Sequencing Technologies – Overview of PCR and Sanger method of DNA sequencing

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Sequencing and Technical Support Manager

inqaba biotec™
Africa's Genomics Company

ABOUT INQABABIOTEC

inqaba biotec™
Africa's Genomics Company

South Africa
2003

Kenya
2010

Nigeria
2015

Ghana
2018

Tanzania
2019

Company Profile

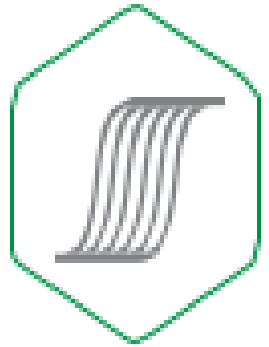
Our Mission: *Catalysing Africa's Prosperity Through Genomics!*

Core Services

DNA SYNTHESIS
(PRIMERS & PROBES)

SANGER DNA
SEQUENCING

SNP GENOTYPING &
METHYLATION ANALYSIS

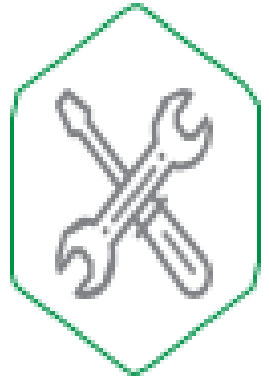


qPCR ANALYSIS

NEXT GENERATION
SEQUENCING (NGS)

BIOINFORMATICS
SOLUTIONS

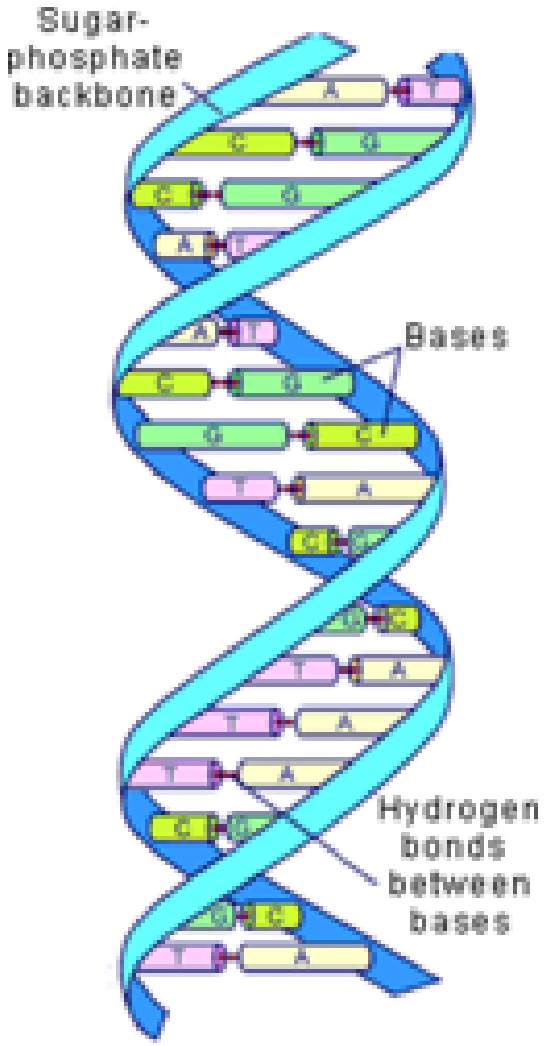
ANIMAL
GENETICS



INSTRUMENTS SERVICE,
REPAIRS & MAINTENANCE

TECHNICAL SESSIONS

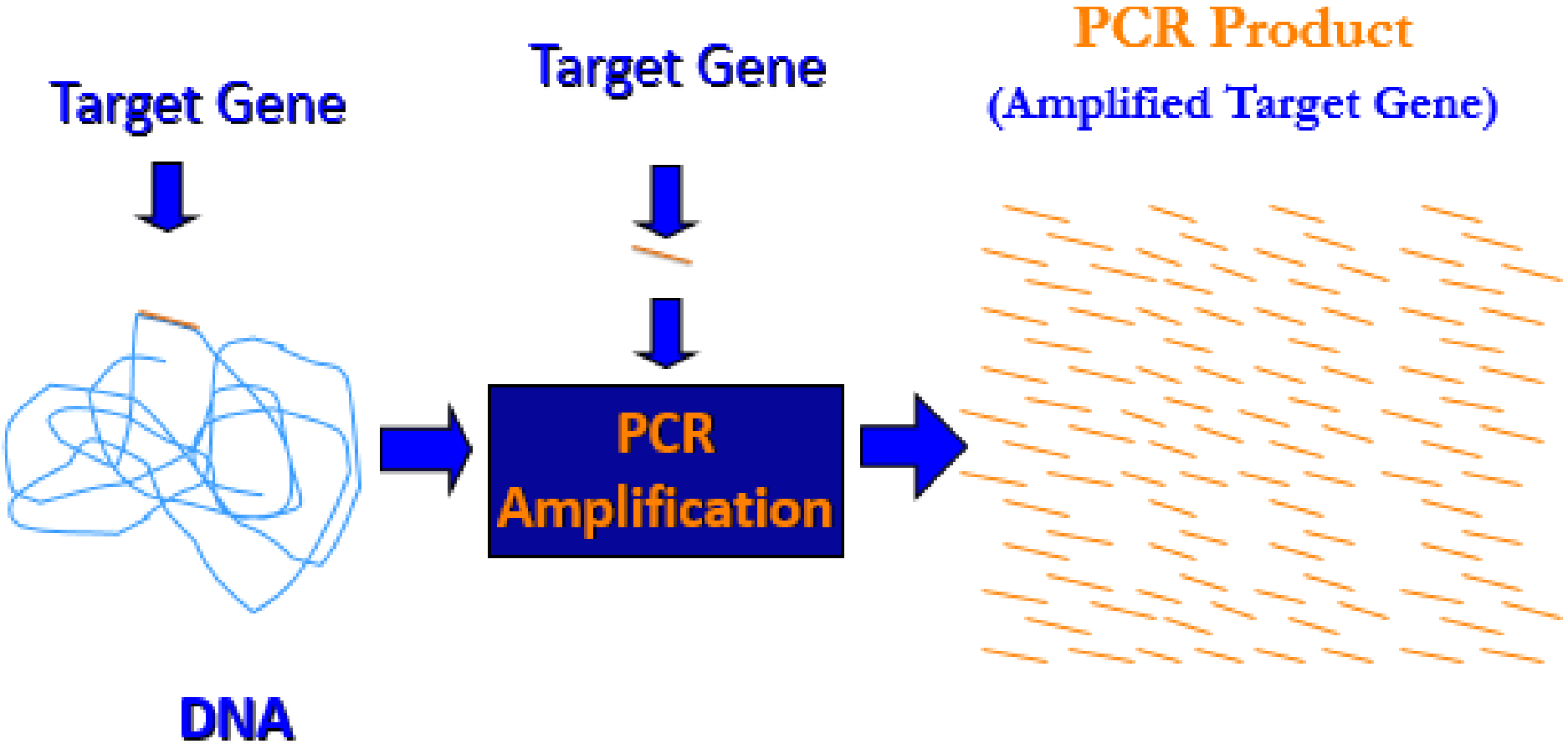
Polymerase Chain Reaction



Polymerase Chain Reaction (PCR) refers to an in-vitro technique which is nucleic- acid based for making numerous copies of a DNA fragment of interest, such as a gene for its identification and characterization. For an example: HPV, BBTV, YMV, insect identification etc.

An in-vivo technique use to make more copies of a virus or any other gene require the use of living organisms such as bacteria in a procedure called cloning and transformation.

Polymerase Chain Reaction

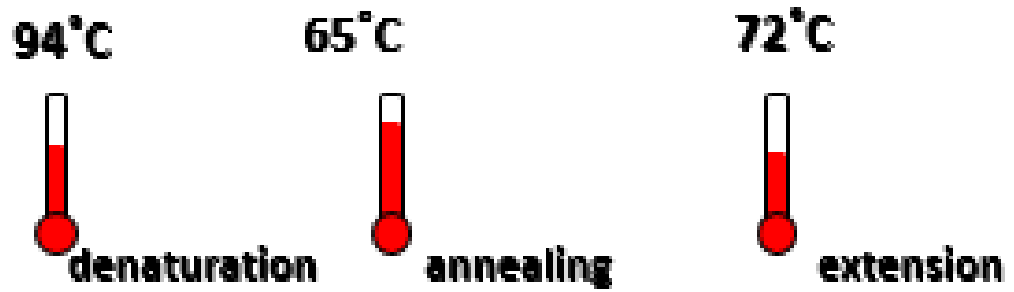


Mechanism

Polymerase Chain Reaction

During PCR, a thermocycler brings the reaction mix to 3 different temperatures analagous to the 3 steps of DNA replication

Denaturation (94˚C) of the DNA template by heat
Annealing (37˚-70˚C) of the primers to the template
Extension (72˚C) of the DNA strand by DNA polymerase



These steps are repeated for 25 to 40 cycles

Denaturation

Denaturation occurs at 94˚C

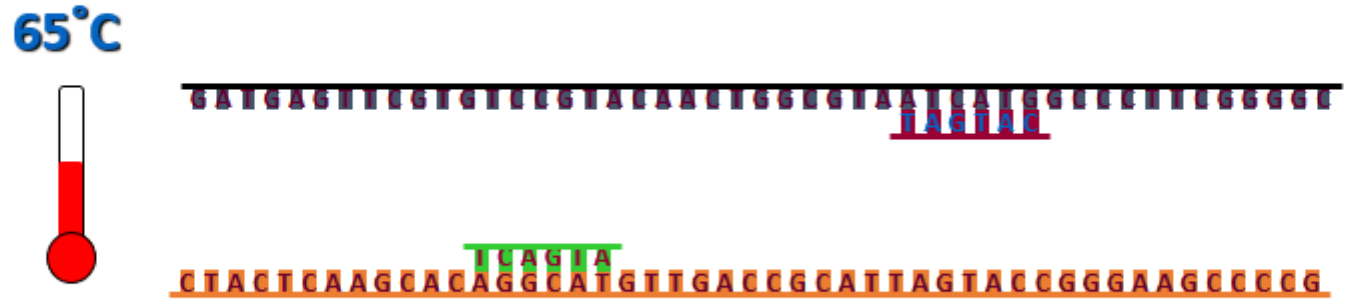
The high temperature is used to break down the hydrogen bonds that hold the two strands together

Annealing

Annealing occurs at 37˚-70˚C

Oligonucleotide DNA primers anneal to their complementary sequences on the template strands

Annealing temperature depends on the melting temperature (T_m) of the primer (dependent on base composition)

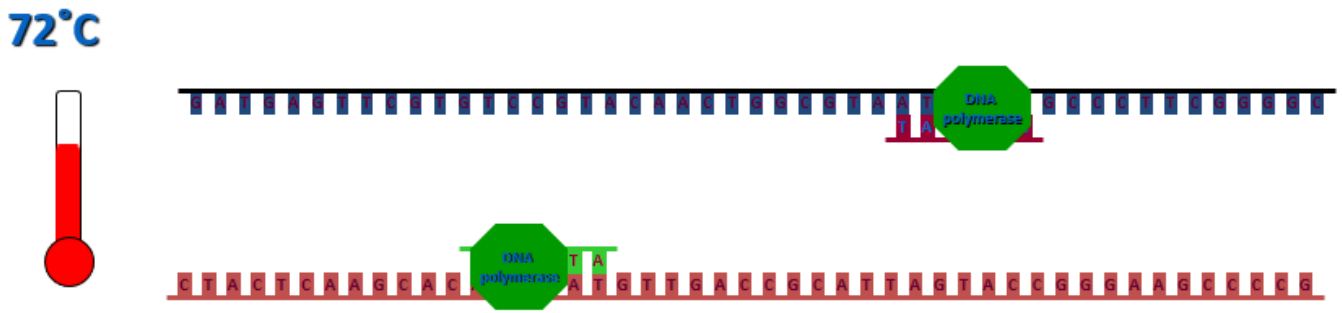


Extension

Extension occurs at 72˚C

DNA polymerase attaches to the primers and extends the new DNA strand

The 3 steps (denaturation, annealing, and extension) are repeated for another 24 to 29 cycles



Target Sequence

A desired target sequence is identified

To isolate the target sequence, primers that flank the region must be constructed

The DNA segment that is then amplified contains the region of interest

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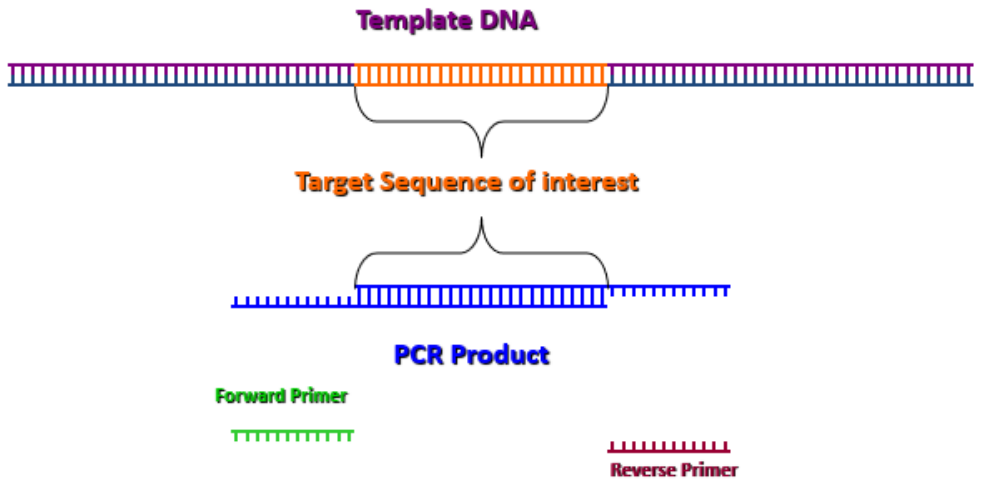
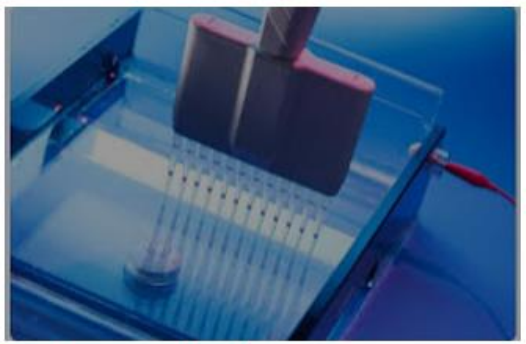
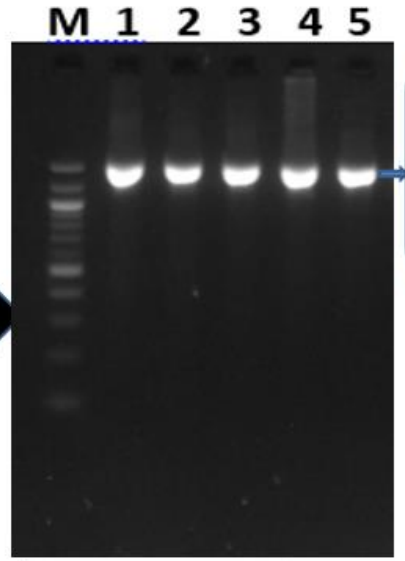


Post-PCR Assay

Gel electrophoresis

Purify PCR products, run on agarose gel electrophoresis, take gel picture under UV light and subject to sanger DNA sequencing





1500 bp- 16s DNA fragment from bacteria cultures resolved on 1.5% gel

Method of gel electrophoresis

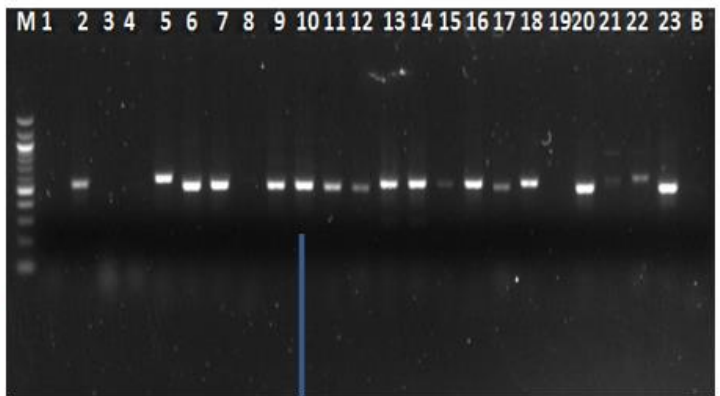
Melt agarose powder in buffer, cool and add DNA stain (Safe Red). Mix thoroughly

Pour into casting tray with comb and allow to solidify

Add running buffer to the gel tank, place gel tray in and load DNA samples and marker

Run gel at constant voltage until band separation occurs

View DNA on UV light box and show results



480-720 bp- ITs DNA fragment from Fungi isolates resolved on 2% gel

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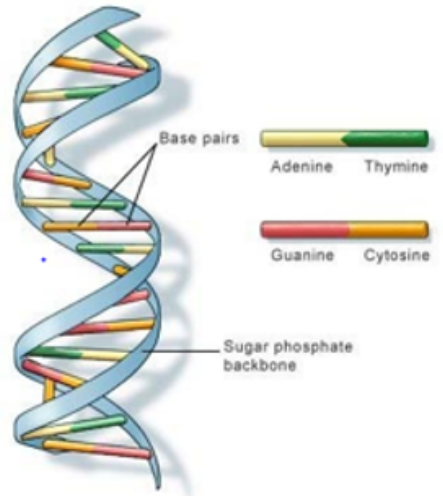
SEQUENCING TECHNOLOGIES

Sequencing Technologies

Determines the order of nucleotide (G, A, T, and C) in a stretch of DNA

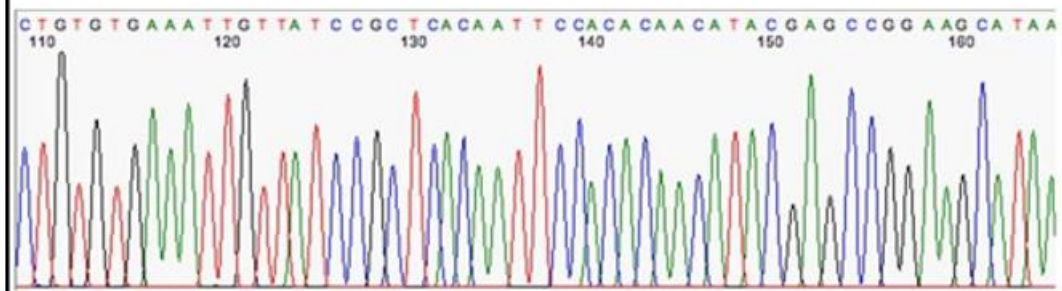


- Sanger Sequencing
- Illumina Sequencing: MiSeq, HiSeq and PacBios
- Nanopore, MinION, Single-molecule

Application of Sequencing

Sanger Sequencing

- Used with individual pieces of DNA
- Great when amount of data needed is small
- High accuracy
- LOW throughput

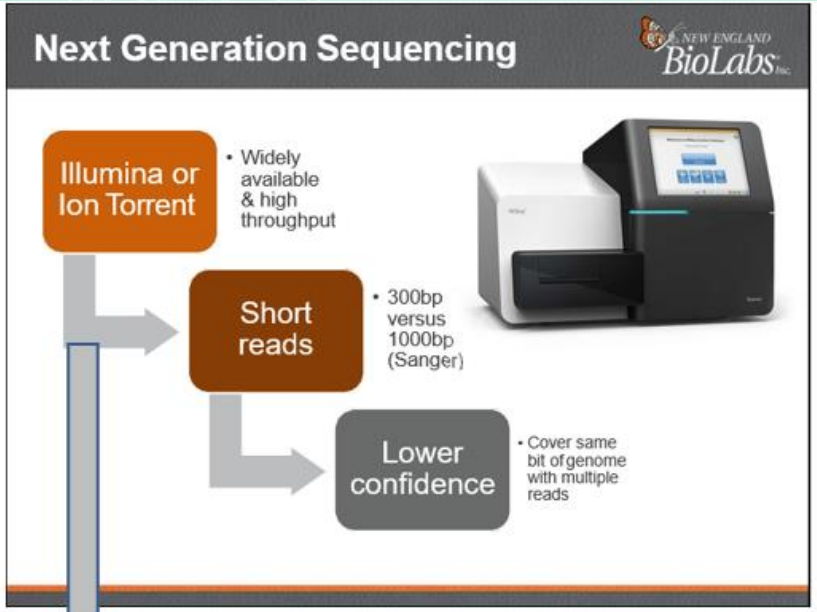


HGP: 2.7 B 1990-2003

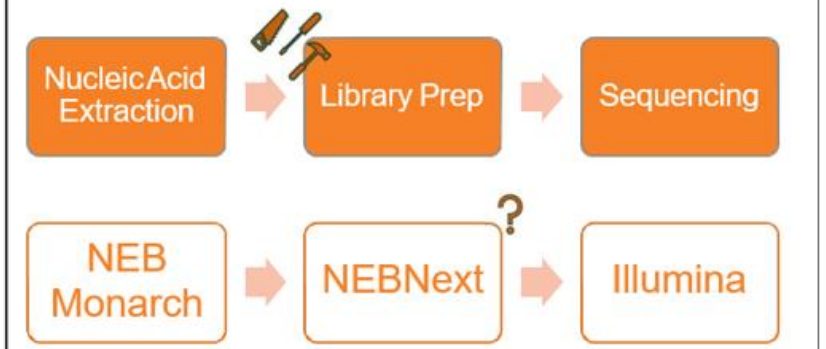
Human genome was completed using Sanger at the cost of 2.7 billion US dollars. It took from 1990 to 2003.
NGS the cost is now 1 K $

Next Generation Sequencing (NGS)

Next Generation Sequencing



Workflow



PACBIO (now part of Illumina)

Oxford NANOPORE Technologies

- Longer reads = great for assembly
- Read quality = low confidence
- Need a LOT of DNA/RNA
- Less well known suppliers

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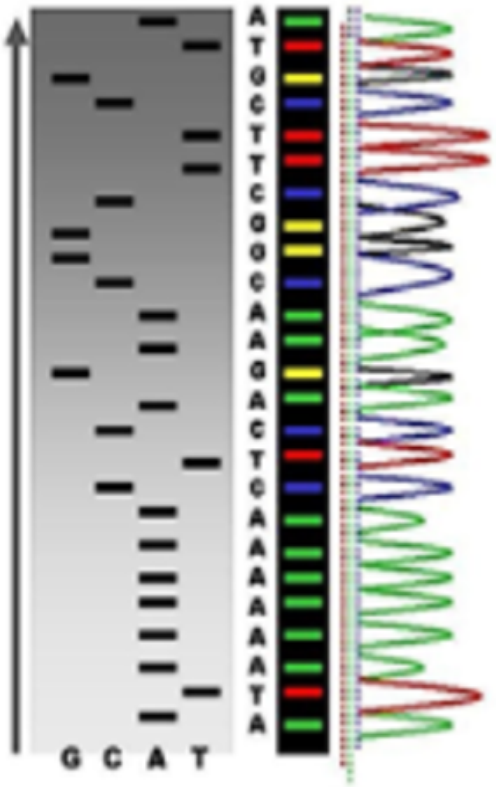
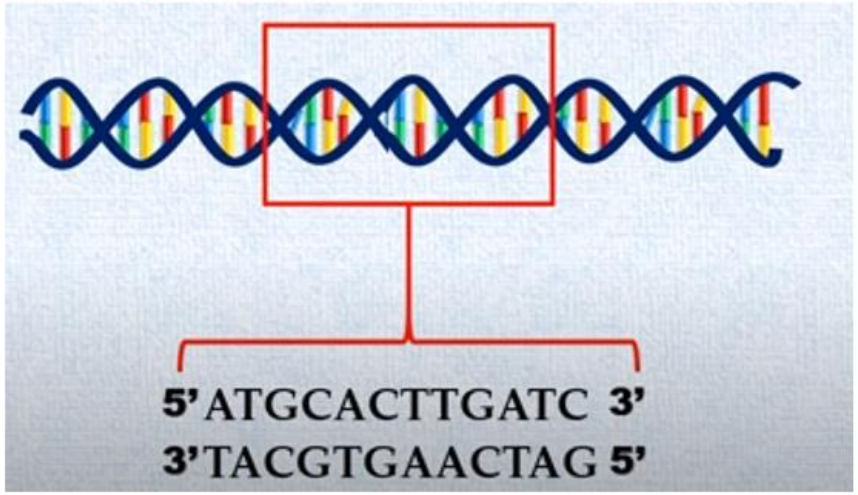
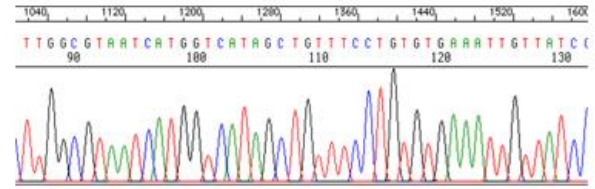


SANGER SEQUENCING

Sequencing Technologies

DNA Sequencing- It is a technique whereby precise order of nucleotides (G, A, T, and C) in a stretch of DNA can be determined





TGAAAGAGGAGCTTGATGAC
ACGGATGAAACTGCCGGTCA
GGACACCCCTCTCAGCCGGG
AAAATGT

Developed by Fred Sanger in 1977 using chemically altered "dideoxy" bases to terminate newly synthesized DNA fragments at specific bases (either A, C, T, or G). These fragments are then size-separated, and the DNA sequence can be read.

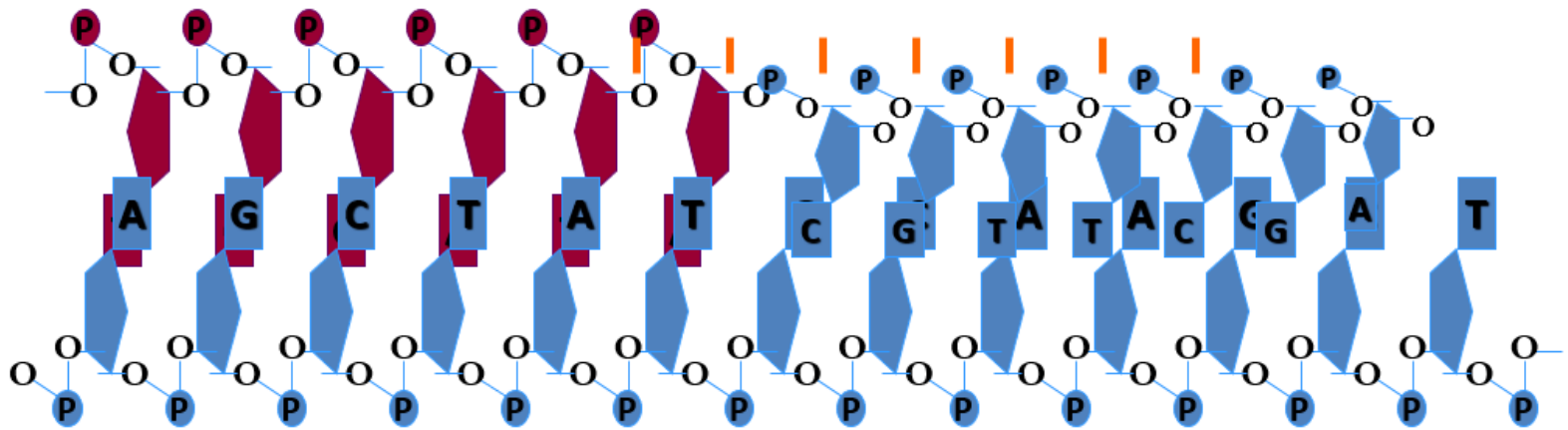
An unknown sequence can be matched up to known sequences published in the Database:

- List all sequences producing significant alignments
- Organism genus/species
- Gene identification
- Identity alignment/match

DNA Polymerization

Modern Sanger sequencing is based on the principles of Di-deoxynucleotides in DNA Polymerization

1) **DNA Template**
2) **Primer**
3) **DNA Polymerase**
4) **dNTPs**
5) **Mg^{2+} ions**



Principles of Sanger Sequencing

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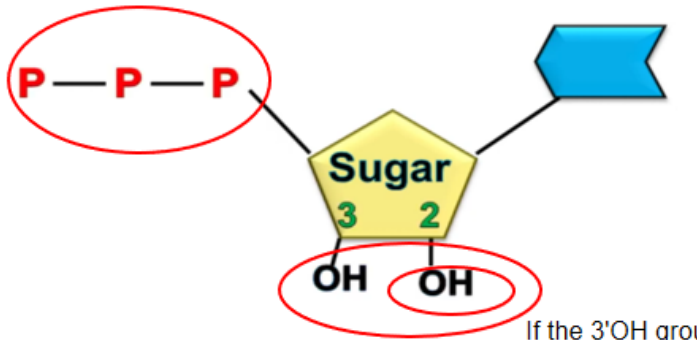
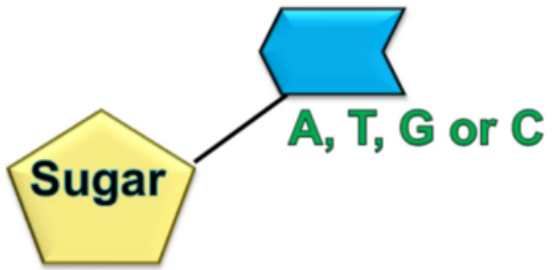
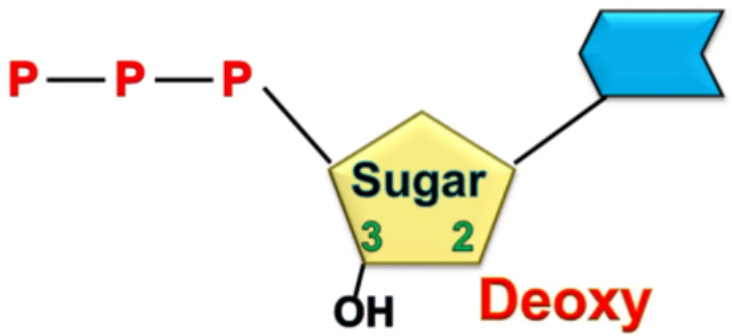


Nucleotide

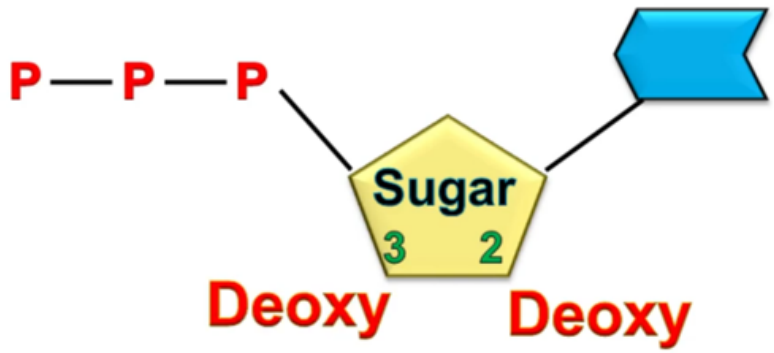


Nucleoside

If the 3'OH group of the nucleotide is absent, then there wont be addition of new nucleotides and the reaction would stop. The incorporation of dideoxy nucleotide and termination of polymerization reaction is a random process. Hence the overall mixture will have several fragements that have experienced the chain termination. If the length of each fragment is known then the location of nucleotides in the DNA can be estimated

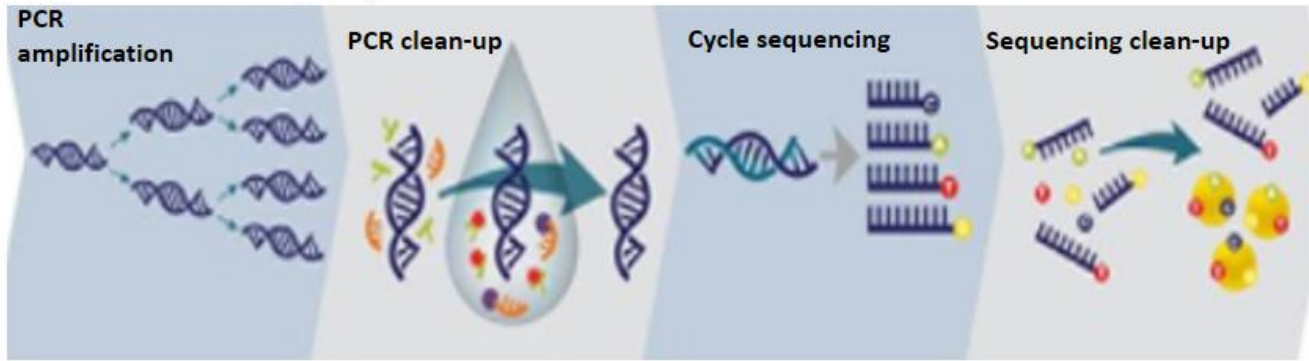


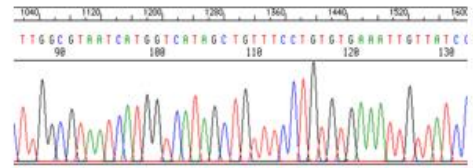
Deoxynucleotide- present in DNA



Di- deoxynucleotide

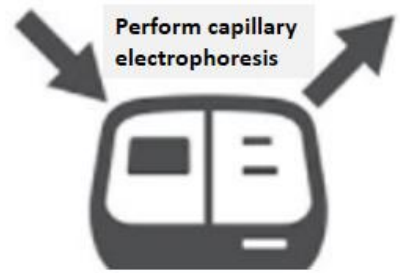
Sanger Sequencing Workflow

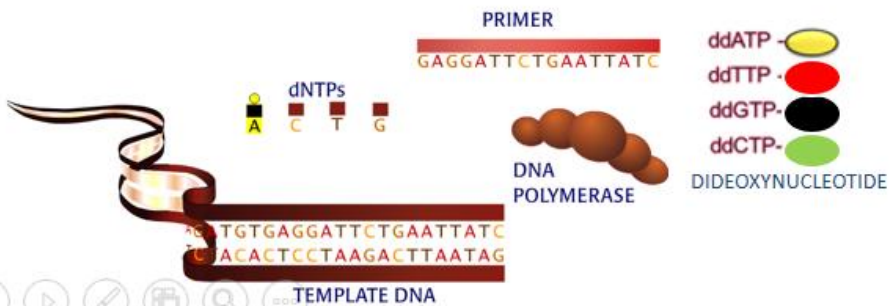




TGAAAGAGGAGCTTG
ATGACACGGATGAAAC
TGCCGGTCAGGACACC

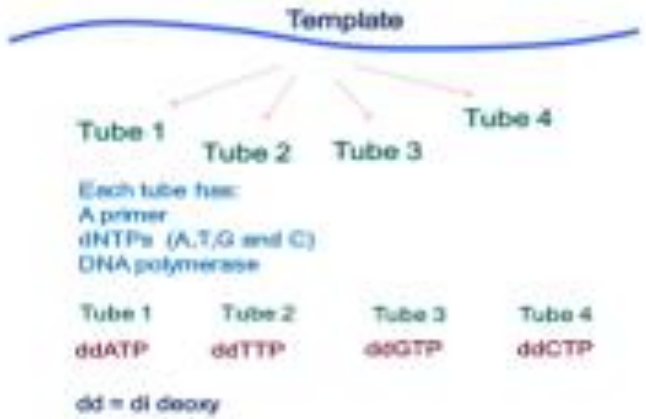
In order to determine the sequence, Sanger sequencing makes use of chemical analogs of the four nucleotides in DNA.These analogs, called dideoxyribonucleotides (ddNTPs), are missing the 3′ hydroxyl group that is required for 5' to 3' extension of a DNA polynucleotide chain. By mixing ddNTPs that have been labeled with a different color for each base, unlabeled dNTPs, and template DNA in a polymerase-driven reaction, strands of each possible length are produced when the ddNTPs are randomly incorporated and terminate the chain.

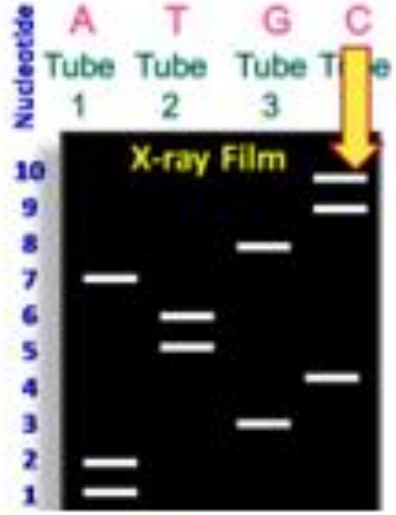


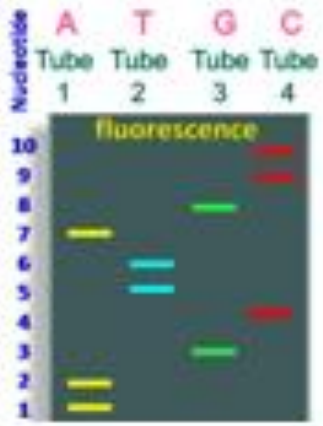


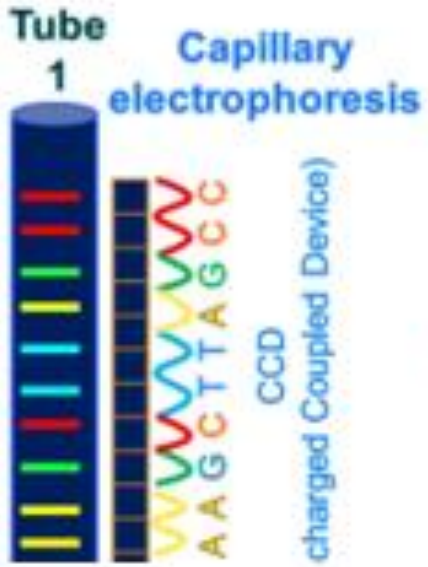
The extension products are then separated by electrophoresis, resolved to single-nucleotide differences in size. The chain-terminated fragments are detected by their fluorescent labels, with each color identifying one of the terminating ddNTPs. The sequence of the template DNA strand can thus be derived by analysis

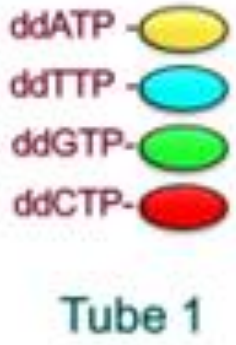
Advancement in Sanger Sequencing











Technical Review

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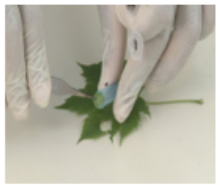
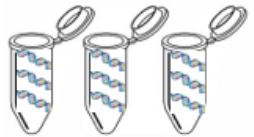
Sample Prep



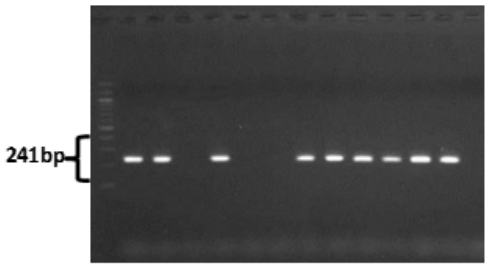
Mortar and pestle

DNA extraction

PCR



Gel electrophoresis



DNA sequencing

ACAGATGTCTTGTAATCCGGCCGTTGGTGG
CATAGGGAAAGGACATTTAGTGAAAGAAAT
TGATGCGATGGGTGGATCGATGGCTTATGC
TATCGATCAATCAGGAATTCAATTTAGAGTA
CTTAATAGTAGCAAAGGAGCTGCTGTTAGA
GCAACACGTGCTCAGGCAGATAAAATATTA
TATCGTCAAGCAATACGTAGTATTCTTGAAT
ATCAAAAATTTTTGTTGGTTATTCA

Bioinformatics

Technical Precautions in Sequencing

✓ Poor Template Quality

Poor template quality is the most common cause of sequencing
problems characterized by:

- Noisy data or peaks under peaks
- No usable sequence data
- Weak signal

Always follow recommended procedures to prepare templates.

✓ Contamination

Potential contaminants include:

- Proteins
- RNA
- Chromosomal DNA

✓ Excessive Reagents

Excess PCR primers, dNTPs, enzyme, and buffer components (from a PCR amplification used to generate the sequencing template)

- Residual salts
- Residual organic chemicals such as phenol, chloroform, and ethanol
- Residual detergents

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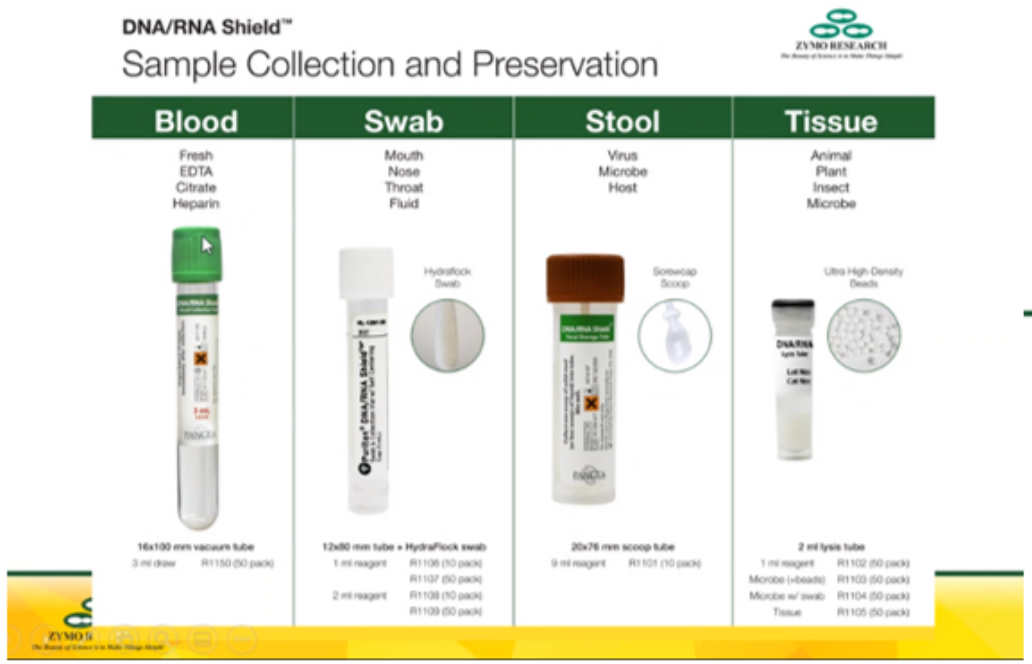
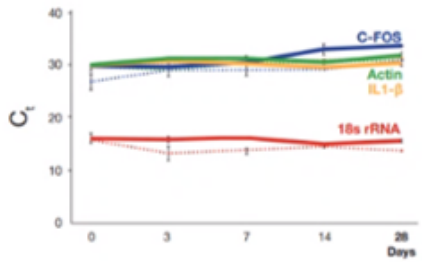
Sample Storage Devices

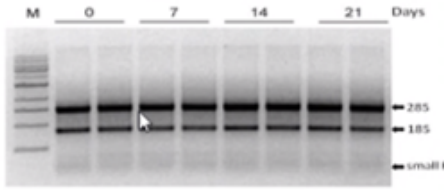
Collection Devices



RNA Stabilization and Storage



RNA in blood is effectively stabilized in DNA/RNA Shield™ at ambient temperature.
RNA was purified from whole blood stored for the indicated time points in DNA/RNA Shield™ and analyzed by RT-qPCR. Solid and dotted lines represent two different donors.



Cellular RNA is effectively stabilized in DNA/RNA Shield™ at ambient temperature.
RNA was purified from cells (HCT 116) stored in DNA/RNA Shield™ at the designated times.

Advantages of Sequencing Technologies

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Sanger Sequencing

Advantages	Disadvantages
Lowest error rate	High cost per base
Long read length (~750 bp)	Long time to generate data
Can target a primer	Need for cloning
	Amount of data per run

Illumina Sequencing

Advantages	Disadvantages
Low error rate	Must run at very large scale
Lowest cost per base	Short read length (50-75 bp)
Tons of data	Runs take multiple days
	High startup costs
	De Novo assembly difficult

PacBio Sequencing

Advantages	Disadvantages
Can use single molecule as template	High error rate (~10-15%)
Potential for very long reads (several kb+)	Medium/high cost per base
	High startup costs

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Final Thoughts

- DNA sequencing is becoming vastly faster and more affordable
- Generating data is no longer the bottleneck, understanding it is
- Bioinformatics types should be in high demand in the near future

Ergonomics

LABSIT –
INTELLIGENT DESIGN,
SUCCESSFUL WORK.

inqaba biotec™

•An ergonomic laboratory chair is a great option for people who need to slide around a space, whether it is because they have to work on multiple samples at once or because they need to provide instructions to the rest of the members of the staff

•Microscopes
Ensure that you can view the eyepiece while sitting or standing in an upright position. This includes the shoulders, back and neck

- Hoods and Biological Safety Cabinets

Keep arms relaxed and by the sides. Back, shoulders and neck should be upright and neutral in position avoiding an awkard position.

Ergonomics

LABSIT –
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SUCCESSFUL WORK.

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LABSIT –
ALWAYS ON HAND
AS A LAB STOOL.

The Labsit lab stool is on hand wherever you need it. It is the uncomplicated solution for short periods of sitting. However the Labsit lab stool is in no way inferior to the swivel chair when it comes to laboratory suitability. It is washable, resistant to disinfectants and robust. The PU-foam upholstery is very comfortable.

ORGANIC FORM
The surface of the seat is designed to offer maximum space. The user can sit on the stool in any direction.

RELEASE
The Labsit lab stool is infinitely height-adjustable. It can be easily operated using the rotating release ring.

Ergonomics

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LABSIT –
BRINGS COLOUR
TO THE LABORATORY.

Design which brings pleasure does not have to be at odds with technical criteria. Labsit is a master of both. Its attractive design brings colour to the workplace and meets all the requirements of a laboratory chair.

Ergonomics

LABSIT –
INTELLIGENT DESIGN,
SUCCESSFUL WORK.

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Instrument Catalogue

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THANK YOU


DEVYSER
Committed
Laboratory Instruments
Molecular Diagnostics
Biolytix
VWR LIFE SCIENCE
HARDY DIAGNOSTICS
Trusted
ZYMO RESEARCH
Sanger DNA Sequencing
Glentham LIFE SCIENCES
CLC bio A QIAGEN Company
Rare & Precious
Cryogatt
PACIFIC BIOSCIENCES
VILBER
Seegene
Next Generation DNA Sequencing
qPCR Analysis
FUJIFILM Value from Innovation
Wako
Agena
Mirus
vircell
Instruments Service, Repairs & Maintenance
Eppendorf Biotech
SNP Genotyping & Methylation Analysis
Cell Culture
Bioo Scientific
IKA
infopia
RONCO
NEW ENGLAND BioLabs Inc.
EDVOTEK
TreffLab
BioConcept
ORF GENETICS
DNA Synthesis (Primers & Probes)
PCR
Integrity
Thermo SCIENTIFIC
JKirsch
eppendorf
Animal Genetics
Bioinformatics Solution
Cleaver
Passionate