GRIA2	EPHS111325-1A	-0.93	Failure	2.71	Failure
6	TRIM58	EPHS101433-1A	1.6	Failure	1.4	Failure
7	SEC	EPHS115450-1A	11.78	0.028%	10.62	0.064%
8	DEC	EPHS115451-1A	5.58	2.091%	10.02	0.096%

Instructions | Raw Data | QC Data Report | Results | Calculations

Fig. 4. QC Data Report worksheet: The figure shows the result of the quality control report and the value in analytical window (W) was abnormal (i.e. less than three) for the all age related genes. Similarly the refractory factor (R) of digestion was not complete for the input DNA means that the digestion efficiency was not high for DNA sample through of the Microsoft excel data analysis.

Although it has been recommended by the manufacturer to use (0.5-1µg) of DNA template, we diluted the input DNA to the minimum amount possible to mimic a real crime case scenario. Hence, the minimum amount which passed the system criteria was 120ng of the template. The final amount of the template will become only 20 ng (i.e. 120/6) per gene. This is because the tube content was divided to six tubes (4 target genes and the controls (SEC and DEC)) as it is mentioned in detail in the enzyme digestion step. This amount of DNA sample is still considered high as it cannot be guarantee to retrieve such high exhibit in the scenes of the crime. Adjusting the system to produce results with less input DNA will be necessary of this technique is to be implemented into forensic work in the future. Furthermore, expand the study to other population and/or different gender (male) to see if the assay will predict the same rate as current study. Further study needs to validate the method with training sample and examine the EpiTect system to see if different tissue type can be discriminated, especially in sexual assault case.

3.5 Analysis of noise factors affecting methylation ratio

Depending on the fact that methylation modification is occurring in different regions of the chromosome [39]. EpiTect methyl II qPCR assay is based on detection of quantified amount of DNA which survived upon enzyme digestion. The CpG loci of our targets are differentially methylated among individuals and the methylation patterns are generally age-

dependent. However, as a result of natural diversity in methylation ratio among people with different ages, even with the same age (but not same environmental exposure and lifestyle impact), there could be potentially some overlap in the observed methylation ratios [31, 40]. Some artifacts such as differences in DNA template concentration for instance (stochastic effect and pipetting error) are associated during experimental steps [41]. Furthermore, recent studies showed that epigenetic patterns can be affected by a wide variety of external influences including diet, exercise, smoking, maternal environment, and more [42, 43].

It has noted; that chronological age is not identical with biological age and it is conceivable that some of the discrepancy between predicted and real age can be attributed to this difference further research might facilitate determination of the biological age for personalized medicine.

3.6 Validation of the method

To assess the statistic result analysis for unknown age samples (training set), the technique of cross validation model or sometime called 'rotation estimation' [44] was used. Thus, 40 samples as training set samples were applied to the equation model and calculate the error rate between real age and predicted age as in Table 7.

Table 7

Evaluation of the prediction potential of the EpiTect assay for training set of 40 blood samples. The prediction result was considered correct if the predict age matched the chronological age ± 11 years.

Age Prediction	**Age Category (year)**			**Total**
	18-39	40-59	≥ 60	
Incorrect (n)	6 (46.1%)	3 (16.6%)	8 (80%)	17 (41.46%)
Correct (n)	7 (53.8%)	15 (83.3%)	2 (20%)	24 (58.54%)

The table of the training set showed that the method is quite good for middle age (40-59 years), but interestingly for elderly women (over 60) the equation was able to predict only

20% of the samples and this result needs further investigation by studying other factors like biomedical and environmental causes.

4 Conclusion

This is the first report describing the use of the EpiTect Methyl II PCR system (QIAGEN, SA-Bioscience) to estimate human age through methylation analysis. Although the system does not have the best age estimation accuracy published to date, it is better than telomere-length-based approaches and much faster and cheaper than DNA methylation quantification technologies that use bisulphite conversion chemistry. The system can be used as a useful tool in forensic work, or non-criminal instances where an objective estimation of age is required (e.g. family law, immigration, and insurance purposes etc.), with only an 11 year error between estimated and chronological age.

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