QIAGEN

Sample & Assay Technologies

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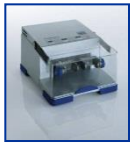
(QIAGEN-CEPHIED)

Covering Every Step
Automated Workflows From Sample To Result

Disruption | Purification | Assay Setup | Detection

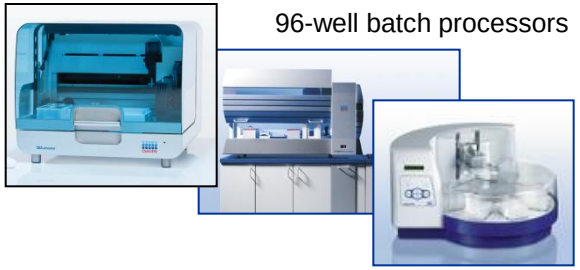


High-throughput

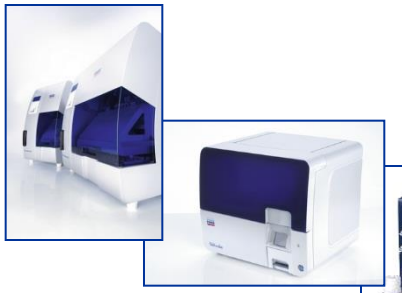
Medium-throughput

Low-throughput

96-well batch processors



Flexible sample numbers

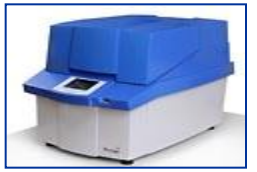


Low throughput processors

Modular assay setup

Integrated assay setup

Real time PCR



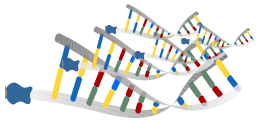
Sequencing

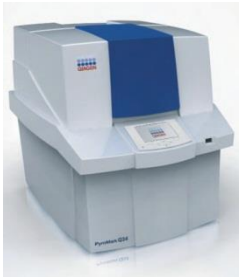
La technologie Pyrosequencing®

- Séquençage par Synthèse*
- Séquence éditée en temps réel
- Simple & robuste
- Pas de gel ni marquage
- Une technologie “non destructive”
- Flexible:
  - débit
  - design d’un essai
  - applications

* Ronaghi M., Uhlén M., Nyrén P. (1998) Real-Time Pyrophosphate Detection for DNA Sequencing. *Science* **281**:363-365

The Principle of Pyrosequencing Technology
Workflow





~ 2h

~ 15 min

~ 10-120 min

Assay Design → **PCR** → **Sample prep** → **Pyrosequencing**

Easily create your own PCR Primer and Sequencing Primer or use pre-designed assays, e.g. PyroMark CpG Assays

Region of interest amplified with a biotinylated primer (~70-500 bp)

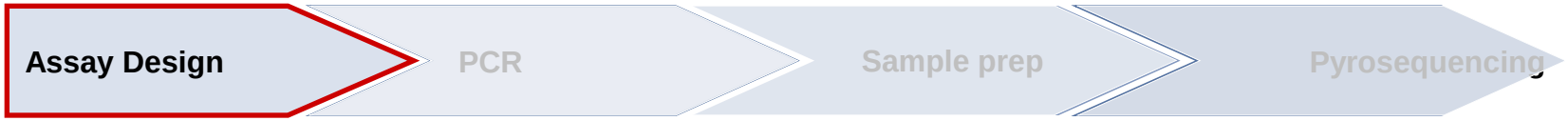
Separation to single-stranded DNA using streptavidin-coated beads.

Annealing of sequencing primer.

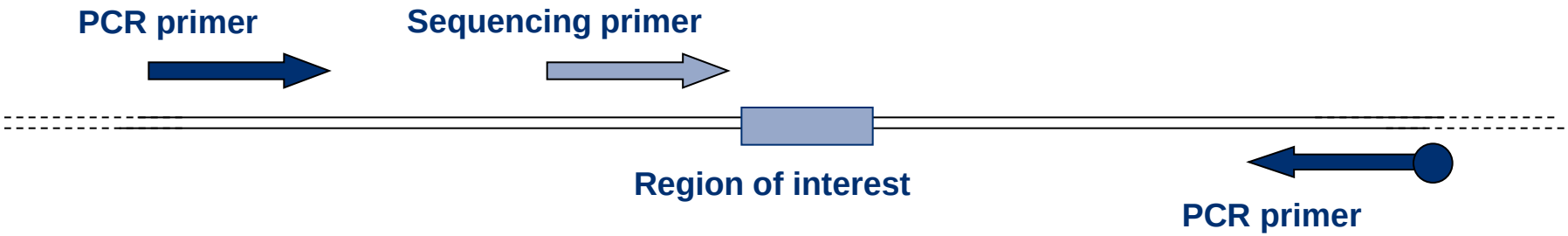
Sequencing-by-synthesis. Sequence data generated from the first base next to the sequencing primer.

Sequence context serves as built in control

The Principle of Pyrosequencing Technology
Assay Design & Ordering

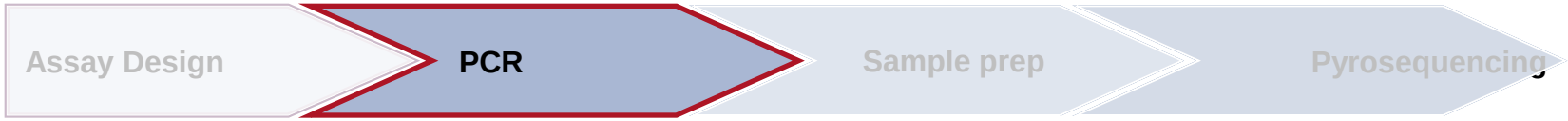


- Customer Assay Design
  - PyroMark Assay Design Software 2.0
  - PyroMark Assay Database*
  - PyroMark Custom Assays[NEW]
- Pre-designed Assays
  - PyroMark CpG Assays[NEW] (genomewide coverage of CpG islands)
  - PyroMark RUO Test (selected CpG or mutation targets)



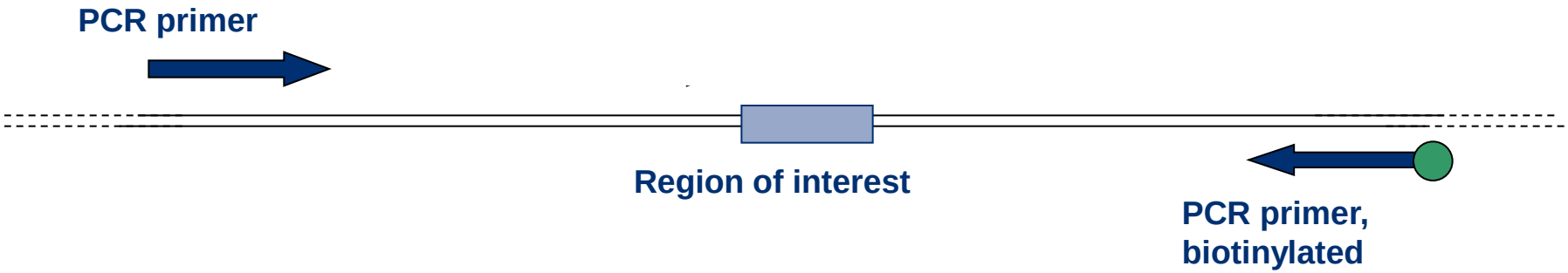
* Free Online Access for customers

Pyrosequencing Workflow
PCR



PCR / RT-PCR

- Can use any PCR machine
- PyroMark PCR Kit / PyroMark OneStep RT-PCR Kit
- Amplify relevant region by PCR (70 - 500 bp)
- Can use very short PCR products if desired (i.e. degraded DNA)
- One primer has to be biotinylated

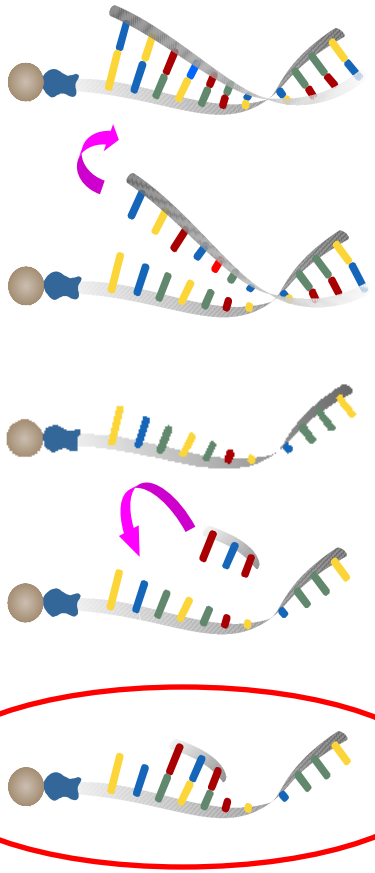


"If you can run a PCR, you can sequence with Pyrosequencing"
Jon Jonasson, University Hospital, Linköping, Sweden

Pyrosequencing Workflow
Sample preparation

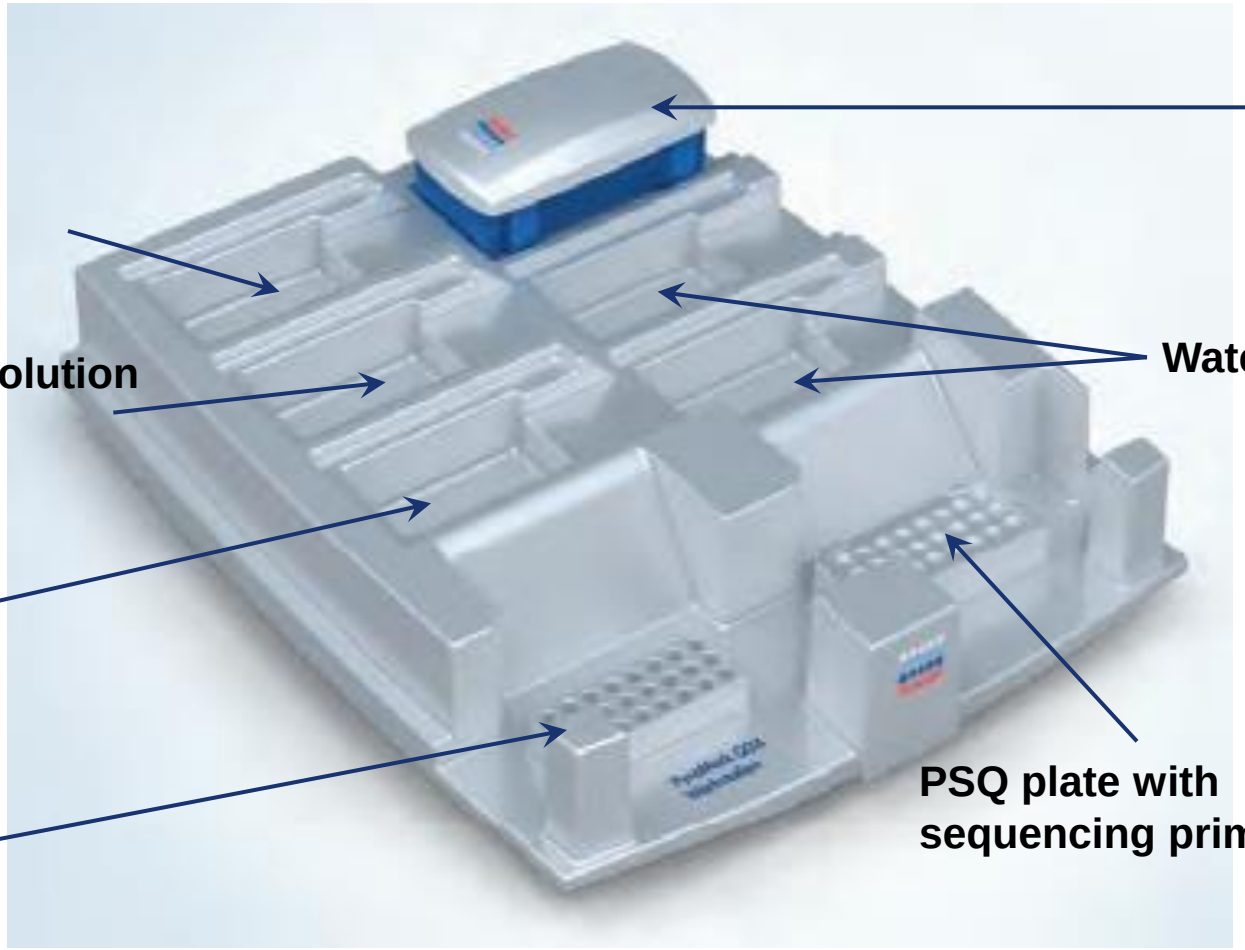
Assay Design → PCR → **Sample prep** → Pyrosequencing

- Immobilize biotinylated PCR products onto streptavidin coated beads
- Separate strands by denaturation in NaOH
- Wash /neutralize the immobilized strand
- Anneal sequencing primer



Single-stranded DNA with annealed sequencing primer as starting molecule for the Pyrosequencing reaction

Pyrosequencing Workflow
Sample Preparation

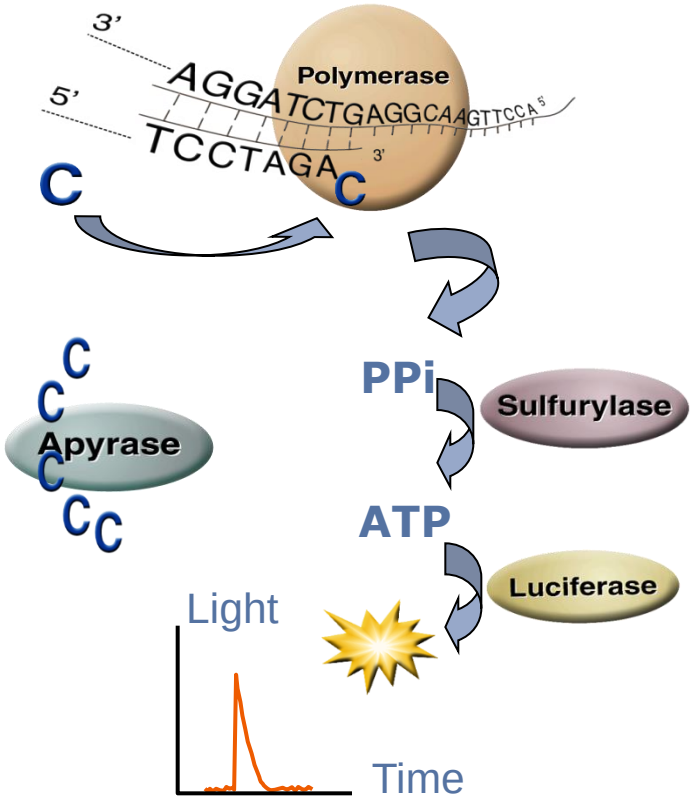


Pyrosequencing Workflow
The principle of the Pyrosequencing reaction

Assay Design → PCR → Sample prep → **Pyrosequencing**

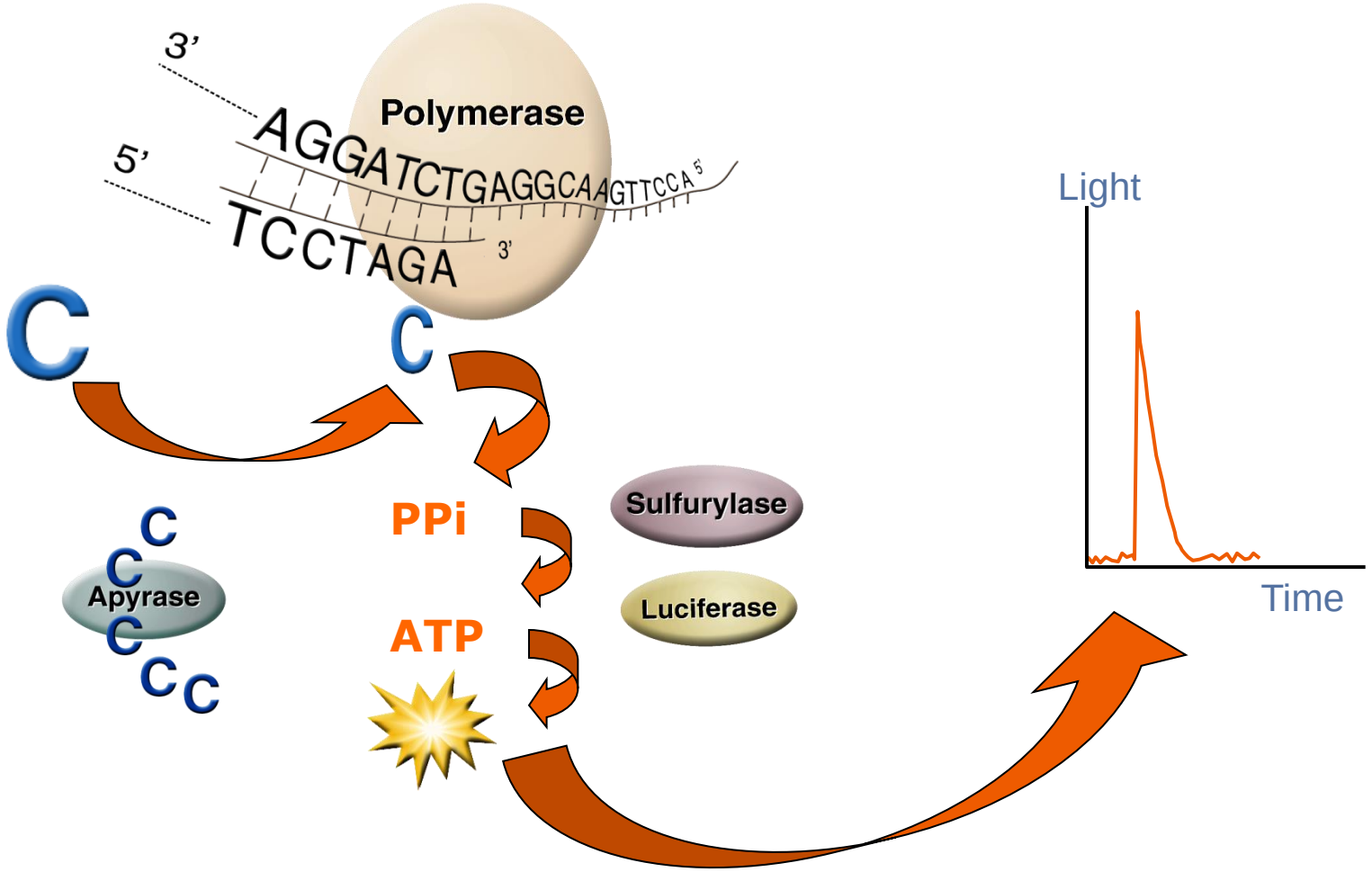
The Pyrosequencing reaction

- One nucleotide (dNTP) is added at a time
- Nucleotide incorporation generates Pyrophosphate (PP_i)
- Pyrophosphate (PP_i) is converted into light seen as peak in the Pyrogram trace
- Excess nucleotides are degraded before the addition of the next base



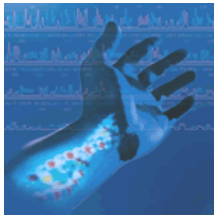
The amount of generated light is proportional to the amount of incorporated nucleotides.

La réaction de Pyrosequencing



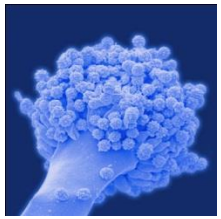
Pyrosequencing

Trois Applications du Pyrosequencing



Genetic Testing

Epigenetics



Microbial Analysis

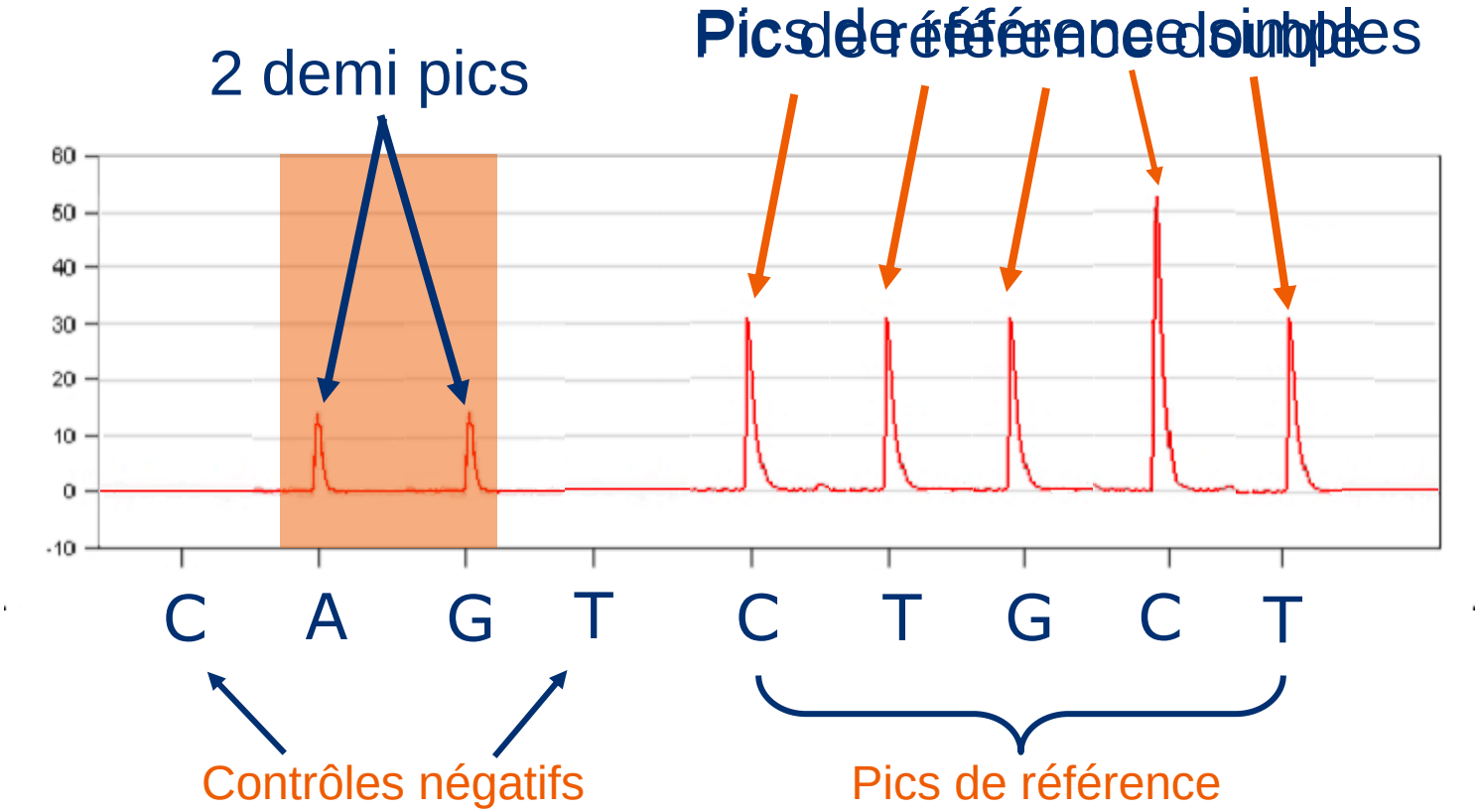
Genetic Testing

une plateforme pour des analyses génétiques multiples

- Analyse de mutation
  - positions connues et inconnues
    - mutations ponctuelles (AG/TC)
    - mutations multiples (AG/TCAG/TCAG/T/AC)
    - mutations di-, tri- and tetra alleliques (GA/C/G/TA)
    - Insertions/Deletions
- Analyse SNP
  - Di-, tri- and tetra allelic SNPs
  - Multiple SNPs
- Quantification d'allèle
  - frequence SNP
  - mutations di-, tri- and tetra allèliques

Séquence à analyser = a/gCTGCCT

Génotype = A/G Hétérozygote

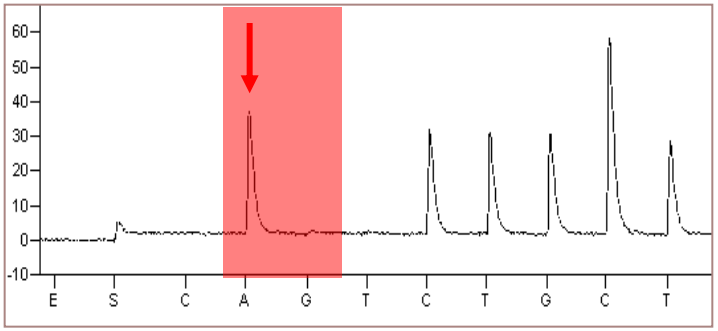


Analyse de SNP

Homozygote A

ACTGCCT
---------TGACGGA---

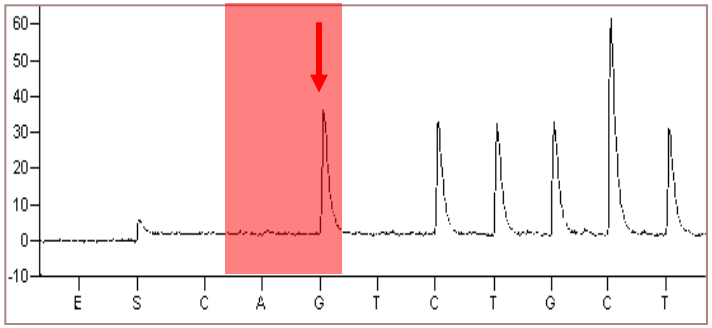
ACTGCCT
---------TGACGGA---



Homozygote G

GCTGCCT
---------CGACGGA---

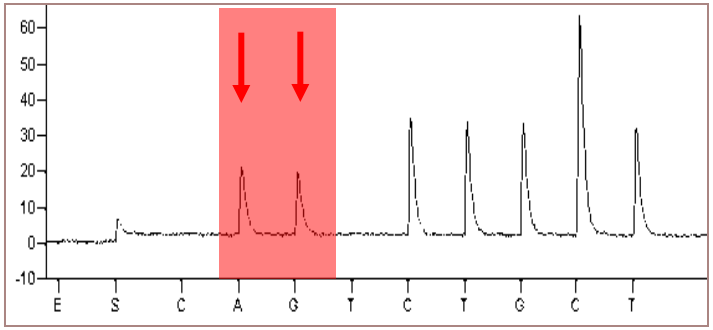
GCTGCCT
---------CGACGGA---



Hétérozygote A/G

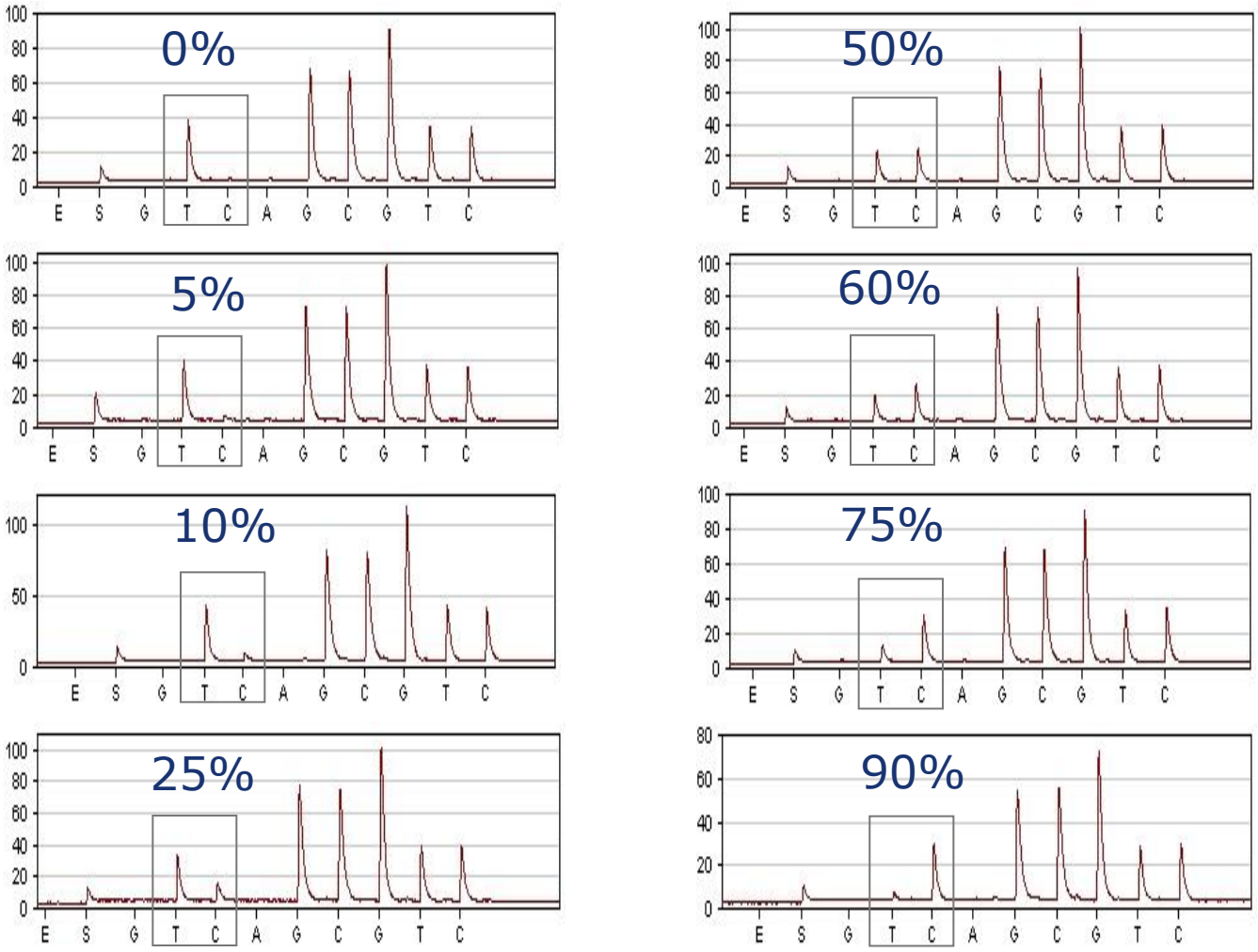
ACTGCCT
---------TGACGGA---

GCTGCCT
---------CGACGGA---



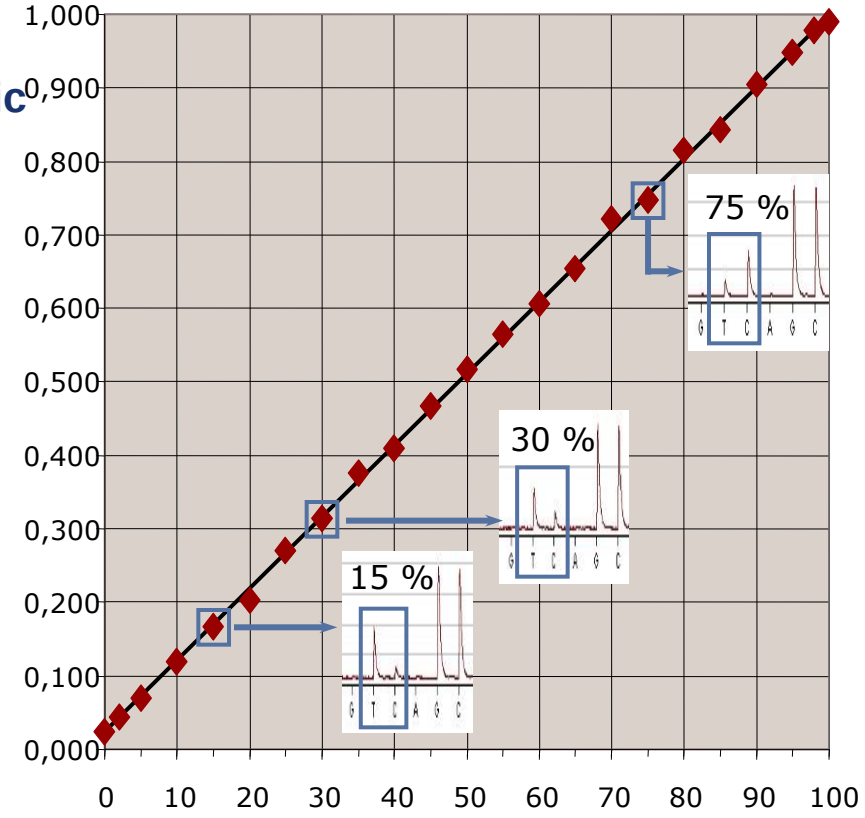
Une distinction claire des génotypes

Quantification



Corrélation quantification-hauteur de pic

Hauteur relative de pic



Fréquence allèlique (%)

Hauteur relative de pic =

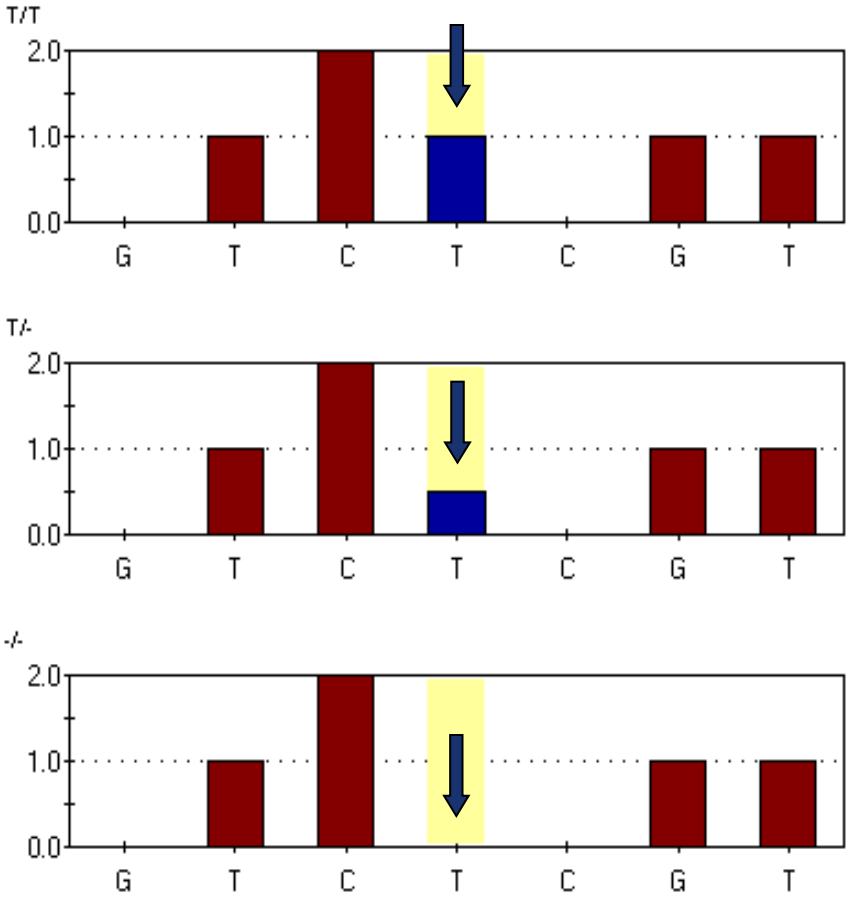
$$= \frac{\text{hauteur pic}_1}{\text{hauteur pic}_1 + \text{hauteur pic}_2}$$

Fréquence allèlique
=
hauteur relative de pic x 100 (%)

Insertion/Deletion – une base délétée

Séquence

TCC[T]GTG



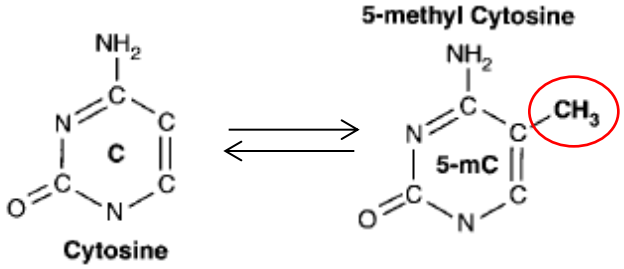
T/T

T/-

-/-

méthylation de l'ADN

- Addition d'un groupe méthyl sur les Cytosines des dinucléotides CpG
- CpGs répartis au niveau d'ilôts CpG
- Les gènes méthylés sont silencieux



Îlots CpG

- Les Îlots CpG définis par la fréquence des dinucléotides CG : 1 CpG pour 10 nucléotides
- Les Îlots CpG sont fréquemment associés aux gènes (régions amont 5')
  - Les Îlots CpG co-localisent avec 60% des promoteurs
  - 70-80% des Îlots CpGs sont méthylés chez l'Humain
  - La plupart des CpGs non méthylés sont localisés dans les Îlots CpG des éléments régulateurs

Lié au Cancer

- Hyperméthylation (↑^{m}C) résulte au "silencing" de gènes suppresseurs de tumeur
- Hypométhylation (↓^{m}C) active des oncogènes (e.g. K-ras, C-myc)

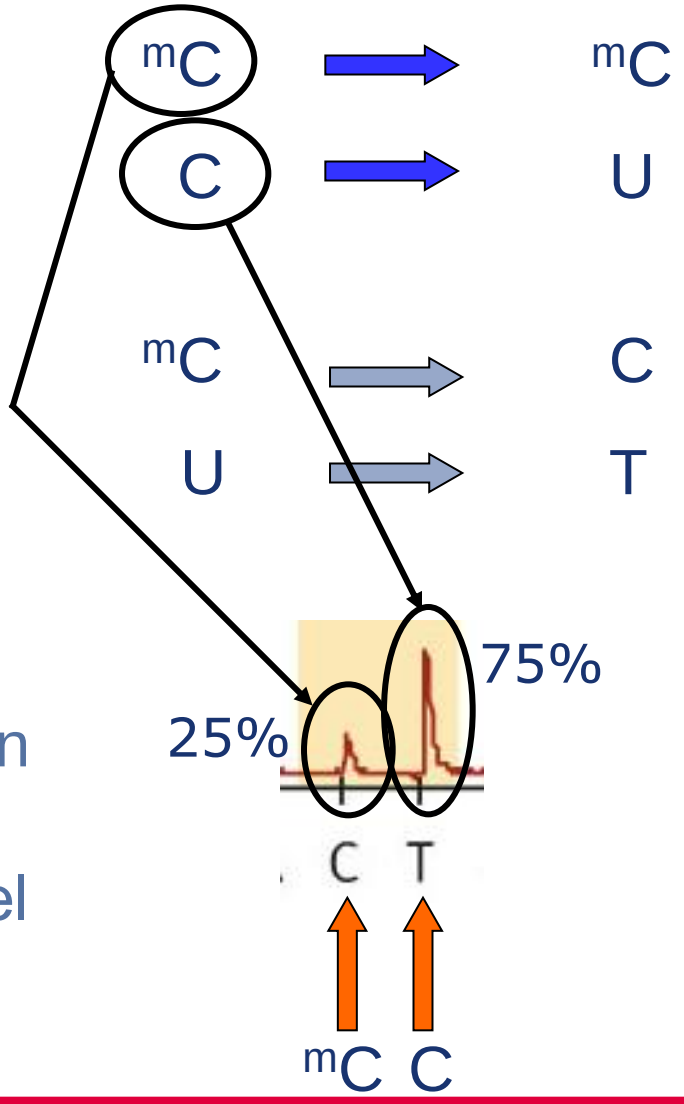
Du traitement bisulfite au pyrosequencing

1. Traitement au Bisulfite

2. Amplification PCR

3. Pyrosequencing

Le degré de methylation est analysé comme un ”SNP C/T” par le logiciel



Exemples d'Aanlyse de méthylation d'un Site CpG

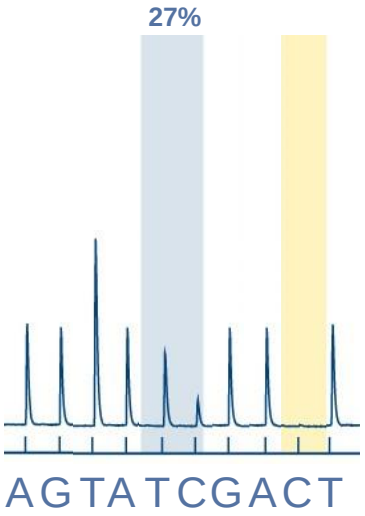
Sequence to be analyzed: **A G T T A C G A C**

A G T T A C^{m}G A C and **A G T T A C G A C**

After Bisulfite conversion: **A G T T A C G A T** and **A G T T A T G A T**

Analyzed sequence: **A G T T A C G A T**

CpG methylation level:



Nucleotides added:

Built-in Quality control: Successful Bisulfite conversion ✓

- Any C not followed by a G is always unmethylated and should be completely converted into T

Design d'un essai

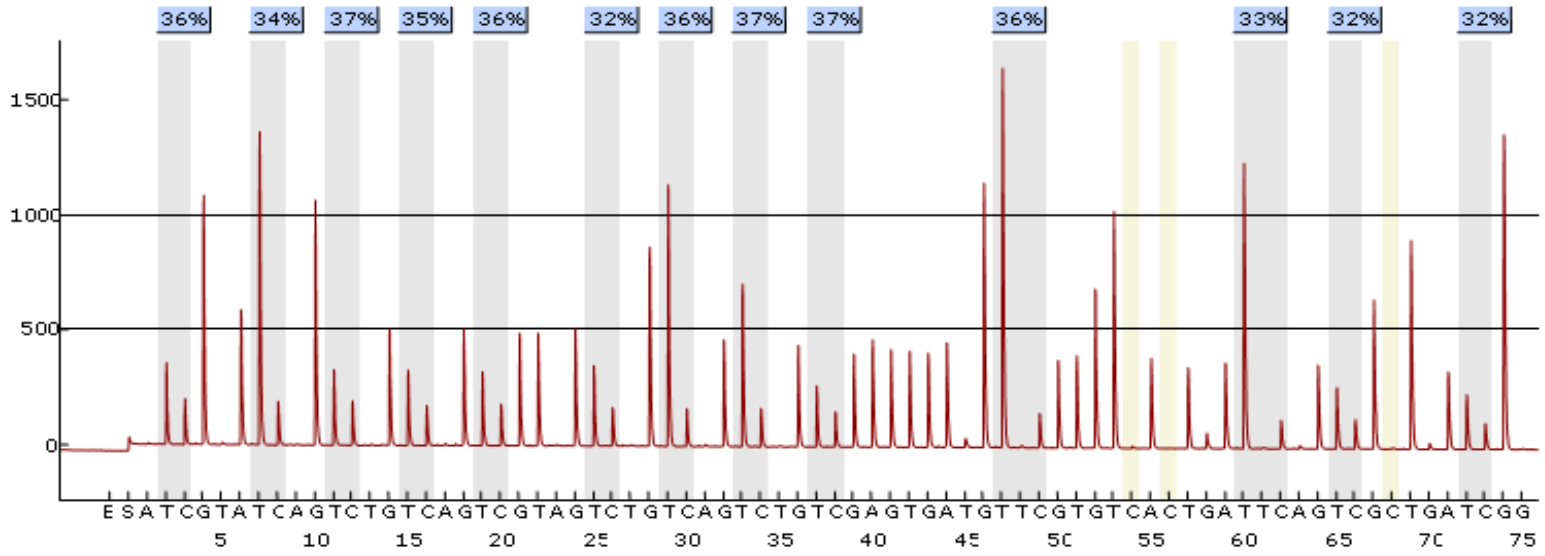
Îlot CpG, *gène p14*

- Tous les primers sont localisés dans des régions non variables, entre les sites CpG.
- Permet l'analyse de plusieurs sites CpG adjacents avec un seul primer de pyroséquençage.

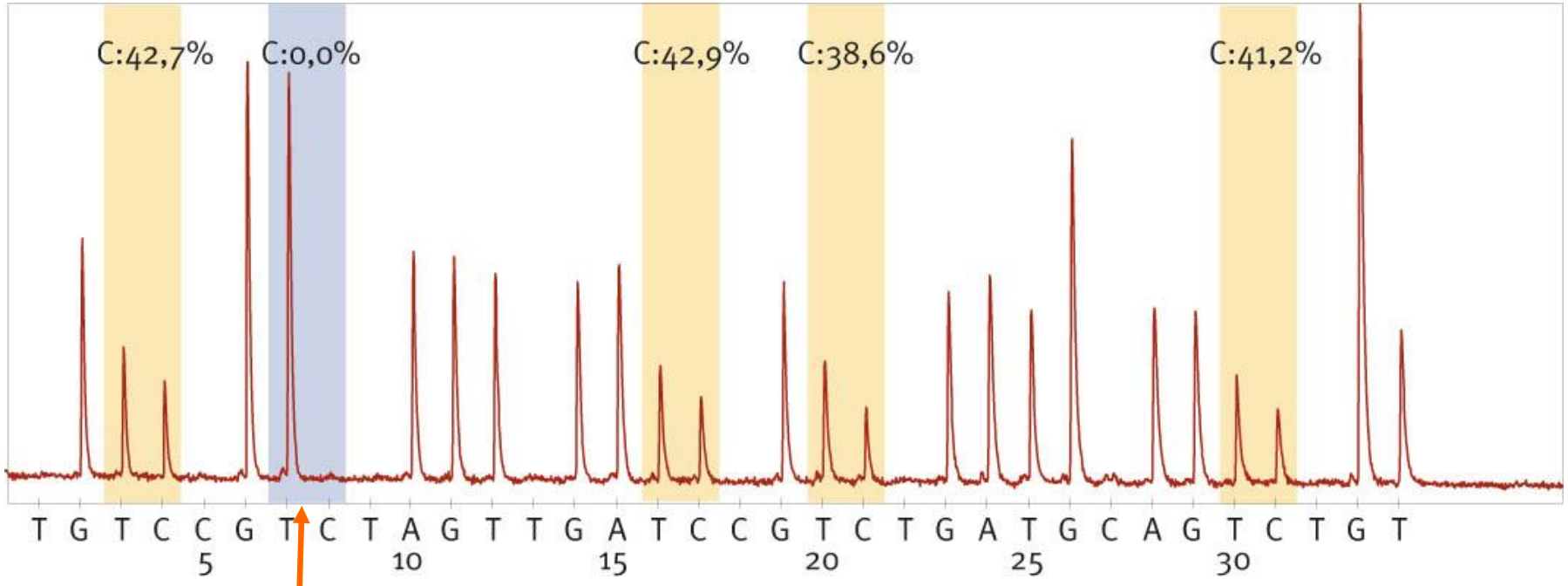
Détail de l’analyse de méthylation

Analyse automatique des résultats

- Taux de méthylation mesurés pour tous les sites CpG analysés
- Contrôle automatique de la qualité du traitement bisulfite



Contrôle qualité de l'action du traitement bisulfite



GCGGTCAGTGACGCGATGGAGCGGGC (Avant traitement bisulfite)

GYGGTTAGTGAYGYGATGGAGYGGGT (Séquence analysée)

N'importe quel C non suivi d'un G permet un contrôle qualité du traitement bisulfite

Pyrosequencing pour l'identification microbienne

Identification Microbienne

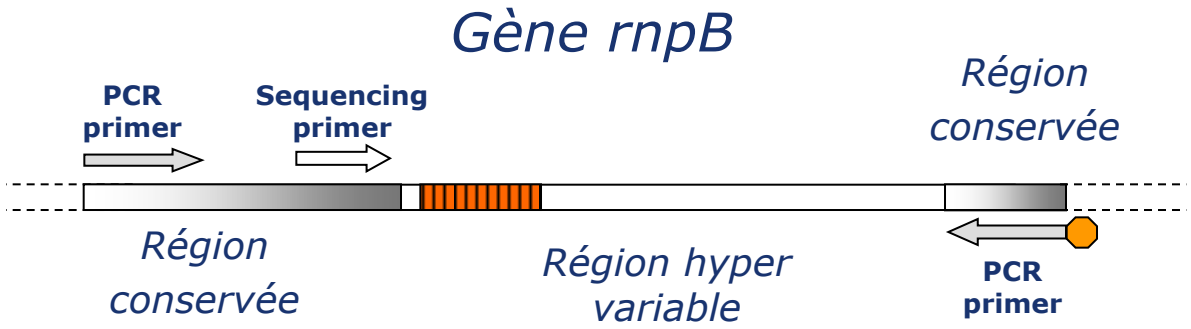
- Bacterie / Virus / Champignons
- Typage microbien

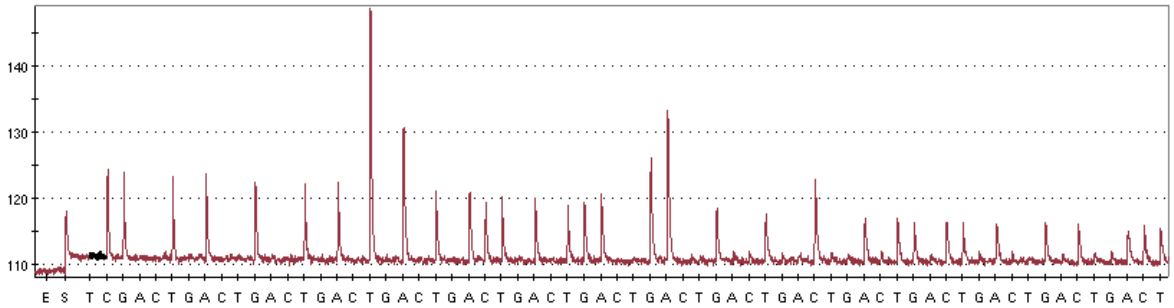
  Exemples
  - Général/universel: 16S bactérien,
  - Panel-based: meningitis, Candida, Gram-plus, Mycobacteria
  - Espèce-specifique: e.g. H. pylori, Bordetella parapertussis/pertussis; Listeria monocytogenes
  - Sous-type-specifique: e.g. B. anthracis, M. tuberculosis, N. gonorrhoeae

Typage de Resistance

Exemples
- mutation résistance au H3N2
- Suivi de résistance du H1N1
- Résistance Rifampicin
- Résistance Linezolid chez *Enterococci*

Identification microbienne- principe





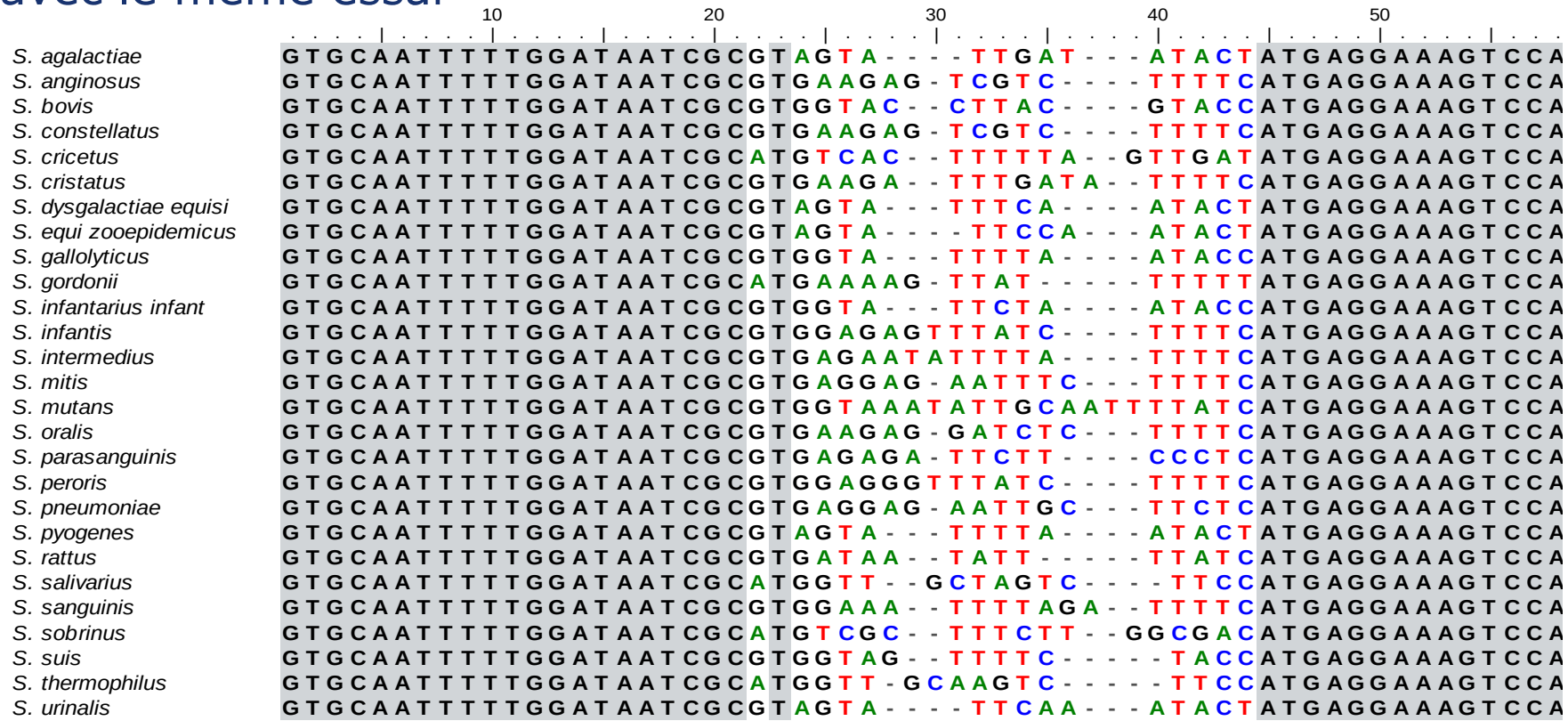
Le PyroMark™ ID génère une séquence en tps réel

Le logiciel IdentiFire™ permet d'interpréter la séquence "signature":

CGTAGTATTTTAATACTATGAGGAAA = ***Streptococcus pyogenes***

Identification avec un même set de primers

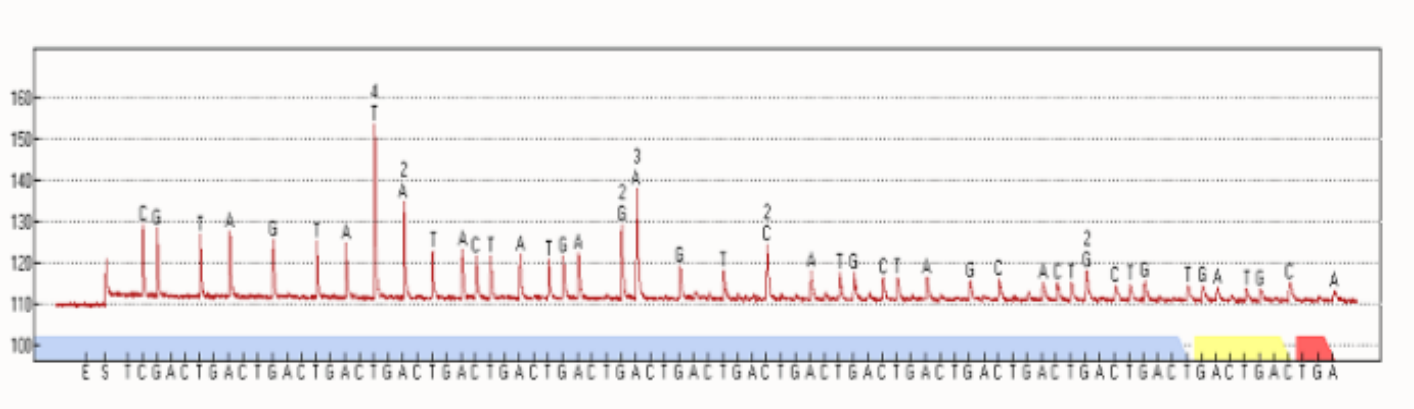
Exemple de *Streptococcus sp.*: plus de 25 espèces sont identifiées avec le même essai



Alignement de la région hyper variable P3 du gène *rnpB*.
Cette région peut être utilisée comme séquence signature des espèces à identifier.

Identification avec IdentiFire™

Result: Streptococcuss_pyogenes T_CCUG4207 **Score:** 100
Quality: Good



Hit 1: **Streptococcuss_pyogenes T_CCUG4207**

Score: 100
Identities: 52/52 (100%)
Gaps: 0/52 (0%)
E-value: 1.39e-047

```
Query    1  CGTAGTATTTTAATACTATCAGGAAAGTCCATGCTAGCACTGGCTGTGATGC  52
           ||||||||||||||||||||||||||||||||||||||||||||||||||||
Library  21 CGTAGTATTTTAATACTATCAGGAAAGTCCATGCTAGCACTGGCTGTGATGC  72
```

"Query" =

séquence obtenue par Pyrosequencing

ID: *Streptococcus pyogenes*