Principal of DNA amplification and sequencing

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polymerase chain reaction

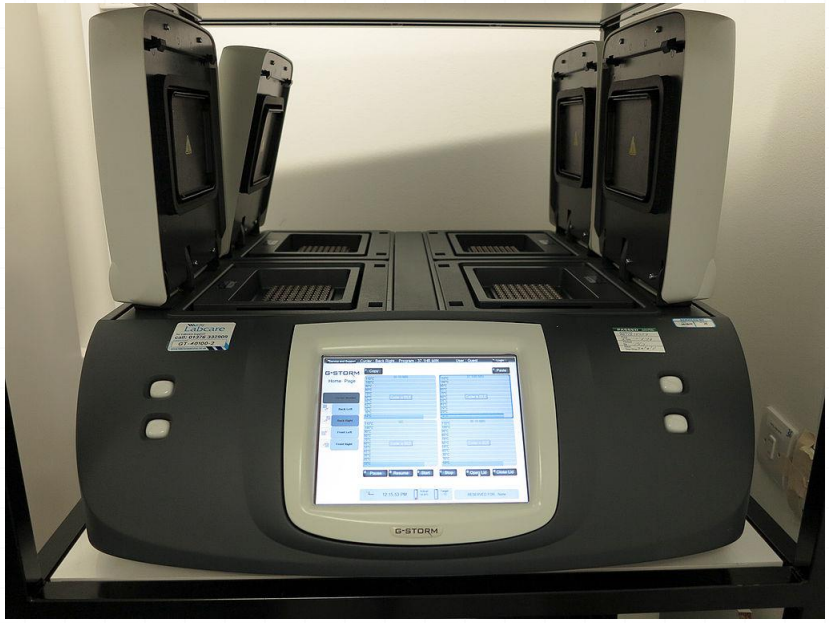
PCR is technology used to amplify a single copy or a few copies of DNA to millions of copies.

The method depends on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

Old machine


BATH 1
BATH 2
BATH 3
INNOVONICS
Please. DO NOT leave this
machine on overnight.

Recently

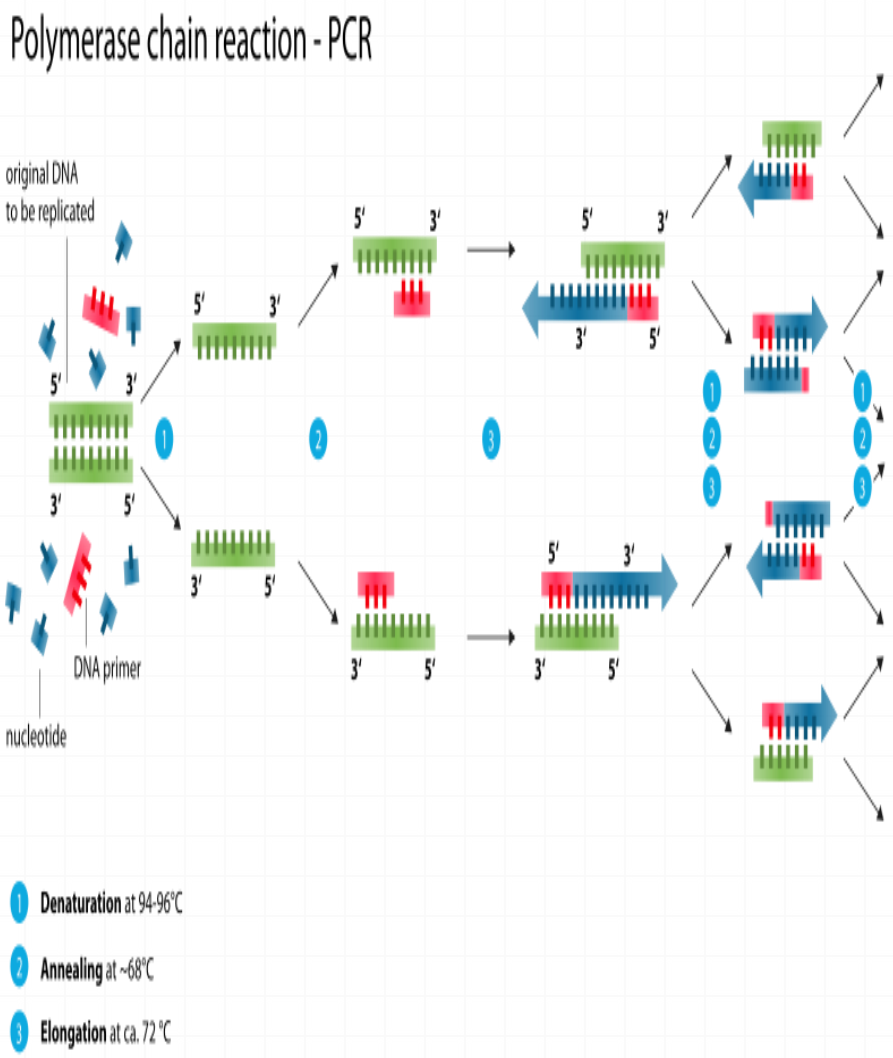


PCR

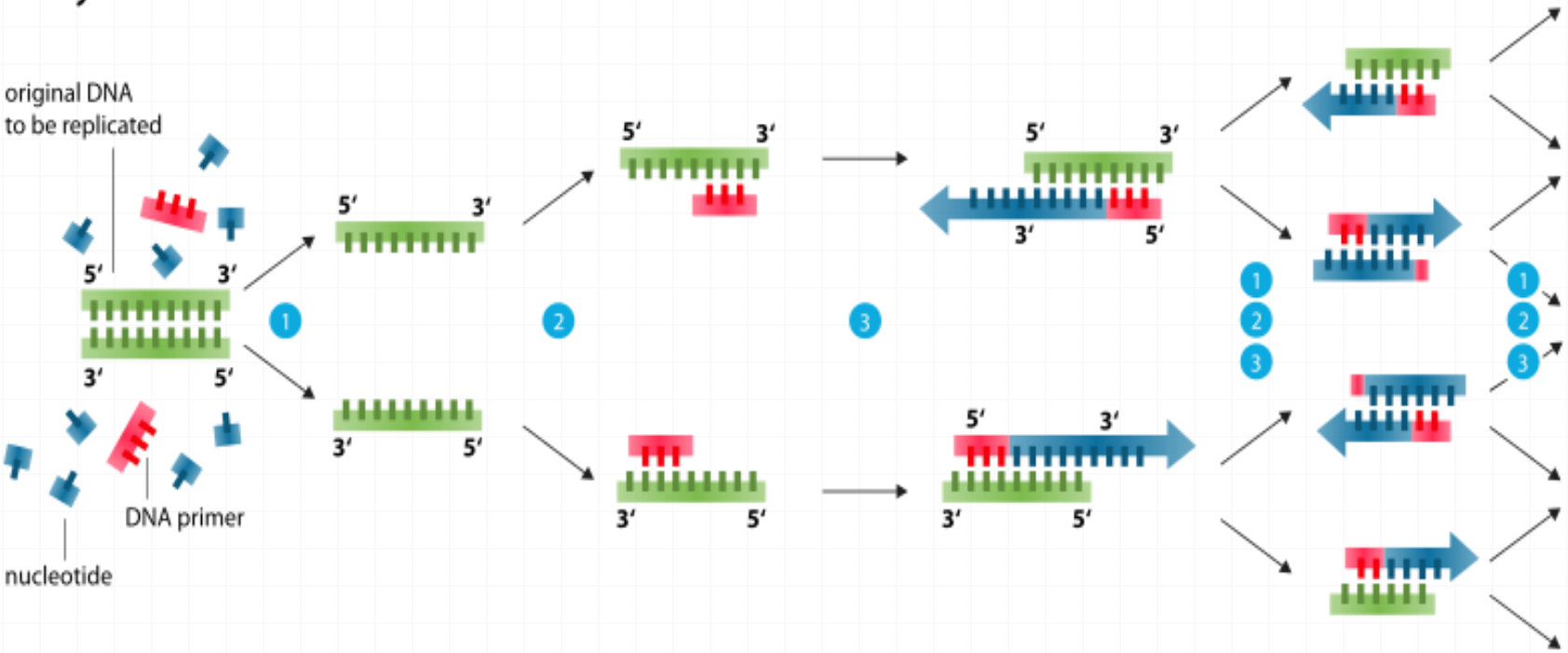
First step, The two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting (Denaturation).

Second step, The temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification.

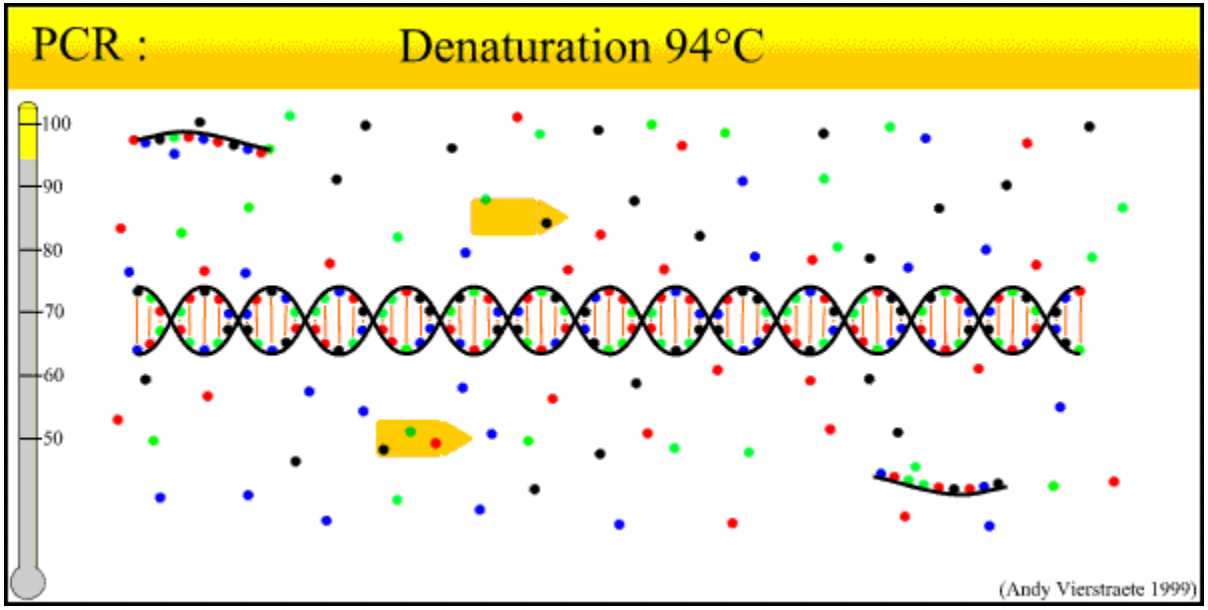
Third step, extension



Polymerase chain reaction - PCR



1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C


PCR :
Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)

DNA sequencing

It is the process of determining the precise order of nucleotides within a DNA molecule.

History

The first DNA sequences were obtained in the early 1970 by academic researchers using laborious methods based on two-dimensional chromatography.

Following the development of fluorescence based sequencing methods with automated analysis.

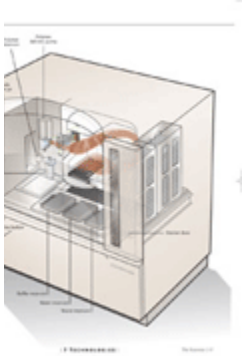
DNA sequencing has become easier and orders of magnitude faster.

DNA sequencing

Knowledge of DNA sequences has become indispensable for basic biological research, and in numerous applied fields such as diagnostic, biotechnology, forensic biology, virology and biological systematics.

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life.

DNA sequencing:



Points of compassion	Ion Torrent PGM	454 GS FLX	HiSeq 2000	SOLiDv4	PacBio	Sanger 3730xl
Manufacturer	Ion Torrent (Life Technologies)	454 Life Sciences (Roche)	Illumina	Applied Biosystems (Life Technologies)	Pacific Biosciences	Applied Biosystems (Life Technologies)
Amplification approach	Emulsion PCR	Emulsion PCR	Bridge amplification	Emulsion PCR	Single-molecule; no amplification	PCR
Data output per run	100-200 Mb	0.7 Gb	600 Gb	120 Gb	100-700 Mb	1.9~84 Kb
Accuracy	**99%**	**99.9%**	**99.9%**	**99.94%**	**88.0% (>99.9% CCS)**	**99.999%**
Time per run	2 hours	24 hours	3–10 days	7–14 days	2-3 hours	20 minutes - 3 hours
Read length	**200-400 bp**	**700 bp**	**100x100 bp paired end**	**50x50 bp paired end**	**5,500-10,000 bp**	**400-900 bp**
Cost per run	$350 USD	$7,000 USD	$6,000 USD (30x human genome)	$4,000 USD	$125-300 USD	$4 USD (single read/reaction)
Cost per Mb	$1.00 USD	$10 USD	$0.07 USD	$0.13 USD	$0.20 - $3.00 USD	$2400 USD
Cost per instrument	**$80,000 USD**	**$500,000 USD**	**$690,000 USD**	**$495,000 USD**	**$695,000 USD**	**$95,000 USD**

Sanger sequencing

Developed by Frederick Sanger and colleagues in 1977, it was the most widely used sequencing method for approximately 25 years.

Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

However, the Sanger method remains in wide use, primarily for smaller-scale projects and for obtaining especially long contiguous DNA sequence reads (>500 nucleotides).

Sanger sequencing

The classical chain-termination method requires:

1. Single-stranded DNA template.
2. DNA primer (Radioactively labeled primer).
3. DNA polymerase.
4. Normal deoxynucleoside triphosphates (dNTPs).
5. Modified di-deoxynucleotidetriphosphates (ddNTPs), these ones responsible for terminate DNA strand elongation.

These chain-terminating nucleotides lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated.

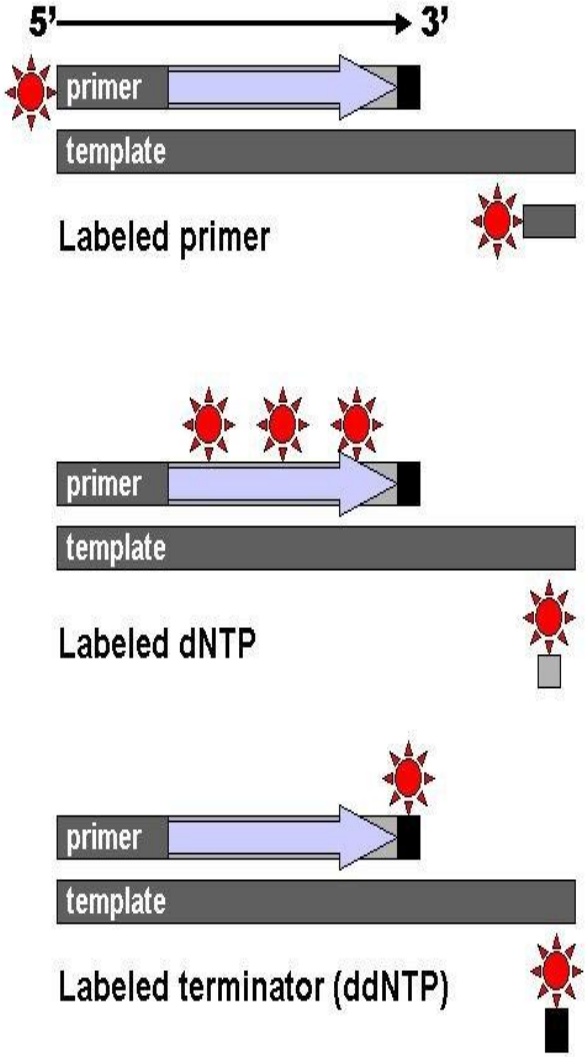
Sanger sequencing

The ddNTPs may be radioactively or fluorescently labeled for detection in automated sequencing machines.

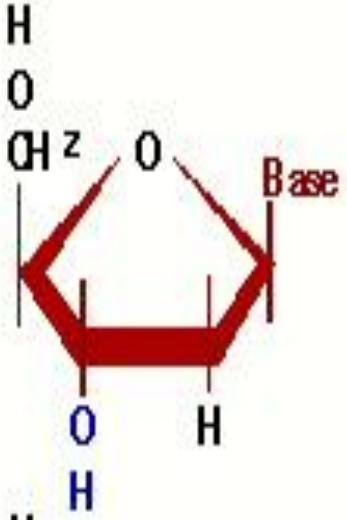
Method:

1. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase.
2. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while three other nucleotides are ordinary ones.

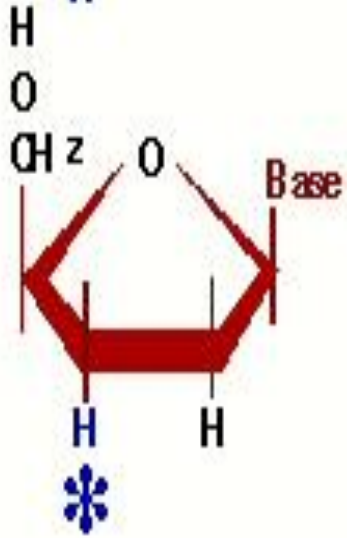
Reading

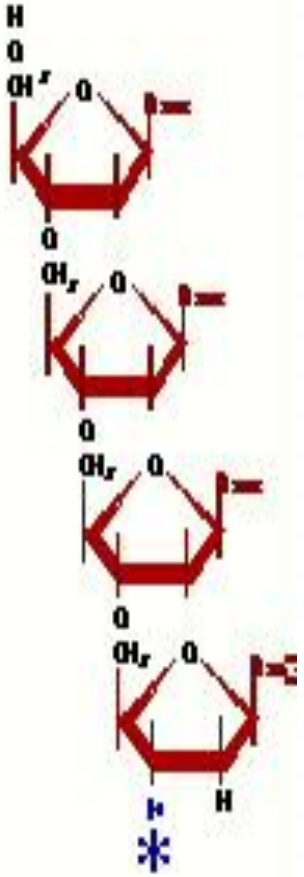


Normal nucleotides:



Dideoxy Chain Terminators:





1. DNA fragments are heat denatured and separated by size using gel electrophoresis.

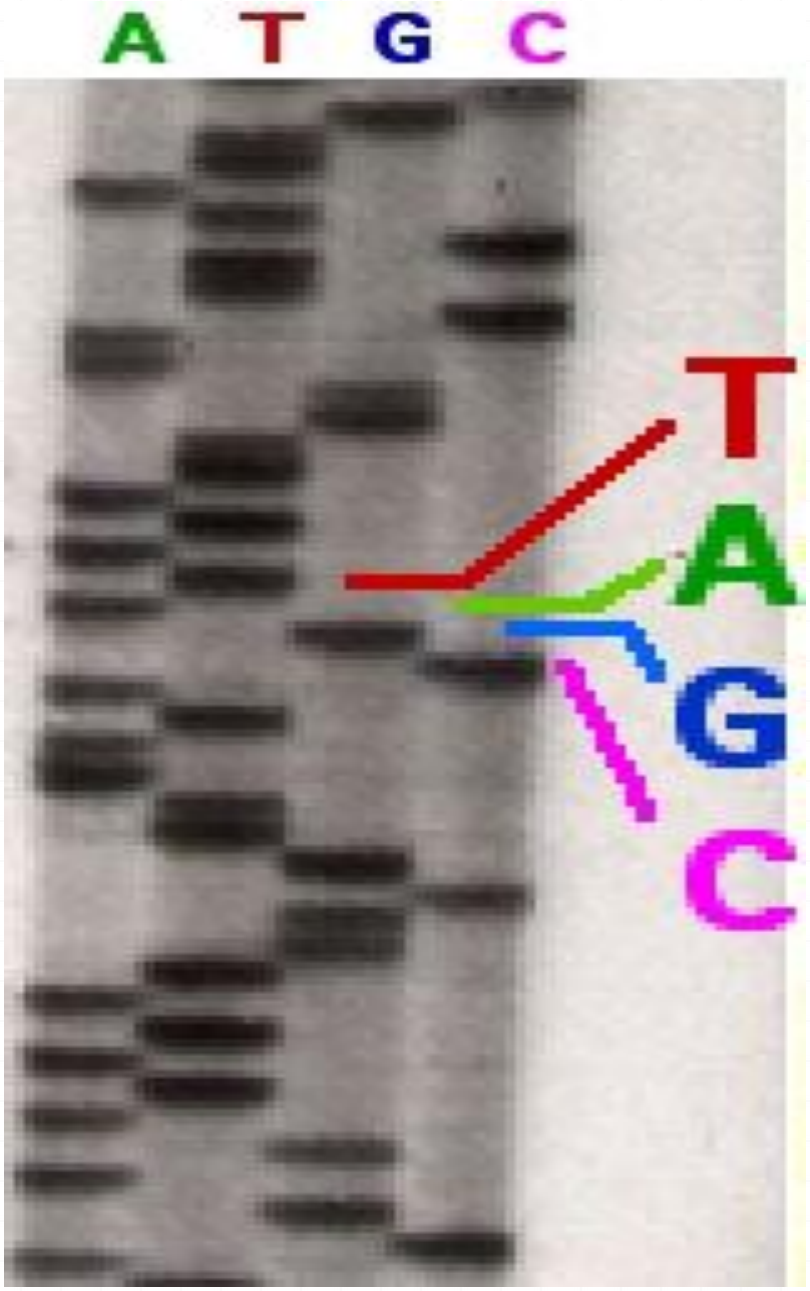
This is frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C).

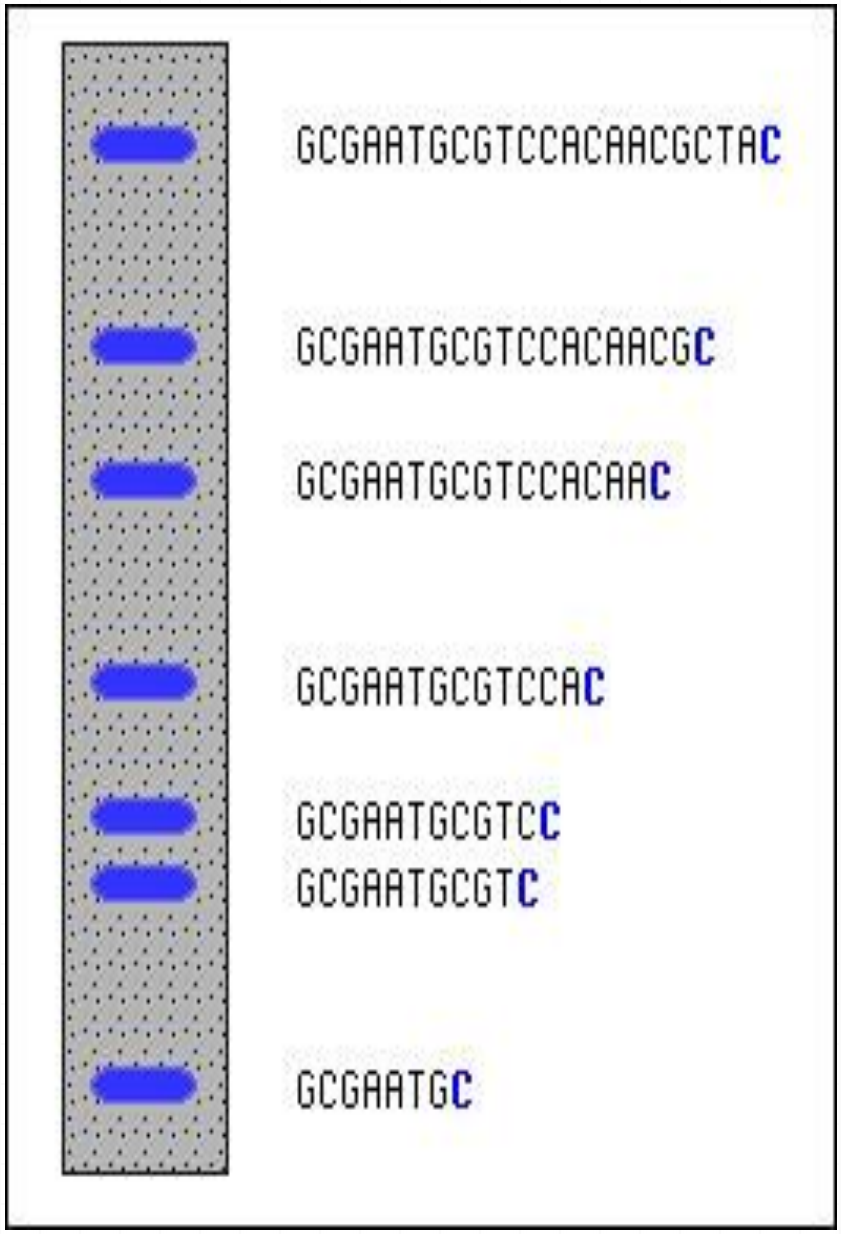
2.The DNA bands may then be visualized by autoradiography or UV light and the DNA sequence can be directly read off the X-ray film or gel image.

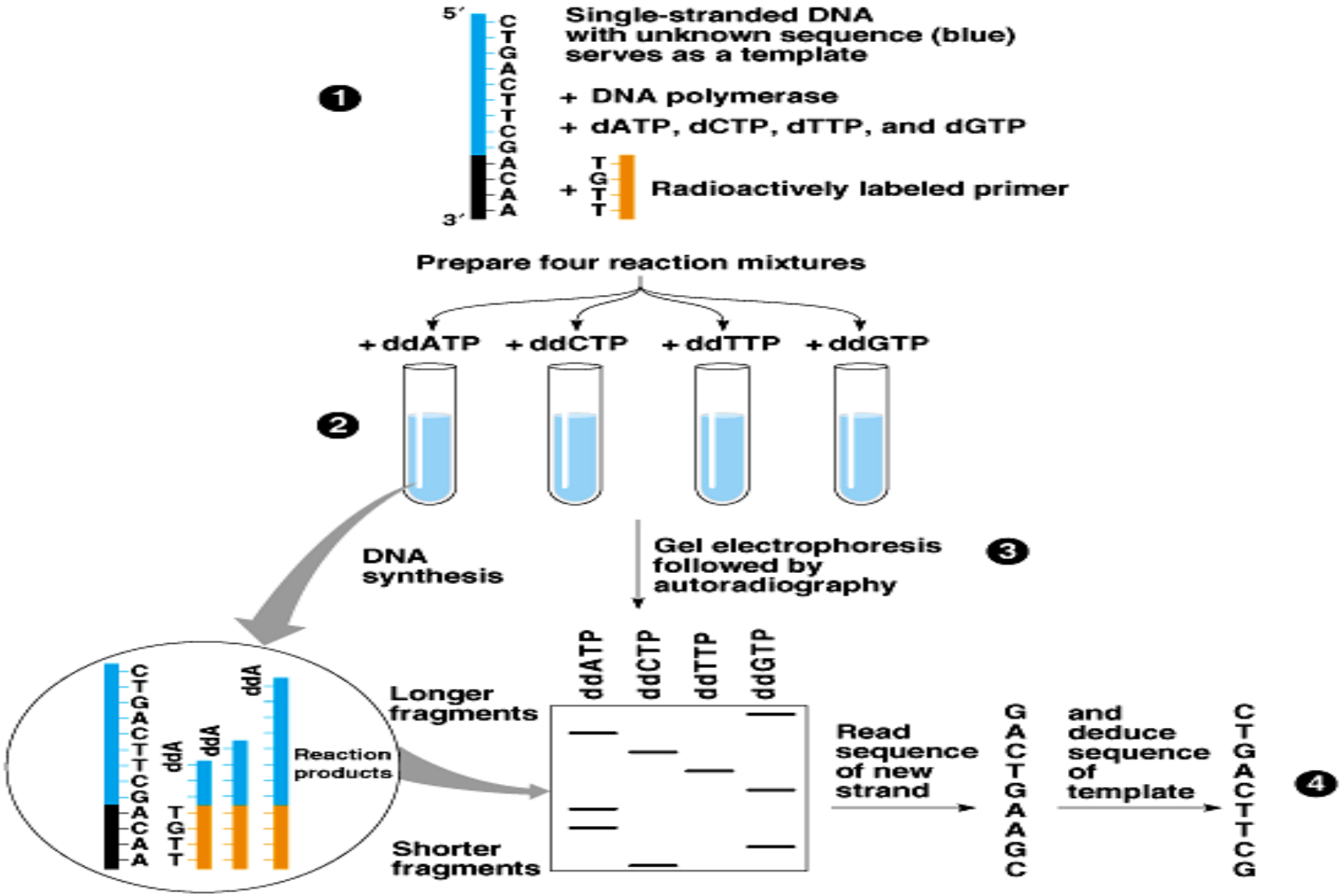
3. The X-ray film was exposed to the gel and the dark bands correspond to DNA fragments of different lengths.

A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide.

4. The relative positions of the different bands among the four lanes, from bottom to top, are then used to read the DNA sequence.


A T G C
T
A
G
C


GCGAATGCGTCCACAACGCTAC
GCGAATGCGTCCACAACGC
GCGAATGCGTCCACAAC
GCGAATGCGTCCAC
GCGAATGCGTCC
GCGAATGCGTC
GCGAATG C



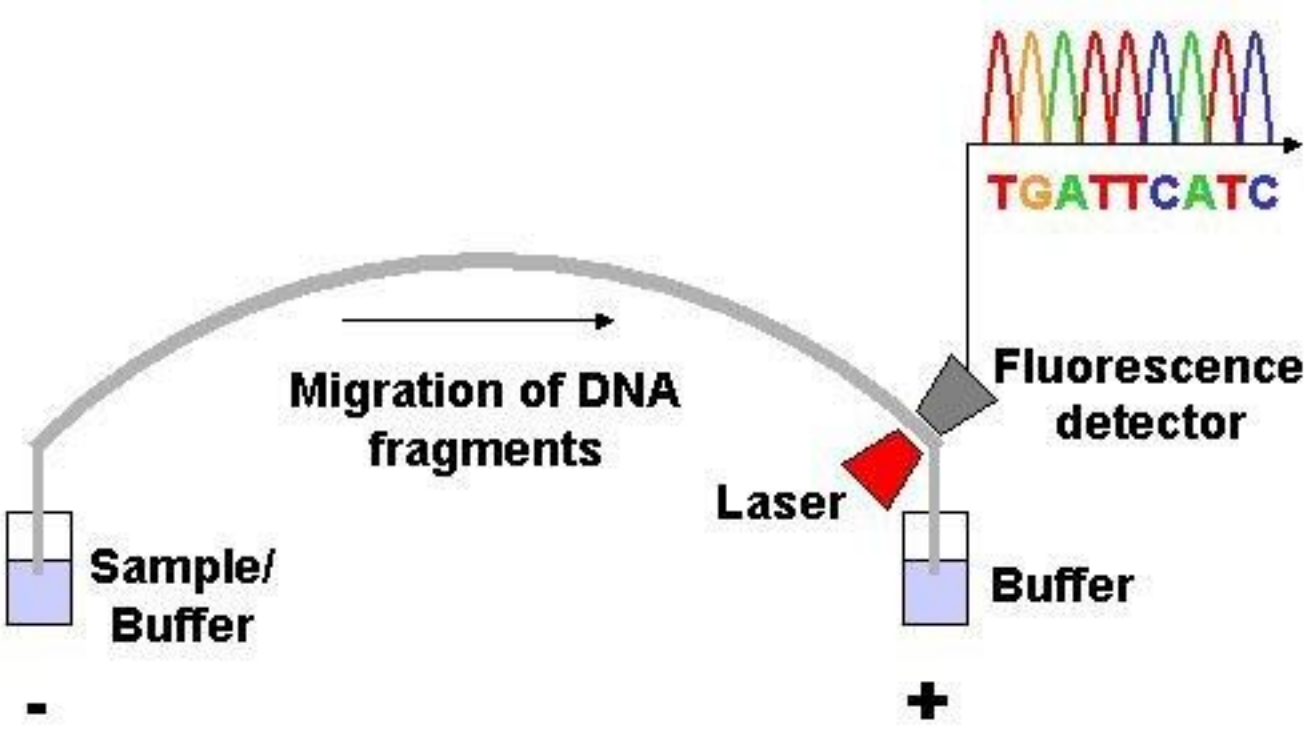
Recently

Dye-terminator sequencing utilizes labeling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than four reactions as in the labelled-primer method.

In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labeled with fluorescent dyes, each of which emit light at different wavelengths.

Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing.

Dye-terminator sequencing



In side the Sequencer

Thermal cycling

In the TC reaction chamber, dye-terminator sequencing reagent, template DNA, and primers are loaded into the TC chamber and thermal-cycled for 35 cycles (at 95°C for 12 seconds and at 60°C for 55 seconds).

Purification

The charged reaction mixture (containing extension fragments, template DNA, and excess sequencing reagent) is conducted through a capture/purification chamber at 30°C via 33-Volts/cm electric field applied between capture outlet and inlet ports.

Sequencing chemistry

Extension fragments are immobilized by the gel matrix, and excess primer, template, free nucleotides, and salts are eluted through the capture waste port.

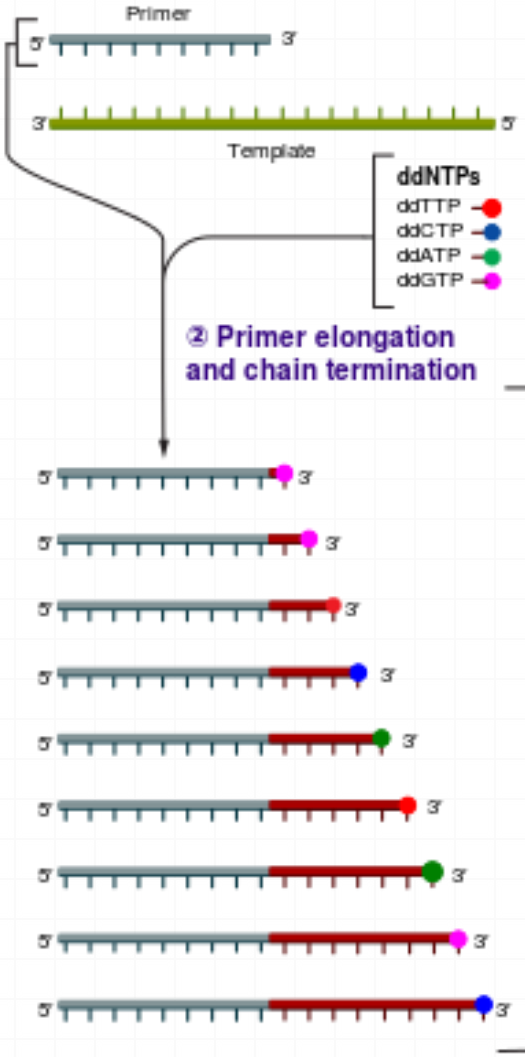
The capture gel is heated to 67-75°C to release extension fragments.

Capillary electrophoresis

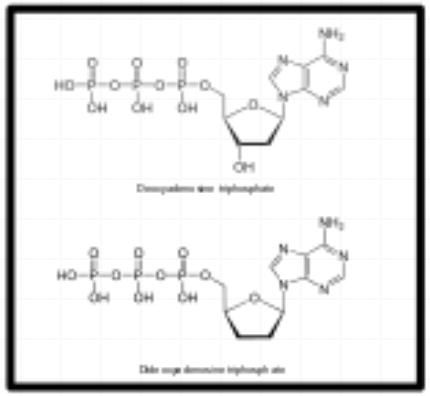
Extension fragments are injected into the CE chamber where they are electrophoresed through a 125-167-V/cm field.

① Reaction mixture

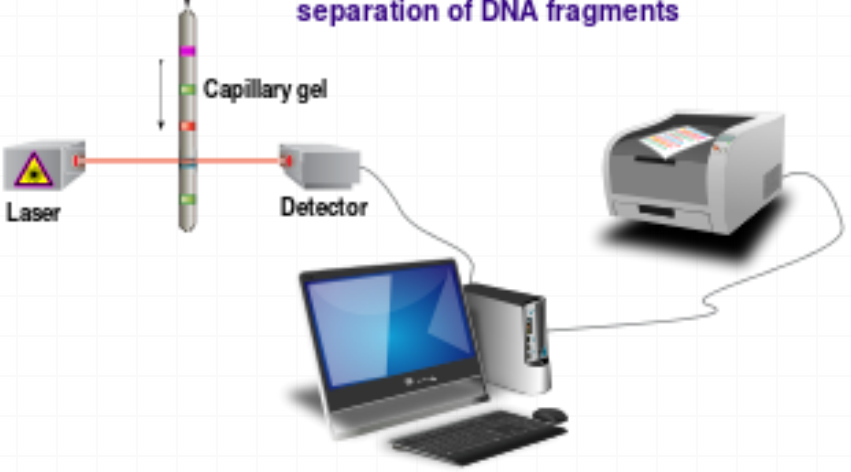
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

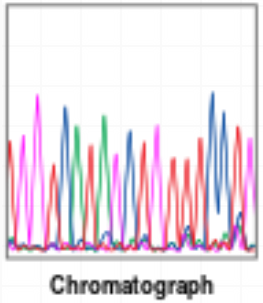


② Primer elongation and chain termination



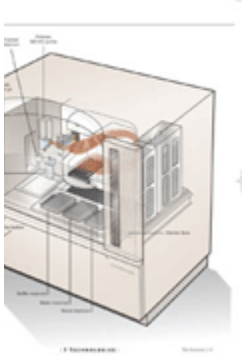
③ Capillary gel electrophoresis separation of DNA fragments



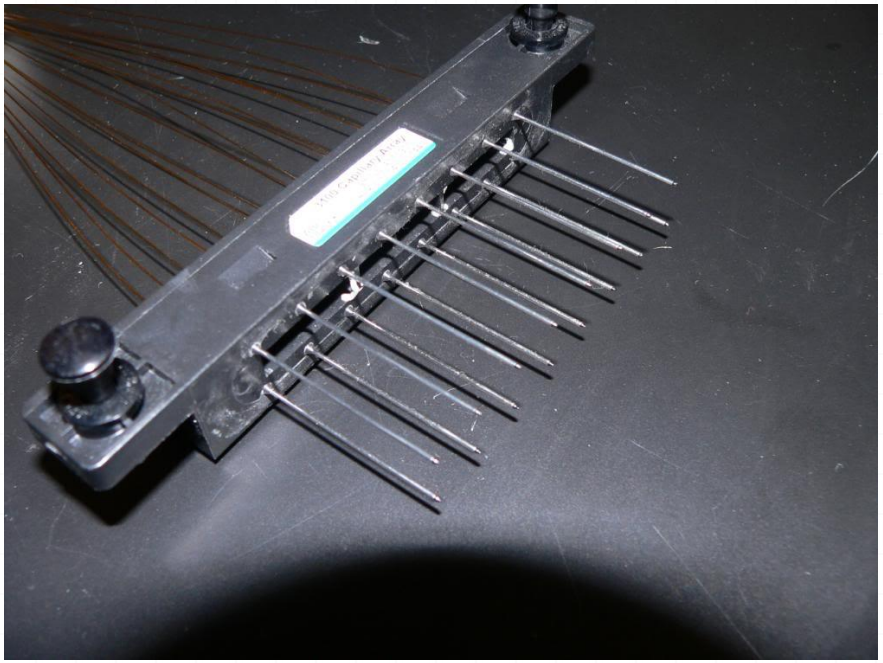


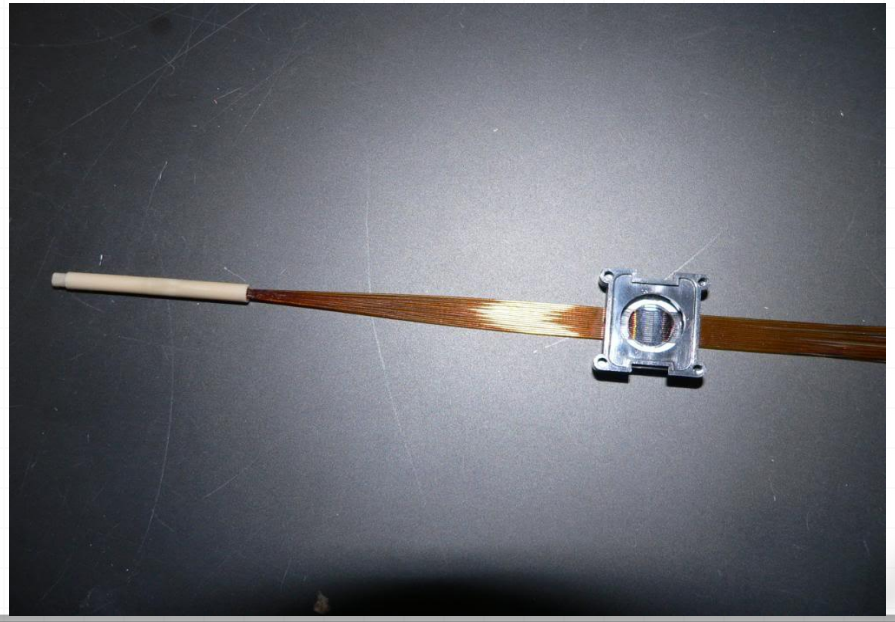
④ Laser detection of flourochromes and computational sequence analysis

DNA sequencing:




HITACHI
POWDER FREE GLOVES ONLY WHEN
USING THE 3100 - INCLUDING LOADING
PLATES, please!!
ABI
PRISM
3100 Genetic Analyzer
FREE inside





Dye-terminator sequencing

Its limitations include:

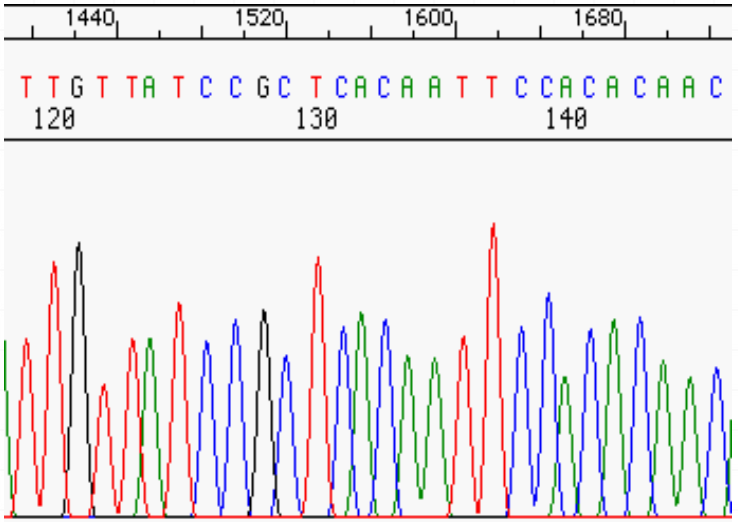
Dye effects due to differences in the incorporation of the dye-labeled chain terminators into the DNA fragment,

Resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis.

This problem has been addressed with the use of modified DNA polymerase enzyme systems and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs".

Sequencing Results

- When you obtain a sequence you should proofread it to ensure that all ambiguous sites are correctly called and determine the overall quality of your data.



- Base Designations
- *"A" designation*—green peaks
- *"G" designation*—black peaks
- *"T" designation*—red peaks
- *"C" designation*—blue peaks
- *"N" designation*—peaks that, for whatever reason, are not clear enough to designate as A, G, T, or C.

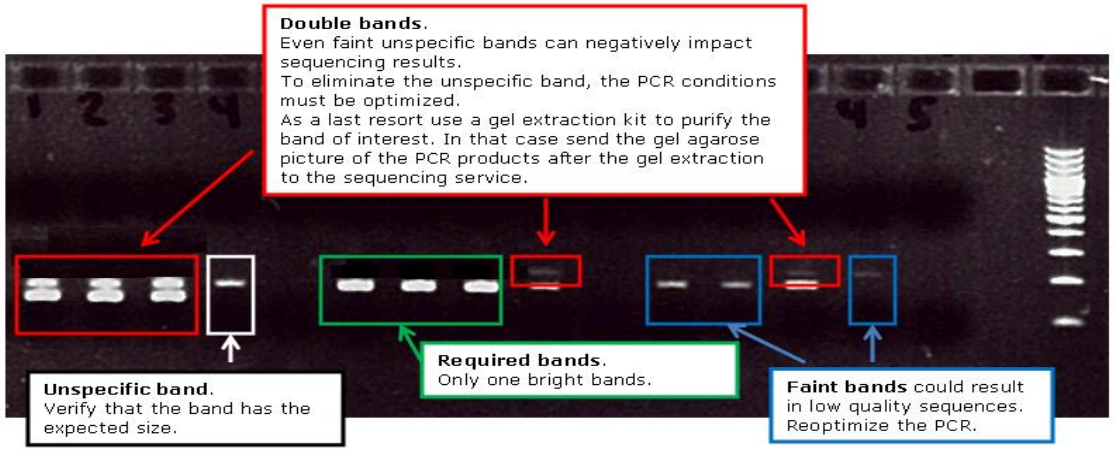
Shotgun sequencing

Shotgun sequencing

Shotgun sequencing is a sequencing method designed for analysis of DNA sequences longer than 1000 base pairs, up to and including entire chromosomes.

This method requires the target DNA to be broken into random fragments.

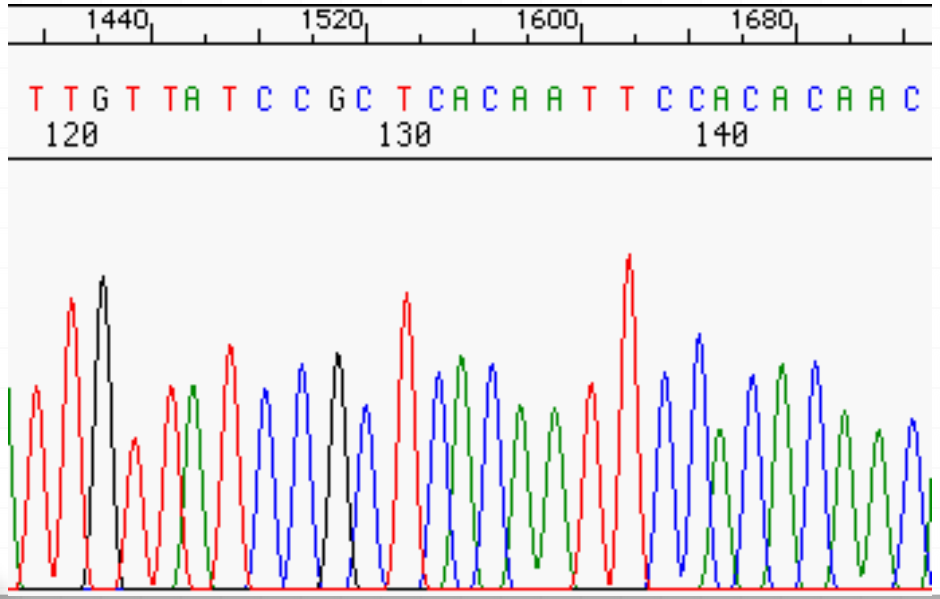
After sequencing individual fragments, the sequences can be reassembled on the basis of their overlapping regions.



Automated DNA Sequencers generate

1- A four-color chromatogram.

2- In addition to a text file of sequence data.



>GXP_210035 loc=GXL_175098|sym=FAM149A|taxid=9606|spec=Homo sapiens|chr=4|ctg=NC_000004|str=(+)|start=187065495|end=187066181|len=687|comm=Promoter Region

GGACGGGCGTGGAAGGGTCCACGTCTTTAGTATGCATGCTTAGATCTAGCGTTCCTGTTGATGGAGTAATGGTTCTCGCA
TTGACCAGATCCGGGGCTTCATTTTTTAAACCTCATTCGTCCACTCCCCACCCCAGCCTGGTGTGCGCACCCTTTGATGG
GGCGGGGATAGGCGAGATGGTCCTGTGGTTCTCTGCCTTCTTCTGGTGAATTAAAATCCGATTTGGAAGAGAGAAGGGCA
GCCAGCACCAGTATGCACAGCCCCCGGCCCCAGAGACCCGGGAAGGAGTAGGGAGGCCGGGCCGTGCGCGGAGGAGTGGC
CGCTGGGTTGGAAACCCGGCCCGGCAGGGAGCGGGGAAGGCGCGCTTTCCCGGAGGTCGGCGCGGGGCCGGGGCCGGGGC
CGGGGCCCGGAGCGGGGATGGGCGGGCGCAGCCGGGATTAGCTGGCGGGCGAGGGCGCAGCGCAGGGAGGAGGAGGGGAG
GCGGCGCCGGCGCGGGCGGGGCGGAGGATCTGGAGAGGGAAGGGGCGTGCGAGCCCCGCGGACCCCGGGCGCGCCCGGGC
CGCCTGAGCTGGGCCAGCCGCGCGGCGGGCGCGGGCGCGGGCGCGGGCGCGGGCGCGGGCGGGTGGGGAGCCCCAGCCCC
GGGGCCGCGGGGGCGCGTGACCGGCTGTCTGCGTGGGGCCCGCGCGC

Thanks a lot

with my Best Regards and My Best wishes

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