UNIVERSITÀ
DEGLI STUDI DI TRIESTE

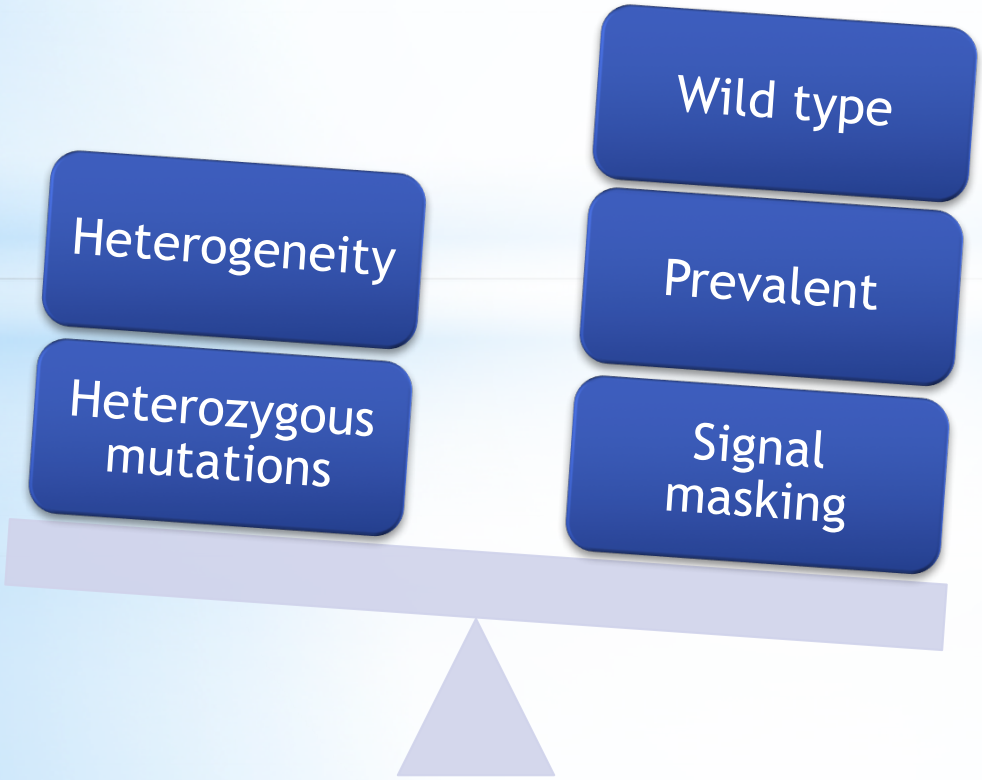
SEQUENCES DETECTION


NGS
MALDI
SANGER SEQ
DNA SEQUENCING FROM FFPE DNA
REAL TIME PCR
PIRO-SEQUENCING

* Background

Tumor tissue

Normal Tissue



!!! Microdissectio to decerase the background from normal tissue

✔ General principles of Sanger's Sequencing

🔍 In the sequencing reaction only one primer is used

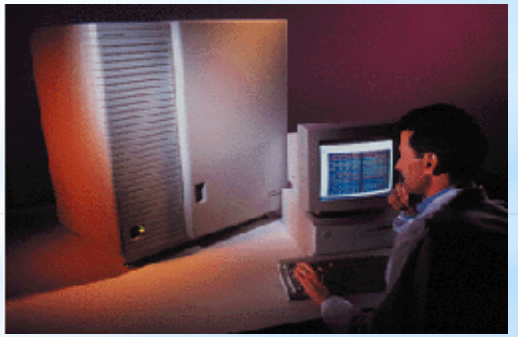
🔍 For each reaction the sequence of a single strand of DNA is obtained

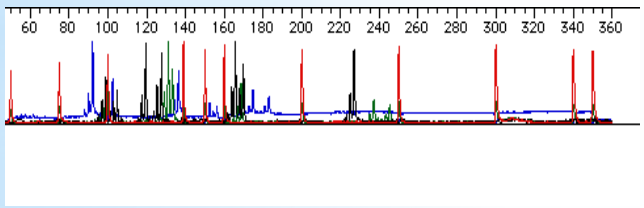
📖 To sequence a DNA fragment the **the flanking regions' sequences are needed**

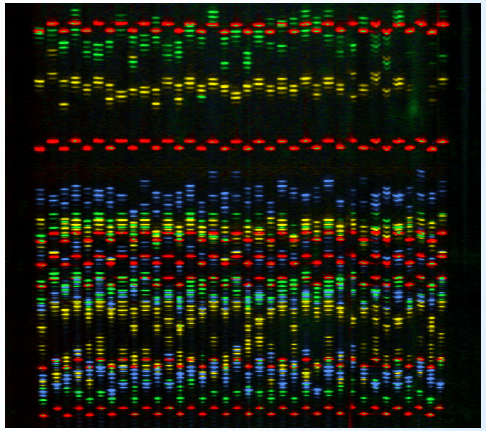
📝 **dNTP and ddNTP (the latter in small amount) are in competition for *incorporation into the growing strand*. This competition allows obtaining partial sequence reactions➔ sequencing**

Use of fluorescent ddNTP

The output is an electropherogram







Electrophoretic Output

✔ Pirosequencing

It is based on the detection of **pyrophosphate** released by the incorporation of a nucleotide during DNA synthesis

$$(DNA)n + dNTP \xrightarrow{\text{polymerase}} (DNA)n+1 + Ppi$$

$$ASP + PPi \xrightarrow{\text{ATP sulfurylase}} ATP + Si$$

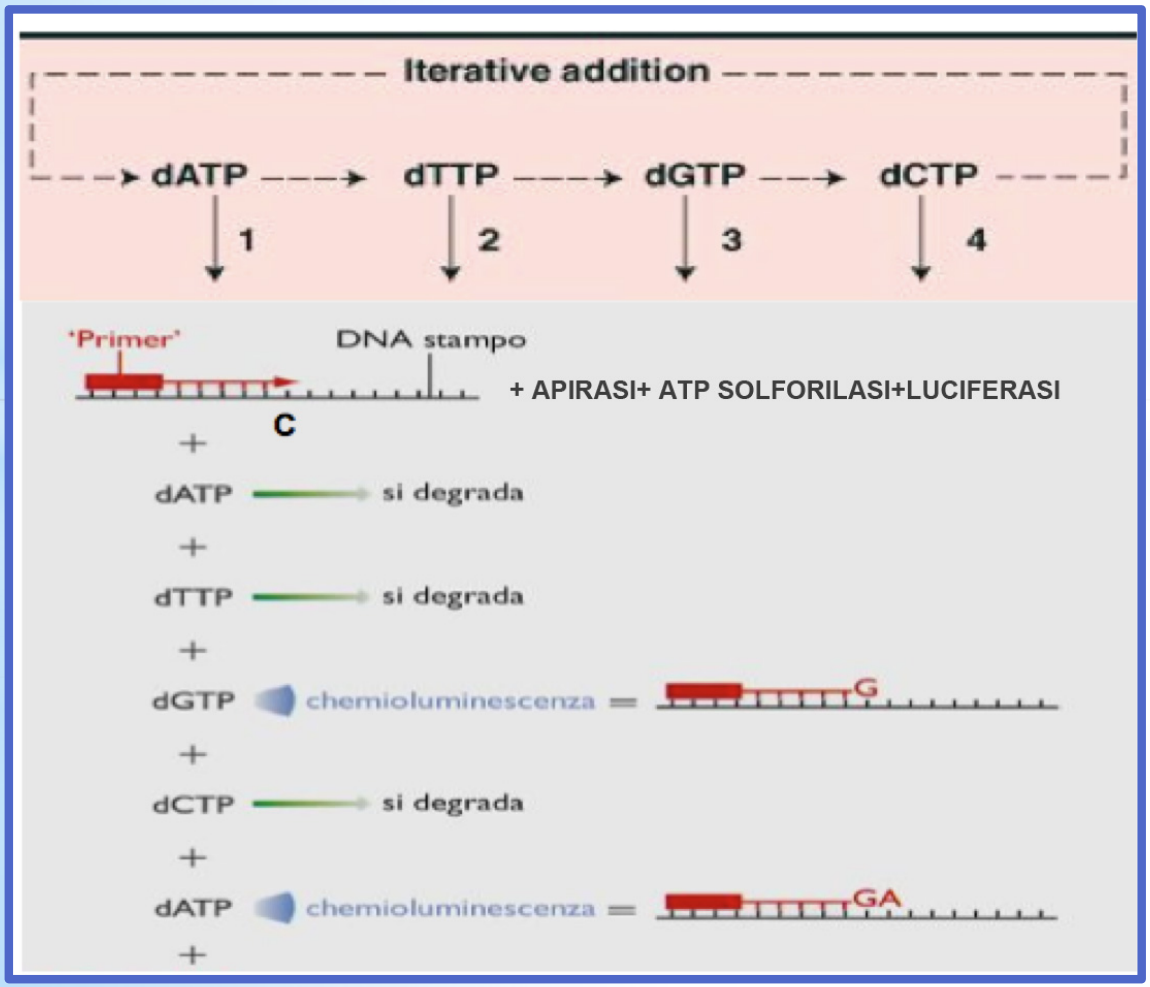
$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{light} + AMP + PPi + \text{oxyluciferin}$$

1. The sequence to be analyzed is amplified by PCR and it is denatured then it is incubated with DNA polymerase, ATP sulforylase, luciferase and apyrrase and to the substrates adenosine sulfophosphate (ASP) and luciferin.
2. DNA polymerase catalyzes the addition of this base only if it is complementary to the template residue. In this case there is concomitant release of inorganic pyrophosphate Ppi
3. Ppi is transformed into ATP by sulfurylase and using the ASP as a substrate. The ATP obtained allows the conversion of luciferin to oxyluciferin by the luciferase with the production of a light signal that is detected by a special photosensitive camera (CCD).
4. The enzyme apyrase degrades the unincorporated dNTPs and the ATP produced by sulfurylase.

✔ Pirosequenziamento

🔓 Only when the degradation is complete the second dNTP is added to continue the polymerization reaction (returning to step 1)

🔎 All 4 dNTP are added cyclically up to the sequence completion

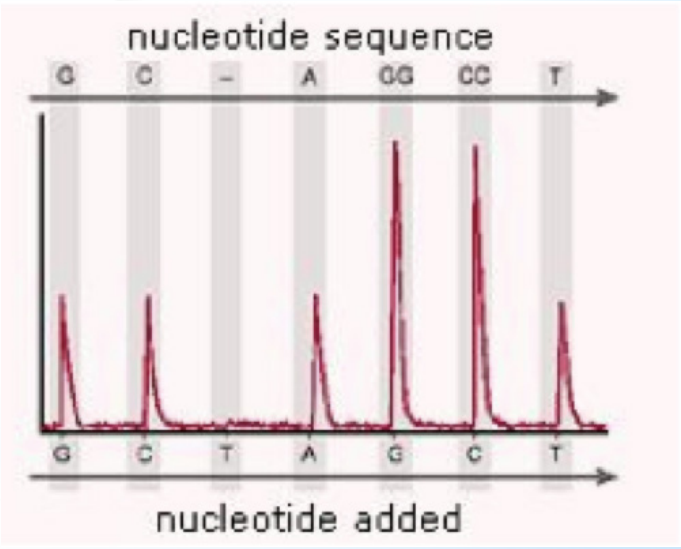


The light signal is recorded in a "**pirogram**". The signal is proportional to the produced ATP and therefore to the incorporated nucleotide.

ATP is not used as dNTP to avoid the signal confusion- whether the detected signal comes from a correct incorporation of the nucleotide or from the intrinsic activity of the ATP. Alternatively, **adenosine-thio-triphosphate** is used, which is recognized by DNA polymerase as being ATP, but not by luciferase.

✔ Pirosequencing

A peak of double intensity, for example, detects that in the same cycle 2 dNTPs (repetition of the same base on the template) were incorporated. Conversely, a null signal indicates that the dNTP added in that cycle is not complementary.

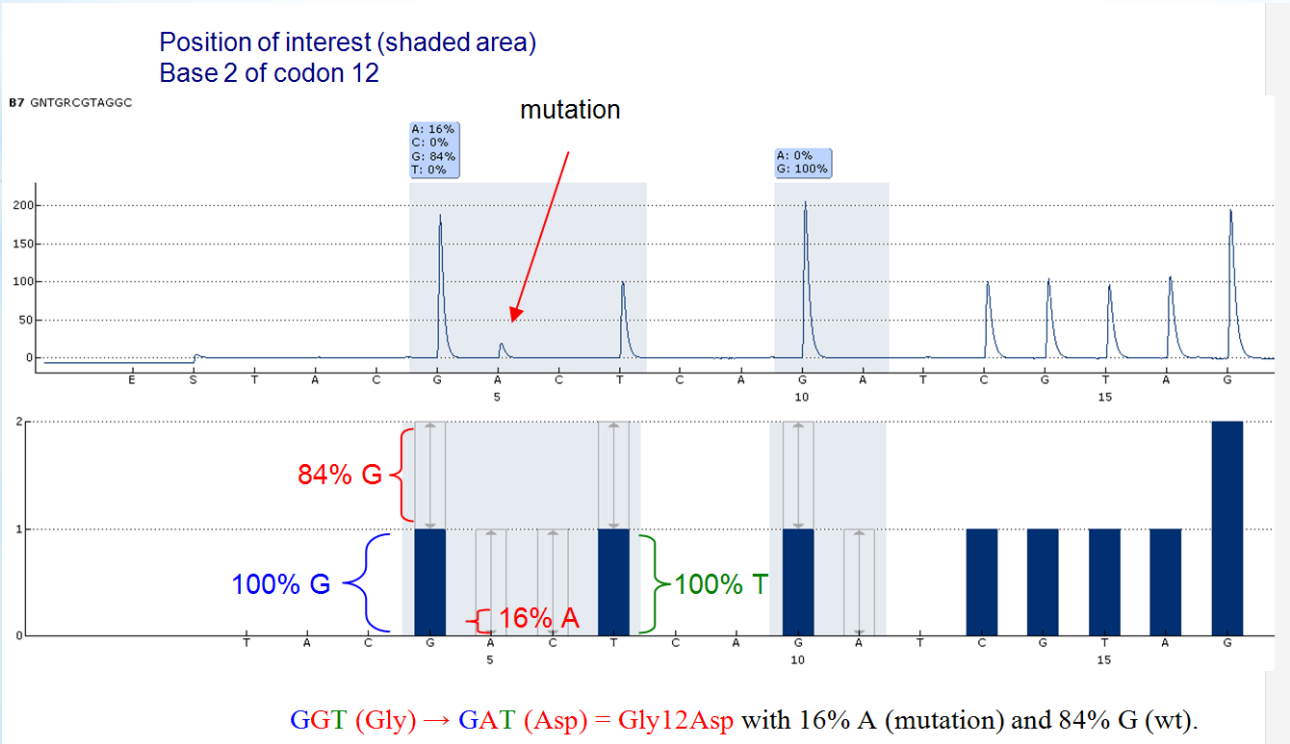


LIMIT: sequencing of relatively short fragments, max 800 bases, but 300 bases recommended.

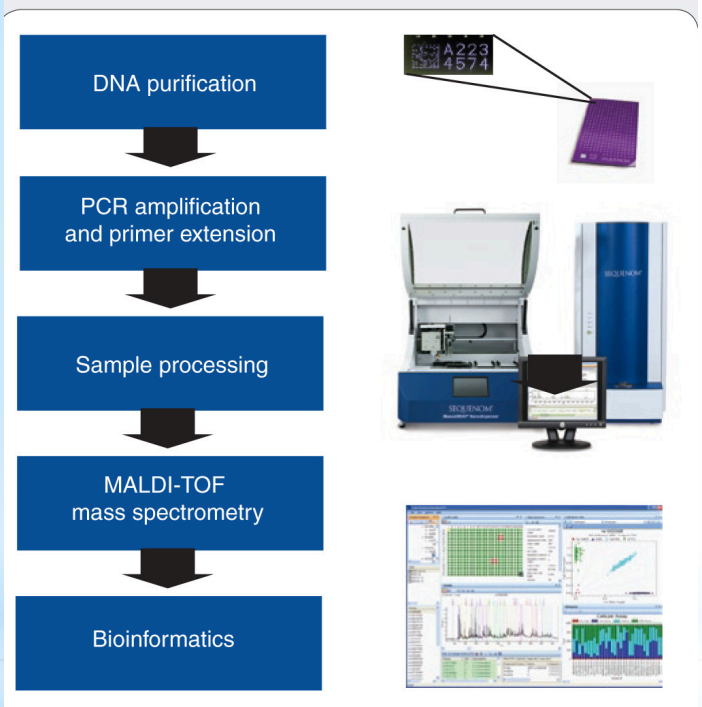
✔ Pirosequencing

* In practice in FFPE

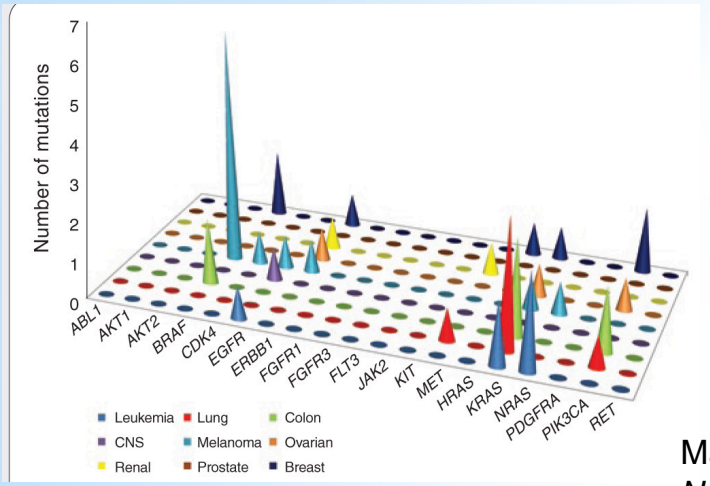
1. Amplification with biotinylated primers and detection of products on an agarose gel.
2. Immobilization of amplicons on sepharose beads
3. Preparation of the ssDNA and primer annealing for sequencing
4. Sample loading and running.

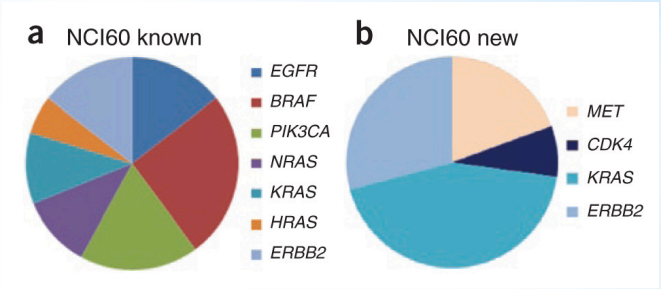


✔ MALDI (sequenom)



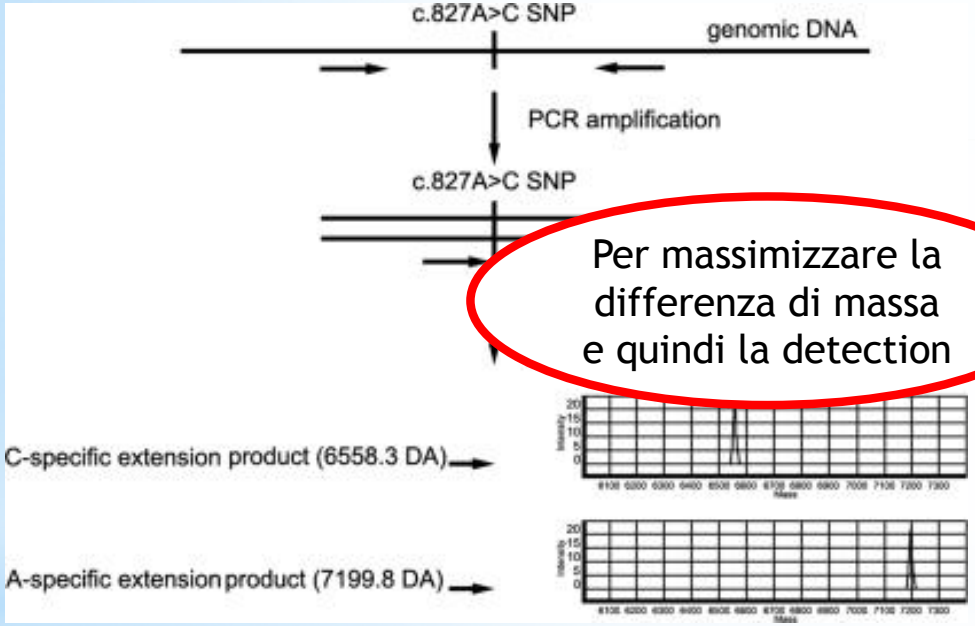
1. PCR amplification including the mutation site.
2. MassEXTEND reaction with “primer extension” adjacent to the site of interest
3. MALDI-TOF to detect differences in mass

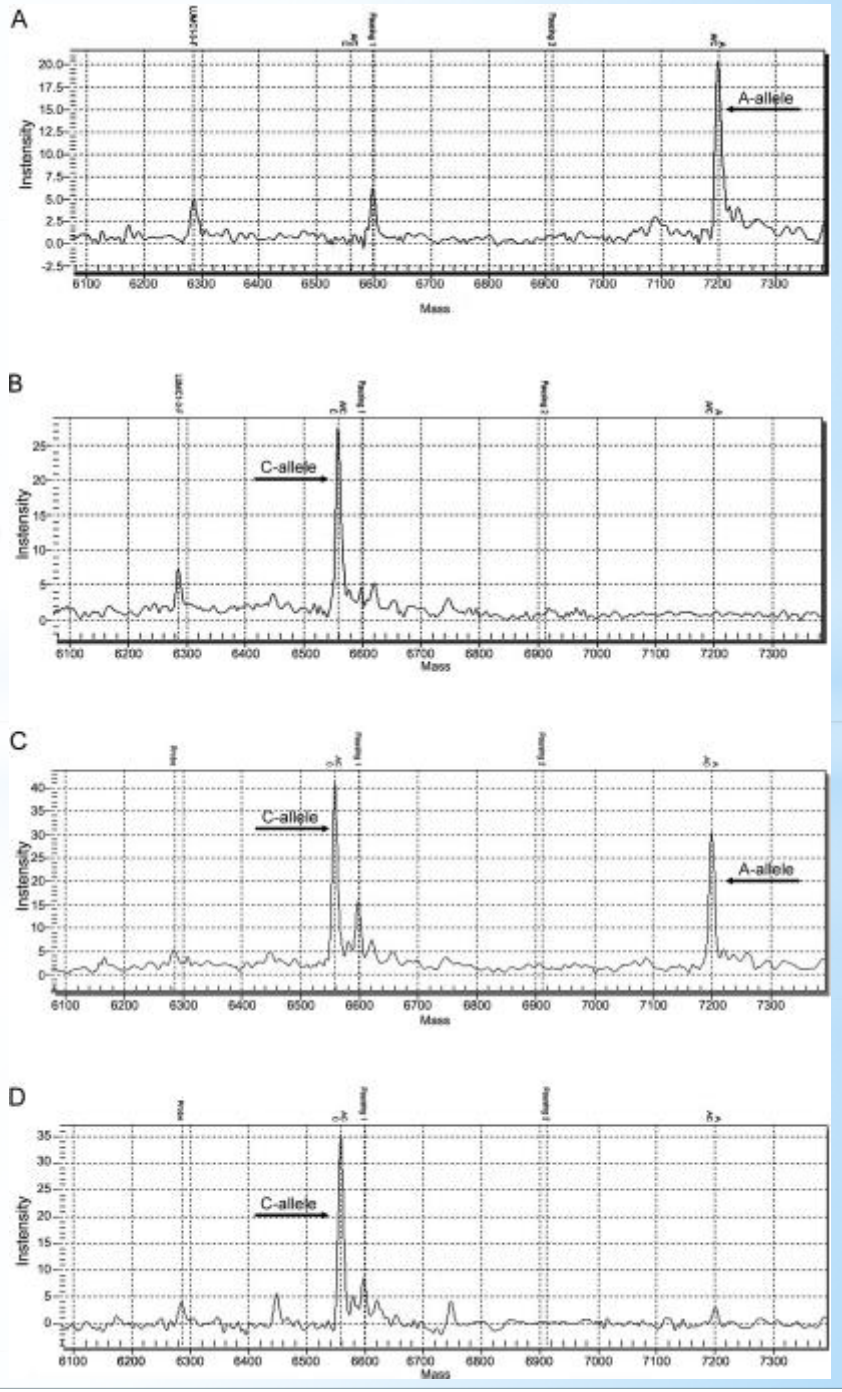




Marisa Pearce, Amy Cullinan, Grant Hogg, Dana Hosseini & Mathias Ehrich
Nature Methods 6, doi:10.1038/nmeth.f.254

✔ MALDI





Extension primer si adjacent to the SNP or mutation.
There are also visibles the extension products for the two SNP or mutated/ wild type
Real-time detection with dedicated software.

J Mol Diagn. 2005 November; 7(5): 623–630.

✔ Realtime PCR

Sequence detection

HRM-High resolution melting analysis

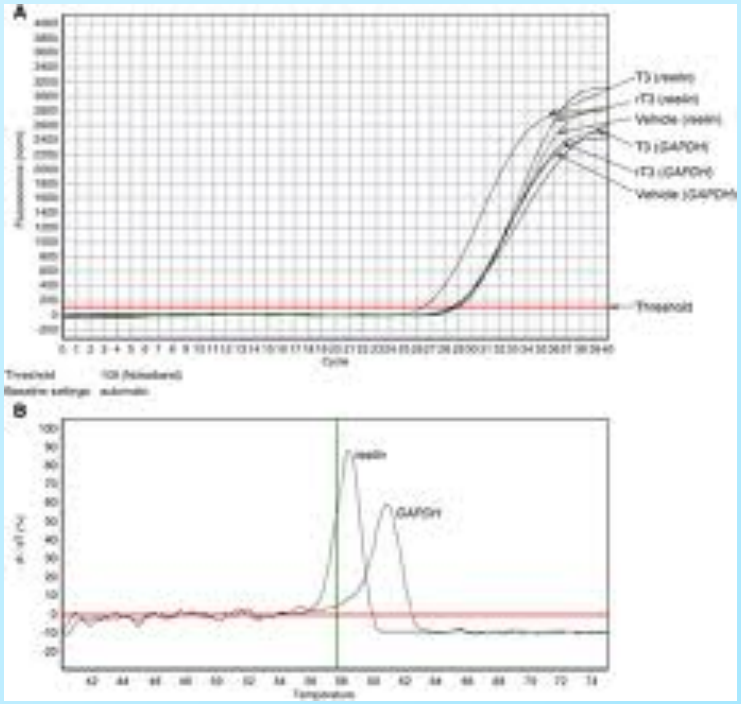
Real Time PCR using specific probes mutated/wild type

STRUMENTAZIONE

Thermocycler interfaced with a system capable of inducing and reading fluorescence. Fluorescence emission stimulation is induced by a laser or led. The detection of λ from 500 to 660 nm allows multiplex PCR analysis with the use of multiple fluorophores in a single tube.

+

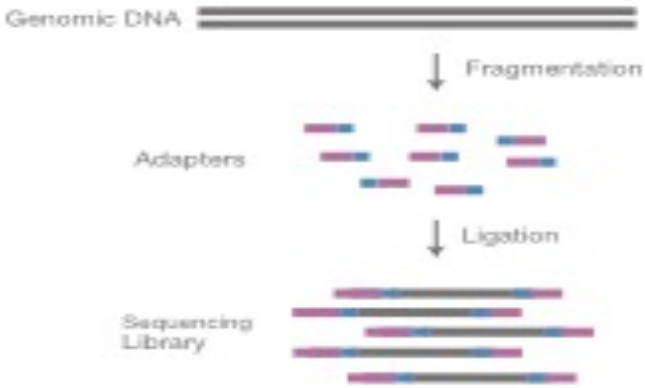
=



Akbani et al., 2015, Cell *161*, 1681–1696
June 18, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cell.2015.05.044

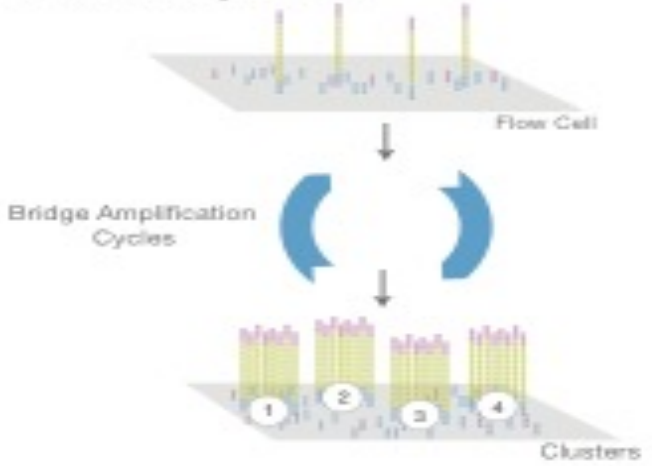
NGS

A. Library Preparation



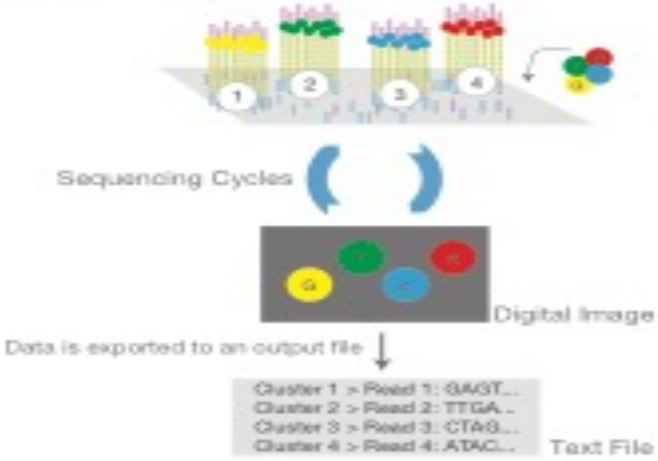
NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.

B. Cluster Amplification



Library is loaded into a flow cell and the fragments hybridize to the flow cell surface. Each bound fragment is amplified into a clonal cluster through bridge amplification.

C. Sequencing



Sequencing reagents, including fluorescently labeled nucleotides, are added and the first base is incorporated. The flow cell is imaged and the emission from each cluster is recorded. The emission wavelength and intensity are used to identify the base. This cycle is repeated "n" times to create a read length of "n" bases.

D. Alignment & Data Anaylsis

Reads are aligned to a reference sequence with bioinformatics software. After alignment, differences between the reference genome and the newly sequenced reads can be identified.

Sequencing Principles

- Sequencing by Synthesis
  - Sanger/Dideoxy chain termination (Life Technologies, Applied Biosystems)
  - Pyrosequencing (Roche/454)
  - Reversible terminator (Illumina)
  - Ion torrent (Life Technologies)
  - Zero Mode Waveguide (Pacific Biosciences) 3rd generation sequencing
- Sequencing by Oligo Ligation Detection
  - SOLiD (Applied Biosystems)
- Direct reading of DNA sequence
  - Nanopore sequencing 3rd generation sequencing
  - Electron microscope 3rd generation sequencing