Application Note

Evaluation of QIAGEN® PyroMark® age estimation technology

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Introduction

Forensic scientists had been searching for approaches to predict age from biological samples left behind at crime scenes. The ability to estimate the age of a suspect is used as an investigative tool by forensic investigators faced with tremendous difficulty in identifying or narrowing down the perpetrators based on the biological evidence left at the crime scene, more so if it involves serial grievous crime offenders. Biological age can also be utilized in fraud cases or identity theft to ascertain the age of the individual in doubt.

There are several DNA based methods that can be applied to estimate human age, such as aspartic amino acid (Asp) racemization which decreases with increasing age, mRNA, DNA rearrangement, or based on telomere length. Nevertheless, DNA methylation is the preferred technique as it has a strong correlation to aging and when coupled with Pyrosequencing® methods utilized in the detection of DNA methylation, provides a valuable tool to estimate age.

The research described here is crucial to evaluate the feasibility and suitability of this method used in Forensic DNA Division in the Department of Chemistry (KIMIA Malaysia) and to determine the accuracy of age estimation of methylated DNA using pyrosequencing system on the QIAGEN PyroMark Q48 Autoprep Instrument, using the published linear regression on the Malay population.

Materials and Methods

Twenty (20) reference samples of varying age were extracted and purified using a validated test method entitled Extraction of DNA from Bloodstains Samples Using the Automate Express™ Forensic DNA Extraction System with PrepFiler Express™ Kits (Thermo Fisher) and quantified with Quantifiler™ Trio on the QuantStudio™ 5 to ensure the extracted DNA was of sufficient quantity for the Fast Bisulfite conversion step.

The extracted DNA was denatured and treated with sodium bisulfite which converts unmethylated cytosines into uracil, while methylated cytosines remain unchanged. This step is very important as DNA methylation percentage on the respective CpG sites (where cytosine is followed by guanine) has the ability to estimate age. This is our main goal in this research.

During amplification, uracil bases are converted into thymine while methylated cytosine remains as cytosine. The amplification of five specific loci, C1orf132, KLF14, TRIM59, ELOVL2 and FHL2, is performed using the PyroMark PCR Kit with specific sets of primers ordered from the **GeneGlobe** website (QIAGEN) and carried out on an Applied Biosystems® GeneAmp® PCR System 9700. These markers were selected based on papers written by Zbiec-Piekarska et al. (1, 2) and Spolnicka et al. (3) referring to development of an age prediction model based on DNA methylation at the age-correlated CpG sites. ▷

The amplified target sequences were run on the PyroMark Q48 Autoprep Instrument with a sequencing-by-synthesis approach. Following the DNA sequence of the single strand DNA, one type of nucleotide is sequentially released and inserted by the DNA polymerase. If the nucleotide is complimentary to the target DNA, pyrophosphate (PPi) is released as a result of intercalation. The PPi is converted into ATP and acts as a substrate for the conversion of luciferin to oxyluciferin that generates visible light. The signal is proportional to the number of nucleotides added which is detected by the instrument camera. The unincorporated nucleotides are degraded by apyrase.

At the end of the sequencing run, the results can be reviewed and analyzed using QIAGEN's Pyrosequencing PyroMark Assay Design Software 2.0.

Calculation of the estimated age published by Zbiec-Piekarska et al. (1) can also be performed using an online tool, **http://biovectis.com/forensic1/age-calculator**. The comparison of the predicted age against the actual age is illustrated in the results section to indicate the accuracy of this evaluated method.

DNA samples

Twenty (20) blood reference samples of varying biological age from 1 to 53 years old were selected and designated as sample numbers 1 to 20. The DNA quantification results are presented in Table 1.

Bisulfite conversion of unmethylated cytosines was carried out using the EpiTect® Fast Bisulfite Kit. The Master Mix for conversion preparation is shown in Table 2. The optimized input amount for high-concentration samples is 100 ng of extracted DNA template in 20 µl.

The bisulfite reaction mix was set up following the recommendations for high-concentration samples and 20 µl of extracted DNA was added. Each reaction – Master Mix plus sample – was prepared in a 200 µl PCR tube and then placed in the thermal cycler and amplified with the conditions defined in Table 3 to achieve complete bisulfite DNA conversion.

Table 1. DNA concentration of the extracted samples with the actual age

Sample number	DNA concentration (ng/µl)	Actual age
1	13.0638	40
2	4.0997	23
3	3.1338	37
4	4.9397	53
5	7.2182	24
6	7.9083	1
7	8.4762	14
8	21.8284	46
9	6.1444	47
10	19.4778	26
11	9.3243	16
12	2.2953	22
13	11.5521	31
14	10.3486	29
15	11.1164	36
16	7.1195	26
17	34.068	47
18	9.0431	35
19	1.4749	22
20	193.5496	48

Table 2. Bisulfite reaction components (from EpiTect Fast Bisulfite Conversion Handbook)

Component	High-concentration samples (1 ng – 2 µg) volume per reaction (µl)	Low-concentration samples (1–500 ng) volume per reaction (µl)
DNA	Variable* (maximum 20 µl)	Variable† (maximum 40 µl)
Rnase-free water	Variable*	Variable†
Bisulfite Solution	85	85
DNA Protect Buffer	35	15
Total volume	140	140

* The combined volume of DNA and RNase-free water must total 20 µl.
† The combined volume of DNA and RNase-free water must total 40 µl.

Table 3. Cycling conditions for the bisulfite conversion

Step	Time	Temperature
Denaturation	5 minutes	95°C
Incubation	15 minutes*	60°C
Denaturation	5 minutes	95°C
Incubation	15 minutes*	60°C
Hold	Indefinite†	20°C

* In some cases, it may be necessary to extend the 60°C cycle time up to 20 minutes to achieve complete bisulfite DNA conversion.
† Converted DNA can be left in the thermal cycler overnight without any loss of performance.

During these experiments, incubation steps were increased from 10 minutes to 15 minutes to favor a complete bisulfite DNA conversion. This time can be further extended up to 20 minutes if needed.

To avoid inhibition following the bisulfite conversion, the samples went through a thorough clean up to remove all bisulfate salts according to the EpiTect Fast Bisulfite Kit protocol.

Independent amplifications using one primer set per amplification were performed for each sample. Amplification Master Mixes are summarized in Table 4.

The three controls used were EpiTect Control DNA (human) methylated and bisulfite converted, EpiTect Control DNA (human) unmethylated and bisulfite converted and Non-Template Control (NTC).

The 115 tubes – 20 samples and 3 controls x 5 markers – were placed in the thermal cycler as per the conditions in Table 5.

The amplified samples were run on the PyroMark Q48 Autoprep Instrument with PyroMark Q48 Advanced CpG Reagents to detect the Pyrosequencing sequence which enables users to calculate the ratio of nucleotides T and C and determine the methylation degree at the designated CpG site in a particular sequence. The percentage of the ratio on the intended markers used for age estimation is given after the run is analyzed using the PyroMark software.

Table 4. Master Mix composition for each primer

	Primer				
	C1orf132	**KLF14**	**TRIM59**	**ELOVL2**	**FHL2**
Component			**Volume (µl)**		
PyroMark PCR Master Mix, 2x			12.5		
CoralLoad® Concentrate, 2x			2.5		
25 mM $MgCl_2$			0.5		
Forward primer	2	0.5	0.75	1.5	0.5
Reverse primer	2	0.5	0.75	1.5	0.5
RNase-free water	3.1	6.1	5.6	4.1	6.1
Template DNA, 10 ng			2.4		
Total volume			**25**		

Table 5: Amplification thermal cycler conditions

Component	Time	Temperature	Additional comments
Initial PCR activation	15 minutes	95°C	HotStarTaq® DNA polymerase is activated by this heating step
3-step cycling: Denaturation	30 seconds	95°C	
Annealing (Touch-up)	30 seconds	Start at 48°C	Cycles 1–10: Increase annealing temperature by +0.5°C per cycle (48°C–52.5°C) Cycles 11–50: Keep annealing temperature at 52°C
Extension	30 seconds	72°C	
Number of cycles	50		
Final extension	10 minutes	72°C	
Hold	Indefinite	4°C	

Results and discussion

The figures below illustrate the analyzed results of Sample 9 for all the intended CpG sites for the different primers.

The percentages are displayed in a colored box giving a first indication on the analysis. Blue colored boxes indicate the peaks are well balanced and above the threshold. Additionally, the software couldn't detect any issue such as incomplete bisulfite conversion or a noisy baseline. Yellow is indicative that sample data requires some attention and red indicates that the bisulfite conversion was not complete or that the peaks are below the defined threshold. Low peaks may be still reliable but should be treated with caution.

Figure 1 and Figure 3 show an indication (orange colored boxes) regarding the bisulfite conversion efficiency. A cytosine (C) not followed by a guanine (G) should not be methylated and therefore should not present a peak, as it became a thymine (T).

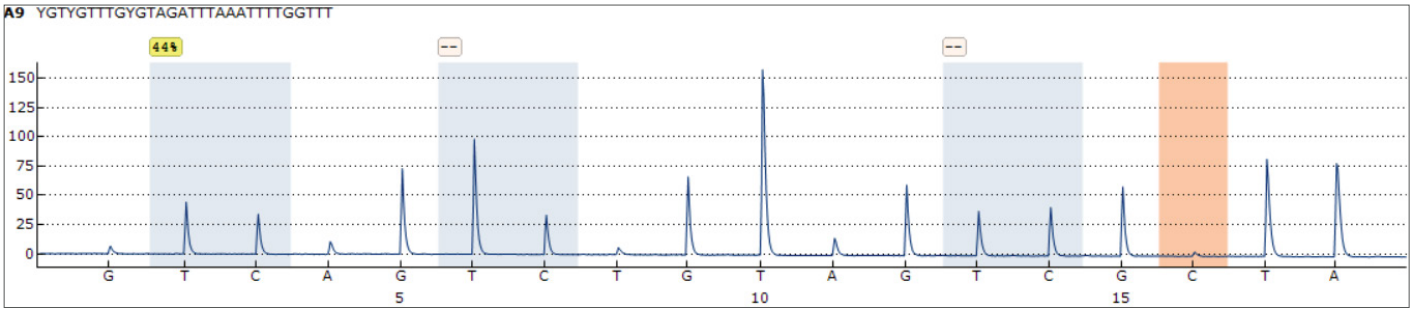

Figure 1. Marker C1orf132, 44% methylated/unmethylated cytosine on CpG site 1.

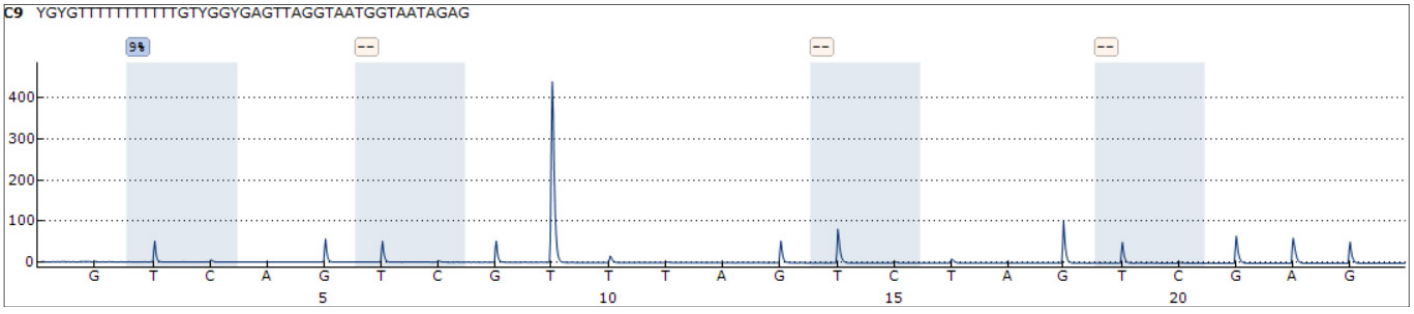

Figure 2. Marker KLF14, 9% methylated/unmethylated cytosine on CpG site 1.

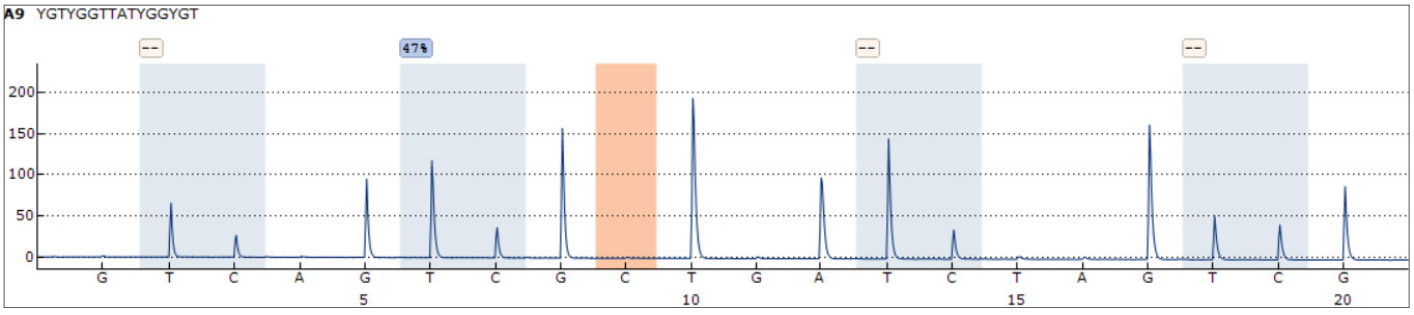

Figure 3. Marker TRIM59, 47% methylated/unmethylated cytosine on CpG site 7.

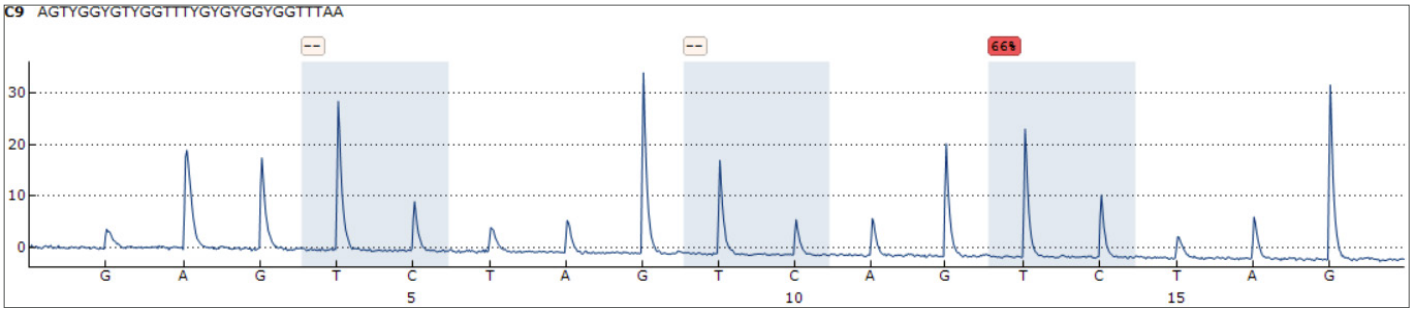

Figure 4. Marker ELOVL2, 66% methylated/unmethylated cytosine on CpG site 7.

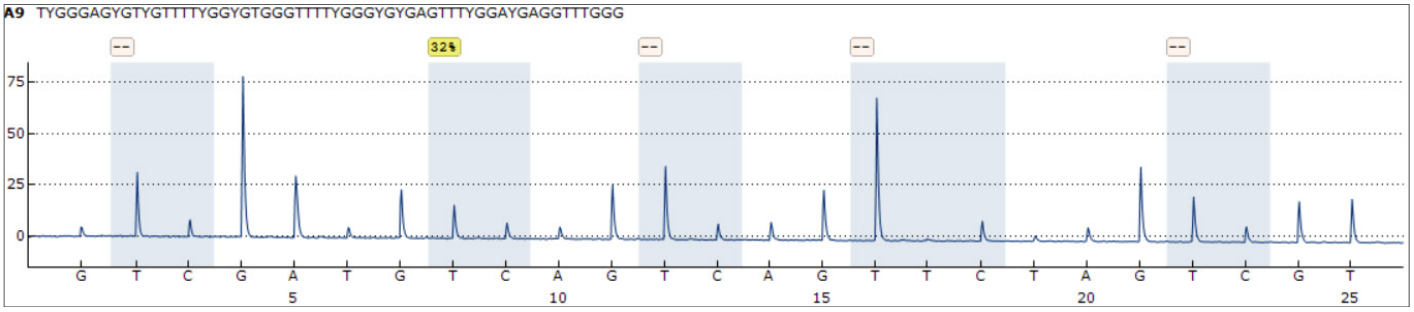

Figure 5. Marker FHL2, 32% methylated/unmethylated cytosine on CpG site 2.

The different percentage results are entered into the Age Calculator algorithm as shown in Figure 6 and the estimated age is calculated. The original equation is as follows:

Predicted age (years) = 3.26847784751817 + 0.465445549010653 X methC7-ELOVL2 – 0.355450171437202 X methC1-C1orf132 + 0.30648854113700 7 X methC7-TRIM59 + 0.832684435238792 X methC1-KLF14 + 0237081243617191 X methC2-FHL2

The Age estimation calculator

The calculator estimates human age based on five epigenetic markers.

Instruction:

1. Enter the percentage of marker methylation level (from 0 to 100%).
2. Press the AgePlex button – the estimated age will be shown in years.

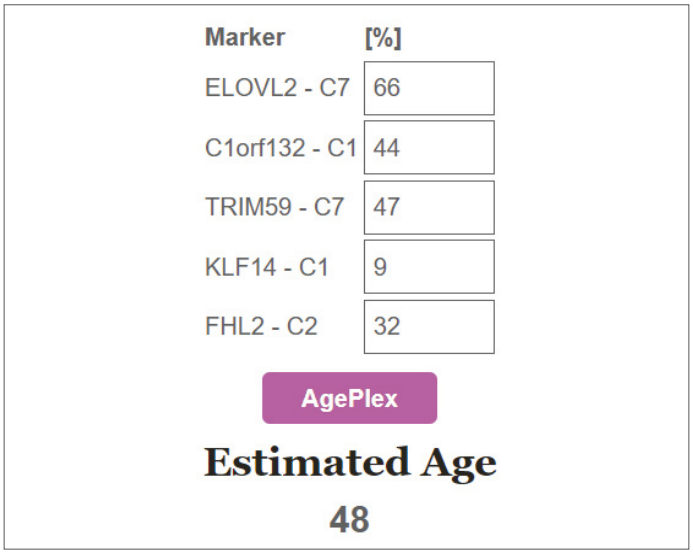

Figure 6. Example of the Age Estimation Calculator (see http://biovectis.com/forensic1/age-calculator).

Table 6. Sample results with actual age, predicted age and absolute difference

Sample number	Actual age	Predicted age	Absolute difference
1	40	37	3.3
2	23	21	2.2
3	37	34	3.0
4	53	47	5.7
5	24	28	4.2
6	1	-8	8.7
7	14	15	1.2
8	46	38	8.2
9	47	48	1.2
10	26	33	7.2
11	16	17	1.0
12	22	25	2.8
13	31	28	3.4
14	29	30	1.4
15	36	34	1.7
16	26	26	0.3
17	47	35	11.8
18	35	30	4.9
19	22	25	2.6
20	48	50	1.9

For 15 out of 20 samples analyzed, the estimated biological age was calculated within ±5 years compared to the actual age of the individuals. The other 5 samples, Samples 4, 6, 8, 10 and 17, show a greater age-gap between the predicted and actual age, with differences ranging between 5- to 12-years.

The mean absolute deviation of all samples is 3.8 years and is therefore comparable to the results published by Zbiec-Piekarska et al. in their initial study on the PyroMark Q24 (1).

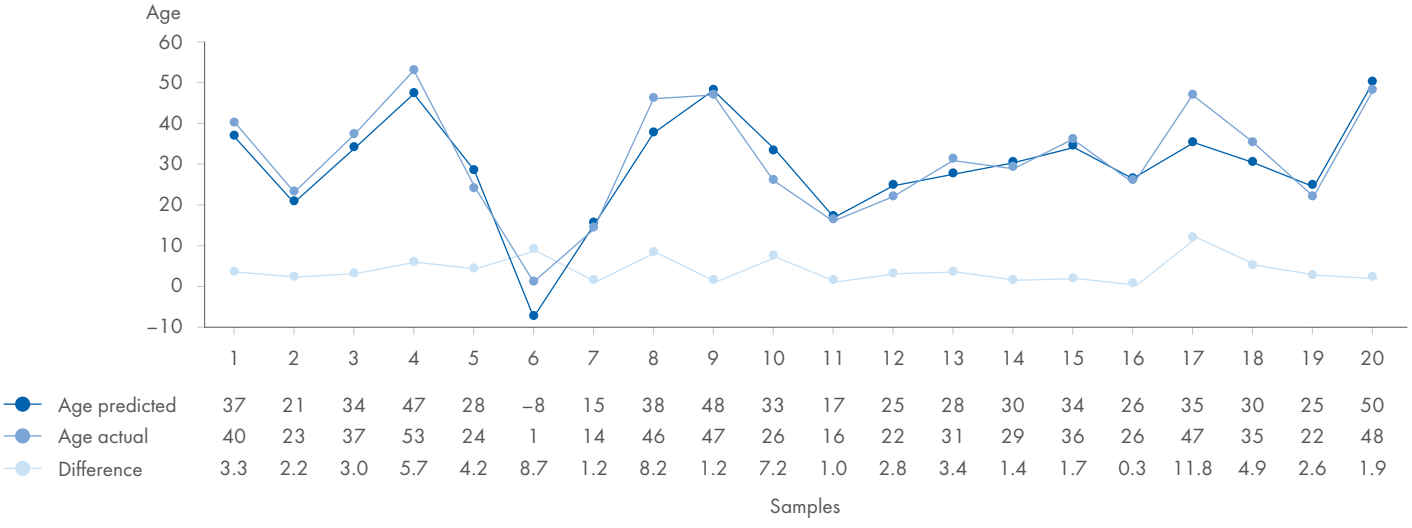

Figure 7. Comparison of Actual Age (in middle blue) against the Predicted Age (in dark blue) and difference (in light blue).

Conclusion

Age estimation was able to accurately predict biological age using the Pyrosequencing method with 5 selected age contributing markers (C1orf132, KLF14, TRIM59, ELOVL2 and FHL2) on methylated DNA run on the PyroMark Q48 Autoprep Instrument and analyzed via Pyrosequencing PyroMark Assay Design Software 2.0.

This method proved to be successful and can be utilized in the forensic field for estimating age from biological samples for investigative purposes.

The research at KIMIA Malaysia will be expanded to cover different types of biological samples such as semen stains and trace DNA. The number of samples analyzed will be increased to investigate the feasibility and robustness of this method. This research will also be expanded to test a wider demographic distribution of individuals to investigate whether the algorithm used can be optimized to fit the methylated DNA sequence of the Malaysian population. The aim of the optimization is to increase the accuracy of age prediction for individuals originating from this geographical region.

Currently, using the formula developed by Zbiec-Piekarska et al., the mean absolute deviation from the 20 samples is 3.8 years, and this is comparable to their published results.

References

1. R Zbiec-Piekarska et al. (2015a) Examination of DNA methylation status of the ELOVL2 marker may be useful for human age prediction in forensic science. Forensic Sci Int. Genet. doi.org/10.1016/j.fsigen.2014.10.002
2. R Zbiec-Piekarska et al. (2015b) Development of a forensically useful age prediction method based on DNA methylation analysis. Forensic Sci. Int. Genet. doi.org/10.1016/j.fsigen.2015.05.00
3. M Spolnicka et al. (2018) DNA methylation in ELOVL2 and C1orf132 correctly predicted chronological age of individuals from three disease groups. Int. J. Legal Med. doi.org/10.1007/s00414-017-1636-0

Ordering Information

Product	Contents	Cat. no.
PyroMark Q48 Autoprep Instrument	PyroMark Q48 Instrument, multistep pipet, software and documentation	9002471
EpiTect Fast DNA Bisulfite Kit (50)	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute® DNA Spin Columns, Carrier RNA, and Buffers	59824
PyroMark PCR Kit (200)	For 200 reactions: 2x PyroMark PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized PyroMark Reaction Buffer containing 3 mM $MgCl_2$ and dNTPs), 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM $MgCl_2$, and RNase-Free Water.	978703
PyroMark Q48 Advanced CpG Reagents (4 x 48)	Reagents for 4 x 48 PyroMark Q48 Autoprep CpG and long-read reactions: PyroMark Advanced Enzyme Mix, PyroMark Advanced Substrate Mix, Denaturation Solution, Annealing Buffer, Binding Buffer, Nucleotides	974022
PyroMark Assay Design SW 2.0	Software for convenient design of PCR and sequencing primers, optimized for Pyrosequencing analysis	9019077

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Learn more about furthering your human identity and forensic investigations with Pyrosequencing at: **https://www.qiagen.com/PyrosequencingHID**.