

DNA METHYLATION ANALYSIS BY PYROSEQUENCING

Standard methylation analysis methods provide only qualitative or semi-quantitative data, which can lead to inaccurate conclusions regarding the effects of epigenetic DNA methylation. Without quantification of methylation levels, it is not possible to distinguish physiologically relevant methylation from background methylation.

Pyrosequencing solves this limitation by generating highly reproducible quantification of methylation frequencies at individual consecutive CpG sites.

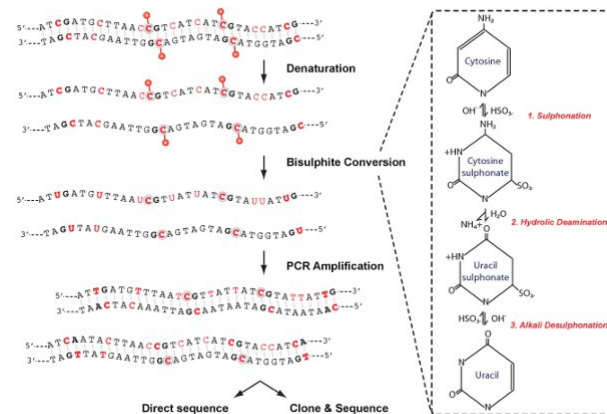
As such, Pyrosequencing can detect and quantify even small changes in methylation levels.

These features have established Pyrosequencing as the gold standard for DNA methylation analysis.

1.1 Bisulfite Conversion

To characterize the methylation status of a DNA sequence via Pyrosequencing, the DNA is first incubated with sodium bisulfite.

This protocol for bisulfite conversion is the "gold standard" for DNA methylation analysis and facilitates identification and quantification of DNA methylation at single nucleotide resolution. The chemistry of cytosine deamination by sodium bisulfite involves three steps.



- 1- **Sulphonation:** The addition of bisulphite to the 5-6 double bond of cytosine.
- 2- **Hydrolyc Deamination:** hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative.
- 3- **Alkali Desulphonation:** Removal of the sulphonate group by an alkali treatment, to give uracil.

Bisulfite preferentially deaminates cytosine to uracil in single stranded DNA, whereas 5-MeC, is refractory to bisulfite-mediated deamination.

Upon PCR amplification, uracil is amplified as thymine while 5-MeC residues remain as cytosines, allowing methylated CpGs to be distinguished from unmethylated CpGs by presence of a cytosine "C" versus thymine "T" residue during sequencing.

	Original sequence	After bisulfite treatment	After PCR amplification
Unmethylated DNA	A-C-G-T-C-G-T-C-A	A-U-G-T-U-G-T-U-A	A-T-G-T-T-G-T-T-A
Methylated DNA	A-C-G-T-C-G-T-C-A	A-C-G-T-C-G-T-U-A	A-C-G-T-C-G-T-T-A

EQUIPMENT

Fixed Equipment

- Thermal Cycler
- Centrifuge
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively

Consumables

- Polypropylene microcentrifuge tubes, 0.2 µl
- Zymo-Spin IC Columns
- Collection Tubes
- Filter pipette tips 10, 200, 1000 µl

REAGENTS

- DNA samples extracted from cellular pellets
- CT Conversion Reagent
- M-Binding Buffer
- M-Wash Buffer

DNA	250 ng	µl ?
H ₂ O		Up to 20 µl

- In a 0.2 mL tube put the DNA and H₂O mix.
- Add 130 µl of the CT Conversion Reagent to 20 µl of DNA and H₂O mix.
- Mix the sample pipetting it up and down, then centrifuge the liquid to the bottom of the tube.
- Place the sample tube in a thermal cycler and perform the following steps:

1. 98°C for 10 minutes
2. 64°C for 2.5 hours
3. 4°C storage for up to 20 hours

- Add 600 μ l of **M-Binding Buffer** to a Zymo-Spin IC Column and place the column into a provided collection tube.
- Load the sample from the thermal cycler into the Zymo-Spin IC Column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.
- Centrifuge at 13000xg for 30 seconds. Discard the flow-through.
- Add 100 μ l of **M-Wash Buffer** to the column. Centrifuge at full 13000xg for 30 seconds.
- Add 200 μ l of **M-Desulphonation Buffer** to the column and let stand at room temperature for 20 minutes. After the incubation, centrifuge at 13000xg for 30 seconds.
- Add 200 μ l of **M-Wash Buffer** to the column. Centrifuge at 13000xg for 30 seconds. Add another 200 μ l of **M-Wash Buffer** and centrifuge for an additional 30 seconds.
- Place the column in a 1.5 mL microcentrifuge tube. Add 20 μ l of H₂O directly to the column matrix. Centrifuge for 30 seconds at 13000xg to elute the DNA.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use.

2.1 Pyrosequencing

Principle of Pyrosequencing

Step 1

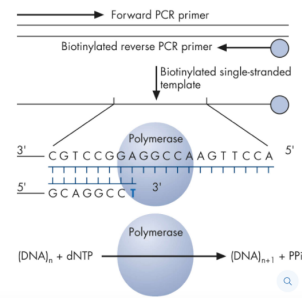
A DNA segment is amplified and the strand to serve as the Pyrosequencing template is biotinylated. After denaturation, the biotinylated single-stranded PCR amplicon is isolated and allowed to hybridize with a sequencing primer.

Step 2

The hybridized primer and single-stranded template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrates adenosine 5' phosphosulfate (APS) and luciferin.

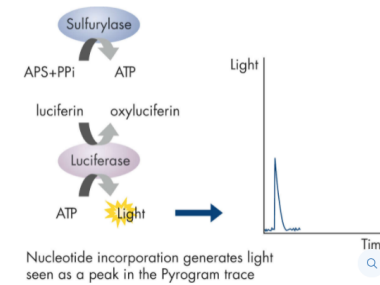
Step 3

The first deoxyribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes addition of the dNTP to the sequencing primer, if it is complementary to the base in the template strand. Each incorporation event is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide



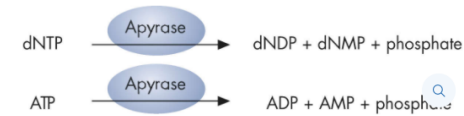
Step 4

ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated



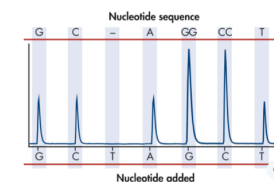
Step 5

Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

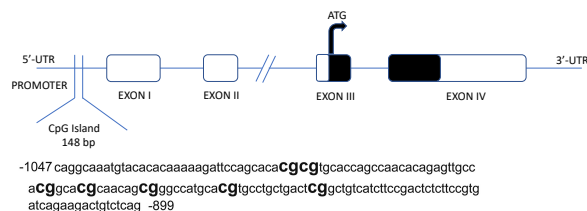


Step 6

Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP α S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.



Human PDYN Promoter region analyzed



Schematic representation of the human *PDYN* gene and the 5' upstream region. ATG is the translation start site. Shown are the locations of the CpG island and Exons I-IV. Coding regions of exons are shown as black. The sequence of the CpG island (chr 20: 1994648–1994795) that was used for DNA methylation analyses is reported. Bold text indicates the 7 CpG sites (out of 9 present in the island) analyzed to evaluate the methylation status.

2.1.1 PCR PyroMark

A biotinylated PCR product serves as the template for the Pyrosequencing reaction. Amplify the DNA to be analyzed by PCR with one of the primers biotinylated.

EQUIPMENT

Fixed Equipment

- Thermal Cycler
- Centrifuge
- Pipettes suitable for measuring 10, 200 and 1000 µl, respectively

Consumables

- Polypropylene microcentrifuge tubes, 0.2 µl
- Filter pipette tips 10, 200, 1000 µl

REAGENTS

- Bisulfite-converted DNA
- PyroMark PCR Master Mix
- CoralLoad Concentrate
- PCR primers mix
- H₂O nuclease-free

- Thaw all required solutions and mix each thoroughly.
- In a 0.2 µl tubes add the following components:

Bisulfite-converted DNA	2.5 µl
PCR Master Mix	12.5 µl
Coral Load	2.5 µl
PCR Primers set	2.5 µl
H ₂ O	5 µl

- Place the PCR tubes in the thermal cycler and starting the following program:

95°C 15 minutes: HotStartTaq DNA Polymerase is activated.
 94°C 30 seconds: Denaturation
 56°C 30 seconds: Annealing
 72°C 30 seconds: Extension
 72°C 10 minutes: Final extension.

45 cycles

- Run the PCR products on 1.8% agarose gel

2.1.2 Sequencing

Setting up a run

- Select PyroMark Q24 Software.
- In the shortcut browser, right-click the folder you want to place the run file in and select “New Run” from the context menu.
- Enter the file name and press “Enter”.
- Select “Instrument Method”.
- Add an assay to each well used, e.g., drag an assay from the shortcut browser to a well or a selection of wells.
- A well is colored according to the assay loaded into the well.

	1	2	3	4	5	6
A	AQ assay 1	AQ assay 2	CpG assay 1	CpG assay 2	SQA assay 1	SQA assay 2

- To enter a sample ID or note, select the cell and enter the text. A selected cell is highlighted with a blue background color.

Assay Name	Assay Name
Sample ID	Sample ID
Note	Note

- Print a list of required volumes of reagents and the plate setup; select “Pre Run Information” from the “Tools” menu.
- Close the run file and copy it to one of the USB sticks supplied.

The run file can now be processed by inserting the USB stick into the USB port at the front of the PyroMark Q24 Instrument.

Fixed Equipment

- PyroMark Q24 Vacuum Workstation
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C

Consumables

- PyroMark Q24 Plate
- 24-well PCR plate or strips
- Strip caps
- PyroMark Q24 Cartridge

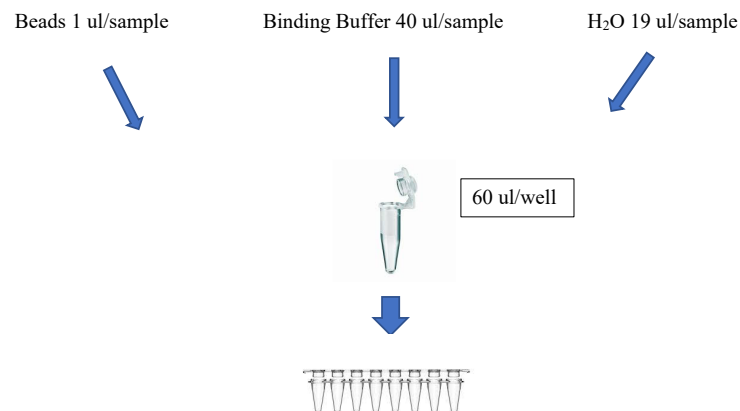
REAGENTS

- Streptavidin Sepharose High Performance
- Sequencing primer
- Ethanol 70%
- PyroMark Binding Buffer
- PyroMark Denaturation Solution
- PyroMark Wash Buffer concentrate
- PyroMark Annealing Buffer
- H₂O Nuclease free
- PyroMark Gold Q24 Reagents (Enzymes, Substrates and Nucleotides)

Immobilizing the PCR product to beads

Biotinylated PCR products are immobilized on streptavidin coated Sepharose beads.

- Make a master mix according to the flowchart.



Note: Before pipetting, gently shake the bottle of streptavidin-coated Sepharose beads to ensure a homogenous suspension.

- Add 20 µl PCR product to each well.
- Dispense 60 µl master mix into each well to give a total volume of 80 µl.
- Seal the wells with strips caps and agitate the strips at 1400 rpm for 10 minutes at room temperature using a mixer.

Note: Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.

Note: During immobilization, prepare the vacuum workstation for the sample preparation.

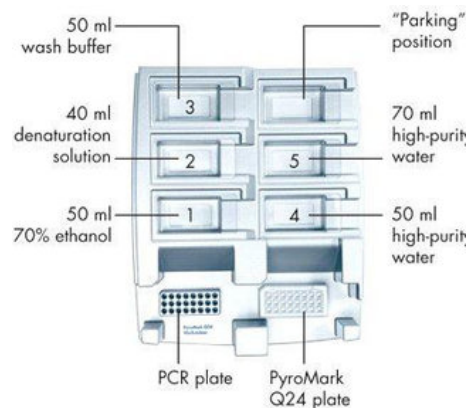
Separation of DNA strands and release of samples into the PyroMark Q24 Plate

Things to do before starting: Prewarm one of the supplied PyroMark Q24 Plate Holders by placing it (without a plate) on a heating block at 80°C.

- Ensure that the PyroMark Q24 Vacuum Workstation has been assembled correctly and securely.
- Fill five separate troughs supplied with the PyroMark Q24 Vacuum Workstation as follows:

Approximately 50 ml ethanol (70%) (1)
 Approximately 40 ml Denaturation Solution (2)
 Approximately 50 ml 1x Wash Buffer (3)
 Approximately 50 ml high-purity water (4)
 Approximately 70 ml high-purity water (5)

A suggested setup is shown below. Refill the troughs to these levels whenever necessary.



Filling the workstation troughs.

- Switch on the vacuum pump.
- Apply vacuum to the tool by opening the vacuum switch.
- Wash the filter probes by lowering the probes into high purity water (trough 5). Flush the probes with 70 mL high purity water.
- Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position
- Refill trough 5 with 70 mL high-purity water.
- Dilute the sequencing primer from 10X to 1X in Annealing Buffer.
- Add 25 µl of the solution to each well of a PyroMark Q24 Plate that is to be used.
- Immediately after immobilization, place the PCR strips) and PyroMark Q24 Plate on the worktable.
- Apply vacuum to the tool by opening the vacuum switch.
- Carefully lower the filter probes into the PCR strips to capture the beads containing immobilized template. Hold the filter probes in place for 15 s. Take care when picking up the tool.
- Ensure that all liquid is aspirated from the wells and that all beads have been captured onto the filter probe tips.

Note: If the wells still contain liquid or white beads remain, the filter probes may need replacing.

- Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.
- Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.
- Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10 s.
- Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.



- While holding the tool over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).
- Release the beads in the plate containing sequencing primer, by shaking the tool gently from side to side.
- With the vacuum switch closed (Off), transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.
- Wash the filter probes by lowering the probes into highpurity water (trough 5) and applying vacuum. Flush the filter probes with 70 mL high-purity water.
- Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
- Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.
- Turn off the vacuum pump.

Annealing of sequencing primer to samples

- Heat the PyroMark Q24 Plate containing the samples at 80°C for 2 min using the PyroMark Q24 Plate Holder and a heating block.
- Remove the plate from the plate holder and allow the samples cool to room temperature (15–25°C) for at least 10 min. The plate can now be processed in the PyroMark Q24 Instrument.

Preparation of PyroMark Cartridge

- Fill the PyroMark Q24 Cartridge according to the Pre-run informations

Human PDYN

Volume Information:

Reagents:

E-mix: 94 µl
S-mix: 94 µl

Nucleotides:

Nucleotide A: 73 µl
Nucleotide C: 66 µl
Nucleotide G: 94 µl
Nucleotide T: 97 µl



Plate setup:

	1	2	3	4	5	6	7	8
A	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge		
B	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge		
C	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge	PDYN Cartridge		



Starting the run

Load the reagent cartridge and the plate:

- When the instrument is not processing, open the instrument lid.
- Open the cartridge gate and insert the filled reagent cartridge with the label facing out. Push the cartridge in fully and then push it down.
- Ensure the cartridge is properly inserted, with the line visible in front of the cartridge, and close the gate.
- Open the plate-holding frame and place the plate on the heating block inside the instrument.
- Close the plate-holding frame and the instrument lid.



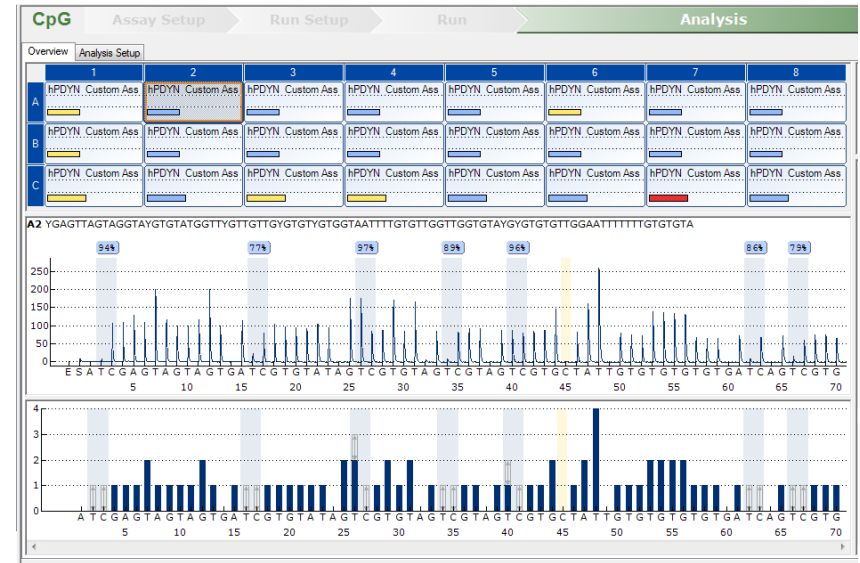
Select the run file and start the run:

- Insert the USB stick containing the run file into the USB port at the front of the instrument.
- Using the screen buttons, select “Run” in the main menu and press “OK”.
- Select the run file and press “Select” to start the run.

The instrument will start dispensing reagents when the pressure in the dispensing unit, the speed of the mixer, and the temperatures of the heating block, process chamber lid, and the coolant liquid have reached their preset levels.

Analyzing the run

- Move the processed run file from the USB stick to a computer running PyroMark Q24 Software.
- Open the run file.
- By selecting an analyzed well in the “Overview” tab, the corresponding Pyrogram is displayed in the Pyrogram area and the well information (including analysis warnings) is listed in the “Well Information” area.



Quality colors

- Blue: Passed
- Yellow: Check
- Red: Failed
- White: Not analyzed*

The methylation percentages are displayed in Pyrogram, 94% for example . The quality assessment is displayed by the background color of the result.