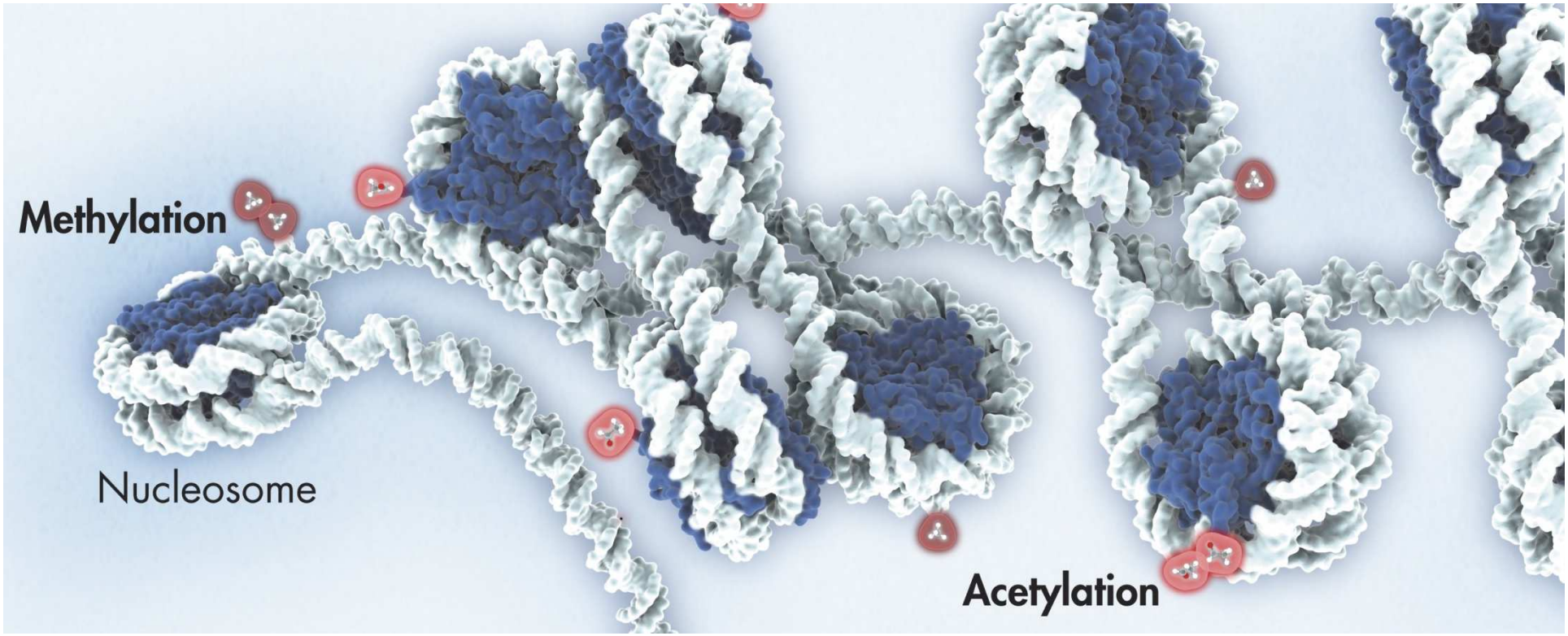


Uncovering the secrets of epigenetics

An introduction into methods and applications

Gerald Schock, Ph.D.
Associate Director Pyrosequencing
QIAGEN GmbH

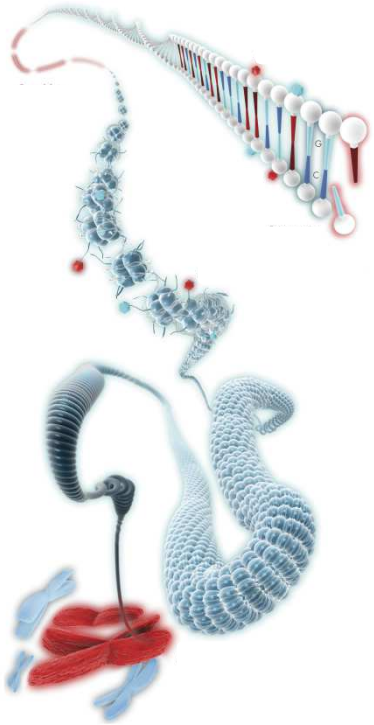
Epigenetics

The study of reversible inheritable influence on gene activity that is not accompanied by a change in the DNA sequence

Epigenetics - Definition and Mechanisms

Epigenetics mechanisms

- **DNA (CpG) Methylation**
- **Histone Modification**
- **micro RNA**



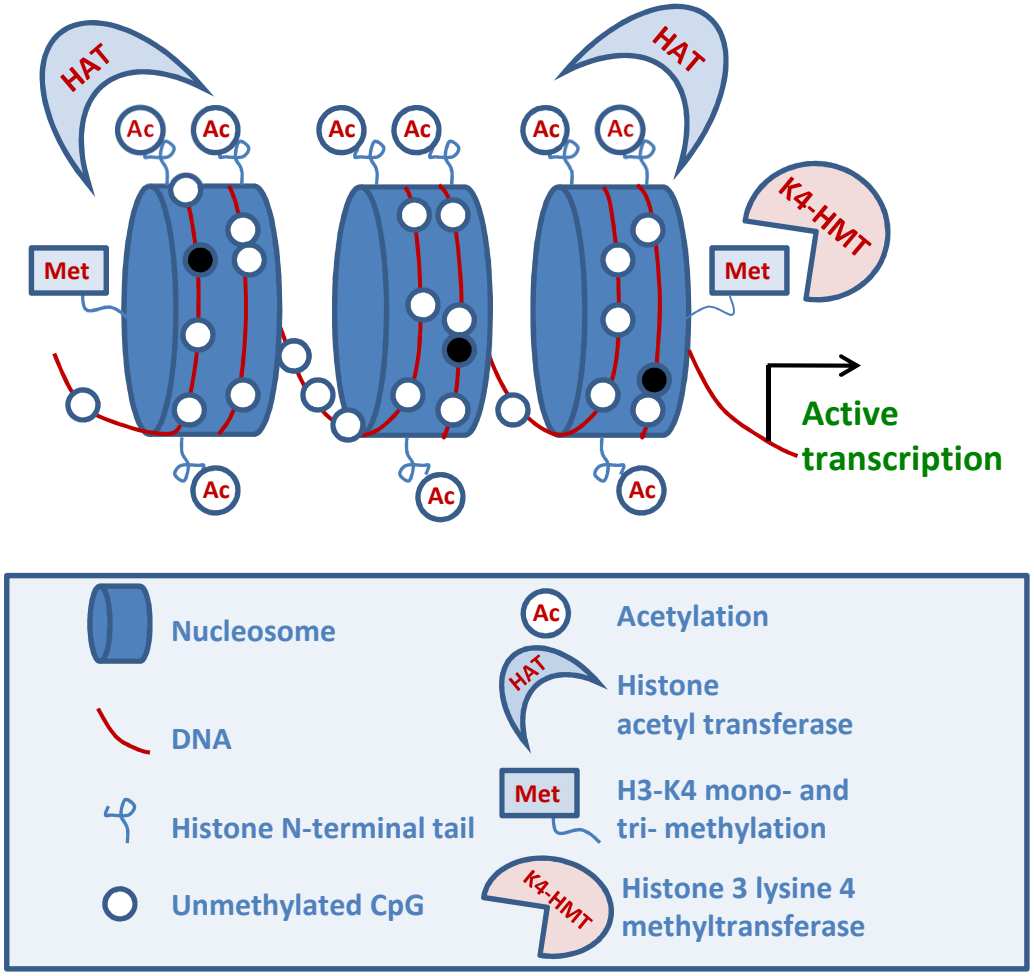


Figure kindly provided by Lasse Sommer Kristensen, Aarhus University, Denmark, Institute for Human Genetics, modified from Kristensen *et al.* 2009. *Eur J Phamacol*

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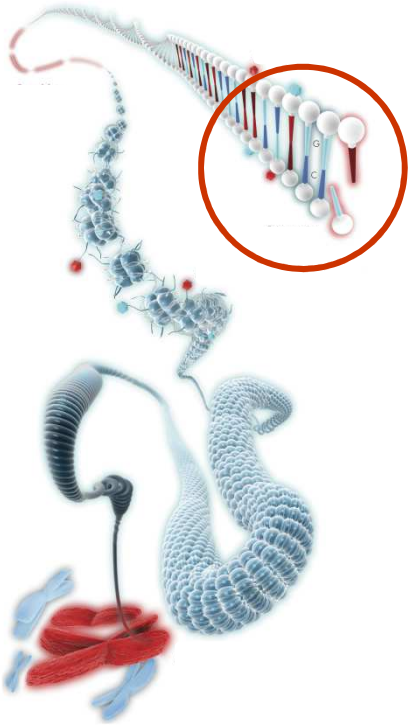
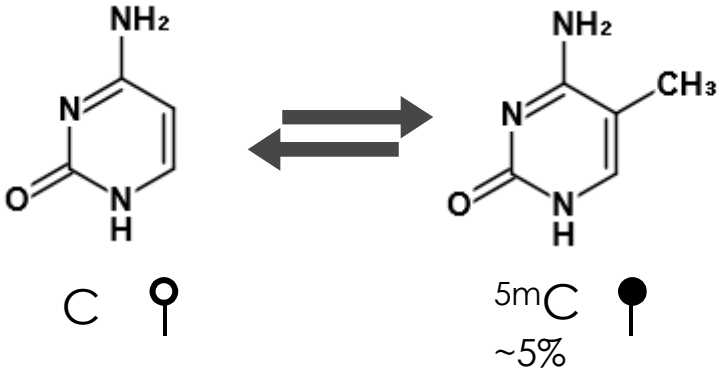
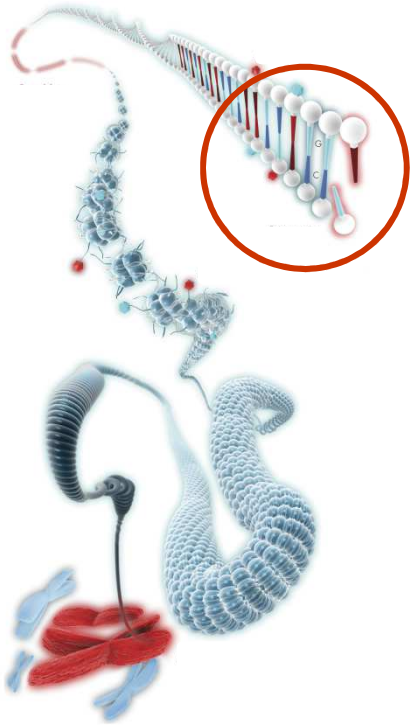


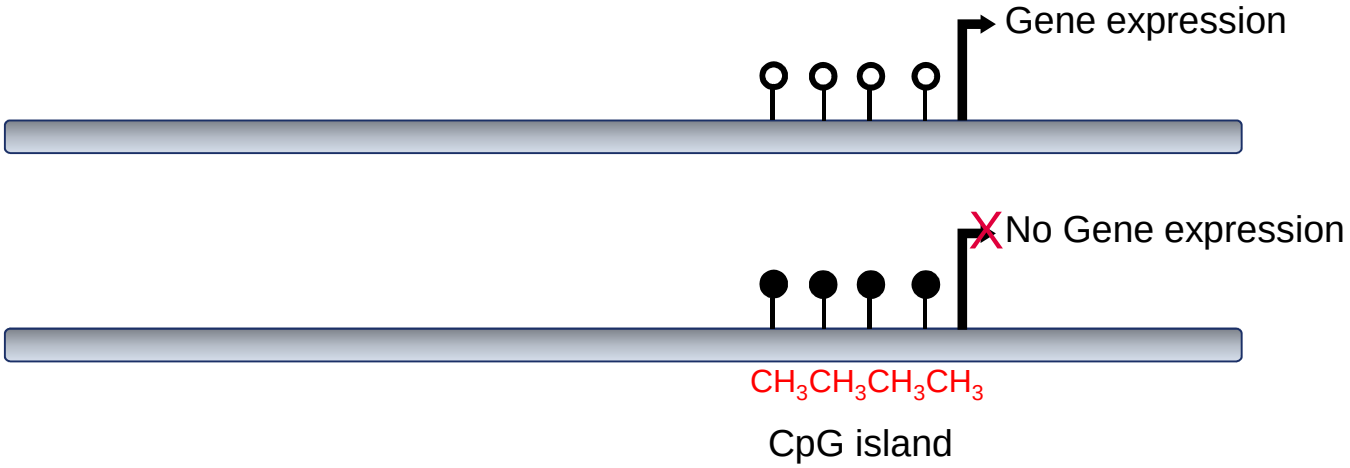


DNA Methylation and the use of epigentic marker analysis



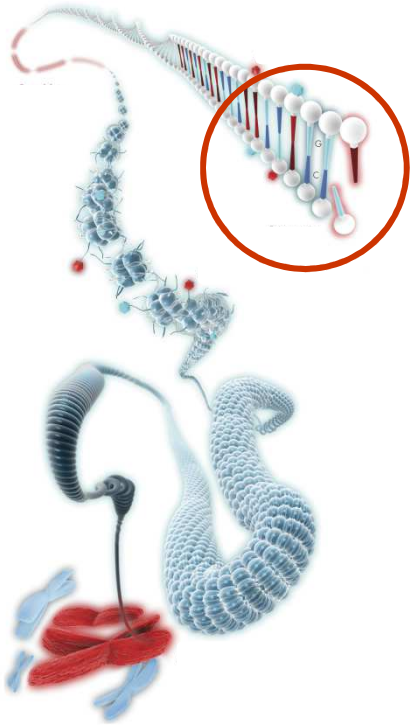
CpG islands

- High frequency of CpG dinucleotides: one CpG per 10 nucleotides
- Often found in promoters and regulatory elements (5' upstream regions)
  - CpG islands co-localize with 60% of all promoters
- Methylated promotors are silenced



Epigenetics - Definition and Mechanisms

DNA Methylation and the use of epigentic marker analysis



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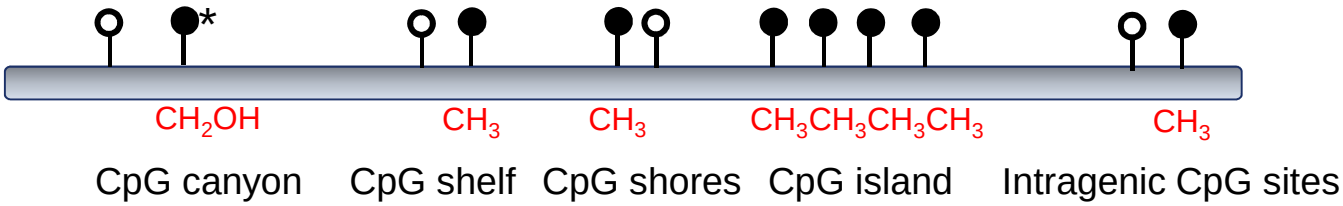
  - CpG islands co-localize with 60% of all promoters
- Methylated promotors are silenced

CpG Shores and Shelves and Canyons

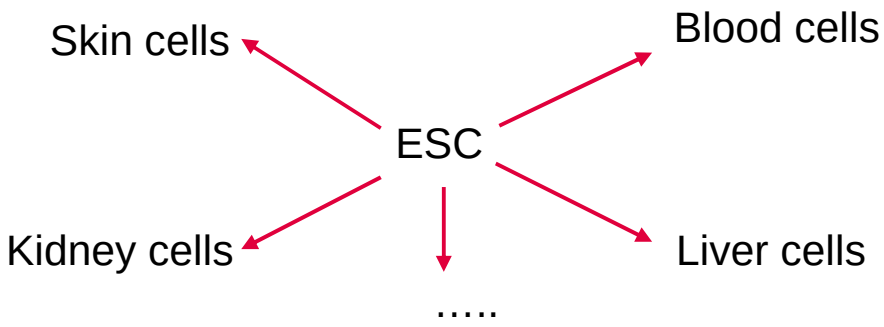
- CpG shores: < 2kb flanking CpG Islands
- CpG shelves: < 2kb flanking CpG shores
- CpG canyons: regions of low methylation
  - CpG canyon borders are landmarked by ^{5hm}C

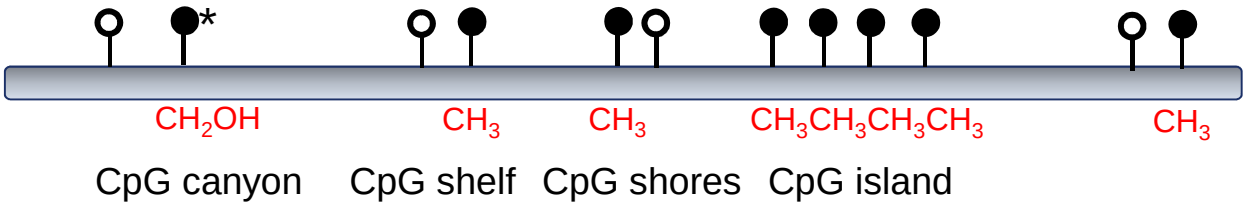


DNA Methylation and the use of epigentic marker analysis

DNA methylation is tissue specific

- DNA methylation differentiates cells of different tissues on its way from ESC* to somatic cells
- Each tissue has its own pattern of gene expression (protein/RNA)
- …and thus its own pattern of DNA methylation.





ESC: embryonic stem cell

Methylation analysis for tissue identification

Analysis of tissue specific DNA methylation using Pyrosequencing

Tissue identification

- Madi *et al** identified a collection of epigenetic markers
  - Each epigenetic marker spans multiple CpG sites
  - All markers show a unique pattern for various tissues
  - A set of four markers was sufficient to differentiate between blood, saliva, epithelial cells, and sperm
- Ongoing studies include new markers for the identification of additional tissues.

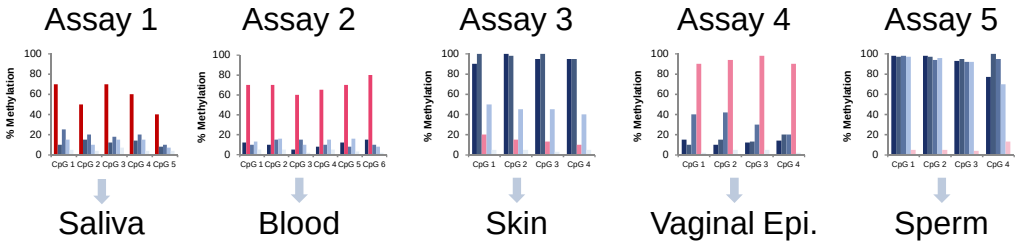
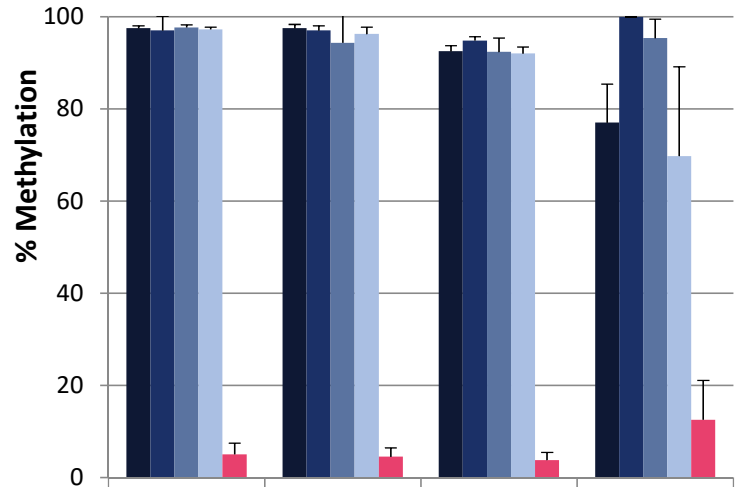


Figure 1: Schematic illustration of the experimental setup. Assays are deveolped which all show different methylation pattern over various CpG sites. These patterns are used to differentiate DNA derived form saliva, blood, skin, vaginal epithelium, and sperm. The values for the graphs for assay 1-4 are illustrative only. The graph for assay 5 shows real data and is explained in figure 2.



	CpG 1	CpG 2	CpG 3	CpG 4
Buccal	98	98	93	77
Blood	97	97	95	100
skin	98	94	92	95
V. Epi.	97	96	92	70
Sperm	5	5	4	13

Figure2: Graphical representation of the mean percent methylation values for buccal swabs, blood, skin, vaginal epithelial cells, and sperm using a methylation specific marker for the identification of sperm (unpublished).
Data kindly provided by Kuppareddi Balamurugan, School of Criminal Justice, University of Southern, Mississippi, USA

*The determination of tissue-specific DNA methylation patterns in forensic biofluids using bisulfite modification and pyrosequencing. Madi, T., Balamurugan, K., Bombardi, R., Duncan, G, and McCord, B. *Electrophoresis* 2012, 33, 1736–1745

Methylation analysis for age estimation

Use of methylation markers for age estimation of an unknown individual based on biological traces

Age estimation

- Certain DNA methylation changes accumulated over time correlate well with chronological aging
- Multiple studies have identified suitable markers
- Zbiec´-Piekarska *et al* [1)] successfully demonstrated on live casework in Poland
  - Markers ELOVL2, C1orf132, TRIM59, KLF14 and FHL2 used
- Spólnicka *et al* [2)] validated the AgePlex assays on the new PyroMark Q48 Autoprep


Application Note

Application Note

Validation of the PyroMark® Q48 Autoprep compared with the PyroMark Q24 system for methylation based age estimation

Magda Spólnicka[1], Natalia Niedzwiecka[2], Maciej Szewczyk[2], and Krzysztof Kucharczyk[2]

[1] Central Forensic Laboratory of the Police, Aleje Ujazdowskie 7, 00-583 Warsaw, Poland
[2] BioVectis, A.Pawińskiego 5a/d, 02-106 Warsaw, Poland

Introduction

An age prediction method has previously been established using Pyrosequencing® and the PyroMark Q24 platform (1). The method is based on determination of the methylation level of a set of markers in genomic DNA extracted from the blood of an individual. Introduction of a new version of the analytical platform, the PyroMark Q48 Autoprep System, prompted us to validate the age prediction algorithm on this new automated higher-throughput platform.

For starting material in the validation we used the same set of over 100 samples of genomic DNA obtained from men and women aged from 2 to 75 years and stored at –70°C for over one year. This set of samples was used previously to validate the PyroMark Q24 age prediction algorithm. All genomic materials were bisulfite converted. Markers located at five loci (ELOVL2 on 6p24.2, C1orf132 on 1q32.2, TRIM59 on 3q25.33, KLF14 on 7q32.3 and FHL2 on 2q12.2) were amplified by singleplex and multiplex PCR, and their methylation level was established with the Pyrosequencing method on the PyroMark Q48 Autoprep platform.

Based on the study results, using the PyroMark Q24 and PyroMark Q48 Autoprep platforms, the mean absolute deviation (MAD) of the standard error of age prediction between the two platforms was 1.7 years. This value is almost two times lower than the MAD of the age estimation error on the PyroMark Q24 platform alone. Based on these data we conclude that the age prediction algorithm developed on the PyroMark Q24 platform can be used for methylation data obtained using the PyroMark Q48 Autoprep without any modification.

Materials

Blood samples were collected in EDTA-blood tubes from volunteers who had signed informed consent statements prior to sample donation. In total approximately 120 unrelated males and females, aged between 18 and 75 years, were analyzed. Additionally, samples from children ▷

Sample to Insight QIAGEN

1) Use of methylation markers for age estimation of an unknown individual based on biological traces. Renata Zbiec´-Piekarska, Magda Spólnicka, Tomasz Kupiec, Zaneta Makowska, Agnieszka Parys-Proszek,Krzysztof Kucharczyk, Keith Elliott, Rafał Płoski, Wojciech Branicki. QIAGEN Application Note 2016

2) Validation of the PyroMark® Q48 Autoprep compared with the PyroMark Q24 system for methylation based age estimation. Magda Spólnicka, Natalia Niedzwiecka, Maciej Szewczyk, and Krzysztof Kucharczyk, QIAGEN Application Note 2017

Workflow for genetic and epigenetic analysis

EZ1

QIAcube

Rotor-Gene Q

QIAcube

Sample

DNA purification

Quantification

Bisulfite conversion

For epigentics only

- QIAamp Kits
- EZ1 DNA Investigator Kit

- Investigator Quantiplex Pro RGQ Kit

- EpiTect Fast DNA Kits

Quantiplex Pro RGQ

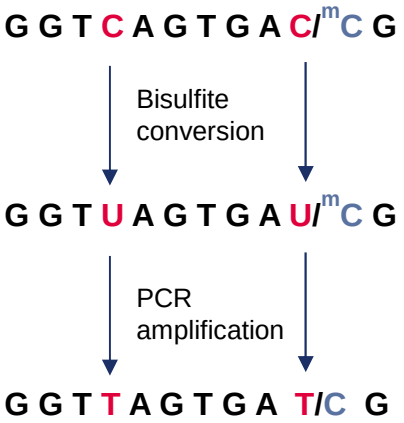
EpiTect Fast DNA

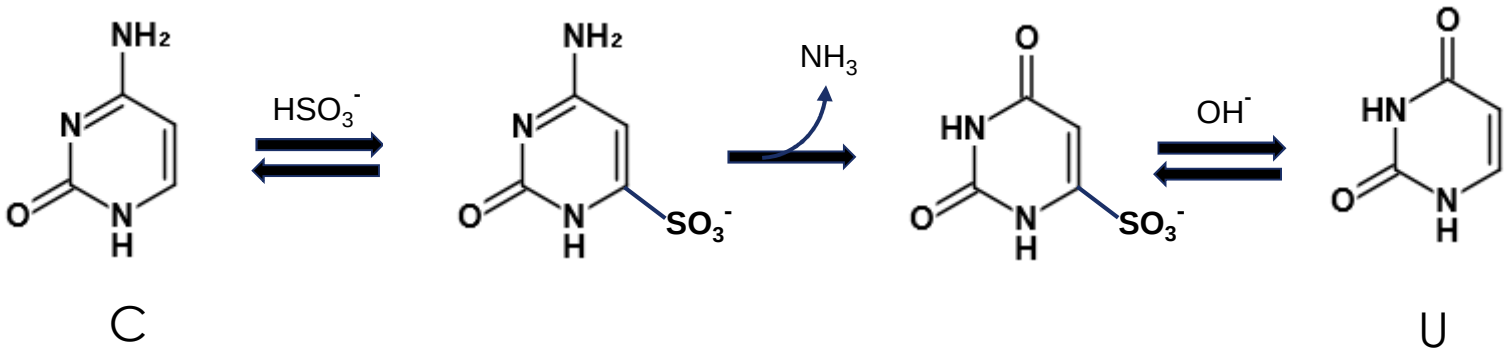
2-3 hrs

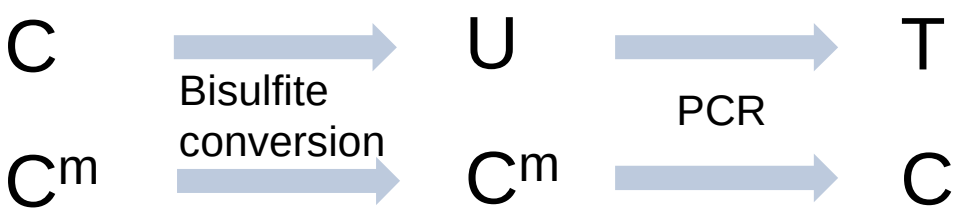
Bisulfite conversion – THE key method for methylation analysis

How to analyze CpG methylation?

- Cannot be measured directly as C and C^m is difficult to differenciate
- Require Bisulfite Conversion treatment:
  - Converts C to U (subsequently to T after PCR)
  - C^m stays unchanged (will be replaced by C after PCR)
  - Ratio C/T is translated into methylation degree of a specific CpG site







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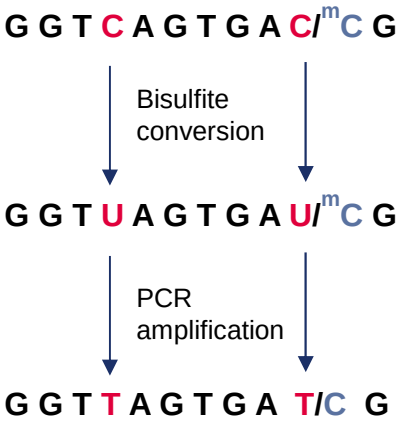
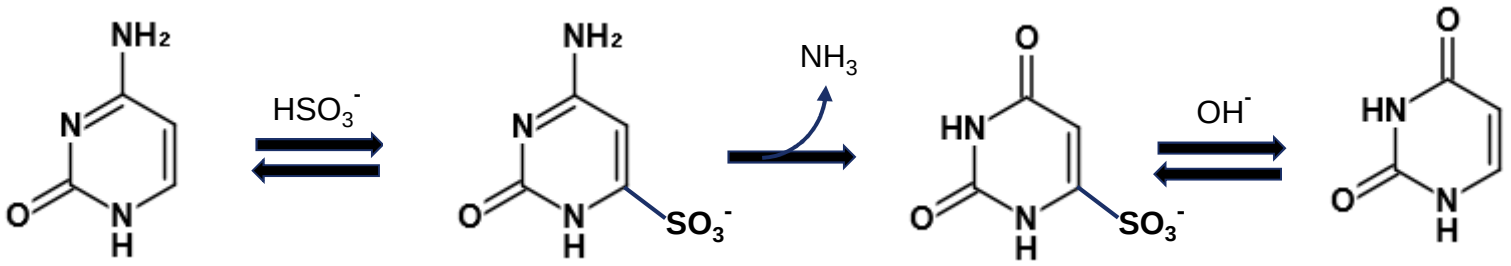




C U

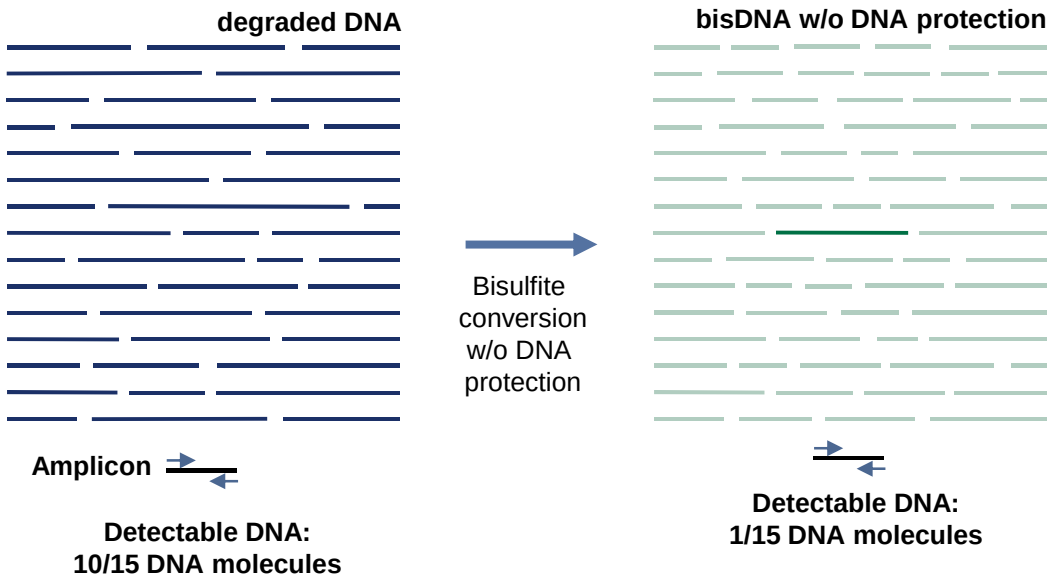
Reaction conditions

- Bisulfite addition is reversible; high salt, acidic (~ pH 5)
- Reaction depends on single stranded DNA
- Desulfonation under strong alkaline conditions
- Harsh reaction conditions (high temperature)

Workflow for epigenetic analysis – Bisulfite conversion

Reaction conditions during bisulfite conversion facilitate DNA degradation

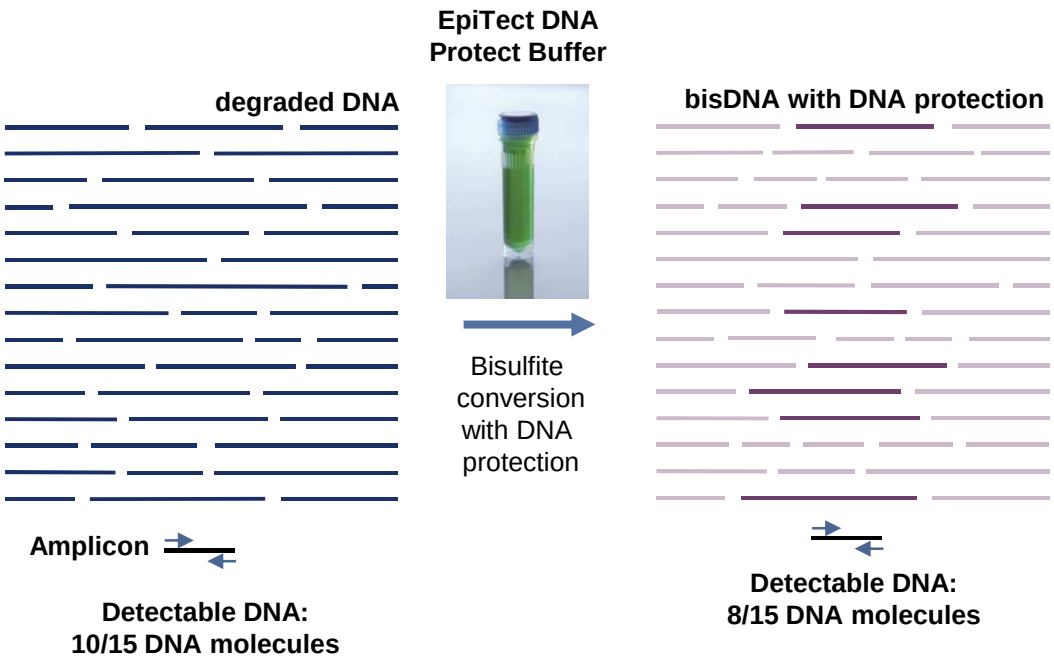
- Results in small molecular bisulfite converted DNA
- Might not be suitable for downstream analyses



Workflow for epigenetic analysis – Bisulfite conversion

Protection of fragmentation during bisulfite conversion

- Results in high molecular bisulfite converted DNA
- Facilitates multiple analyses, even from degraded DNA



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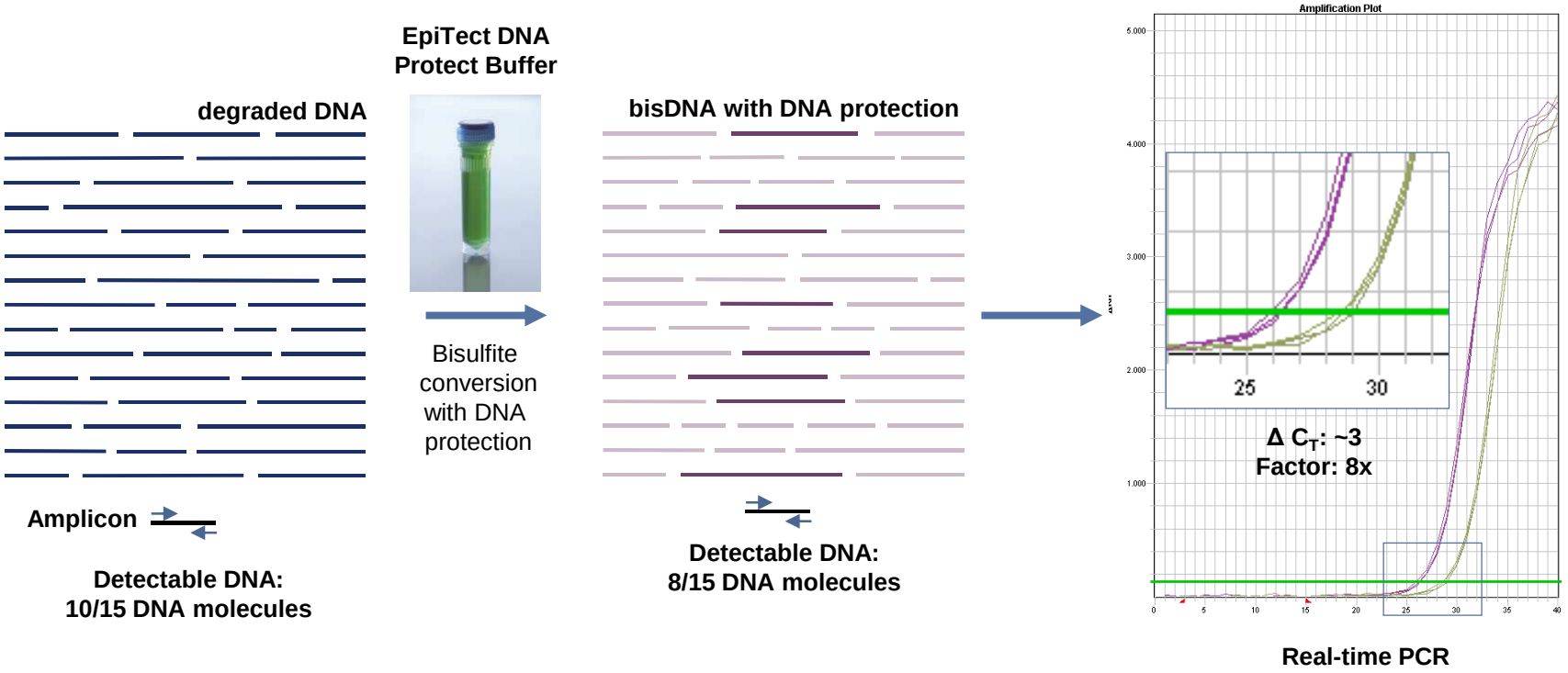

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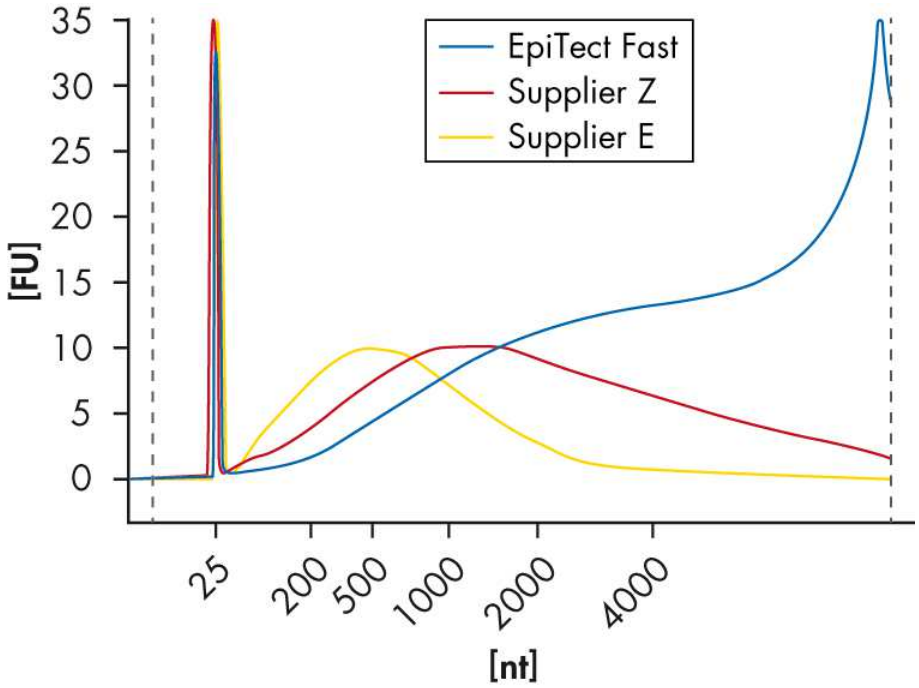


Analysis of DNA fragment sizes after bisulfite conversion. Following bisulfite conversion with the EpiTect Fast DNA Bisulfite Kit, 1 µg of genomic DNA showed the lowest level of fragmentation, with a size distribution ranging from several hundred base pairs to over 6 kb, compared to other methods with peaks at 500 bp and 1 kb, respectively.

Workflow for epigenetic analysis – Bisulfite conversion

Protection of fragmentation during bisulfite conversion

- Results in high molecular bisulfite converted DNA
- Facilitates multiple analyses, even from degraded DNA
- Two protocols available
  - 1 ng–2 µg DNA in a volume of up to 20 µl
  - 1–500 ng in a maximum volume of 40 µl

EpiTect Fast Bisulfite protocol have shown to work from as little as 10 cells (as little as 60 pg of DNA).

Workflow for genetic and epigenetic analysis


EZ1


QIAcube


Rotor-Gene Q


QIAcube

Sample

DNA purification

Quantification

Bisulfite conversion

For epigentics only

Sanger

NGS

Pyro-sequencing

Real time-PCR

Arrays

- QIAamp Kits
- EZ1 DNA Investigator Kit

- Investigator Quantiplex Pro RGQ Kit

- EpiTect Fast DNA Kits


Quantiplex Pro RGQ


EpiTect Fast DNA

2-3 hrs

Pyrosequencing solutions for forensic applications


EZ1


QIAcube


Rotor-Gene Q


QIAcube


QIAxcel Adv.


PyroMark Q48 Autoprep

Sample

DNA purification

Quantification

Bisulfite conversion

For epigentics only

Pre-amplification

QC

Pyro-sequencing

- QIAamp Kits
- EZ1 DNA Investigator Kit

- Investigator Quantiplex Pro RGQ Kit

- EpiTect Fast DNA Kits

- PyroMark PCR Kit

- PyroMark Q48 Autoprep
- PyroMark Q48 Advanced Reagents


Quantiplex Pro RGQ


EpiTect Fast DNA

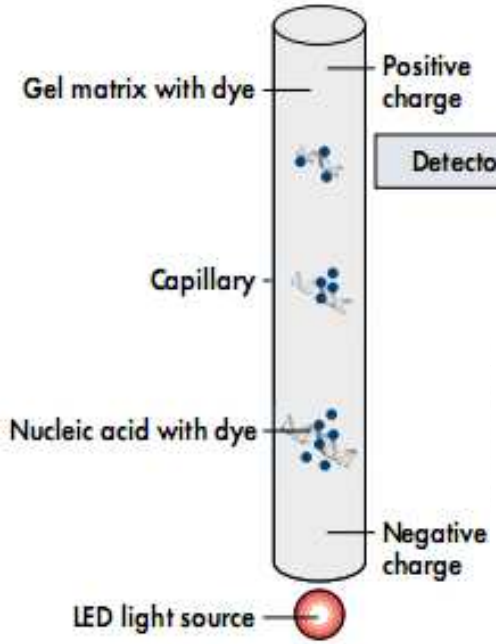

PyroMark PCR Kit


PyroMark Q48 Advanced Kit

2-3 hrs

0,5 days

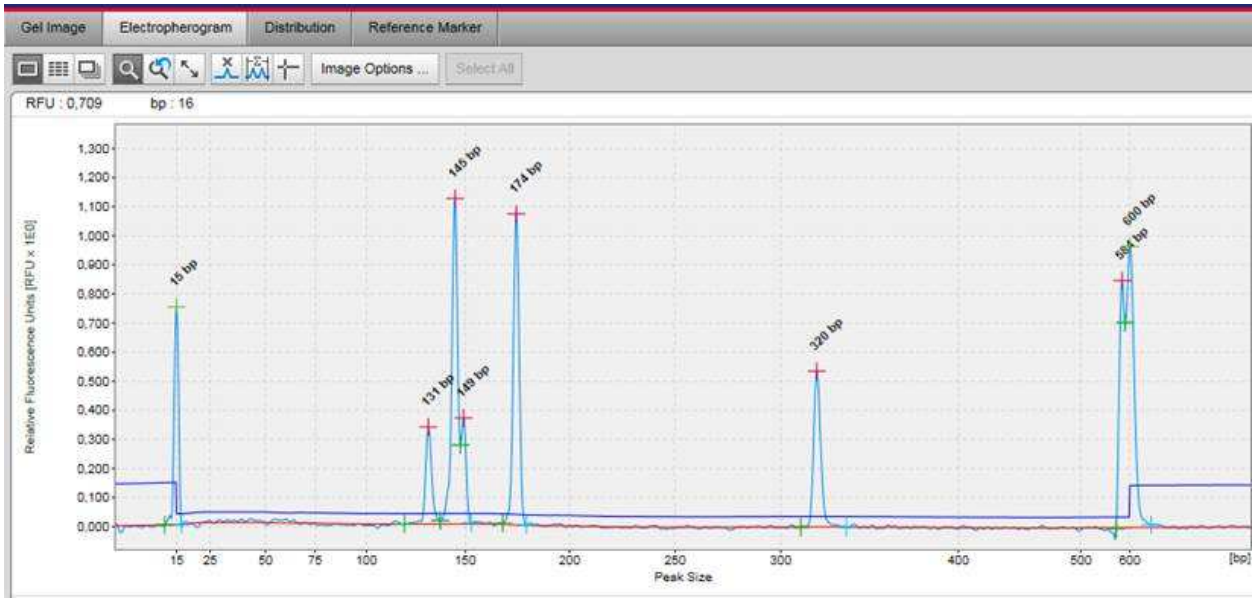
The QIAxcel Advanced capillary electrophoresis principle



A1: Human sample 1
A2: Human sample 2
A3: Human sample 3
A4: NTC
A5: EpiTect unmethylated control DNA
A6: EpiTect methylated control DNA
A9: Alignment marker

The QIAxcel Advanced capillary electrophoresis principle

ScreenGel Analysis of a QIAxcel Advanced run using products from PyroMark PCR kit with AgePlex PCR primers mix



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For epigentics only

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Pyro-sequencing

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- PyroMark PCR Kit

- PyroMark Q48 Autoprep
- PyroMark Q48 Advanced Reagents


Quantiplex Pro RGQ


EpiTect Fast DNA


PyroMark PCR Kit


PyroMark Q48 Advanced Kit

2-3 hrs

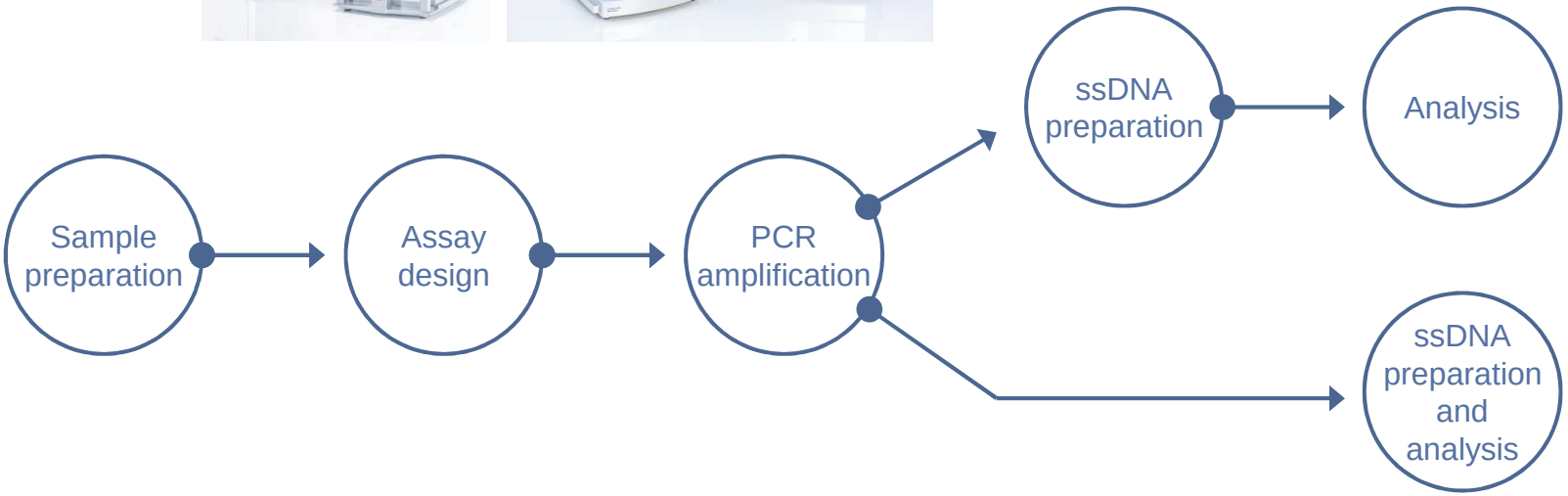
0,5 days

PyroMark Q48 Autoprep – Workflow

Workflow comparison of available Pyrosequencing platforms

- PyroMark Q24 Vacuum Workstation
- PyroMark Q96 Vacuum Workstation

- PyroMark Q24
- PyroMark Q24 Adv.
- PyroMark Q96 ID

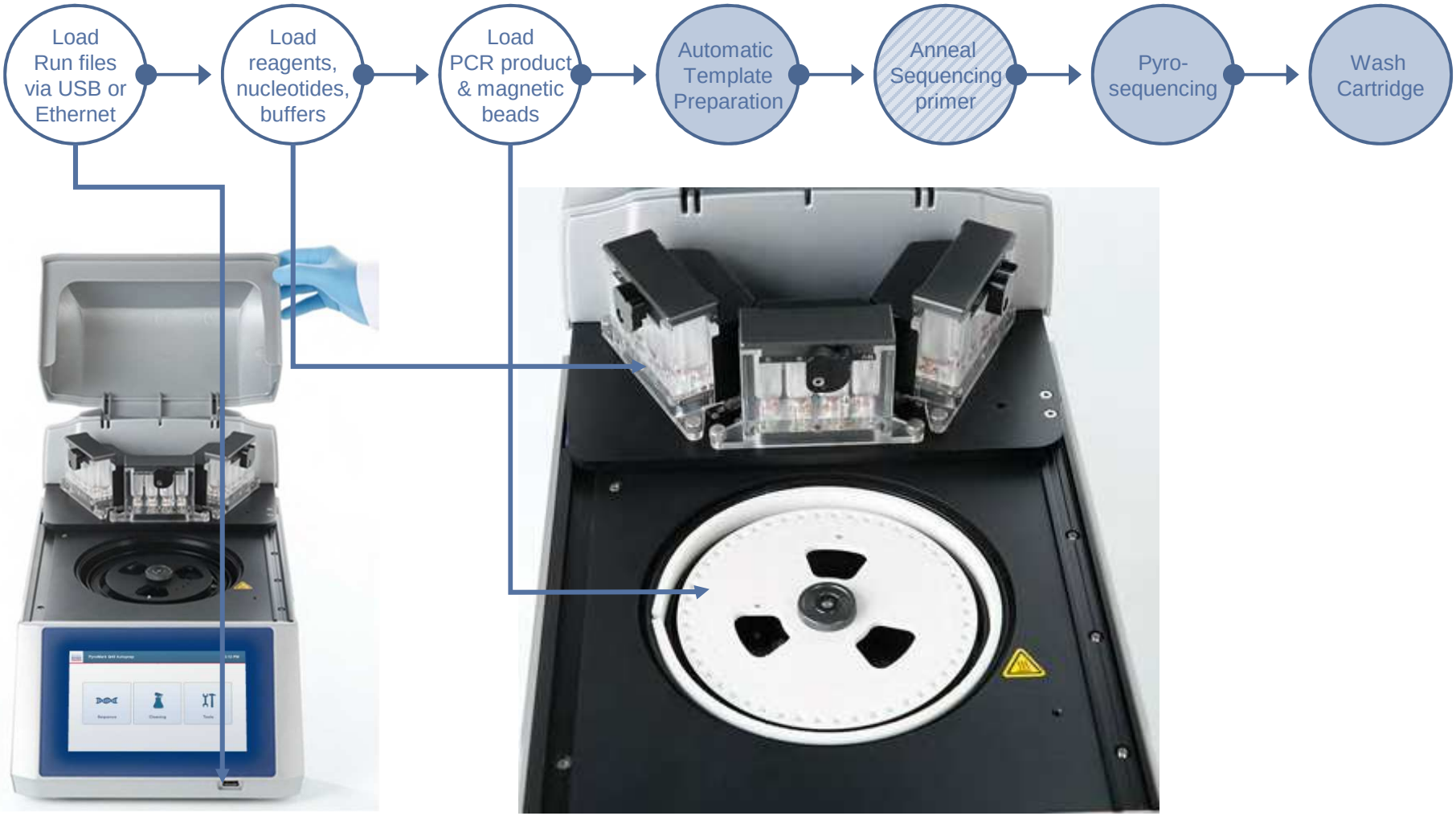


- PyroMark Q48 Autoprep[NEW]

PyroMark Q48 Autoprep:
Simplified workflow combined with advanced Pyrosequencing

PyroMark Q48 Autoprep – Protocol

Automatic template preparation fully integrated in PyroMark Q48 Autoprep workflow



Pyrosequencing solutions for forensic applications


EZ1


QIAcube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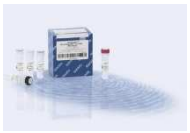
Quantiplex Pro RGQ


EpiTect Fast DNA


PyroMark PCR Kit


PyroMark Q48 Advanced Kit

2-3 hrs

0,5 days

QIAGEN's workflow for forensic methylation analysis in a single day!