Chapter 17

Pyrosequencing Primer Design for Forensic Biology Applications

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Abstract

The polymerase chain reaction (PCR) is used to copy DNA in vitro for a variety of applications including amplifying a target DNA, mutating a base, adding tags, and sequencing by synthesis applications. Next-generation sequencing (NGS) is a DNA sequencing technology that has been applied to screening cancer and tissue variants, deep sequencing, and gene expression analysis, and more recently, it has been applied to DNA typing for human identification, estimating age, and detecting and differentiating body fluids. Body fluids are normally identified using color tests, microscopy, and immunochromatographic assays. Pyrosequencing is an NGS approach that has been applied to body fluid analysis. The pyrosequencing assays can detect one or several mixed body fluids by analysis of their tissue-specific differentially methylated regions (tDMRs). Here, the process of designing pyrosequencing primers for forensic biology applications is described.

Key words Molecular biology, Polymerase chain reaction (PCR), Primer, Next-generation sequencing (NGS), Pyrosequencing, Sequencing by synthesis (SBS), Methylated DNA, Body fluid analysis

1 Introduction

Pyrosequencing is a sequencing by synthesis (SBS) next-generation sequencing (NGS) technique that was developed by Mostafa Ronaghi and coworkers in 1996 [1]. It has been applied to identifying DNA single-nucleotide polymorphisms (SNPs), mutations, unknown sequence variants, and insertion–deletions (InDels) [2, 3]. It has also been used to analyze DNA extracted from blood to predict age [4, 5] and body fluids to detect and identify them in forensic biology assays [6–11]. Pyrosequencing is a reasonably cheap and fast method for sequencing short segments of DNA using primers designed to be specific for the target region directly upstream of the variable region [2, 3].

In pyrosequencing, light emission signals base addition in the sequencing reaction [2, 3]. Prior to pyrosequencing, a target region of the DNA is amplified with polymerase chain reaction

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(PCR) primers, one of which is biotinylated [3]. The amplicons are introduced to magnetic streptavidin-coated beads, where they are immobilized through the formation of a biotin–streptavidin complex [3]. Next, the double-stranded DNA fragments are denatured in a basic solution resulting in single strands [3]. Following wash and neutralization steps, a pyrosequencing primer is added. It anneals to the complementary location in the immobilized single-stranded DNA leading to the formation of a double-stranded stretch [3]. The DNA polymerase enzyme attaches to the double-stranded region formed by the primer binding [2, 3]. In the pyrosequencing reaction, each deoxyribonucleotide triphosphate (dNTP) base (dCTP, dGTP, dTTP and deoxyadenosine-5′-(α-thio)-triphosphate (dATPαS) instead of dATP) is dispensed one at a time in a predetermined pattern [2, 3]. When the base released complements the next base in the single strand, the base hydrogen bonds to its complement in the immobilized strand next to the base at the 3′ end of the primer [2, 3]. DNA polymerase attaches the base covalently to the primer, and pyrophosphate (PPi) is released in the reaction [2, 3]. Incorporation of the base starts an enzyme cascade [2, 3]. ATP sulfurase enzyme uses the PPi to form ATP in the presence of adenylyl sulfate [2, 3]. Luciferase enzyme uses the ATP produced (but not dATPαS) to produce light [2, 3]. The light and pyrogram peak signals that the complementary base was incorporated, indicating that the introduced base is next in the sequence [2, 3]. If no light is produced, the base was not incorporated; the pyrogram will register a flat line for that dispensation event [3]. The light is detected using a photomultiplier tube (PMT) [3]. A charge-coupled device (CCD) camera detects the light, and a peak is an output in the form of a pyrogram in the instrument software [3]. If the same base is incorporated consecutively in the sequence, a double-height peak will register in the pyrogram [3]. Before the next base is dispensed, residual ATP and dNTP are degraded by apyrase and washed away [3]. Pyrosequencing can sequence and quantitate base incorporation at each site in the target region up to approximately 140 bases [3].

Upon sequencing the human genome in 2001, an additional variable and hereditary layer on the DNA sequence called epigenetic information was found [12]. Scientists observed that genomic DNA can be modified through chemical modification of selected cytosine nitrogenous bases [12]. Attaching a methyl group at the 5′-carbon of cytosine forms 5′-methylcytosine or methylated cytosine [12, 13]. DNA methylation posttranscriptional modifications influence gene function in eukaryotes including tissue-specific gene regulation, aging, carcinogenesis, and X chromosome inactivation [13]. Methylation in promoter regions typically acts to repress gene transcription but, in some cases, causes transcription promotion [12]. Methylation is dynamic and can also be caused by the environment and disease [12]. Methylated cytosines tend to be found

in cluster regions called CpG islands in which methylated cytosines are followed by guanines in the 5′ to 3′ direction [12]. Methylated CpG islands account for approximately 2% of the human genome [13] but 7% of CpG islands, depending upon how they are defined [12].

Pyrosequencing can be used to probe DNA methylation levels at CpG sites [3]. To differentiate between unmethylated and methylated cytosine, the extracted DNA can be treated with bisulfite [13]. At low pH, bisulfite converts unmethylated cytosine to uracil [13]. In PCR, the uracil is copied as thymine. To analyze the methylation pattern in CpG loci, cytosine and thymine will both be dispensed in the dispensation sequence, and the incorporation of one or both will be detected [3]. Tissue-specific differentially methylated regions (tDMRs) have been investigated for forensic applications [14–17]. Tissue samples and mixtures can be partially methylated. The pyrogram can be used to quantify the extent of methylation (percentage) at that site based upon the light produced [3, 18].

Pyrosequencing assays have been designed using epigenetic methylation markers for body fluids, including semen, saliva, blood, and vaginal fluid [6–11]. The BCAS4 marker has been found to be specific for saliva, ZC3H12D has been found to be specific for semen, cg06379435 has been found to be specific for blood, and PFN3A and VE_8 have been found to be specific for vaginal epithelial cells [7, 8, 10, 11]. Assays can be developed to include up to four different sequencing primers [3]; a multiplex assay for semen, blood, saliva, and vaginal cells has been developed [10].

For the assay to work, pyrosequencing primers need to be specific for the region upstream of the CpG, SNP, or de novo target region to be sequenced [3]. Although researchers may use previously designed primers, this limits their targets to the loci covered by the kit or product that is commercially available. Designing new primers affords researchers the flexibility to analyze any target and go beyond what is published or commercially available and is the key to sequencing new loci. The primers can be designed manually or using software such as the commercially available PyroMark™ (Qiagen, Hilden, Germany) software. The primers are selected for a variety of characteristics including length, formation of primer dimers, and if they overlap with a known SNP or CpG site. The software can save time, and programs will offer numerous candidate assays. To probe multiple body fluids, a primer multiplex can be designed and optimized experimentally to sequence the target loci in samples of interest.

In this chapter, the process of designing pyrosequencing primers is described. A human body fluid tDMR target will be the example in this protocol.

2 Materials

2.1 Websites and Software

1. A computer or device connected to the Internet is required to download the target sequence unless it is already available locally. Websites that are sources of genome and SNP data include NCBI Genome (https://www.ncbi.nlm.nih.gov/genome/), Ensembl (https://uswest.ensembl.org/index.html), SNPedia (https://www.snpedia.com/index.php/SNPedia), and the UCSC Genome Browser (https://www.genome.ucsc.edu/cgi-bin/hgGateway) (*see* **Note 1**).
2. The NCBI Standard Nucleotide Basic Local Alignment Search Tool (BLAST) BLAST Nucleotide (BLASTn) Web page (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) can be used to assess primer specificity (*see* **Note 1**).
3. The user has the option of designing pyrosequencing primers manually or using the software. If software such as PyroMark™ is preferred, the user needs to obtain and install it before beginning primer design. For manual design, the OligoAnalyzer™ Tool Web-based software (https://www.idtdna.com/calc/analyzer) has features for evaluating primer length, hairpin, and primer dimer formation (*see* **Notes 1** and **2**).
4. The Web-based UCSC In silico PCR tool, https://genome.ucsc.edu/cgi-bin/hgPcr, can be used to perform in silico PCR using the designed PCR primer set (*see* **Note 1**).

2.2 Obtaining a DNA Sequence for the Region to be Sequenced

A target region DNA sequence is needed for primer design. To obtain the sequence, follow the steps below. If the sequence has been obtained, proceed to Subheading 3.1. The *Homo sapiens* breast carcinoma-amplified sequence 4 (BCAS4) locus will serve as the example, and the UCSC Genome Browser will be used to download the sequence of the gene region and upstream and downstream regions.

1. Open the UCSC Genome Browser. From the front page, select "Genome Browser." Next, select the Human Assembly "Dec. 2013 GRCh38/hg38," and in the Position/Search Term box, input "AL031680.20" and select the "Go" button. This browser loads GENCODE Genes, NCBI RefSeq genes, and ENSEMBL annotation sets. The GENCODE is intended to reflect the protein-coding and functional features of genes in the genome [19].
2. Scroll down to "Regulation" and select "CpG Islands." On the next page, toggle Display "hide" to "show" and select "submit" and "refresh" (*see* **Note 3**). The "CpG Islands" header is

now visible in green. The number ("92") indicates how many CpG islands have been characterized. Right-click on the central green "CpG Islands" header. In the Configure CpG Islands box that appears, click the "+" to expand "All tracks in this collection" and select "unmasked CpG." Change the display mode to "pack" and "ok" and "CpG 27" appears (Fig. 1). Alternatively, one can scroll down to "Regulation" and select the "CpG Island" heading and select "pack" next to "unmasked CpG" there and select "submit" to display "CpG 27."

3. To get the DNA sequence for the CpG islands, right-click on the green CpG: 27 number and select "Get DNA for CpG: 27." Under "Sequence Retrieval Region Options," more bases can be added upstream and downstream of the CpG island region. I added 100 bases upstream and downstream (*see* **Note 4**).
4. Select sequence formatting options "All upper case" and "to lower case" (defaults). Select "get DNA." The CpG region is shown in the box (*see* **Note 5**). Figure 2 shows the retrieved text sequence for the BCAS4 target. The sequence is the upper strand in the 5′ to 3′ direction.

3 Methods

3.1 Design of Pyrosequencing Primers

The example will focus on the use of the Qiagen PyroMark® Assay Design software [20, 21] but also introduce manual primer design. You will need the locus of interest obtained from Subheading 2 or previously obtained to design the primers.

1. Open the PyroMark® Assay Design software on your computer. To begin a new assay, select "New" in the "File" menu.
2. To create an assay for tDMRs, select the Assay Type "Methylation Analysis (CpG)." In the "Sequence editor" window box that reads "Upper Strand (5′ to 3′)," paste the DNA sequence obtained from the UCSC Genome Browser (or other source) by right-clicking and selecting "Copy Entered Sequence" or using the "Import" function to import a FASTA file. If the amplification primer sequences, amplicon length, and amplicon sequence are known, they can be entered into the window.
3. For the CpG assay, select the box to display the "Converted Sequence" to show the sequence after bisulfite conversion; it will be more T-rich (*see* **Note 6**).
4. Next, highlight the target region by clicking and dragging the mouse over the sequence. Right-click on the sequence and select "Target Region -> Set Target Region."

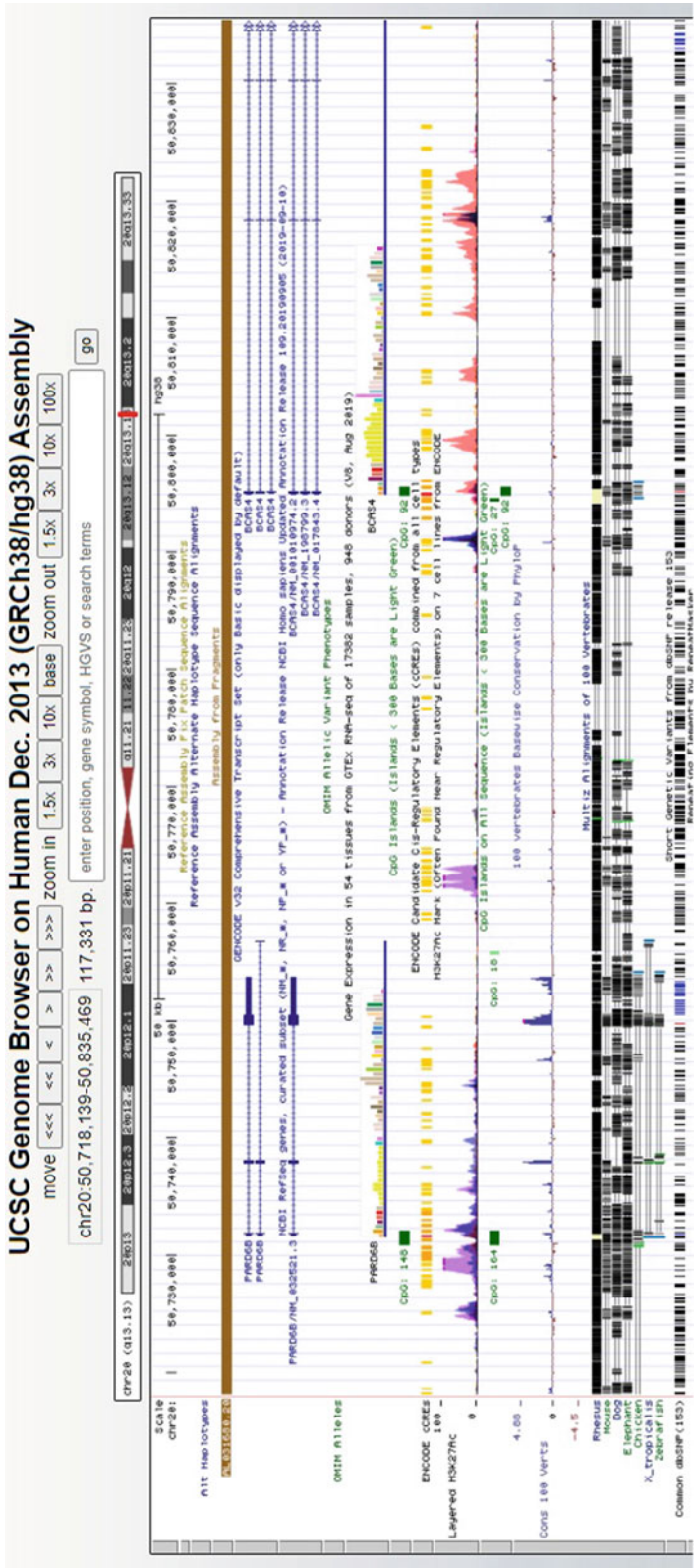

Fig. 1 BCAS4 CpG islands shown in green in the UCSC Genome Browser

```
>hg38_dna range=chr20:50794228-50794759 5'pad=100 3'pad=100 strand=+
repeatMasking=none
AGGGTCTATCTAGGCCGGCCTCCGAGGGCATGGAGGGAGTGGGTGCGGTT
GTGAAATGTAGTGCGCTCAATAGTTTCCTGGTGAAGTTTATTTTAAAATC
CGCACCGAAGAGGAAGACGAGGACCGTCACACTCGGCCTTCCCTAAATTC
CAGGACCCTCCGCCCGATGCAAACTAGATGCTTTAGTAGGATGGGAACGG
GTGGGGGGCGGGCGGCTTTGGGCTTCCTCTAAGCTAGCGCCTCTCTAACC
CGGACGCCCGTTAGAATCACCCGGGGAGTTTTAGAAACTACCGATGCCCA
AGCCCCACTCCGAAGGATTCCAACTTAATCGGCCTGGTGCGAGGCCTGGC
TTCCGGGCTTTTAAAAGCTTCCCGGGGATTCTATTTTACGGCCGGGTCCG
GGGCCGGGAGCCTGTACTCTACCGGGATTCCGATGGGGAGGGGTGGCTTG
CCCCAATAGTTCTCAAATTTAGCTTTGGGTCAACAATCTGGTTGGAACCA
CCTAACAAACATCAAAAGATCCTGATGCCCAG
```

Fig. 2 BCAS4 CpG island-containing sequence retrieved with the UCSC Genome Browser

Forward PCR Primer	AGTGGGTG**A**GGTTGTGAAATGT
Reverse PCR Primer	CCCATCCTACTAAA**A**CATCTA**A**TT
Pyrosequencing Primer	**A**GT**T**T**T**AATAGTTT**TT**TGGTG

Fig. 3 The published, tested primers for BCAS4 are shown [10]. They vary slightly from the database sequence because they reflect the thymines that replaced the unmethylated cytosines following bisulfite treatment and PCR. Variations from the NCBI sequence are highlighted in bold

5. Select the blue "Play" button. The generated primers and assays are displayed in the box (*see* **Note 7**). The primer sets include the forward and reverse primers and the sequencing primer. The "Graphic View" displays the primer set binding (*see* **Note 8**). The sequencing primer position should be directly preceding the variable position (if it is known), which in this example is the CpG island region. During bisulfite conversion, unmethylated "C" bases will be converted to a "U" base and be converted to a "T" base in cDNA synthesis using PCR. The primer with "C" will bind the complementary base "G" at the methylated CpG site, and the primer with "A" will bind the complement "T" from the unmethylated sequence posttreatment (*see* **Note 9**).
6. A "Pyrosequencing Assay Design Analysis Report" can be generated, which contains additional assay information. The biotin tag is displayed as a circle on the primer to which it will be attached. Save this file as it contains the "Sequence to Analyze" (*see* **Note 10**).
7. Once the assay is selected, it is the completed PyroMark™ assay build for pyrosequencing (*see* **Note 11**). The pyrosequencing primer and PCR primers designed using the PyroMark Assay Design software version 2.0 for the BCAS4 example [10] are shown in Fig. 3. The locations in the NCBI sequence are shown in Fig. 4.

Target NCBI GenBank Nucleotide Sequence
(Accession AL031680.20)

```
            Forward PCR Primer Region  Pyrosequencing Primer region
76221 GGAGGGAGTG GGTGCGGTTG TGAAATGTAG TGCGCTCAAT AGTTTAATGG TGAAGTTTAT
      CCTCCCTCAC CCACGCCAAC ACTTTACATC ACGCGAGTTA TCAAAGGACC ACTTCAAATA

            Pyrosequencing Region
76261 TTTAAAATCC GCACCGAAGA GGAAGACGAG GACCGTCACA CTCGGCCTTC CCTAAATTCC
      AAATTTTAGG CGTGGCTTCT CCTTCTGCTC CTGGCAGTGT GAGCCGGAAG GGATTTAAGG


76321 AGGACCCTCC GCCCGATGCA AACTAGATGC TTTAGTAGGA TGGGAACGGG TGGGGGGCGG
      TCCTGGGAGG CGGGCTACGT TTGATCTACG AAATCATCCT ACCCTTGCCC ACCCCCCGCC
                            Reverse PCR Primer Region*
```

Fig. 4 Genome sequence for the NCBI GenBank Accession Number AL031680.20 for the BCAS4 locus region on human chromosome 20 with bold and boxed PCR primers and underlined pyrosequencing primer region upstream of the tDMR region. The pyrosequencing assay region is italicized, and the tDMRs are bold italicized

8. Alternatively, the pyrosequencing and PCR primers can be designed manually. As described above, the target amplicon must first be amplified with a set of PCR primers in which one the primers is biotinylated, so these must be designed. The position of the pyrosequencing primer should be directly preceding the variable position, or in this example, the CpG islands. Locate the region of the 5′ (upper strand) directly before (upstream) the CpG region of interest. The pyrosequencing primer should anneal to a region upstream of the CpG loci of interest but within the region amplified by PCR primers and have a low primer dimer propensity with the PCR primers also [22–25]. Consider the sequence after treatment with bisulfite. Unmethylated "C" bases in the top strand will be converted to "T" bases and need an "A" base in the reverse primer to be complementary. Unmethylated "C" bases in the bottom strand will be converted to "T" bases and need a complementary "A" base in the forward primer. Design the PCR and pyrosequencing primer for each target. The primers should have a length of 18–30 bases and have a low propensity for primer dimer formation [22–25]. To aid in the analysis of these, the OligoAnalyzer™ Tool can be used. Upon pasting the primer sequencing into the OligoAnalyzer™ Tool Web-based software (https://www.idtdna.com/calc/analyzer), the user can select the "Analyze" button to obtain the primer length and other features, "Hairpin" to compute any hairpin formation and melt temperatures, "Self-Dimer" to compute primer dimer formation, and "Hetero-Dimer" to compute primer dimer formation with other primer sequences [22–25] (*see* **Notes 12** and **13**).

```
>chr20:50794265+50794423 159bp AGTGGGTGCGGTTGTGAAATGT
TCCCATCCTACTAAAGCATCTAGTT

AGTGGGTGCGGTTGTGAAATGTagtgcgctcaatagtttcctggtgaagt
ttattttaaaatccgcaccgaagaggaagacgaggaccgtcacactcggc
cttccctaaattccaggaccctccgcccgatgcaAACTAGATGCTTTAGT
AGGATGGGA
```

Fig. 5 Sample in silico PCR results using the BCAS4 primers

3.2 Evaluating Nonspecific Priming by NCBI BLAST Nucleotide

1. Primer specificity can be checked by pasting the primer sequences from Fig. 3 into the box in the NCBI BLASTn webpage (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). There is an option to input the organism name, in this case, *Homo sapiens* for the Search Set. Use the default parameters for the other options. Select the "BLAST" button at the bottom to begin the computation. It may take a few minutes for the results to be displayed in the browser.
2. The results include descriptions and links to the aligned regions. The first two hits for the BCAS4 forward primer were for chromosome 20, the known location of BCAS4. There was also a 100% coverage hit on chromosome 15 for the third hit and hits with less coverage in other regions of the genome.

3.3 Performing In Silico PCR

1. Open the browser for the UCSC In silico PCR tool (https://genome.ucsc.edu/cgi-bin/hgPcr).
2. Copy and paste the forward and reverse PCR primer sequences (in the 5′ to 3′ orientation) into the appropriately labeled boxes. Use the defaults, which is the same assembly (human genome, Dec. 2013 assembly) used to build the primers. Select "submit."
3. The output indicates the amplicon will be 159 base pairs and amplifies the desired target on chromosome 20 (Fig. 5).

3.4 Obtaining Pyrosequencing Primer Reagents

1. Primers may be purchased commercially from a variety of manufacturers including Qiagen and Integrated DNA Technologies (IDT). The assay can be purchased as a PyroMark™ Custom Assay from Qiagen (*see* **Notes 14** and **15**).
2. Upon receiving the Qiagen custom primer sets, they must be reconstituted by adding 550μL TE^{-4} to each (*see* **Note 16**). Two microliters (2μL) of each sequencing primer (4μM concentration) are used in each sequencing reaction.
3. The target loci must first be bisulfite-treated and amplified using the biotinylated PCR primer set. The PyroMark™ PCR Kit can be used for 25μL reactions. PCR amplification of the

target region can be assessed by gel electrophoresis before pyrosequencing (*see* **Note 17**). The Qiagen tissue ID protocol can be used for preparing the sample(s) and performing the pyrosequencing [3, 26] (*see* **Note 18**).

4. For analysis after pyrosequencing, the percent methylation for each CpG site is automatically calculated by the PyroMark® Q24 software version 2.0.6 and is displayed as a pyrogram.

4 Notes

1. These links were active at the time of this writing.
2. A free user account is required for use.
3. Toggling the CpG display shows the CpG islands that are otherwise not visible in the masked display.
4. It is recommended to add more bases upstream and downstream of the CpG islands to have more flexibility in designing the primers.
5. Download the sequence locally by copying and pasting it into a Note or Word Document window and save. The nitrogenous bases found in DNA are designated using IUPAC codes A, T, G, C, N, R, and Y, referring to adenine, thymine, guanine, cytosine (A/T/G/C), purine (G/A), and pyrimidine (T/C), respectively. Avoid regions in which the base is not defined (e.g., N, R for purine, and Y for pyrimidine) for primer design for best performance.
6. The software converts the unmethylated cytosine (C) bases to thymine (T) and colors them red and highlights the CpG site methylated cytosines in bold.
7. The software generates over 100 different assays in ranked order on a 100 point scale. The primers are scored on several criteria, including the potential for mispriming, primer length, and propensity for primer dimer formation. In general, the higher the primers' score, the better they should perform in the PCR and sequencing reactions. A primer set with a score of 70 or above typically performs well.
8. Do not select a primer set for use if the primers bind over a CpG site as it may be methylated or unmethylated. If a CpG site must be included, the primers can be produced to have both potential variants, such as a "C" and an "A" at that position.
9. Bisulfite conversion is harsh and will fragment DNA. Keeping the PCR amplicon size small (<200 bases) is best for successful amplification of the target. If a target is longer than 200 bases, it can be split into two or more targets for amplification and sequencing.

10. A biotin tag is required to tether the amplicon for pyrosequencing [3]. It can be placed on either a forward or reverse PCR primer.
11. It is best to keep the PCR amplicon length to 200 base pairs or less for pyrosequencing targets (80–200 base pairs is ideal), although some assays may work with amplicons up to 500 base pairs.
12. Especially in manual design, but also in automated design, primer design can take time. Each potential assay needs to be carefully considered to yield the optimal assay. The pyrosequencing primer can be shifted within the upstream region until the user criteria are met. The specificity needs to be maintained even with a very T-rich sequence posttreatment with bisulfite.
13. In silico pyrosequencing primer design poses no chemical safety hazards.
14. The Qiagen assay for body fluid marker BCAS4 for saliva is cat. no. PMC0002408, C20orf227 for blood is cat. no. PM00197323, cg06379435 for blood is cat. no. PMC0085498, FGF7 for sperm is cat. no. PMC0004067, and ZC3H12D cat. no. PM00124145 is for sperm.
15. The PyroMark™ Assay database can be used to search for an existing assay. Predesigned primers can be identified and obtained from Qiagen.
16. Prior to pyrosequencing, users should read the safety data sheets (SDS) for the reagents and reaction mixes and safety recommendations provided by the manufacturer for preparing the pyrosequencing reaction mixtures and using the instrument.
17. Production of the desired amplicons can be checked prior to pyrosequencing using a 2% agarose gel with appropriate size standards, stained with SYBR green I, and visualized using a UV light source [23].
18. Pyrosequencing has been performed on a PyroMark® Q48 instrument using the PyroMark PCR Kit (cat. no. 978705) according to the manufacturer's protocols using the primer sets listed in **Note 14** which include the BCAS4 locus [3, 26, 27].

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References

1. Ronaghi M, Karamohamed S, Pettersson B et al (1996) Real-time DNA sequencing using detection of pyrophosphate release. Anal Biochem 242:84–89
2. Kircher M, Kelso J (2010) High-throughput DNA sequencing-concepts and limitations. BioEssays 2(6):524–536
3. PyroMark® Q48 autoprep user manual, June 2020. https://www.qiagen.com/us/resources/download.aspx?id=650a0c13-3b8e-4a77-b433-6b1e50b9525a&lang=en
4. Silva DSBS, Antunes J, Balamurugan K, Duncan G, Alho CS, McCord B (2015) Evaluation of DNA methylation markers and their potential to predict human aging. Electrophoresis 36:1775–1780
5. Daunay A, Baudrin LG, Deleuze JF, How-Kit A (2019) Evaluation of six blood-based age prediction models using DNA methylation analysis by pyrosequencing. Sci Rep 9(1):8862
6. Madi T, Balamurugan K, Bombardi R, Duncan G, McCord B (2012) The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Electrophoresis 33:1736–1745
7. Balamurugan K, Bombardi R, Duncan G, McCord B (2014) Identification of spermatozoa by tissue-specific differential DNA methylation using bisulfite modification and pyrosequencing. Electrophoresis 35 (21–22):3079–3086
8. Antunes J, Silva DS, Balamurugan K, Duncan G, Alho CS, McCord B (2016) Forensic discrimination of vaginal epithelia by DNA methylation analysis through pyrosequencing. Electrophoresis 37(21):2751–2758
9. Silva DSBS, Antunes J, Balamurugan K, Duncan G, Alho CS, McCord B (2016) Developmental validation studies of epigenetic DNA methylation markers for the detection of blood, semen and saliva samples. Forensic Sci Int Genet 23:55–63
10. Gauthier QT, Cho S, Carmel JH, McCord BR (2019) Development of a body fluid identification multiplex via DNA methylation analysis. Electrophoresis 18-19:2565–2574
11. Alghanim H, Balamurugan K, McCord B (2020) Development of DNA methylation markers for sperm, saliva and blood identification using pyrosequencing and qPCR/HRM. Anal Biochem 611:113933
12. Bernstein BE, Meissner A, Lander ES (2007) The mammalian epigenome. Cell 128 (4):669–681
13. Lilischkis R, Kneitz H, Kreipe H (2001) Methylation analysis of CpG islands. Methods Mol Med 57:271–283
14. Rakyan VK, Down TA, Thorne NP, Flicek P, Kulesha E, Graf S, Tomazou EM, Bäckdahl L, Johnson N, Herberth M, Howe KL, Jackson DK, Miretti MM, Fiegler H, Marioni JC, Birney E, Hubbard TJ, Carter NP, Tavaré S, Beck S (2008) An integrated resource for genome-wide identification and analysis of human tissue-specific differentially methylated regions (tDMRs). Genome Res 18 (9):1518–1529
15. Slieker RC, Bos SD, Goeman JJ, Bovee JVMG, Talens RP, van der Breggen R, Suchiman HE, Lameijer EW, Putter H, van den Akker EB, Zhang Y, Jukema JW, Slagboom PE, Meulenbelt I, Heijmans BT (2013) Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array. Epigenet Chromatin 6:26
16. Lee HY, Park MJ, Choi A et al (2012) Potential forensic application of DNA methylation profiling to body fluid identification. Int J Legal Med 126:55–62
17. Park J-L, Kwon O-H, Kim JH, Yoo H-S, Lee H-C, Woo K-M, Kim S-Y, Lee S-H, Kim YS (2014) Identification of body fluid-specific DNA methylation markers for use in forensic science. Forensic Sci Int Genet 13:147–153
18. Dejeux E, El Abdalaoui H, Gut IG, Tost J (2009) Identification and quantification of differentially methylated loci by pyrosequencing™ technology. Methods Mol Biol 507:189
19. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, Aken BL, Barrell D, Zadissa A, Searle S, Barnes I, Bignell A, Boychenko V, Hunt T, Kay M, Mukherjee G, Rajan J, Despacio-Reyes G,

Saunders G, Steward C, Harte R, Lin M, Howald C, Tanzer A, Derrien T, Chrast J, Walters N, Balasubramanian S, Pei B, Tress M, Rodriguez JM, Ezkurdia I, van Baren J, Brent M, Haussler D, Kellis M, Valencia A, Reymond A, Gerstein M, Guigó R, Hubbard TJ (2012) GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res 9:1760–1774

20. PyroMark Assay Design SW 2.0 quick-start guide—(EN). https://www.qiagen.com/us/resources/download.aspx?id=231a6894-57d4-4f0b-81f2-eaa56a2b6bd8&lang=en
21. https://toptipbio.com/cpg-pyromark-assay-design/
22. Elkins KM (2011) Designing PCR primer multiplexes in the forensic laboratory. J Chem Educ 88:1422–1427
23. Elkins KM, Kadunc RE (2012) An undergraduate laboratory experiment for upper-level forensic science courses: the use of TPOX single locus primers to amplify human DNA by real-time PCR with SYBR green detection. J Chem Educ 89(6):784–790
24. Elkins KM (2012) Forensic DNA biology: a laboratory manual. Academic, Waltham
25. Elkins KM (2015) Primer design for PCR reactions in forensic biology. In: Basu C (ed) Methods in molecular biology, vol 1275: PCR primer design. Humana Press, New York, pp 17–30
26. Powell M, Lee AS, St. Andre P, McCord B. (2018) Tissue source attribution using the PyroMark® Q48 autoprep system: sperm identification in forensic casework. Qiagen applications note. https://www.qiagen.com/us/resources/download.aspx?id=ddaa262e-f3ec-4ac7-9bac-aaf3ec8968cf&lang=en
27. O'Hern CP, Klavens A, Elkins KM, Zeller CB (2020) Unpublished results.