User-Developed Protocol

Pyrosequencing® method for the forensic identification of tissue source using QIAGEN's PyroMark® Q24 Advanced system

This protocol has been adapted from the publication "The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and Pyrosequencing".

This protocol has not been thoroughly tested and optimized by QIAGEN.

IMPORTANT: Please read the "Safety Information" and "Important Notes" sections in the associated QIAGEN handbook before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate safety data sheets (SDSs), available from the product supplier.

The protocol is comprised of five steps:

1. DNA extraction
2. Quantification
3. Bisulfite conversion
4. Polymerase chain reaction
5. Pyrosequencing

DNA extraction

Equipment and reagents to be supplied by user

For EZ1 Advanced XL users

- EZ1 Advanced XL System (cat. no. 9001874)
- EZ1 Advanced XL DNA Investigator Card (cat. no. 9018699)
- EZ1 DNA Investigator Kit (cat. no. 952034)
- Buffer MTL (cat. no. 19112)
- Spin Baskets (Kerafast®, Inc.)

Important points before starting:

- If using the EZ1 DNA Investigator Kit for the first time, read the "Important Notes" in the *EZ1 DNA Investigator Kit Handbook*.
- The Large-Volume EZ1 DNA Purification Protocol is used, and requires Buffer MTL, which is not included in the EZ1 DNA Investigator Kit. Buffer MTL should be purchased separately.

Preparation

1. Prepare a 1:2 dilution of Buffer G2. Calculate the total amount needed by multiplying the number of samples, plus 2, by 490 µl.
2. Using sterile scissors, cut the cotton part of the swab into an appropriate 2 ml sample tube, supplied with the EZ1 DNA Investigator Kit.

Digestion

1. Add 490 µl of the 1:2 diluted Buffer G2.
2. Add 10 µl Proteinase K.
3. Vortex and incubate at 56 ºC for more than 2 h, vortexing at least every 30 min to improve cell lysis.

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DNA purification

1. Use a spin basket and centrifuge to collect the lysate from the swab into the 2ml sample tube.
2. Add 400 µl of Buffer MTL to each sample tube containing lysate.
3. Vortex and spin briefly.
4. Load the sample tubes into the proper locations on the EZ1 Advanced XL.
5. Load tips, elution tubes and reagent cartridges onto the instrument (see *EZ1 DNA Investigator Kit Handbook* for instructions).
6. Run the Large Volume Protocol with a 50 µl elution volume of water.
7. Once complete, remove samples and freeze at –20 ºC for longer periods of time, or store at 4 ºC if quantification is performed on the same day.

Quantification

DNA quantification can be performed by various methods, including the use of the following QIAGEN products:

- Investigator Quantiplex® Kit (cat. no. 387016)
- Investigator Quantiplex HYres Kit (cat. no. 387116)

Bisulfite conversion

Equipment and reagents to be supplied by user

- Epitect® Fast DNA Bisulfite Kit (50) (cat. no. 59824)
- Ethanol (Molecular Biology Grade, 96–100%)*
- Pipets and pipet tips (we recommend pipet tips with aerosol barriers to prevent cross-contamination)
- 0.2 ml PCR tubes
- Thermal cycler with heated lid
- 1.5 ml microcentrifuge tubes for elution steps
- Microcentrifuge

Important points before starting:

- Prior to starting the bisulfite conversion, all samples and reagents need to be brought to room temperature.
- Add 30 ml ethanol (96–100%) to Buffer BW and store at room temperature.
- Add 27 ml ethanol (96–100%) to Buffer BD and store at 2–8°C.
- Add 310 µl RNase-Free Water to Carrier RNA, and store in aliquots at –20°C.
- Each aliquot of EpiTect Bisulfite Reaction Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, dissolved EpiTect Bisulfite Reaction Mix can be stored at –20°C for up to 4 weeks, without any loss of performance.
- DNA Protect Buffer should turn from green to blue, after addition to DNA-EpiTect Bisulfite Reaction Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

* Do not use denatured alcohol, which contains other substances, such as, methanol or methylethylketone.

Procedure

1. Based upon sample concentration, pipette the appropriate volume to obtain 1–500 ng DNA, into a 0.2 ml (PCR) tube.
2. Add Molecular Biology Grade Water, up to 40 µl total volume per tube.
3. Add 85 µl of bisulfite solution.
4. Add 15 µl of DNA protect buffer.
5. Vortex and spin briefly in the centrifuge to remove any droplets that may have collected inside the lid.
6. Place tubes in a thermal cycler and run the following program (See Note 1):

Step	Time	Temperature
Denaturation	5 min	95 ºC
Incubation	20 min	60 ºC
Denaturation	5 min	95 ºC
Incubation	20 min	60 ºC
Hold	∞	20 ºC

7. Briefly centrifuge the tubes and transfer the solution to 1.5 ml tubes.

Cleanup of converted DNA

1. Add Carrier RNA solution to Buffer BL. Calculate the volume of Buffer BL and Carrier RNA solution required, by multiplying Buffer BL (350 µl) and carrier RNA (3.5 µl) by the number of samples to be processed, plus 2, to account for pipetting loss.
2. Add 310 µl of the Buffer BL-Carrier RNA mix to each tube.
3. Vortex and spin briefly in the centrifuge.
4. Add 250 µl of ethanol (96-100%) to each sample.
5. Vortex for 15 s and spin briefly in the centrifuge.
6. Transfer the contents of each tube to labeled MinElute® DNA spin columns.
7. Centrifuge the columns for 1 min at 12000 x *g*, or maximum speed. Discard the liquid at the bottom of the tubes (flow-through), and place the columns back into the collection tubes.
8. Invert Buffer BW several times before use, and add 500 µl to each spin column. Repeat step 14.
9. Add 500 µl of Buffer BD to each tube. Close the tubes and incubate for 15 min at room temperature; make sure Buffer BD is not open longer than required. Repeat step 14.
10. Add 500 µl of Buffer BW to each spin column and repeat step 14.
11. Repeat step 17.
12. Add 250 µl of ethanol (96–100%) to each spin column and repeat step 14.
13. Place the spin columns into new 2 ml collection tubes. Centrifuge for 1 min at 12000 x *g*, or maximum speed, to remove any remaining liquid.
14. Incubate the spin columns for 5 min at 60 ºC with the lids open, to evaporate any residual ethanol.
15. Place the spin columns into clean labeled 1.5 ml tubes (not included in the kit).
16. Add 10 µl of Buffer EB onto the center of the membrane in the spin column. Incubate for 1 min at room temperature and centrifuge for 1 min at 12000 x *g*, or maximum speed.
17. Repeat step 23 (See Note 2)
18. Store the converted DNA at –20 ºC

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Polymerase Chain Reaction

Equipment and reagents to be supplied by user

- PyroMark PCR Kit (200) (cat. no. 978705)
- PyroMark CpG Assays:
  - BCAS4 (cat. no. PMC0002408)
  - C20orf227 (cat. no. PM00197323)
  - FGF7 (cat. no. PMC0004067)
  - ZC3H12D (cat. no. PM00124145)
  - cg06379435 (cat. no. PMC0085498)
- 0.2 ml PCR tubes
- Pipets and pipet tips (aerosol resistant)
- Thermal cycler

Important points before starting:

- Thaw the PyroMark PCR Master Mix, the CoralLoad® Concentrate, a Molecular Biology Grade Water aliquot, the aliquot of the PyroMark assay primers and the bisulfite-modified DNA to be amplified.
- To determine the number of reactions, add the number of DNA samples with 2 for No Template Controls (NTC, no DNA is added, just water) plus 1 (to account for pipetting loss).

Procedure

1. Prepare a master mix by adding 12.5 µl of PyroMark PCR Master Mix, 2.5 µl of CoralLoad Concentrate, 2.5 µl of PyroMark assay primers A and B and 5.5 µl of Molecular Biology Grade Water, per reaction. To determine the volumes to add, multiply each volume given by the number of reactions determined in step 2.
2. Aliquot 23 µl of the mix prepared in 3 to the appropriate number of 0.2 ml (PCR) tubes.
3. Add 2 µl of Molecular Biology Grade Water to the NTC tubes and 2 µl of bilsulfite-converted DNA to the remaining tubes.
4. Centrifuge the tubes and load them into a thermal cycler. Program the thermal cycler as follows (See Note 3):

Step	Time		Temperature
Initial	15 min		95 ºC
Denaturation Annealing* Extension	45 cycles	30 s 30 s 30 s	94 ºC 55 ºC 72 ºC
Final Extension	10 min		72 ºC
Hold	∞		4 ºC

* For custom primers the annealing temperature is usually set to T_m –5 ºC.

5. When the PCR is complete, remove the tubes and store at –20 ºC.

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Pyrosequencing

Equipment and reagents to be supplied by user

- PyroMark Q24 Vacuum Workstation (cat. no. 9001516)
- PyroMark Q24 Advanced Software (cat. no. 9022779)[†]
- PyroMark Q24 Advanced CpG Reagents (cat. no. 970902)
- PyroMark Q24 Plate (cat. no. 979201)
- PyroMark Q24 Cartridge (cat. no. 979202)
- PyroMark Q24 Vacuum Prep Troughs (cat. no. 979206)
- PyroMark Q24 Plate Holder (cat. no. 9022273)
- PyroMark Q24 Vacuum Prep Filter Probe (cat. no. 979010)
- PyroMark Denaturation Solution (cat. no. 979007)
- PyroMark Wash Buffer concentrate (cat. no. 979008)
- Plate mixer for immobilization to beads
- Heating block capable of reaching 80°C
- 24-well PCR plate or strips
- Adhesive foil or strip caps
- Streptavidin Sepharose® High Performance (34 µm, 5 ml, GE Healthcare; cat. no. 17-5113-01)
- Sequencing primer (available with PyroMark CpG Assays, page 4)
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Ethanol (70%)

Important points before starting:

- Switch on the PyroMark Q24 Advanced instrument at least 30 minutes before use.
- Thaw the PCR products, an aliquot of biotinylated PCR primers (this aliquot is only used for Pyrosequencing and should never be used for amplification, to avoid contamination from PCR products to pre-amplification DNA) and the sequencing primer.
- Equilibrate the diluted wash buffer, the denaturation buffer, the annealing solution, the binding solution, the streptavidin-coated beads and the PyroMark Q24 Advanced CpG reagents, at room temperature.
- Place the metal tray for the PyroMark Q24 plate on a heating plate, set at 80 ºC.

Procedure

1. Prepare the bead mixture by adding 40 µl of binding buffer, 29 µl of Molecular Biology Grade Water and 1 µl of beads per reaction. Multiply each volume per the number of samples, plus 1, for pipetting loss.
2. Add 70 µl of this mixture to 22 wells of a 24-well PCR plate. Leave the last two wells empty for negative controls and do not put beads mixture into these wells. (See Note 4)
3. Add 10 µl of PCR product into the corresponding well of the 24-well PCR plate.
4. Close the wells with a strip cap and mix at 1400 rpm for at least 15 min (See Note 5).
5. Remove from mixing only when ready to use the vacuum station.
6. Add 20 µl of 1 x sequencing primer in annealing buffer to 22 wells on the PyroMark Q24 plate.

[†] The PyroMark Q24 Advanced SW is included in the PyroMark Q24 Advanced system. If older PyroMark systems are used (e.g., PyroMark Q24) the system can be upgraded with a simple software upgrade. More information is available at **https://www.qiagen.com/de/resources/technologies/pyrosequencing-resource-center/upgrading-to-pyromark-q24-advanced/**

7. The last two wells are reserved for the primer controls. Add the sequencing primer into one well, and add a mixture of biotinylated PCR primer (2 µl) and sequencing primer (20 µl) into the other well.
8. Place the PyroMark Q24 plate on the vacuum preparation workstation and fill all the trays of the station with the corresponding solutions.
9. Prepare the template for the plate on the PyroMark Q24 Advanced software.
10. Check the volumes to add to the cartridge for each nucleotide, enzyme mix and substrate mix. Add the volumes to the cartridge and place it on the PyroMark Q24 Advanced instrument.
11. Switch on the vacuum switch in the suction probe, and place it on the prime tray with distilled water, aspirating approximately 70 ml.
12. Remove the 24-well PCR plate from agitation, take the lids off (See Note 6) and place in the vacuum preparation workstation.
13. Place the suction probe into the PCR plate wells with the vacuum switched on, carefully capturing all the solution from the wells for about 15 s.
14. Wash the beads/samples with 70% ethanol for 5 s.
15. Continue to the tray with Denaturing solution, flush for 5 s.
16. Aspirate for 10 s on the Washing Buffer tray and tilt the suction probe to beyond vertical 90º for a few s.
17. Place the vacuum suction probe on top of the PyroMark Q24 plate without touching the liquid.
18. Switch off the vacuum and lower the handle on the PyroMark Q24 plate.
19. Shake the suction probes gently from side to side for 30 s to 1 min, to release the beads on the PyroMark Q24 plate containing sequencing primers.
20. Rinse the vacuum suction probe on the rinse tray containing distilled water, by agitating for 10 s.
21. Aspirate 70 ml distilled water on the prime tray.
22. Move the suction probe beyond 90º vertical for a few s and disconnect the vacuum.
23. Place the plate on the metal tray and heat at 80 ºC for 5 min.
24. Within a 30 s interval, place the sequencing plate on the PyroMark Q24 Advanced instrument, close the lid and start the run.
25. When the run is complete, discard the PyroMark Q24 plate and the unused contents of the cartridge by inverting it, and rinse the cartridge with deionized water.
26. Fill up each well of the cartridge with water, and by applying pressure to the top with your fingers, force the water to pass though the small needles at the bottom.
27. Repeat step 26 at least 3 times for each well.
28. Let it dry at room temperature.
29. Remove all trays from the vacuum preparation workstation and rinse with deionized water. Dry at room temperature.

Data analysis

1. The PyroMark Q24 Advanced software will perform the methylation percent analysis at each variable CpG sites and the data is displayed as a Pyrogram®.
2. If any yellow or red warning is displayed, due to a height variation on a homopolymer, deselect that peak as a reference peak.
3. Get the percent methylation value for each variable position and make an Excel® spreadsheet with values for all tissues tested for each primer set.

Notes

Note 1: The QIAGEN protocol suggests the possibility of increasing the 60 ºC incubation time to 20 min, to guarantee a complete bisulfite conversion. An increase in DNA fragmentation or any other problems were not observed; therefore, this time was adopted into the standard protocol in order to avoid incomplete conversion that would give false results during Pyrosequencing analysis.

Note 2: To improve yield, 10 µl of elution buffer was added twice, which is the minimum amount recommended by QIAGEN. In addition, a 1 min incubation was performed after each elution.

Note 3: The default annealing temperature on the PyroMark PCR kit is 56 ºC. An optimization of the PCR protocol was performed by evaluating which temperatures, within the range of 54 ºC to 58 ºC, would give more PCR product. Although the results for the temperatures of 55 ºC and 56 ºC were close to each other, it was decided to use 55 ºC as an annealing temperature. A similar procedure is recommended to be performed for all new primers that will be adopted as markers for body fluid determination, to ensure a good yield of PCR product. Q-Solution®, which is included in the kit, was not used, because it diminished the PCR efficiency for the samples that were tested.

Note 4: A 96-well non-skirted PCR plate may be obtained commercially, and cut into a 24-well plate format (8 x 3), to fit into the vacuum station.

Note 5: The shaker should be able to shake at 1400 rpm and hold 24-well PCR plates. Also, a 2 mm orbital diameter is preferable to other shakers.

Note 6: The lids of the PCR plates must be removed with caution, to avoid drops from one reaction contaminating the next well of the plate. A compromise between tapping the plate on the bench top to make drops on the lid descend, and avoiding the delay of starting the vacuum procedure, must be reached, to avoid the beads from depositing on the bottom of the plate.

References

1. 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.
2. 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**, 3079–3086.
3. Antunes, J., Balamurugan, K., Duncan, G., McCord, B. (2015) Tissue-specific DNA methylation patterns in forensic samples detected by Pyrosequencing. Methods in Molecular Biology **1315**, 397–409.