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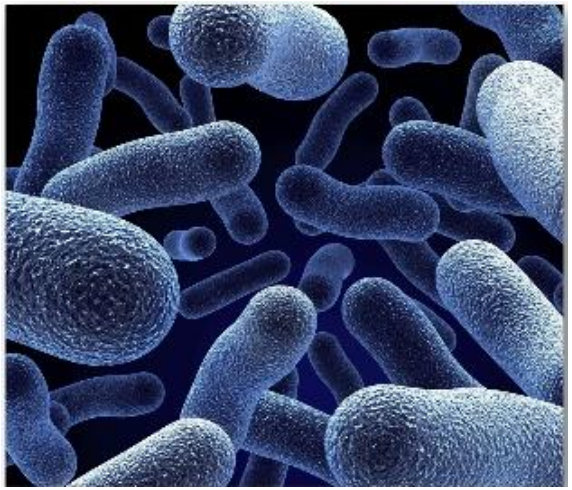
Molecular biology and Microbiology



Lecture 1a

Department of Medical laboratories

Theodoros Rampias
08/05/ 2017

Technological
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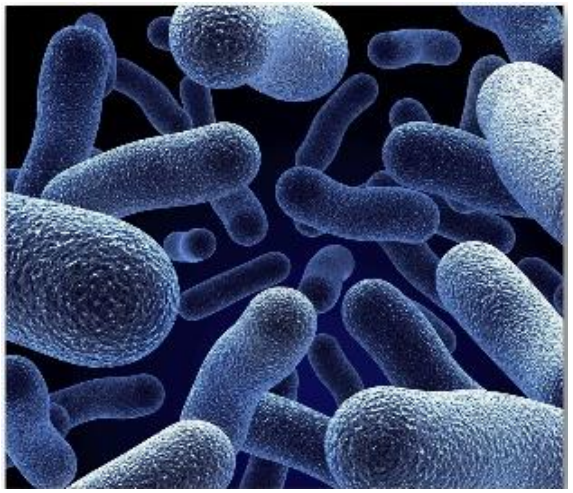
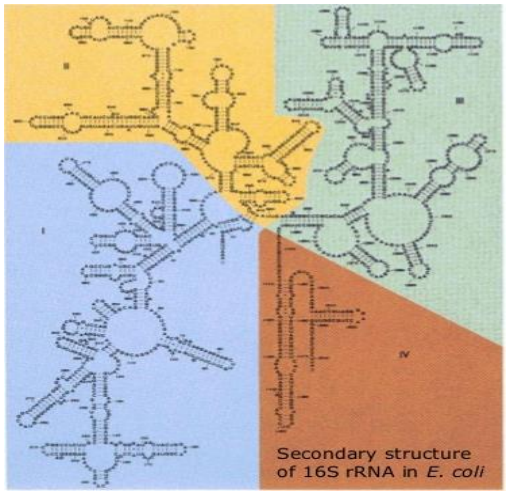
Molecular typing in Microbiology





Lecture 1b

Department of Medical laboratories

Theodoros Rampias

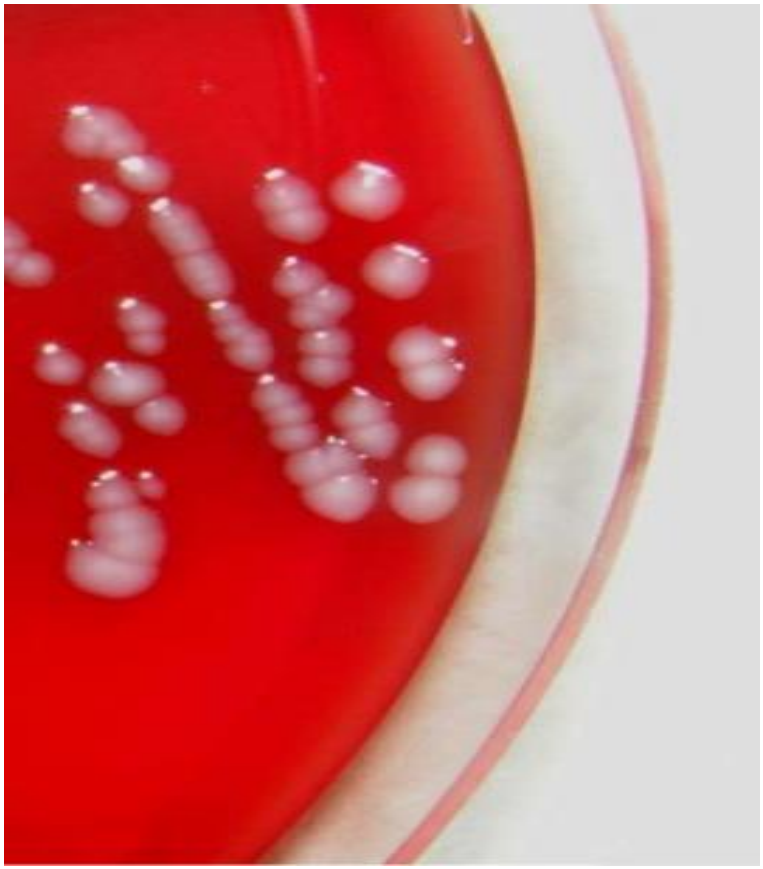
08/05/ 2017

- Clinical microbiologists are engaged in the field of diagnostic microbiology to determine whether **pathogenic microorganisms** are present in **clinical specimens collected from patients with suspected infections**. **If microorganisms are found, these are identified**

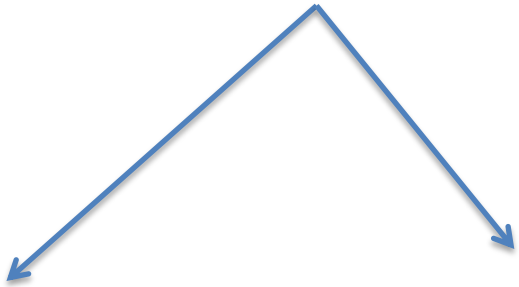
- During the past 3 decades, technical advances in the field of diagnostic microbiology have made constant and enormous progress in various areas, including **bacteriology**, **mycology**, **mycobacteriology**, **parasitology**, and **virology**.

- The diagnostic capabilities of modern clinical microbiology laboratories have improved rapidly and have expanded greatly due to a **technological revolution in molecular aspects of microbiology**.

- In particular, rapid techniques for nucleic acid amplification and characterization combined with automation have significantly broadened the diagnostic arsenal for the clinical microbiologist.



- **<u>Identification of pathogens</u>**
- **<u>Study of Antibiotic resistance</u>**

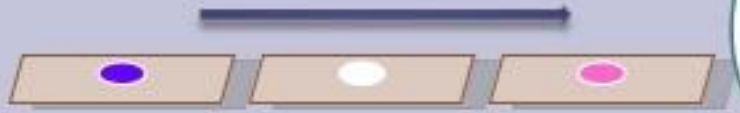


Phenotypic methods
-Microscopy/staining
-Biochemical reactions
-Serological reactions
-Susceptibility to anti-microbial agents

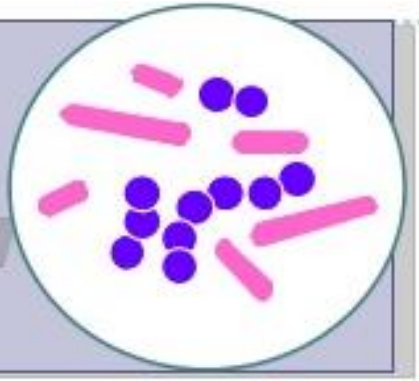
Genotypic methods
-Molecular Biology techniques

microscopy

unstained or stained with e.g. Gram stain



Stain Decolorise Counterstain



culture

on plates or in broth

identification by biochemical or serological tests on pure growth from single colony

sensitivities

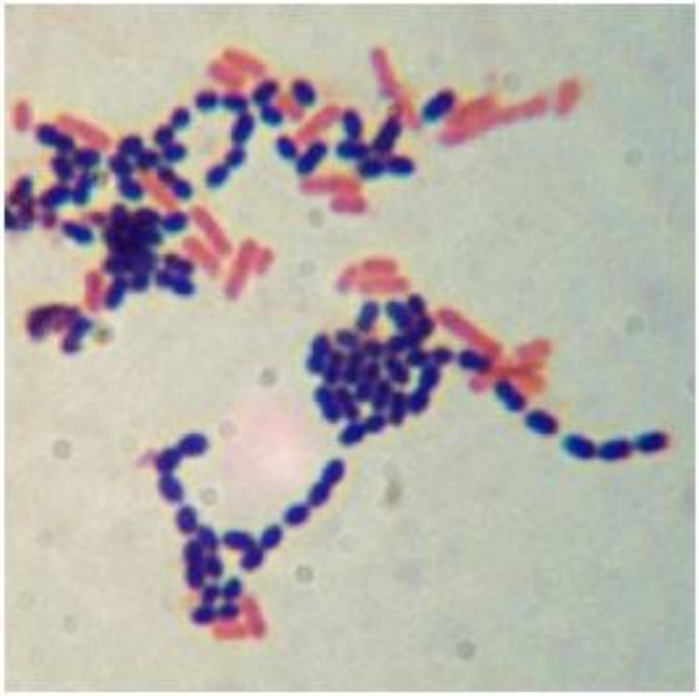
by disc diffusion methods, breakpoints or MICs

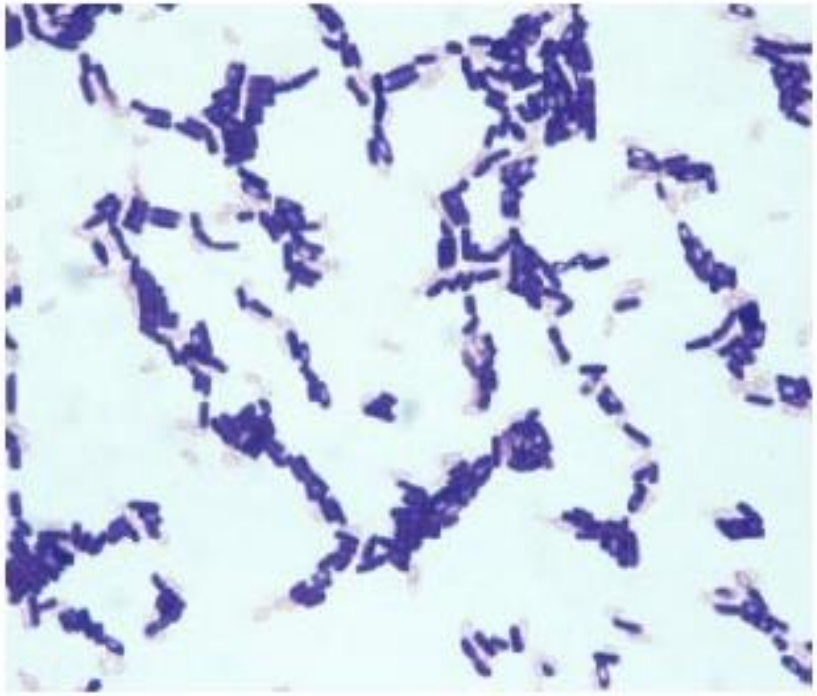
Serodiagnosis

DNA technologies

Routine laboratory methods

- Gram Stain





Routine laboratory methods (cont.)

- Biochemical characteristics

Catalase test

Indole test

Fermentation of sugars and H2S production

SUSCEPTIBILITY TESTING METHODS

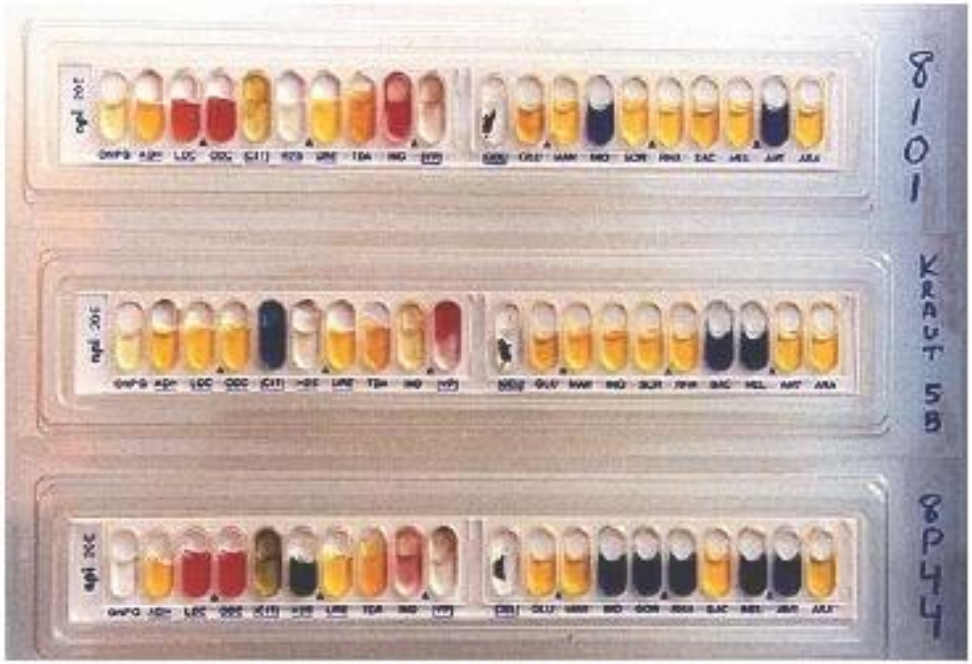
Inoculate
MH plate

Place disks
on agar plate

Incubate plate
18-24 hr, 35 C
Measure and record
zone of inhibition
around each disk

Routine laboratory methods (cont.)

- Commercial identification systems
  - API , Rapid ANA

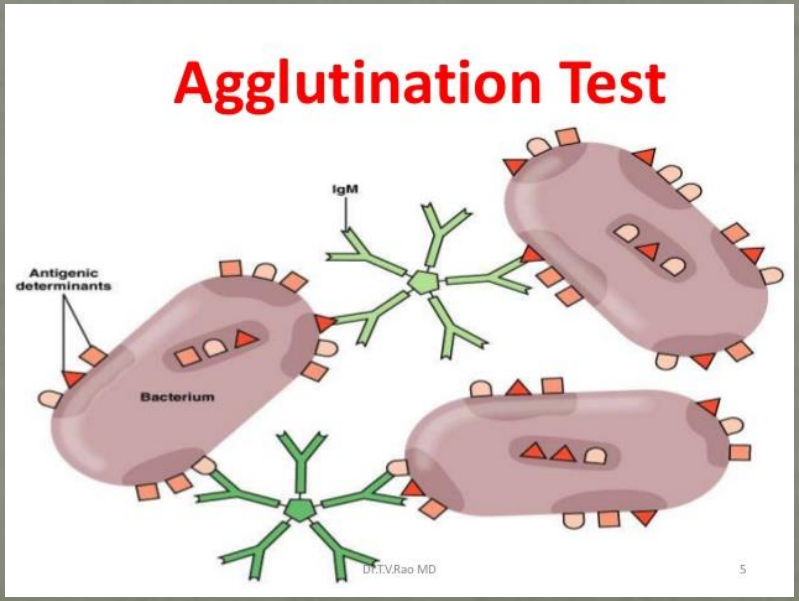


API identification system

Rapid ANA identification system

Serotyping

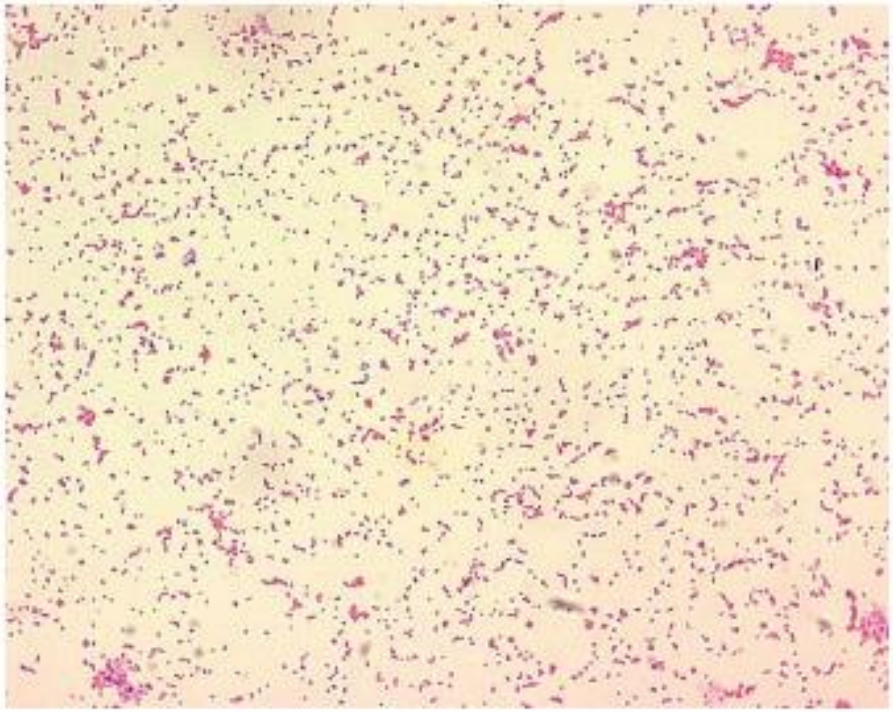
- Based on antigenic determinants expressed on the cell surface
- referred as 'Serotypes'



Problems with phynotypical methods

- Some bacterial isolates stain poorly or exhibit unique catabolic or growth patterns

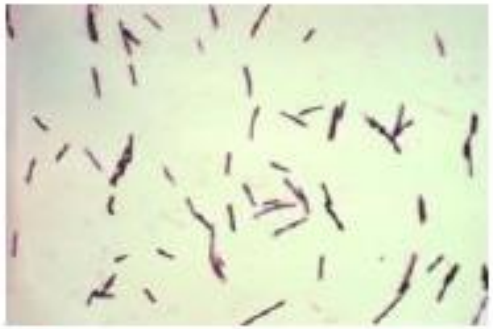
Growth after 48H



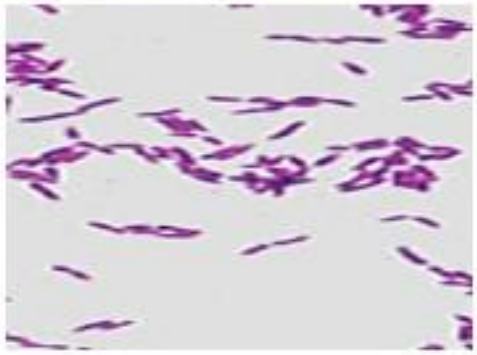
Ambiguous Gram stain result

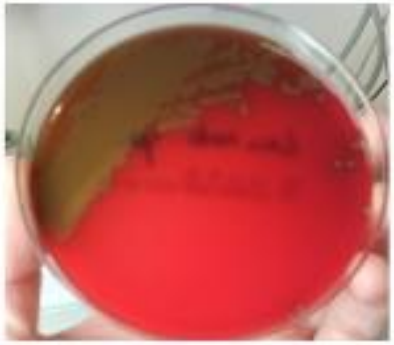
Lactobacillus sp. versus *Clostridium tertium*

- Common characteristics: slender gram-positive rods, Alpha hemolytic, grow in aerobic conditions, catalase neg., oxidase neg.



Clostridium tertium

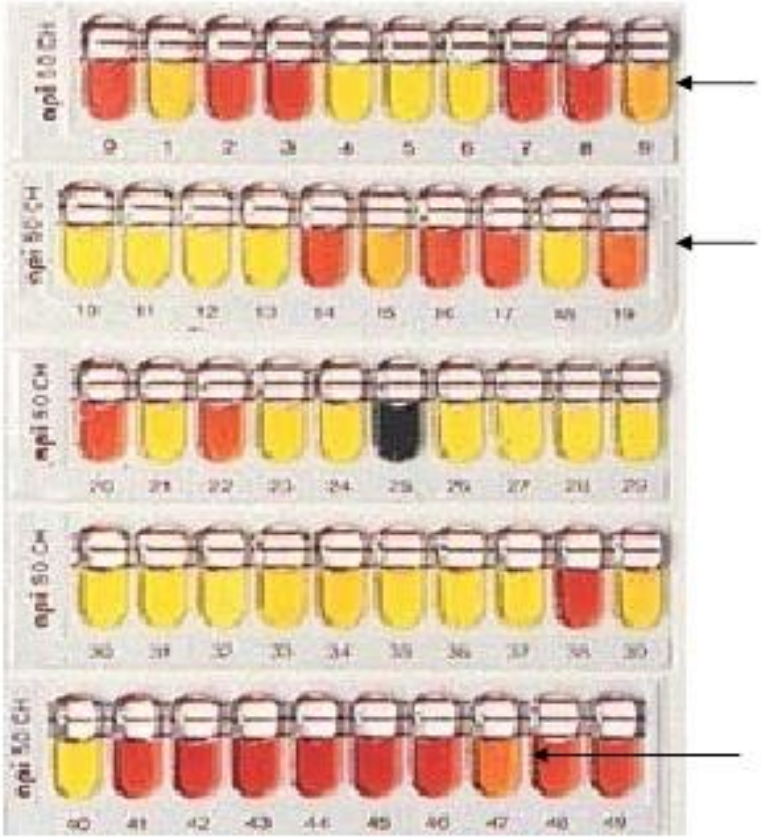


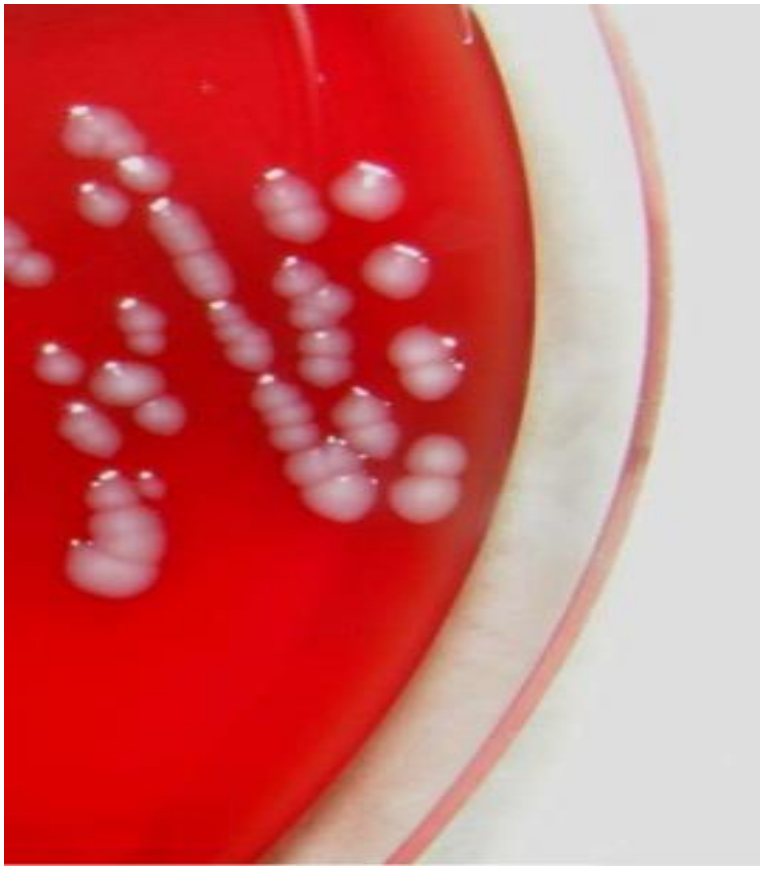


Lactobacillus sp.

Problems with phonotypical methods

- Biochemical readings are subject of variation and dependent on individual interpretation and expertise.





Identification of pathogens

Phenotypic methods
-Microscopy/staining
-Biochemical reactions
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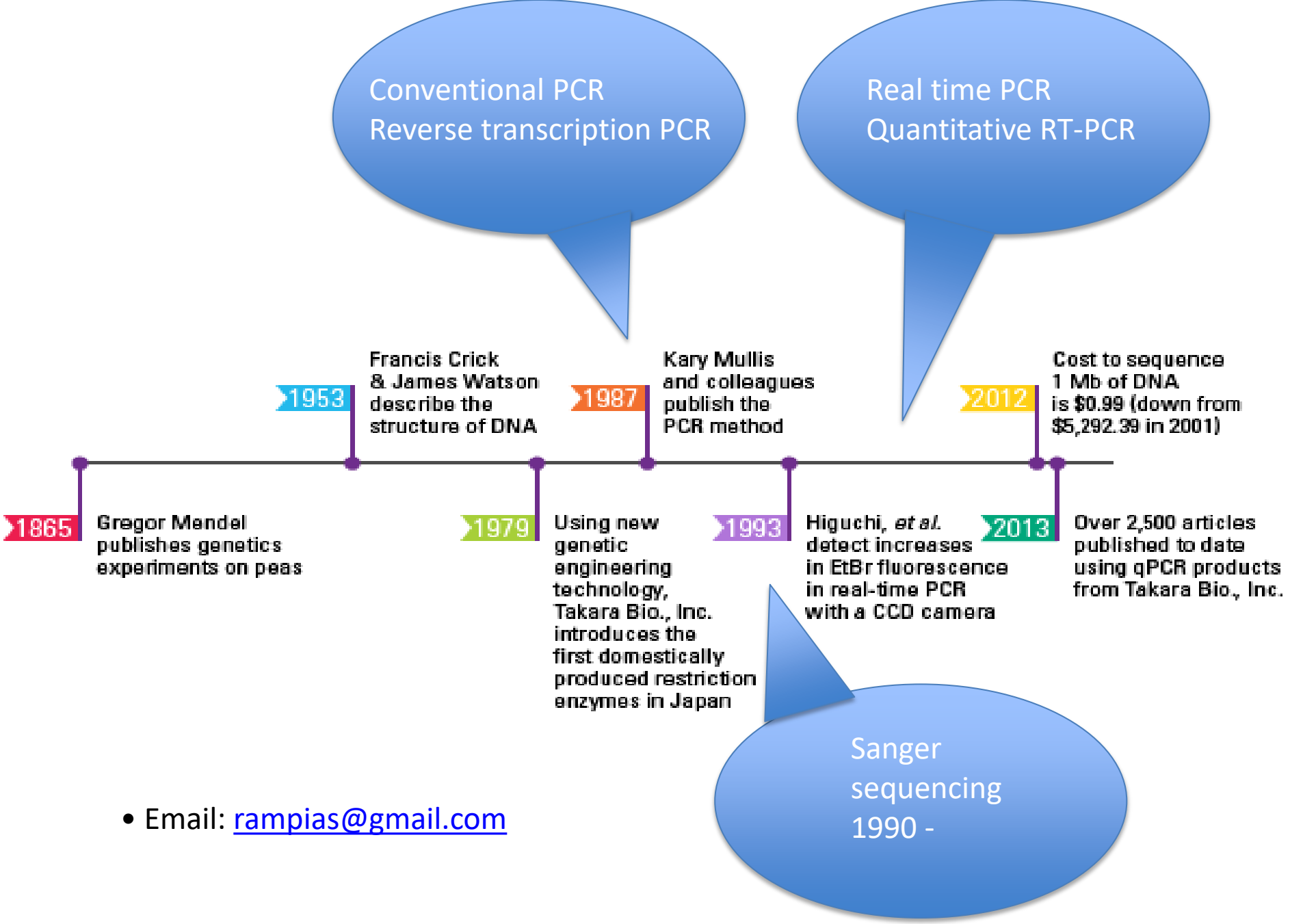
-Susceptibility to anti-microbial agents

Genotypic methods
-Molecular Biology techniques

Targets the DNA

Molecular typing

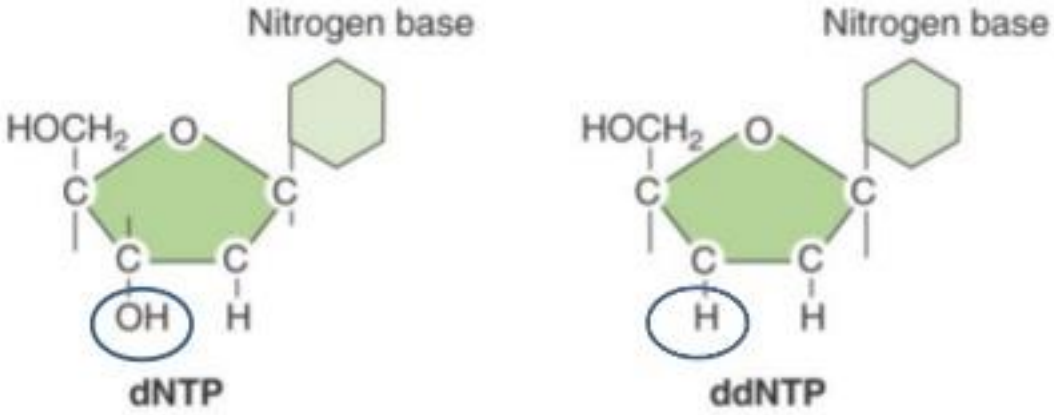
- Stable target
- Objective
- No specific growth requirements
- Easier to standartize

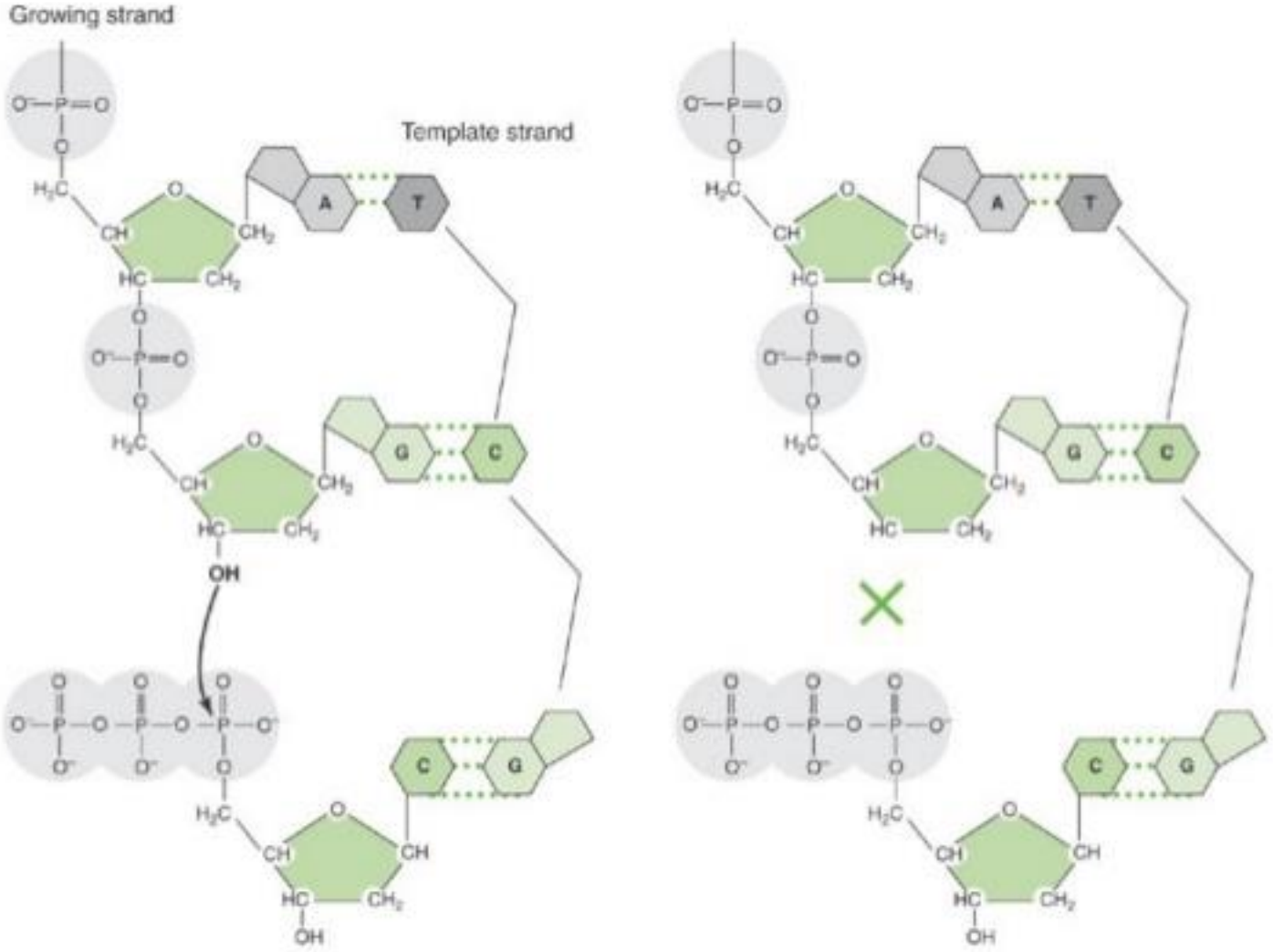


- Email: rampias@gmail.com

Sanger; Chain Termination Sequencing

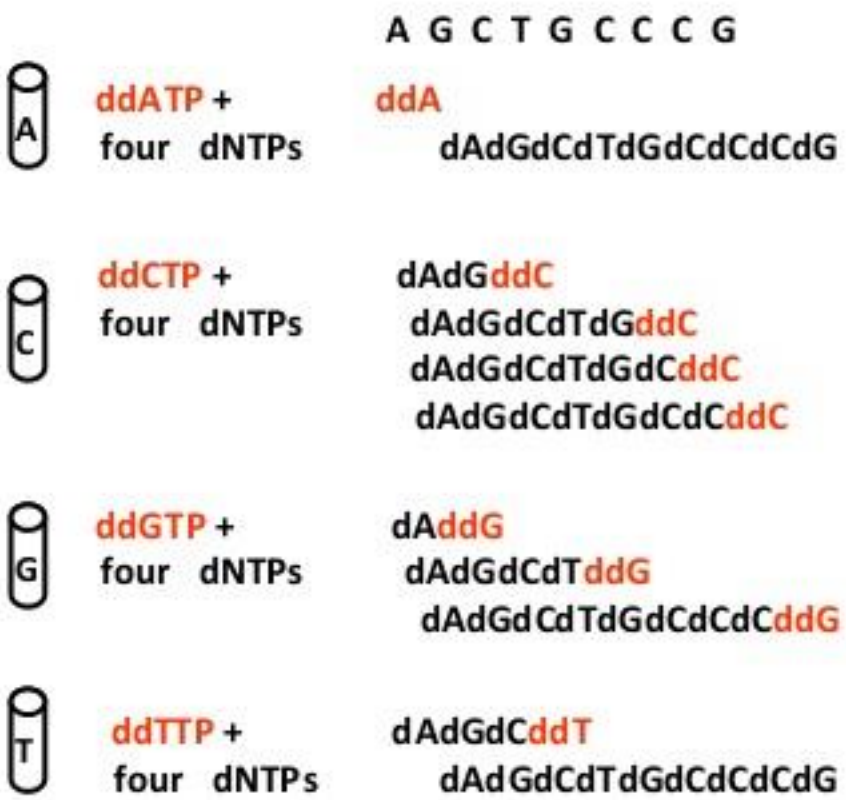
- It is PCR based method
- A modified DNA replication reaction
- Growing chains are terminated by dideoxynucleotides





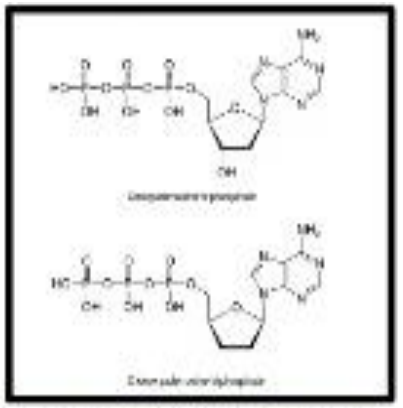
The 3′-OH group necessary for formation of the phosphodiester bond is missing in ddNTPs

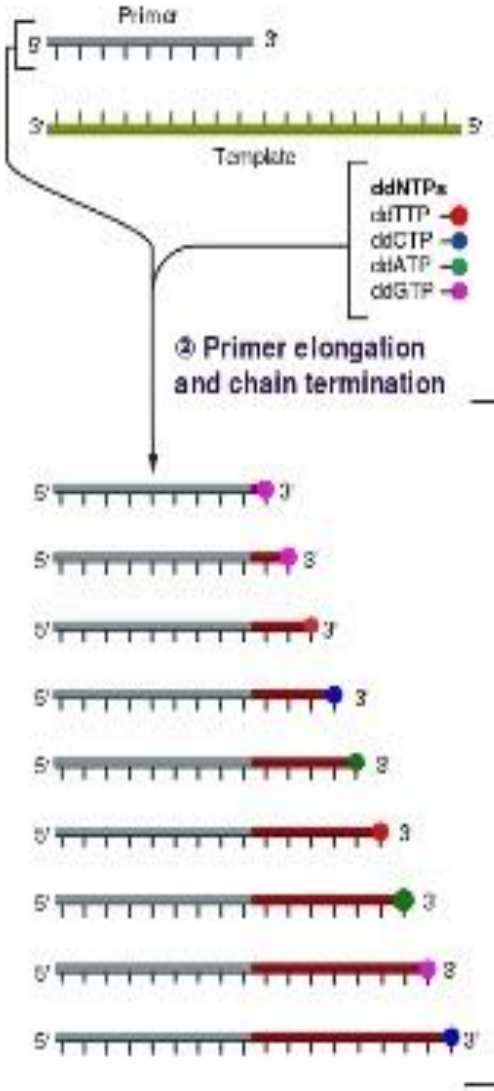
Sanger; Chain Termination Sequencing



① Reaction mixture

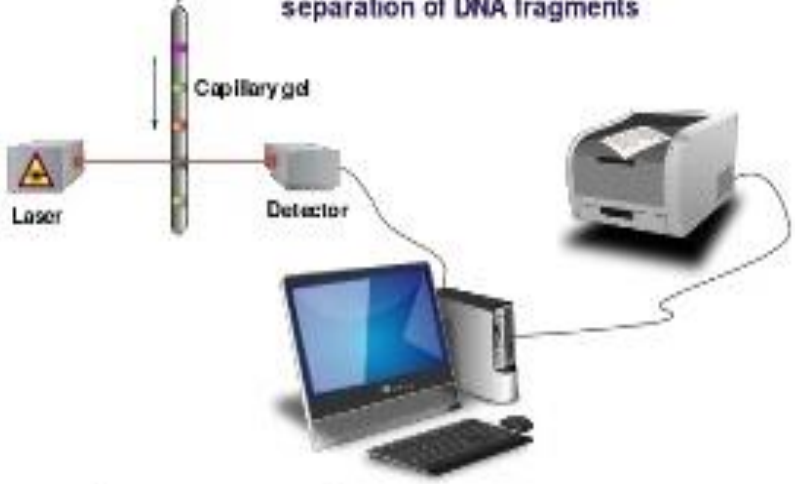
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)

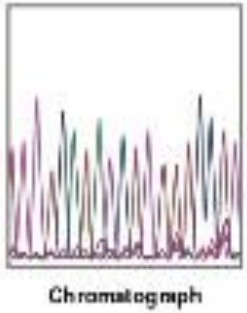




② Primer elongation and chain termination

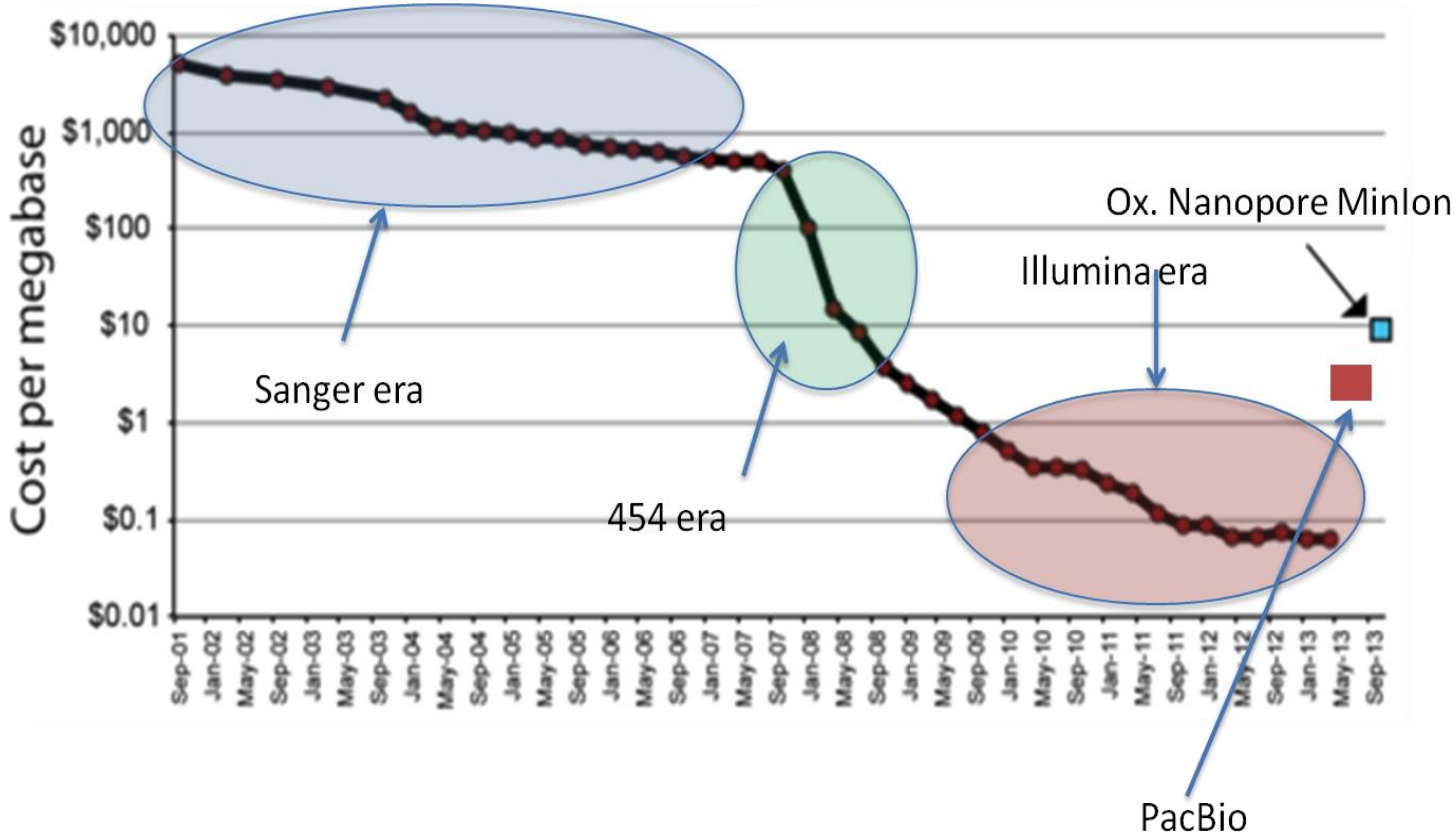
③ Capillary gel electrophoresis separation of DNA fragments





④ Laser detection of flourochromes and computational sequence analysis

Cost per megabase

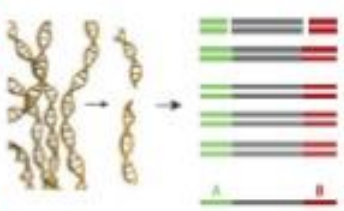


Second Generation Sequencing

- Developed to increase throughput of Sanger sequencing
- Can sequence many molecules in parallel
  - Does not require homogenous input
  - Sequenced as clusters
- Sequencing by synthesis
  - Bases are added, signals scanned, and then washed
  - Cycle repeated (30-2000x)

Next-generation DNA sequencing

1. Library preparation
2. Clonal amplification
3. Cyclic array sequencing



DNA fragmentation and in vitro adaptor ligation

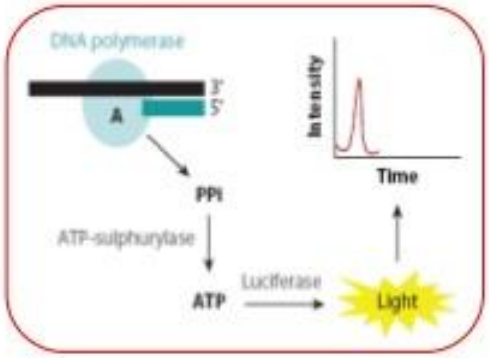
emulsion PCR

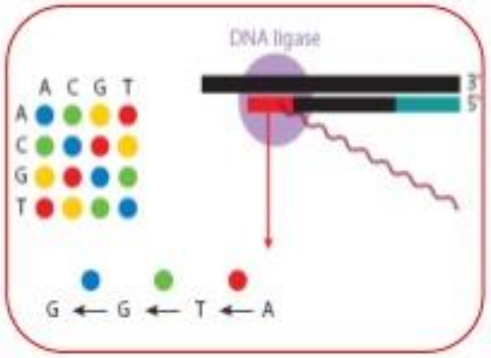
bridge PCR

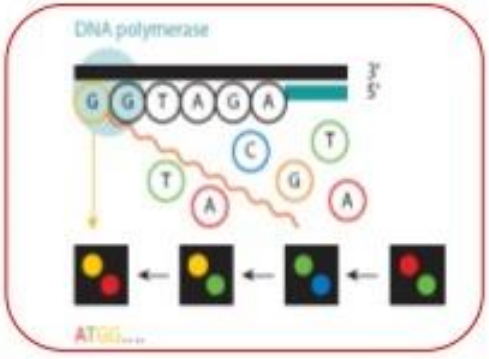
Pyrosequencing

Sequencing-by-ligation

Sequencing-by-synthesis





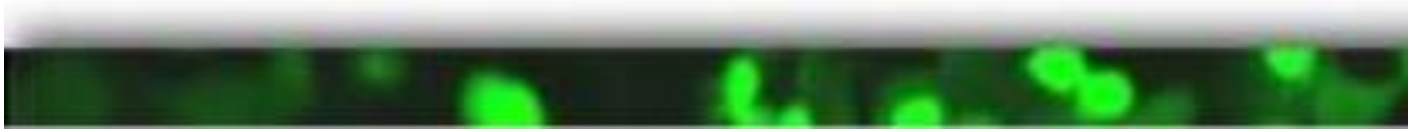


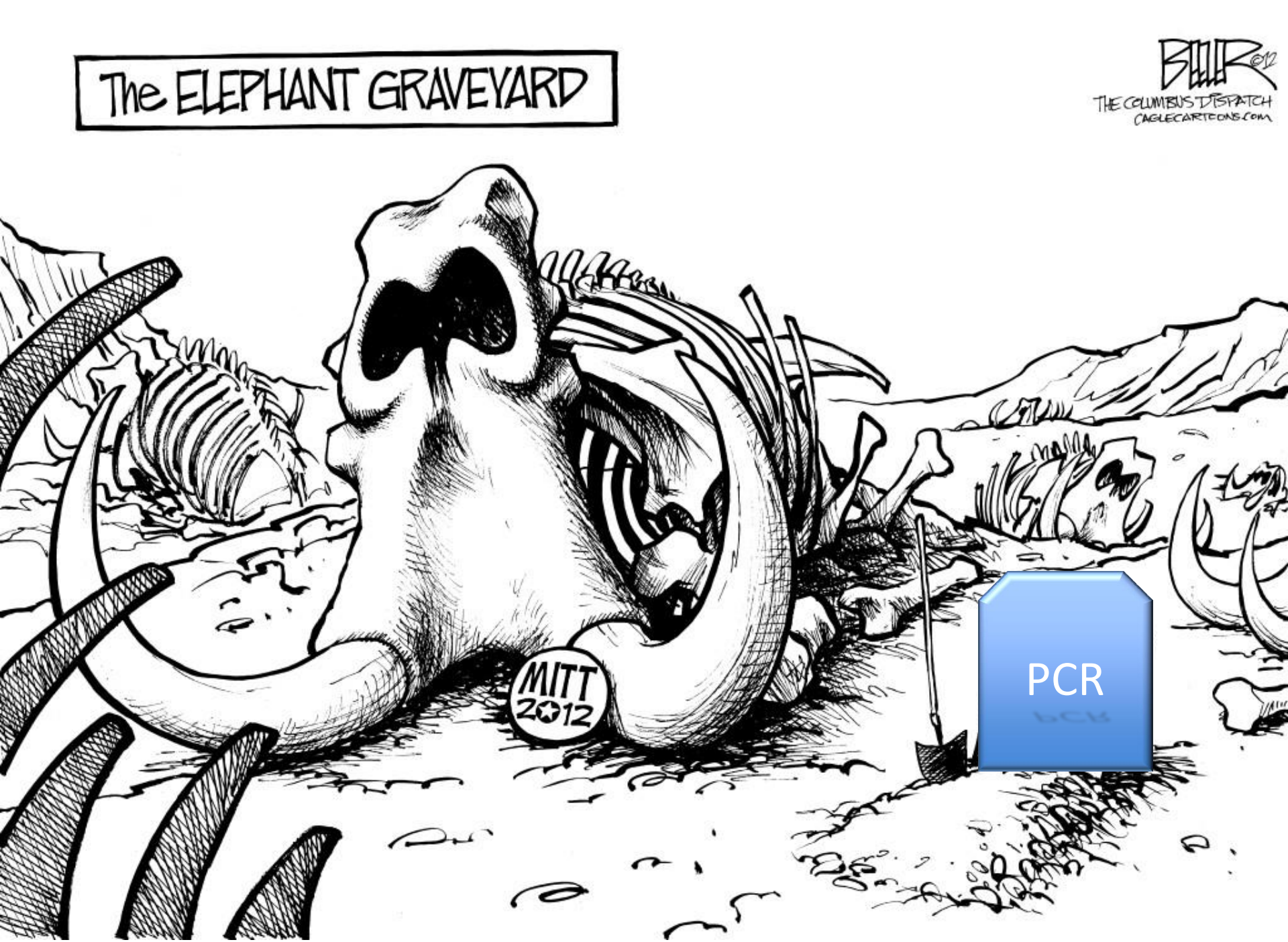
454 sequencing

SOLiD platform

Solexa technology

- Human genome took 10 years to complete and cost $3 billion dollars
  - Done by laboriously cloning overlapping segments of the human genome into bacmid libraries and Sanger sequencing each one
  - Genome assembled using computers to line up over lapping sequences
- Current estimate is around $4000
  - Can be completed in a week
  - Companies like Complete Genomics say they have already sequenced thousands of human genomes
- Future
  - Long read sequencers will make agricultural sequencing more viable
  - Whole genome sequencing for human diagnostics will become routine
  - Increasing the catalog of organismal genomes will improve our understanding of evolution and development




The ELEPHANT GRAVEYARD
BLAIR ©12
THE COLUMBUS DISPATCH
CAGLECARTOONS.COM
MITT 2012
PCR

What is 'omics'?

- OMICS
  - The term "omic" is derived from the Latin suffix "ome" meaning mass or many. Thus, OMICS involve a mass (large number) of measurements per endpoint. (Jackson et al., 2006)
- Integration of OMICS data
  - Efficient integration of data from different OMICS can greatly facilitate the discovery of true causes and states of disease, mostly done by softwares (Andrew et al., 2006).

What is 'omics'?

- In biological context , suffix –omics is used to refer to the study of large sets of biological molecules (Smith et al., 2005)
- The realization that DNA is not alone regulate complex biological processes (as a result of HGP, 2001), triggered the rapid development of several fields in molecular biology that together are described with the term OMICS.
- The OMICS field ranges from
  - Genomics (focused on the genome)
  - Proteomics (focused on large sets of proteins, the proteome)
  - Metabolomics (focused on large sets of small molecules, the metabolome).

TYPES OF OMICS

- Genomics
- Computational genomics
- Epigenomics
- Functional genomics
- Immunomics
- Metagenomics
- Pathogenomics
- Regenomics
- Personal genomics
- Proteomics
- Psychogenomics

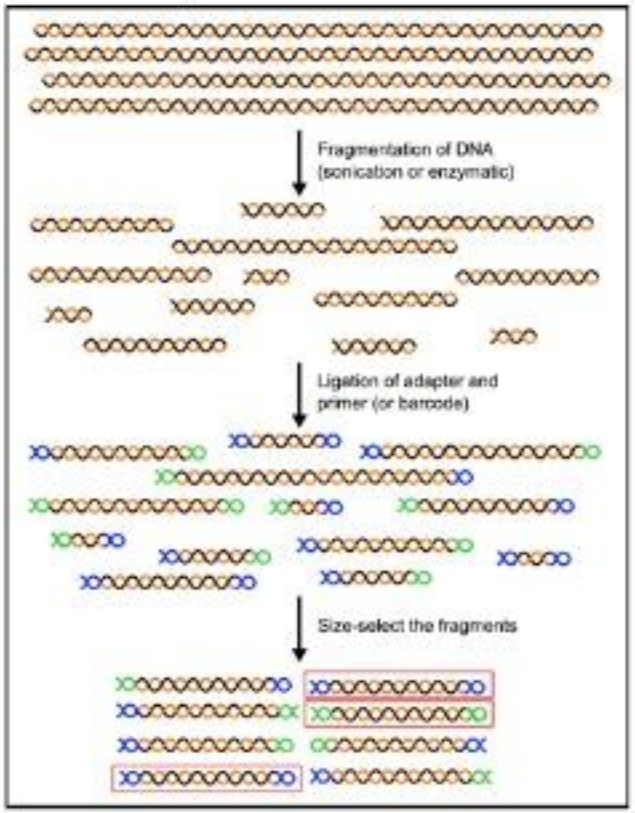
Overview of the different OMICS technologies

Technology	Molecules of interest	Definition	Temporal variance	Disease influence
Genotyping	DNA	Assessment of variability in DNA sequence in the genome	None	No
Epigenomics	Epigenetic modifications of DNA	Assessment of factors that regulate gene expression without changing DNA sequence of the genome	Low / Moderate	Probable
Gene expression profiling	RNA	Assessment of variability in composition and abundance of the transcriptome	High	Yes
Proteomics	Proteins	Assessment of variability in composition and abundance of the proteome	High	Yes
Metabolomics	Small molecules	Assessment of variability in composition and abundance of the metabolome	High	Yes

(Jelle et al., 2010)

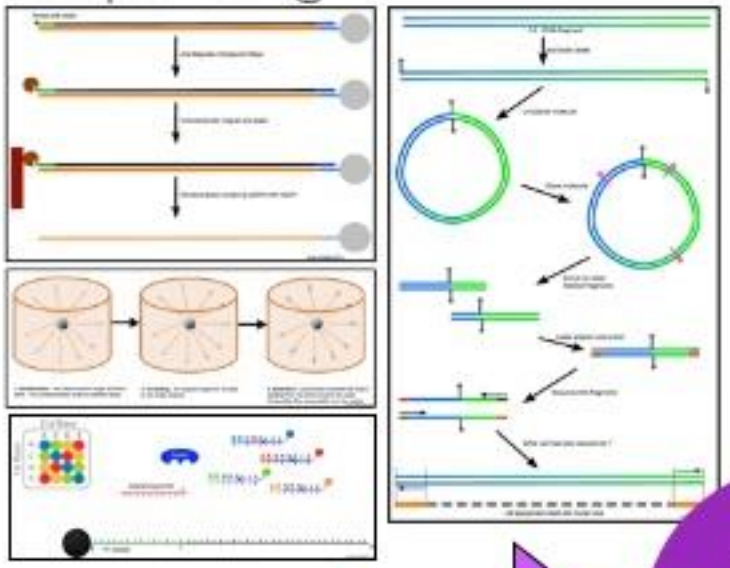
NGS

Library Construction



http://users.ugent.be/~avierstr/nextgen/nextgen.html

Sequencing

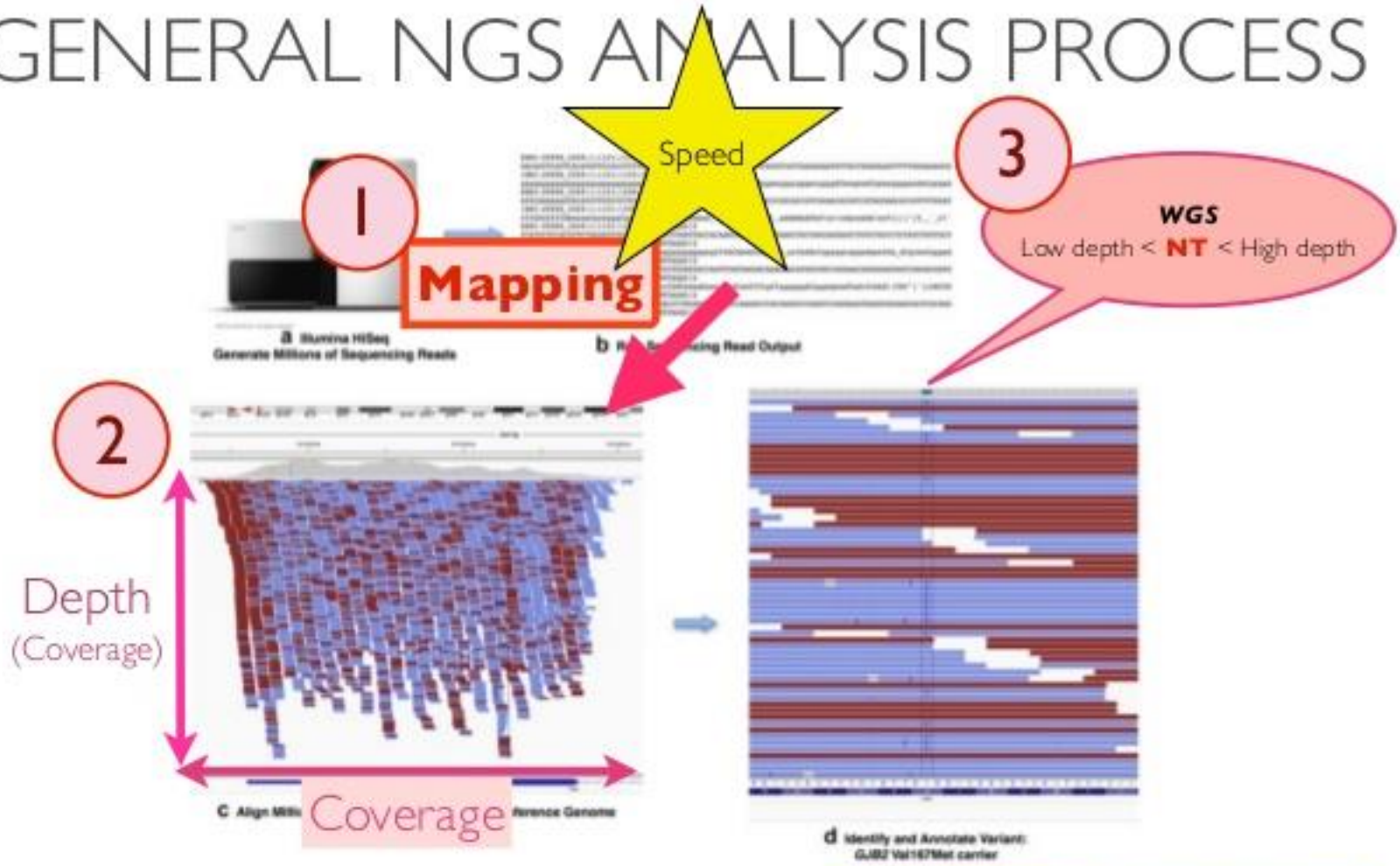


Raw Reads

Theragen bio institute

TotalOmics solutions

GENERAL NGS ANALYSIS PROCESS



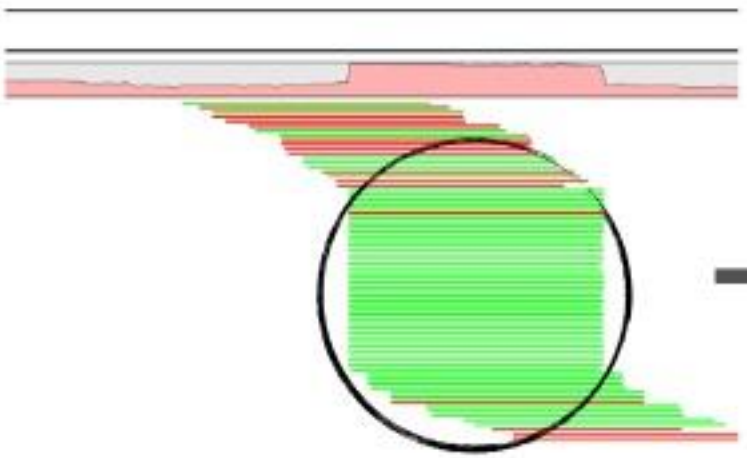
Shearer AE, Hildebrand MS, Sloan CM, Smith RJ. Deafness in the genomics era. Hear Res. 2011 Dec;282(1-2):1-9. doi: 10.1016/j.heares.2011.10.001. Epub 2011 Oct 8.

Theragen bio institute

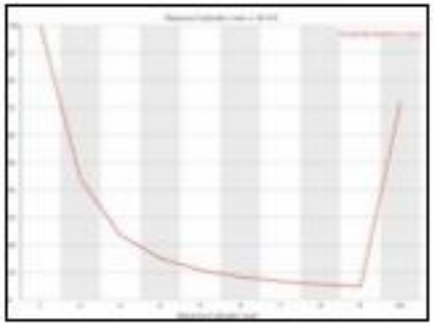
TotalOmics solutions

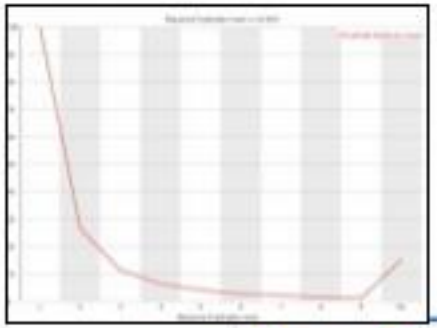
PCR DUPLICATION

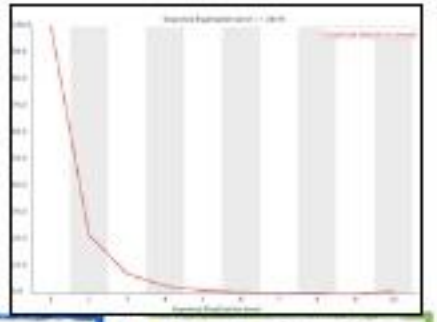
http://www.clcbio.com/clc-plugin/duplicate-reads-removal-plugin/



remove







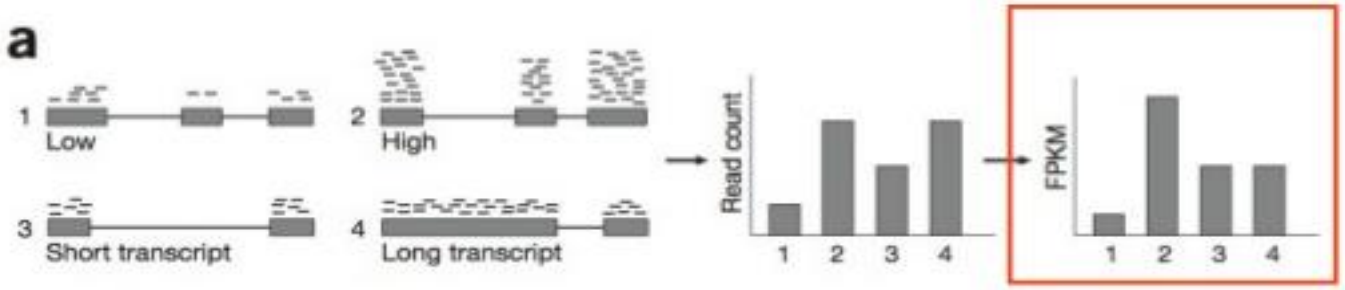
Theragen bio institute

TotalOmics solutions

RNASeq

NORMALIZATION

- Read counts need to be properly normalized to extract meaningful expression estimates
  - First, RNA fragmentation during library construction causes longer transcripts to generate more reads compared to shorter transcripts present at the same abundance in the sample
  - Second, the variability in the number of reads produced for each run causes fluctuations in the number of fragments mapped across samples



Garber M, Grabherr MG, Guttman M, Trapnell C. Computational methods for transcriptome annotation and quantification using RNA-seq. Nat Methods. 2011 Jun;8(6):469-77.

PCR

Αλυσιδωτή Αντίδραση Πολυμεράσης (PCR)

- Η αλυσιδωτή αντίδραση πολυμεράσης είναι μέθοδος παραγωγής μεγάλου αριθμού αντιγράφων συγκεκριμένων μικρών αλληλουχιών DNA – λογαριθμική αύξηση

PCR: 1985, Βραβείο Nobel για τον Kary Mullis in 1993

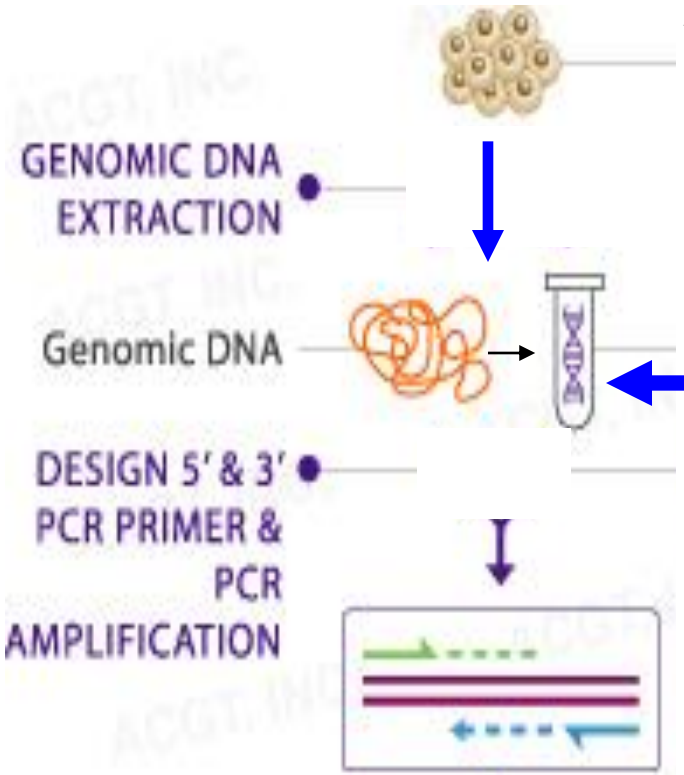
Three Aspects of PCR

- Specificity
- Efficiency
- Fidelity

Αναλώσιμα

PCR απαιτούνται:

- Magnesium chloride: .5-2.5mM
- Buffer: pH 8.3-8.8
- dNTPs: 20-200µM
- Primers: 0.1-0.5µM
- DNA Polymerase: 1-2.5 units
- Target DNA: **1–10 µg/ml**



χρησιμοποιείται ένα ζεύγος εκκινητών (primers) που έχουν συμπληρωματική αλληλουχία με τα άκρα του DNA στόχου

+ $MgCl_2$

+ Buffer διάλυμα

+ dNTPs

+ Taq polymerase

+ εκκινητές

ηλεκτροφόρηση

Ένας χώρος → χρήση θαλάμων βιολογικής ασφάλειας, χρήση απολυμαντικών και UV

- **Όχι σε χώρο καλλιεργειών μικροοργανισμών!**
- Χρονικός χωρισμός!

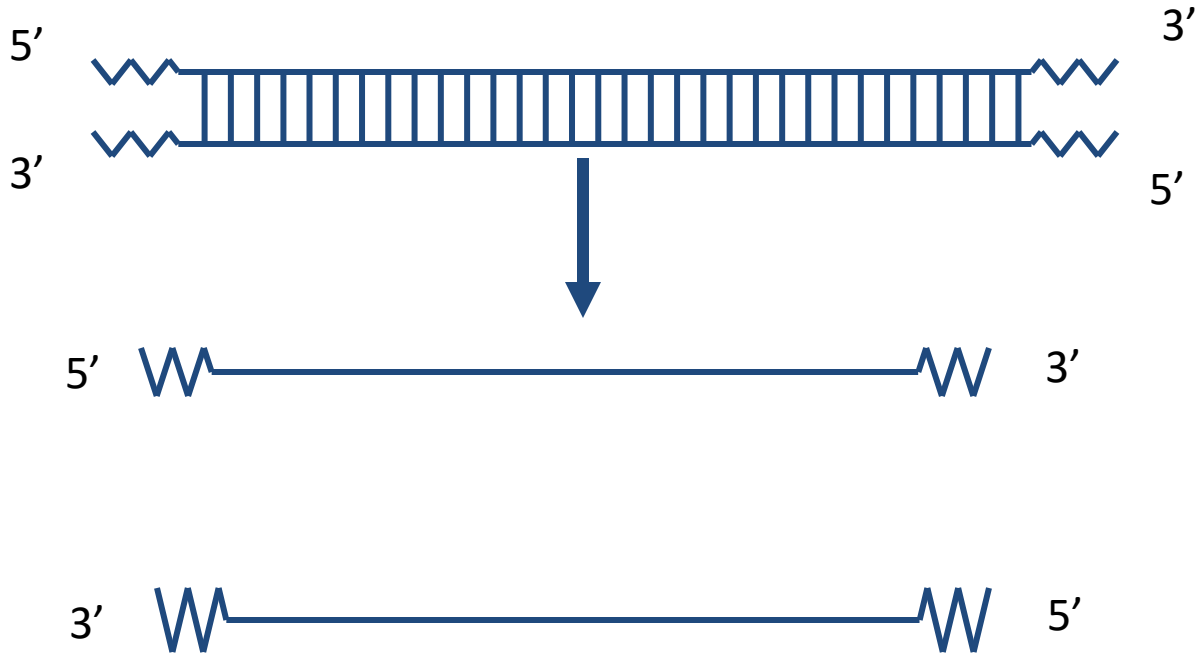
ΘΕΡΜΑΝΣΗ (94^{o}C) ΓΙΑ ΤΗΝ ΑΠΟΔΙΑΤΑΞΗ ΤΩΝ DNA ΑΛΥΣΙΔΩΝ

ΨΥΞΗ (52-61^{o}C) ΓΙΑ ΤΗΝ ΥΒΡΙΔΟΠΟΙΗΣΗ ΤΩΝ ΕΚΚΙΝΗΤΩΝ ΣΤΟ DNA ΥΠΟΣΤΡΩΜΑ

ΘΕΡΜΑΝΣΗ (72^{o}C) ΓΙΑ ΤΗΝ ΕΝΕΡΓΟΠΟΙΗΣΗ ΤΗΣ *Taq* ΠΟΛΥΜΕΡΑΣΗΣ, ΠΟΥ ΠΟΛΥΜΕΡΙΖΕΙ ΤΟ DNA

ΕΠΑΝΑΛΗΨΗ 35 ΚΥΚΛΟΙ

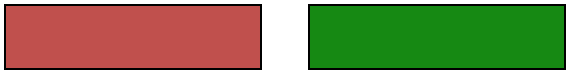
ΑΠΟΔΙΑΤΑΞΗ DNA



94ºC

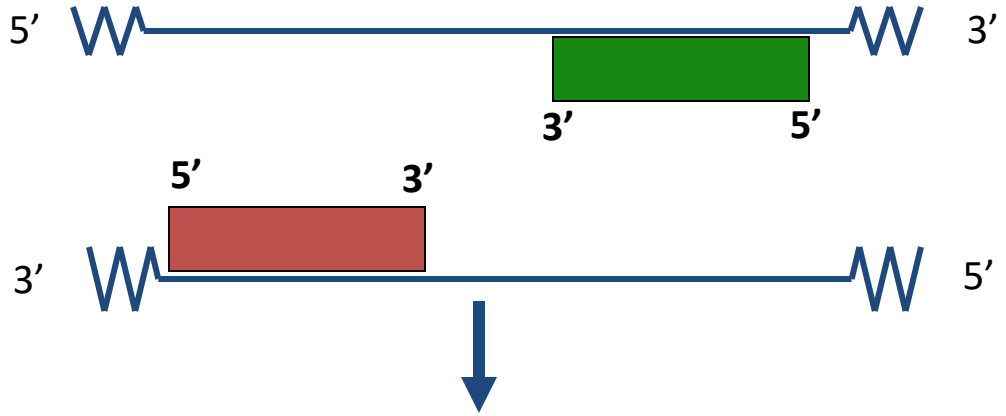
ΥΒΡΙΔΟΠΟΙΗΣΗ ΕΚΚΙΝΗΤΩΝ

ΕΚΚΙΝΗΤΕΣ

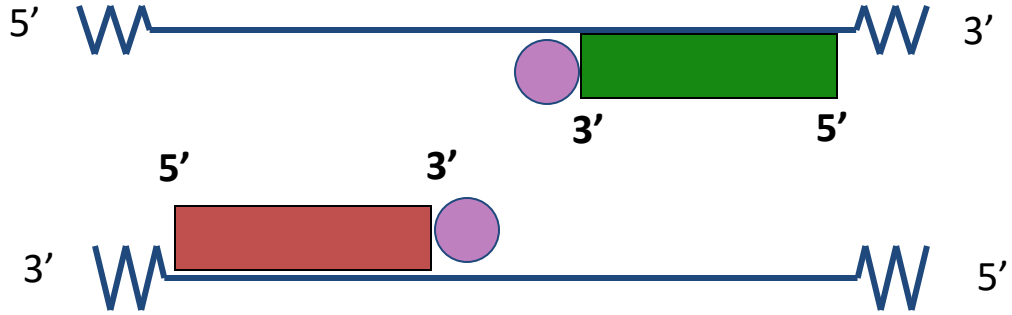


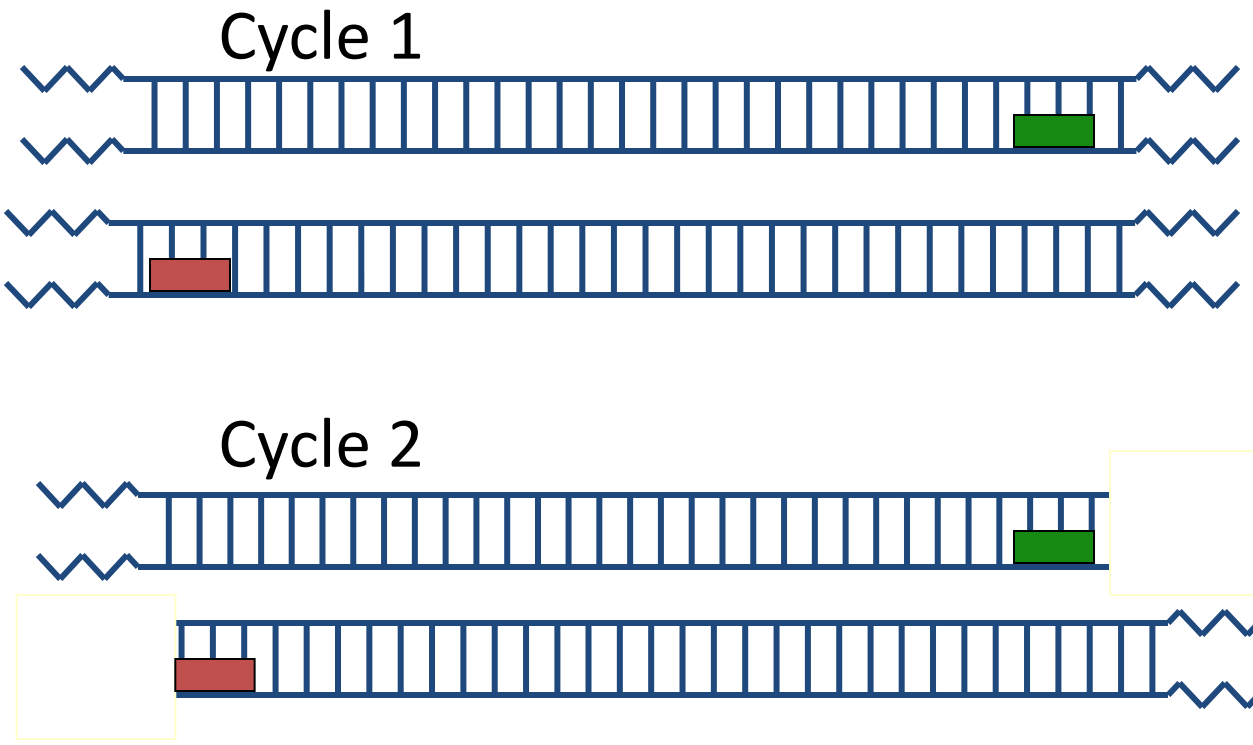
Η *Taq* ΠΟΛΥΜΕΡΑΣΗ ΑΝΑΓΝΩΡΙΖΕΙ ΤΟ 3’ ΑΚΡΟ ΤΟΥ ΕΚΚΙΝΗΤΗ ΚΑΙ ΤΗΝ ΜΗΤΡΙΚΗ ΑΛΥΣΙΔΑ

ΟΙ ΕΚΚΙΝΗΤΕΣ ΥΒΡΙΔΟΠΟΙΟΥΝΤΑΙ ΣΤΟΥΣ 52^{o}C

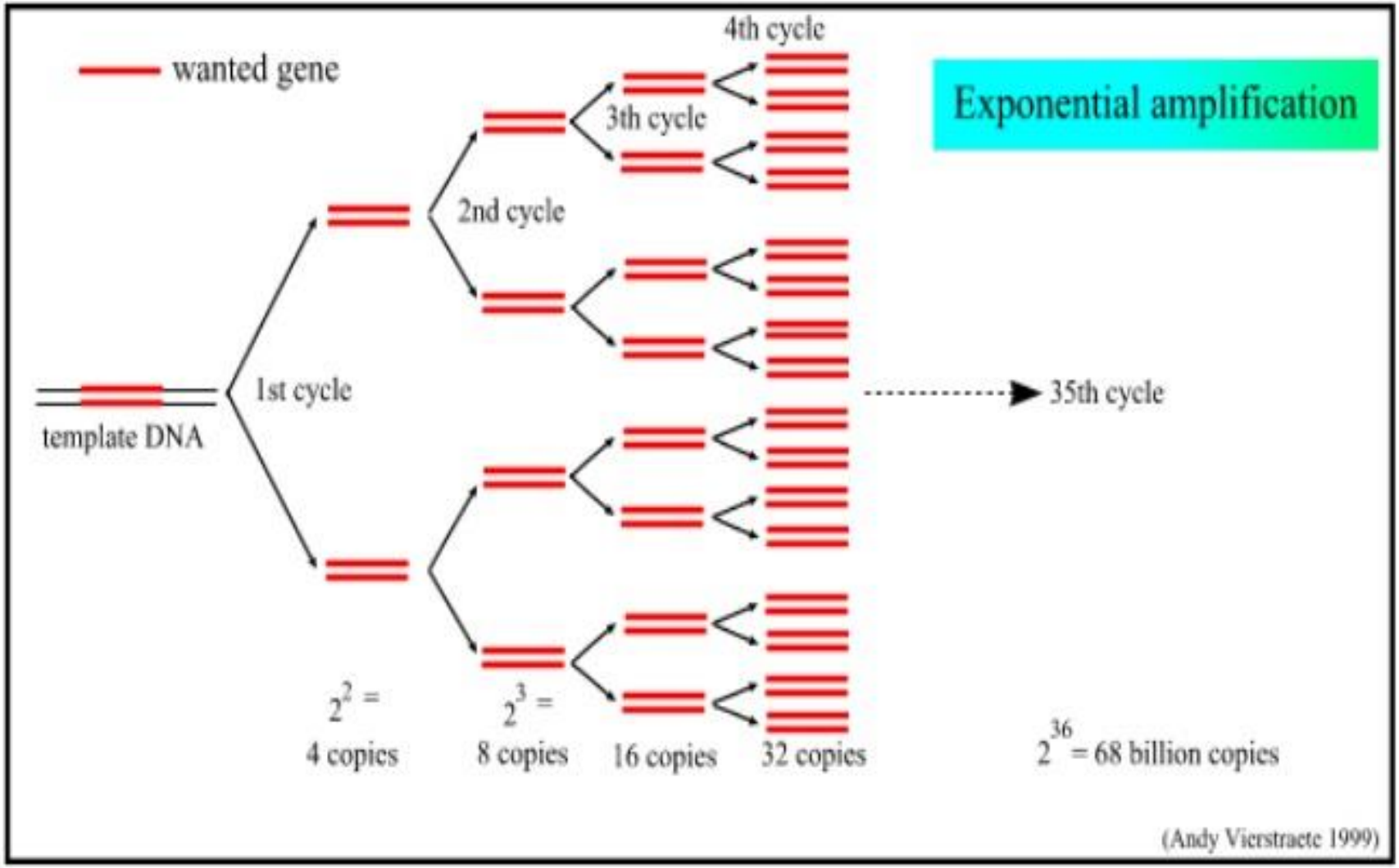


Η *Taq* ΠΟΛΥΜΕΡΙΖΕΙ ΣΤΟΥΣ 72^{o}C

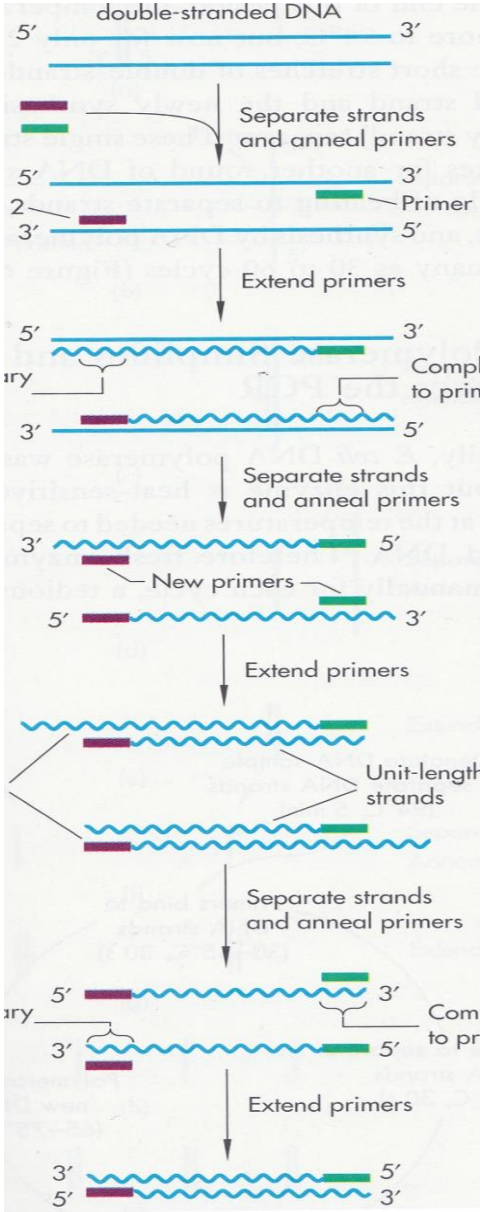


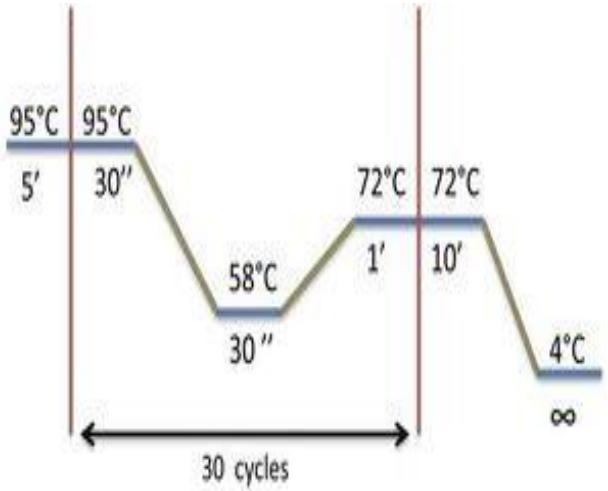


ΤΟ ΑΚΡΙΒΕΣ ΜΕΓΕΘΟΣ ΤΟΥ PCR ΠΡΟΙΟΝΤΟΣ ΚΑΘΟΡΙΖΕΤΑΙ ΣΤΟΝ ΤΡΙΤΟ ΚΥΚΛΟ


wanted gene
Exponential amplification
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^2 =
4 copies
2^3 =
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

Αλυσιδωτή Αντίδραση Πολυμεράσης (PCR)





- Βασίζεται στην ιδιότητα των αλυσίδων του DNA

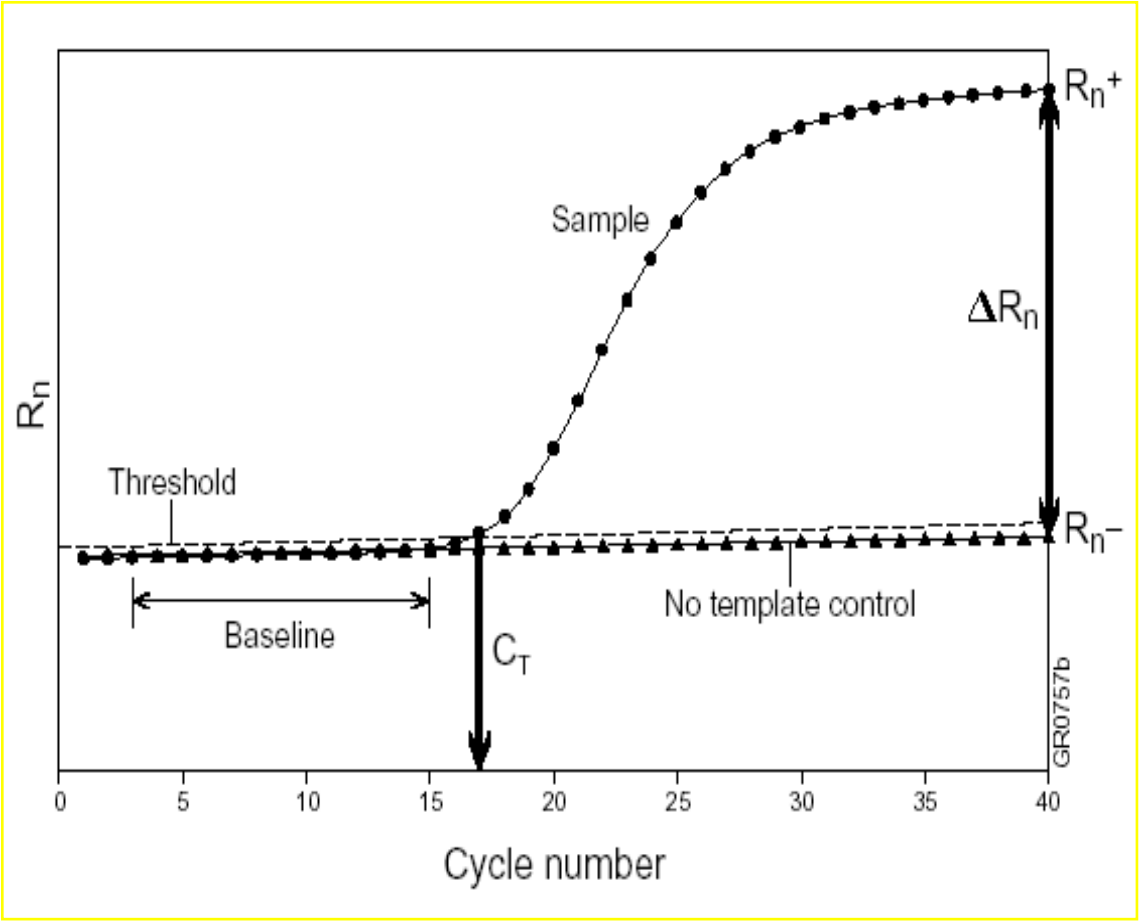
  α) να αποχωρίζονται σε υψηλή θερμοκρασία και να επανενώ νονται σε χαμηλότερη θερμοκρασία και

  β) να αντιγράφονται
- Παράγονται εκατομμύρια αντιγράφων

Κινητική της αντίδρασης της PCR

Component	Final Concentration
10x *Taq* Buffer (2.5 mM Mg^{2+})	1x
dNTPs Mix (10 mM)	0.2 mM
Tubulin forward (10 µM)	300 nM
Tubulin reverse (10 µM)	300 nM
Human Template DNA (26 ng/µL)	26 ng
Taq DNA Polymerase (5 U/µL)	1 U
Molecular Biology Grade Water	--

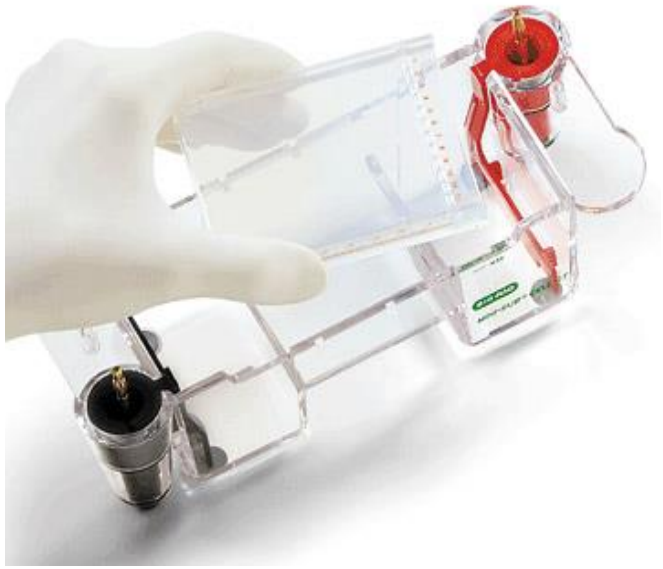
Table 3: Reaction setup for Tubulin.



Agarose
Electrophoresis

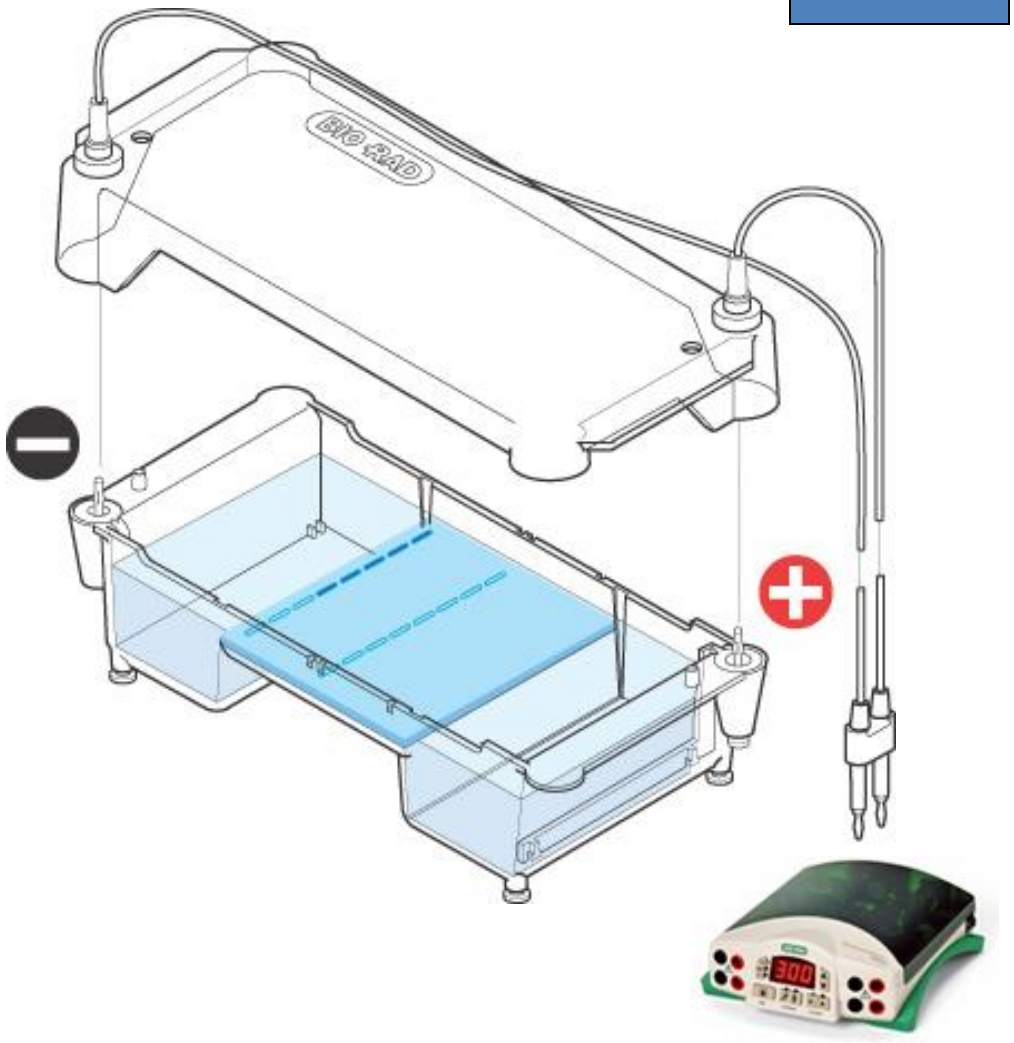
Place gel in gel box

Pour buffer in box until gel wells are covered.



Place 20ul of samples into appropriate wells

Set up electrophoresis chamber by putting top in place and connecting it to the power supply



Agarose

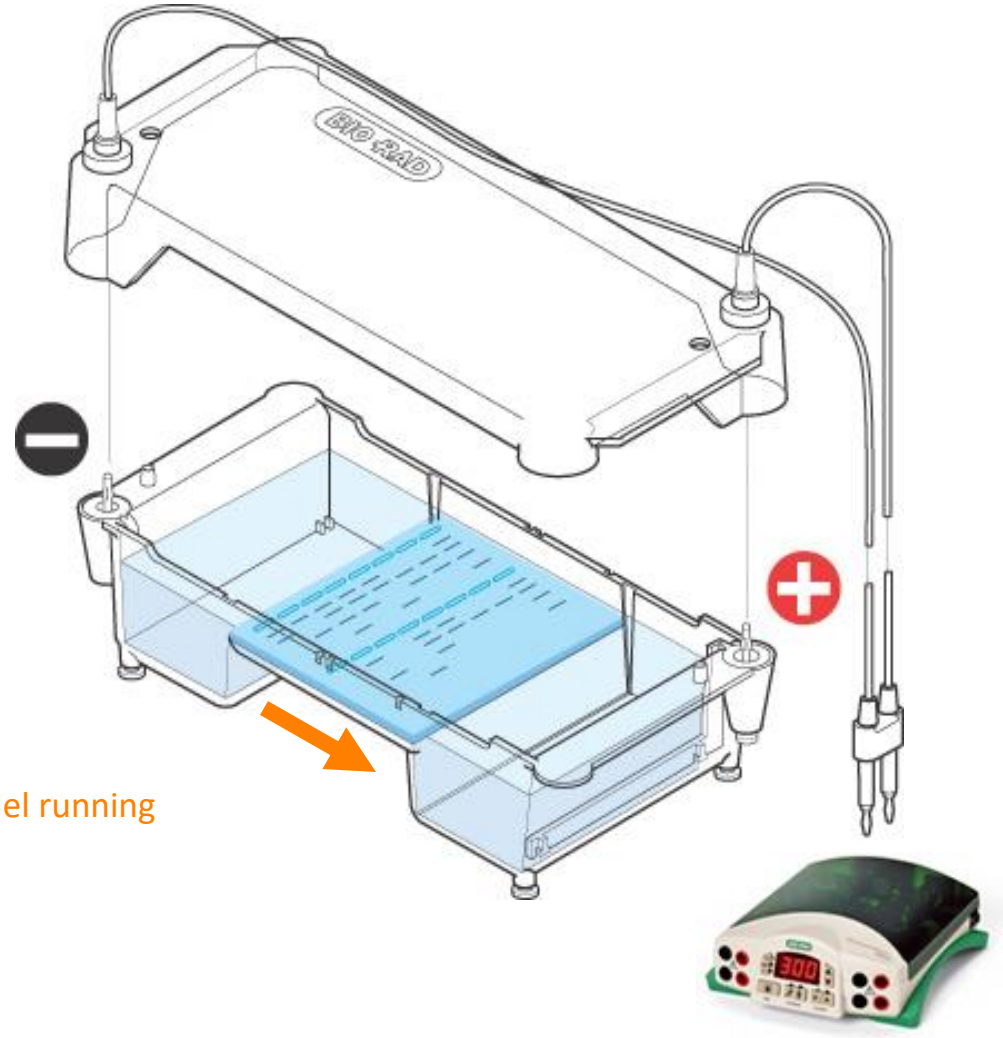
Electrophoresis

Running

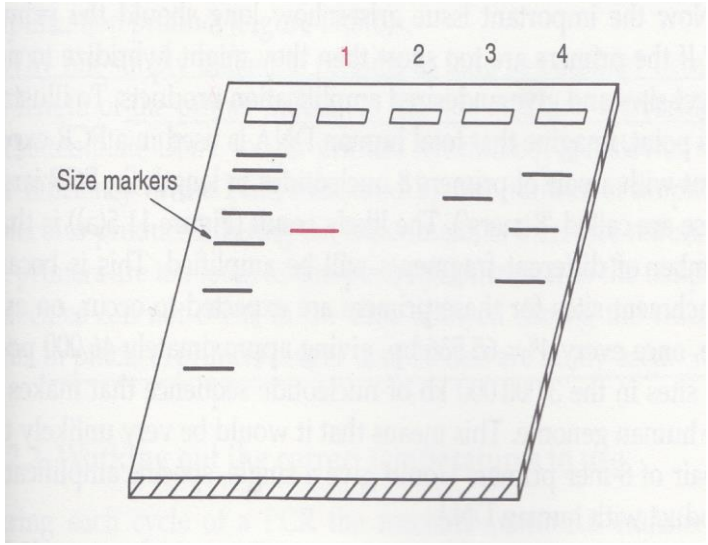
Agarose gel sieves **DNA fragments according to size**

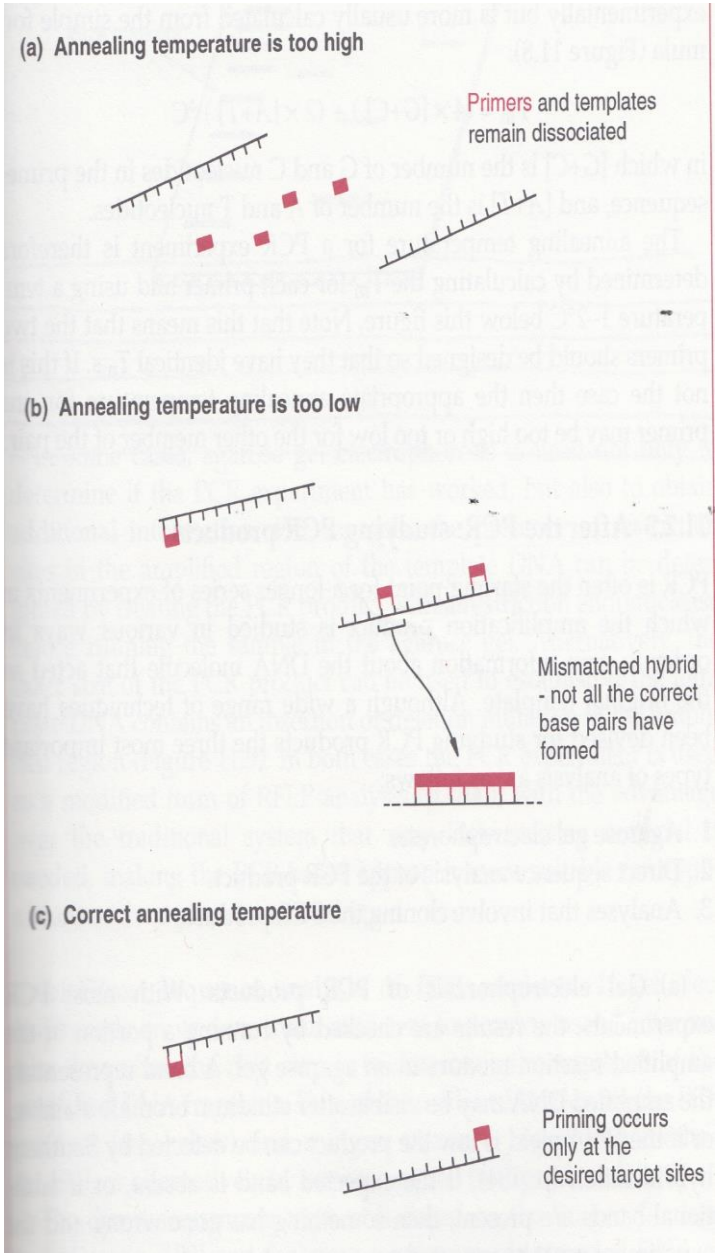
- **Small fragments move farther than large fragments**

Use a 3% gel to separate small fragment sizes



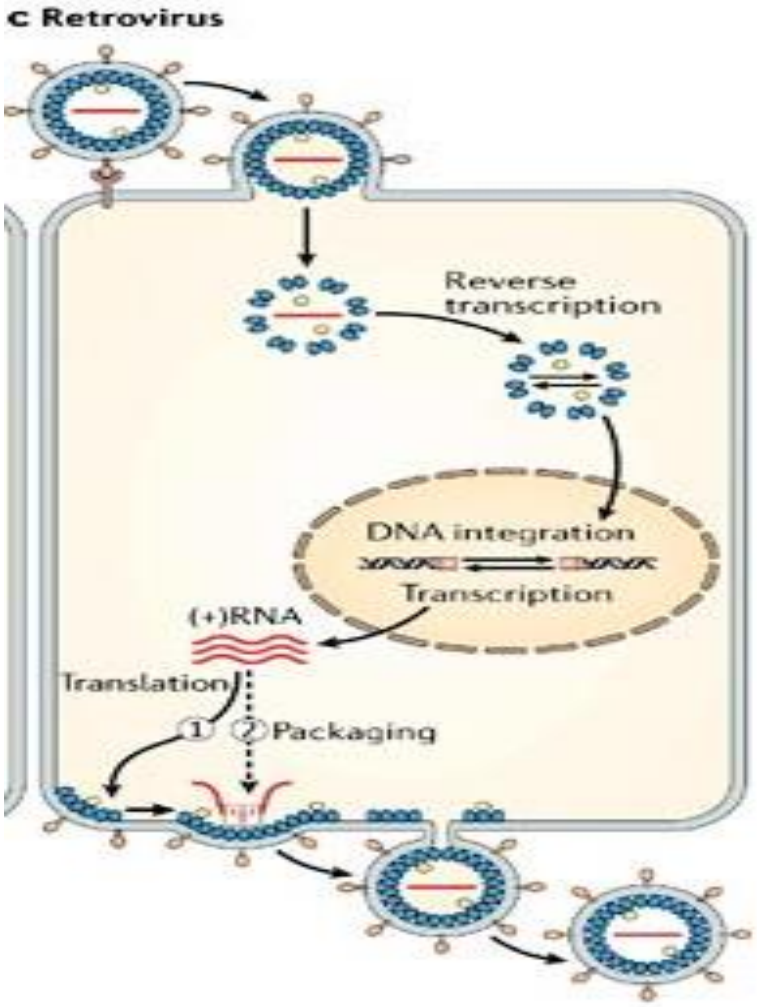
Gel running


1
2
3
4
Size markers


(a) Annealing temperature is too high
Primers and templates remain dissociated
(b) Annealing temperature is too low
Mismatched hybrid - not all the correct base pairs have formed
(c) Correct annealing temperature
Priming occurs only at the desired target sites

Conventional RT-PCR

RT-PCR and quantitative RT-PCR for studying RNA viruses



OVERVIEW

Tissue, blood

↓

extract RNA

↓

copy into cDNA

(reverse transciptase)

↓

do real-time PCR or RT-PCR

↓

analyze results

What does the term “RT-PCR” stand for?

Involves two processes:

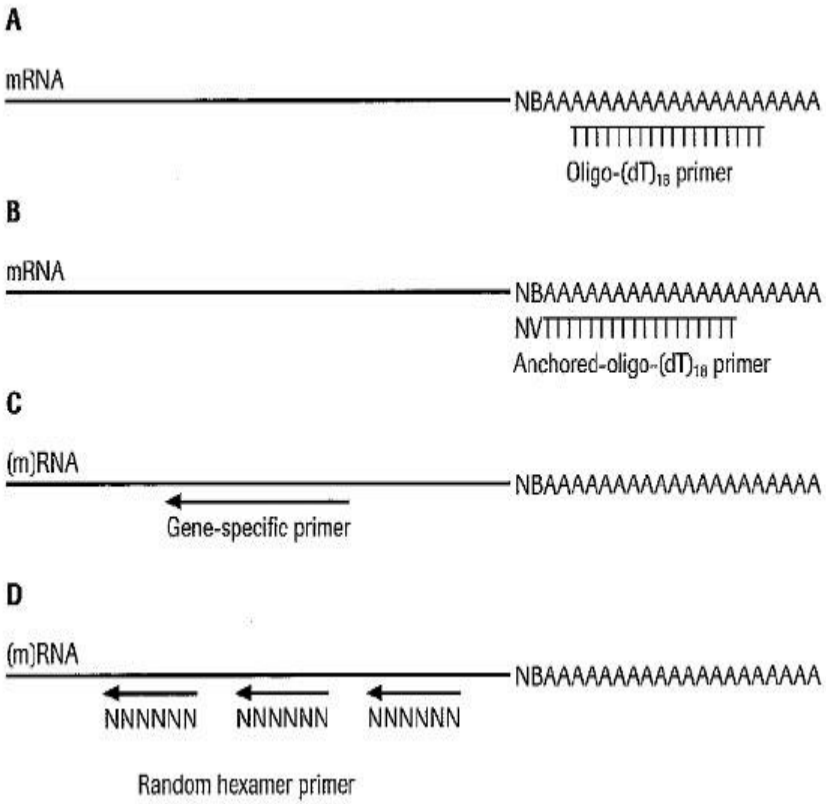
RT – Reverse Transcription

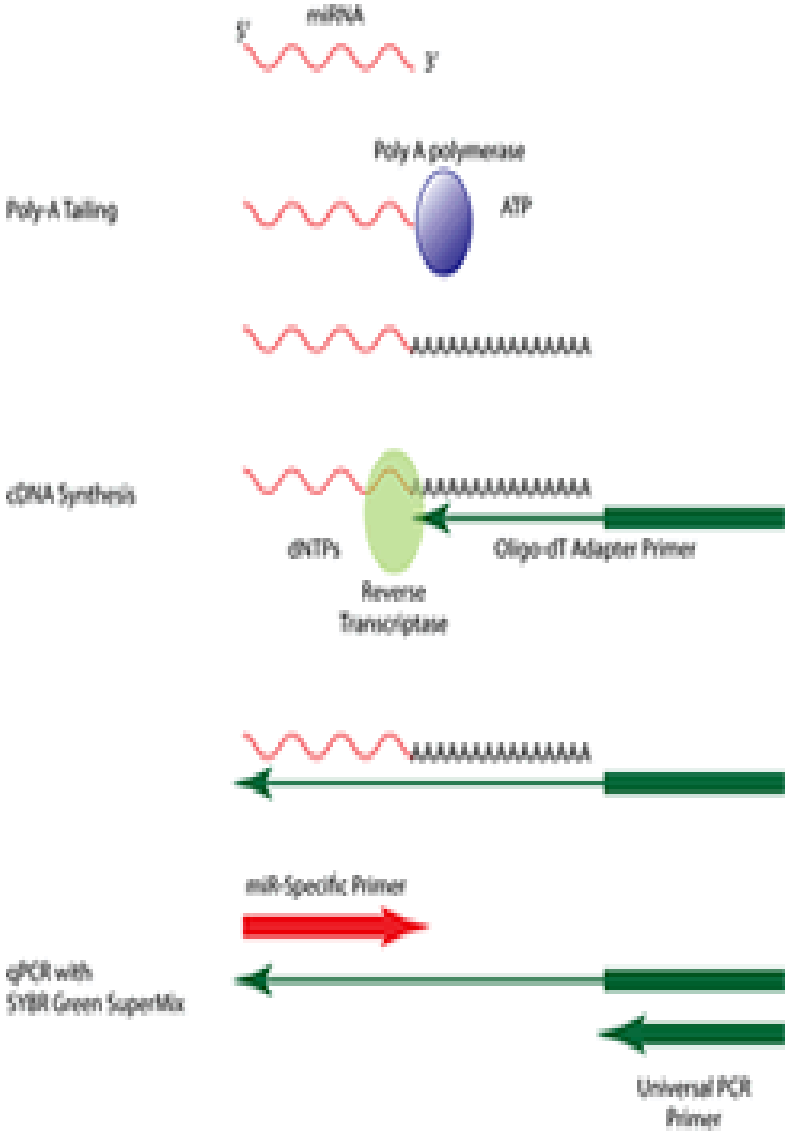
During this step we synthesize single stranded DNA from RNA template

PCR – Polymerase chain reaction

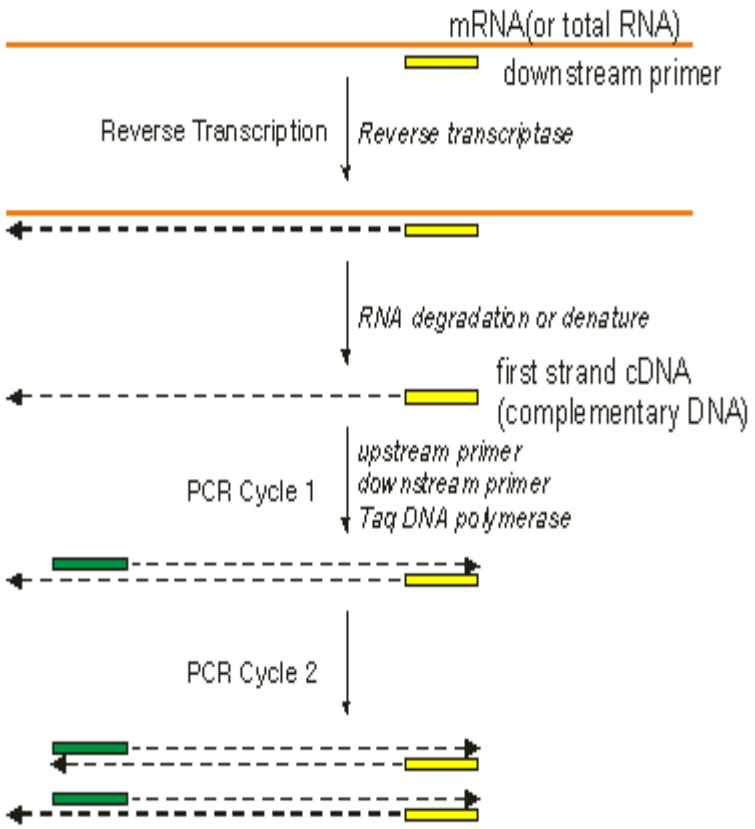
Using gene-specific primers we amplify a certain part of our gene of interest to get enough amount for further analysis

cDNA synthesis



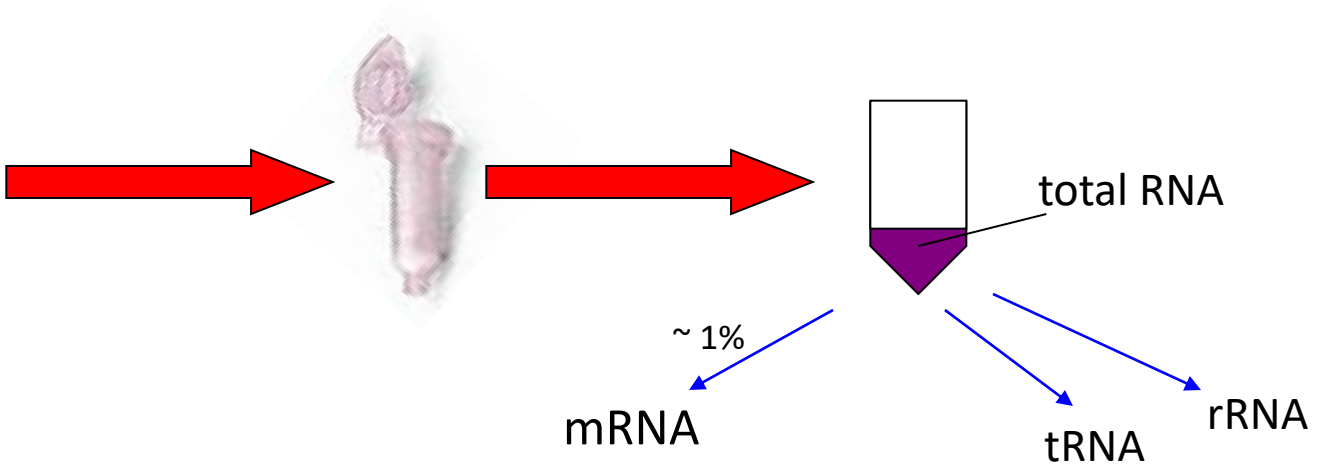


Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

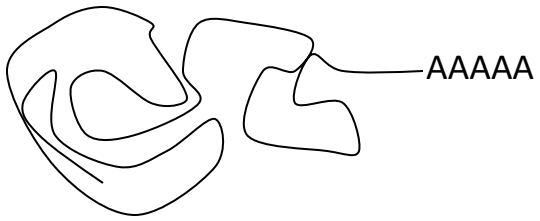


Let's start!

RNA isolation



- Most of the RNA is unimportant for us (tRNA, rRNA)
- mRNA population consists of about 3-5000 different kind
- Strong secondary structure – enzyme cannot work



Only mRNA has a poly-Adenin tail at the 3' end

Sampling and Template Preparation

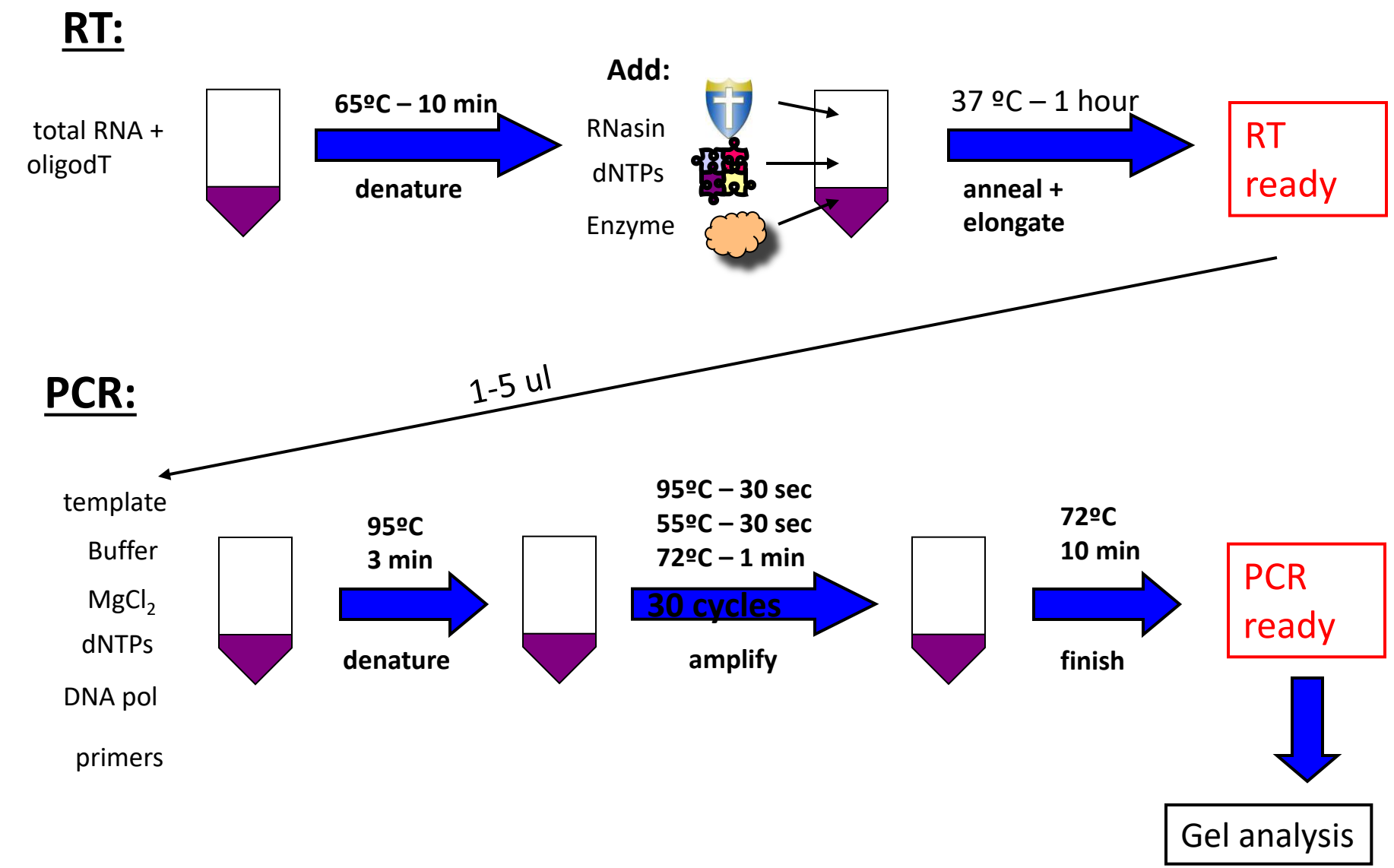
- Important to be familiar with general principles of working with RNA:
  - Avoid RNAses
  - Always wear gloves when handling reagents or equipment that will be used in the RNA extraction and reverse transcription procedures
  - RNAse-free water can be commercially purchased or nanopure water can be treated with diethyl pyrocarbonate (DEPC)

http://www.promega.com/~/media/files/resources/product%20guides/rna%20analysis%20notebook/workingwithrna.ashx?la=en

IMPORTANCE OF RNA QUALITY

- Should be free of protein (absorbance 260nm/280nm)
- Should be undegraded (28S/18S ~2:1)
- Should be free of DNA (DNAse treat)
- Should be free of PCR inhibitors
  - Purification methods
  - Clean-up methods

RT–PCR at the bench



Real time PCR

Advantages of Real-Time PCR

- Increased dynamic range of detection
- High technical sensitivity
- High precision
- No post-PCR processing
- Detection is capable down to a 2-fold change
- Collects data in the exponential growth phase of PCR
- An increase in reporter fluorescent signal is directly proportional to the number of amplicons generated
- Minimize risk of cross contamination.

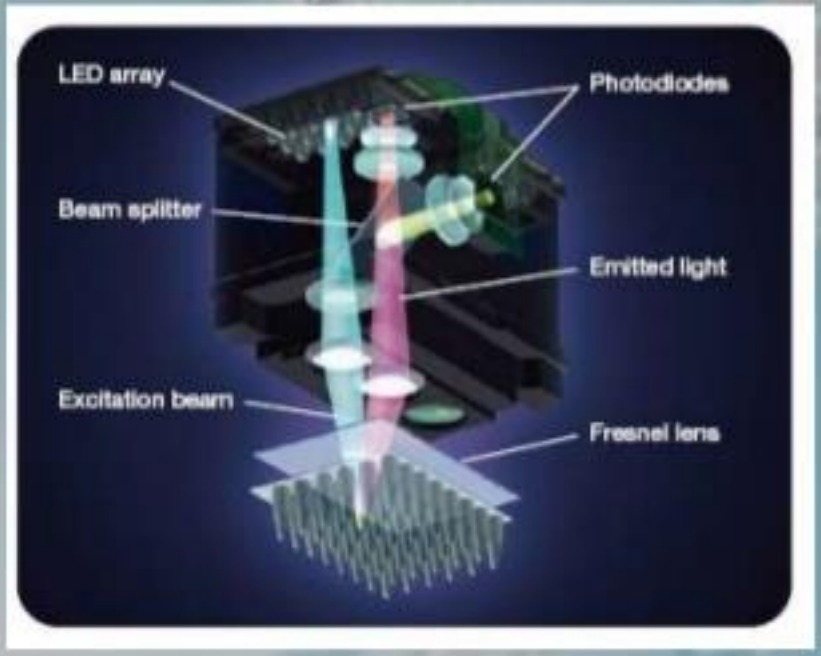
Real Time PCR

- Real-time PCR: DNA amplification analysis is monitored simultaneously over the course of thermocycling, the amplification product is detected as it accumulate.

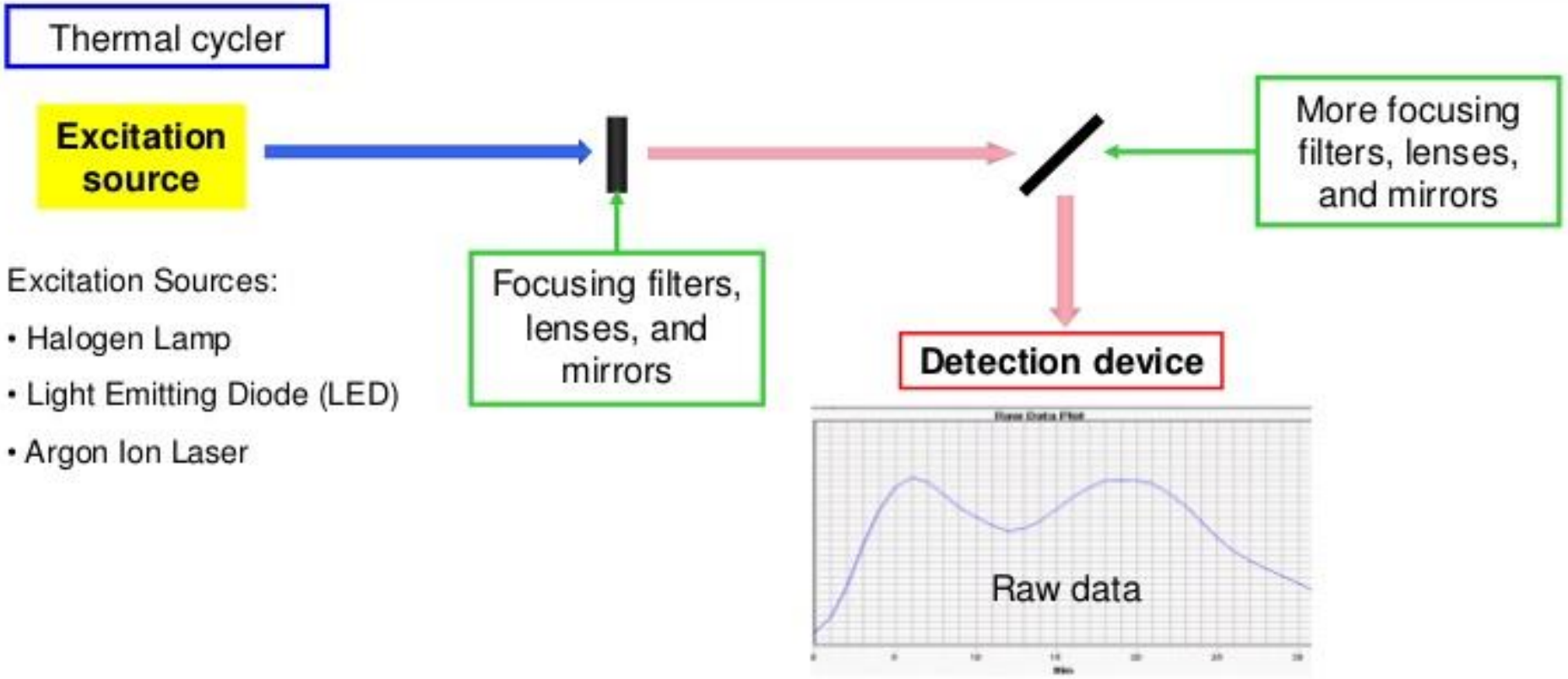
- Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time).

A good example is the MiniOpticon real-time instrument.

Optical Module
Thermal Cycler Base



How does a real-time PCR cycler work?



Excitation Sources:

- Halogen Lamp
- Light Emitting Diode (LED)
- Argon Ion Laser

Detection Devices:

- Charge Couple Device (CCD Camera)
- Photomultiplier Tube (PMT) * Rotor-Gene Q
- Photodiode

Real Time PCR

- Real-time PCR quantitates the initial amount of the template most specifically, sensitively. The higher the starting copy number of the nucleic acid target, the sooner significant increase in fluorescence is observed.

Principles of Real-Time Quantitative PCR Techniques

Intercalator based

The simplest and cheapest principle is based on interaction of double stranded DNA binding dyes (Sybr green).

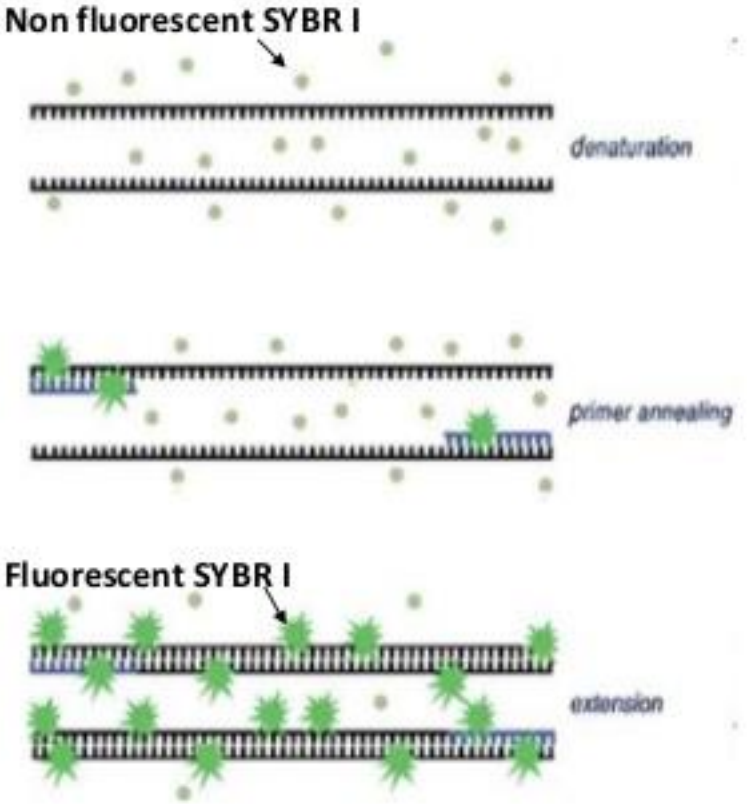
Probe based PCR

The principles are based on the introduction of an additional fluorescence labeled oligonucleotide (Taqman, Molecular Beacons, Hybridization Probes,..) .

SYBR Green I

- DNA binding dye that insert itself into dsDNA.
- During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence.
- Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.

Reporter chemistries: SYBR® Green I assay



SYBR I binds to double-strand DNA but not single-strand DNA. Little fluorescence emitted from SYBR I in solution

SYBR I upon binding to double-strand DNA emits fluorescence very brightly

The SYBR I signal intensities correlate with DNA amplified (amplicon amount) and thus the initial **sample** input amounts

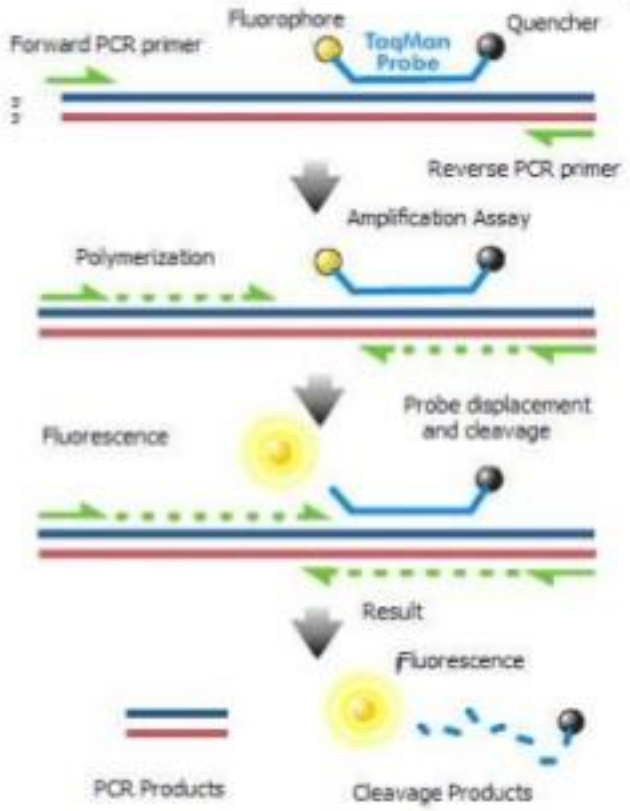
- Simple and cost saving
- **High specificity is required** when using SYBR Green since SYBR I binds all double-strand DNA (non-specific or primer dimer)

TaqMan Probe

- Target specific probe
- 5' reporter and 3' quencher
  - Reporters: FAM,TET,VIC,JOE
  - Quenchers: TAMRA, MGB.
- The probe conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact.

Reporter chemistries

Hydrolysis-based probe – Taqman® probe assay



The fluorescence of the reporter dye is suppressed by the quencher

Primer binding followed by extension

Probe cleavage by Taq to free the reporter dye thus the fluorescence intensity correlates with the initial sample input amounts
Taq has 5' → 3' exonuclease activity

Each amplicon needs a sequence-specific probe (cost and time)

Sybr Green PCR/ TaqMan Assay

Sybr Green PCR	
Advantage	Inexpensive, easy to use, and sensitive. No probe is required, which reduces assay setup and running costs.
Disadvantage	Sybr Green PCR assay both specific and non specific PCR products are both detected, therefore the assay require careful optimization of the PCR conditions and clear differentiation between specific and nonspecific PCR products using melting curve analysis (Dissociation Graph).
TaqMan Assay	
Advantage	TaqMan probes add specificity to a PCR reaction. Non specific amplification due to mis-priming or primer dimer artifact does not generate signal. Allow the development of multiplex reaction.

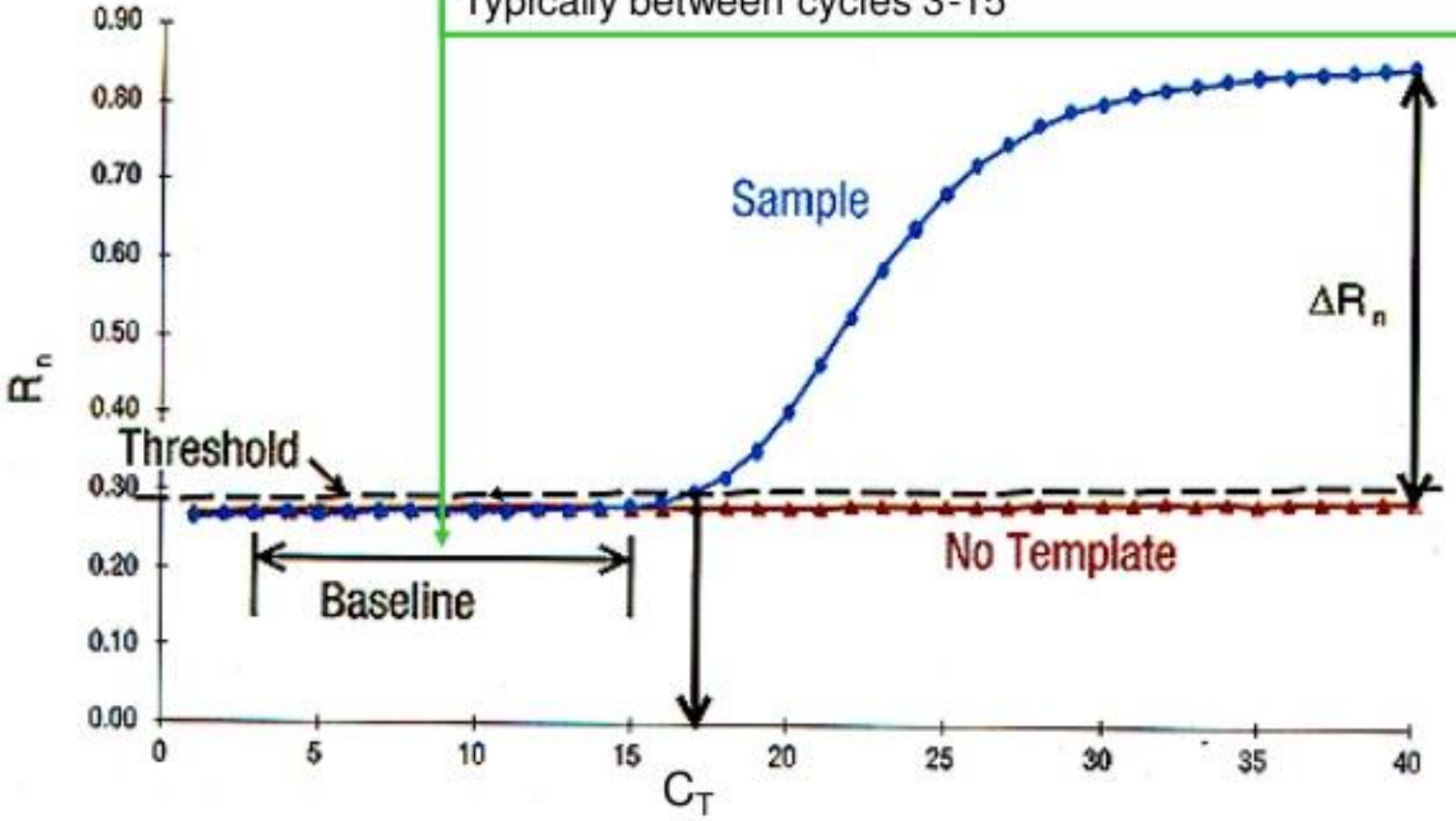
Mechanism

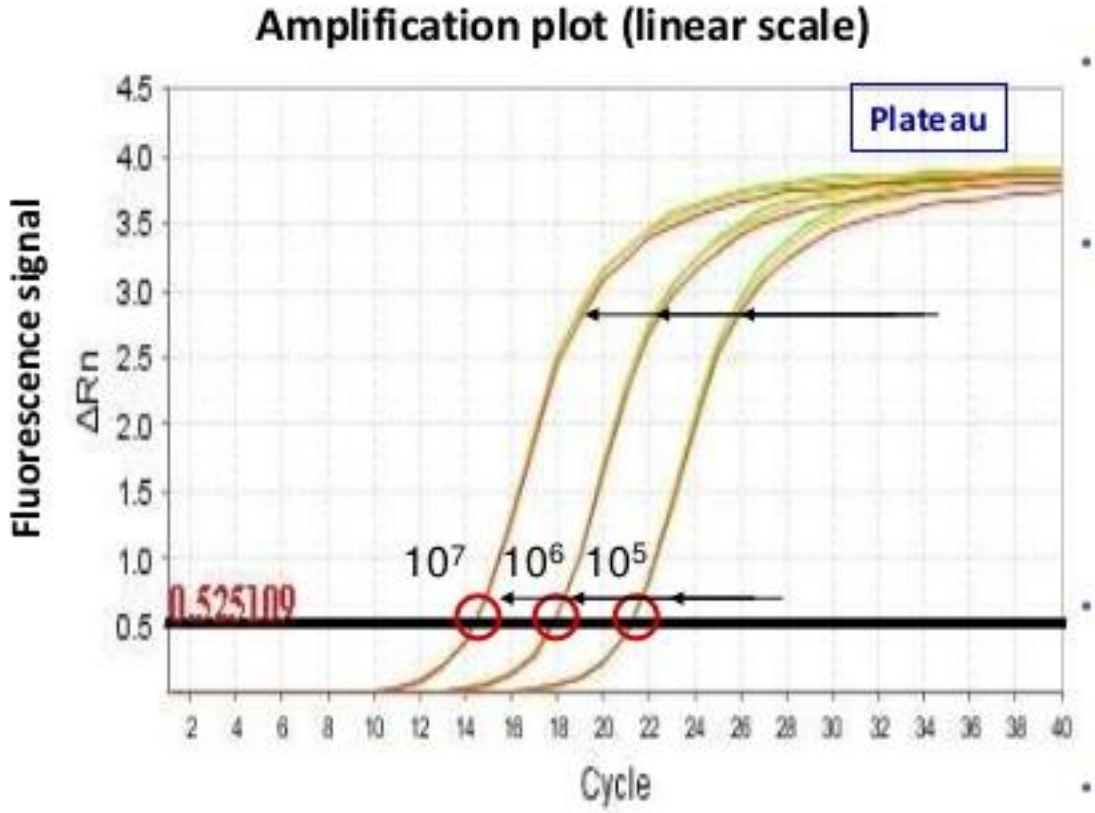
- All technologies are based on the measurement of florescence during the PCR. The amount of emitted fluorescence is proportional to the amount of PCR product and enables the monitoring of the PCR reaction.
- The resulting PCR curve is used to define the exponential phase of the reaction, which is a prerequisite for accurate calculation of the initial copy number at the beginning of the reaction.

Baseline

Baseline

The initial cycles prior to amplification in which there is little change in fluorescent signal.
Typically between cycles 3-15

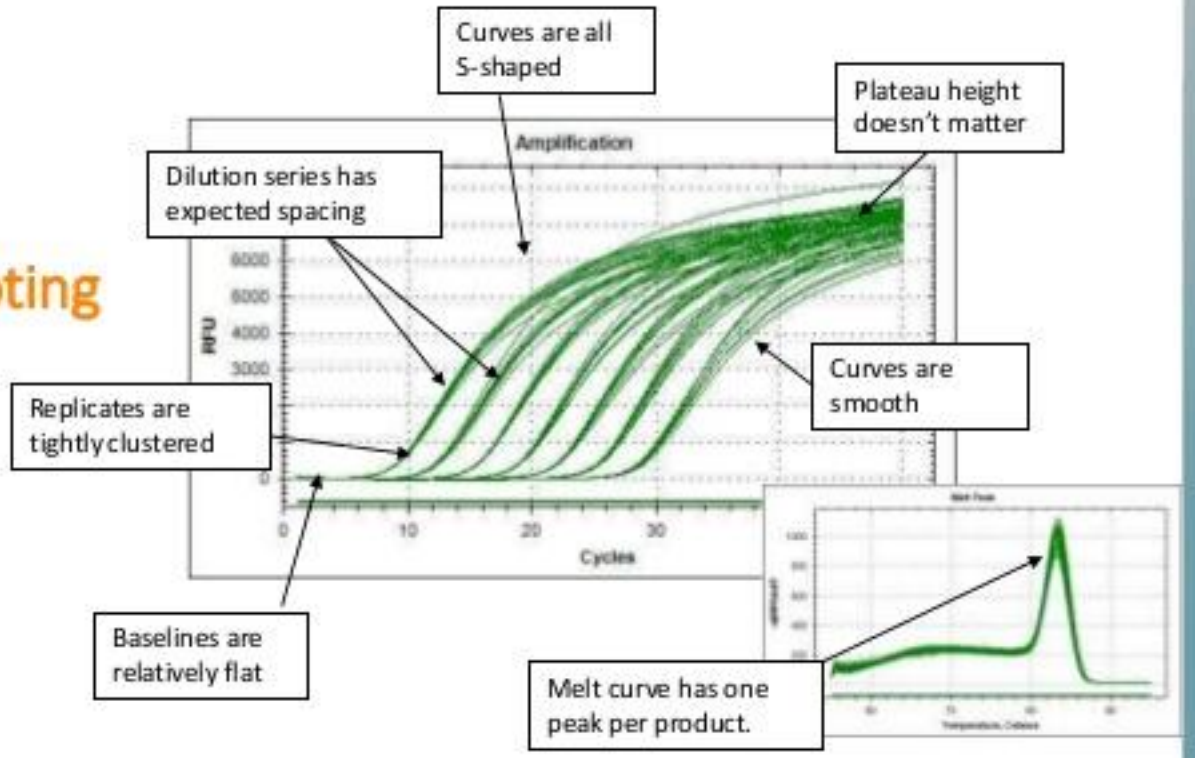




- End-point PCR data collection at plateau (gel analysis)
- Reactions start varying due to reagent depletion and decreased PCR efficiencies (enzyme activity, more product competing for primer annealing)
- Real-time PCR does early phase detection at the exponential state
- Precisely proportional to input amounts

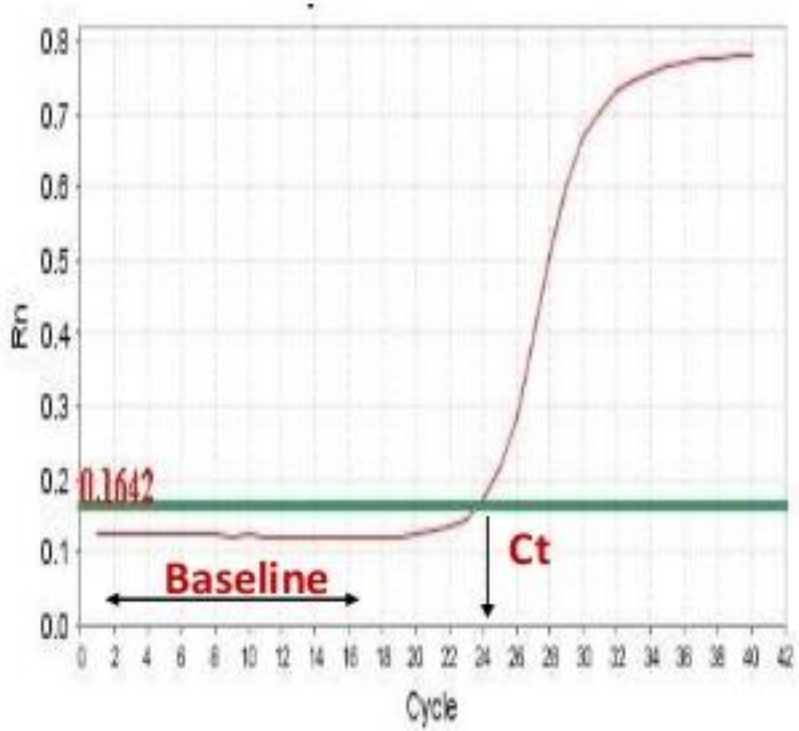
Trouble-Shooting

- A successful real-time PCR experiment will have the following characteristics:



Analyzing qPCR curves: how to define baseline

Linear amplification plot



Automated baseline option

- Instrument establishes baseline

Manual baseline option

- Use linear view of the plot
- Establish baseline beginning at cycle two and subtracting two cycles from earliest amplification seen
- Usually the baseline falls between cycles 3-15

How Real-Time PCR Quantitation Assays Work

- In the initial cycles of PCR, there is little change in fluorescence signal (The baseline).
- An increase in fluorescence above the baseline indicates the detection of accumulated target (The amplification plot).
- A fixed fluorescence threshold can be set above the baseline.
- The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

A measure of *when*

the C_T

C_T = threshold cycle: the calculated fractional cycle number at which the PCR product crosses a threshold of detection

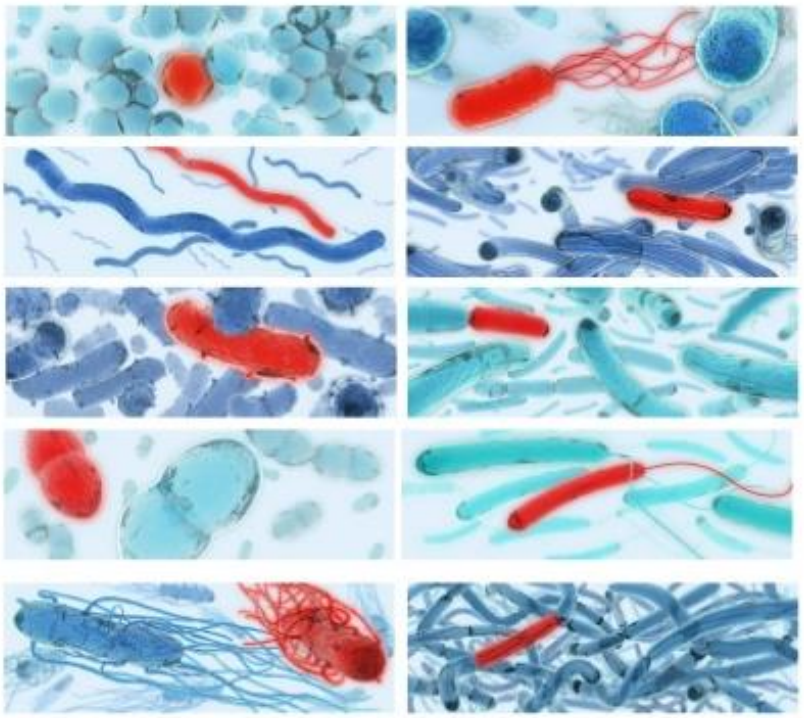
Quantitation Assay

- It measures (quantitates) the amount of a nucleic acid target during each amplification cycle of the PCR.

- The target may be:
  - DNA,
  - cDNA, or
  - RNA.

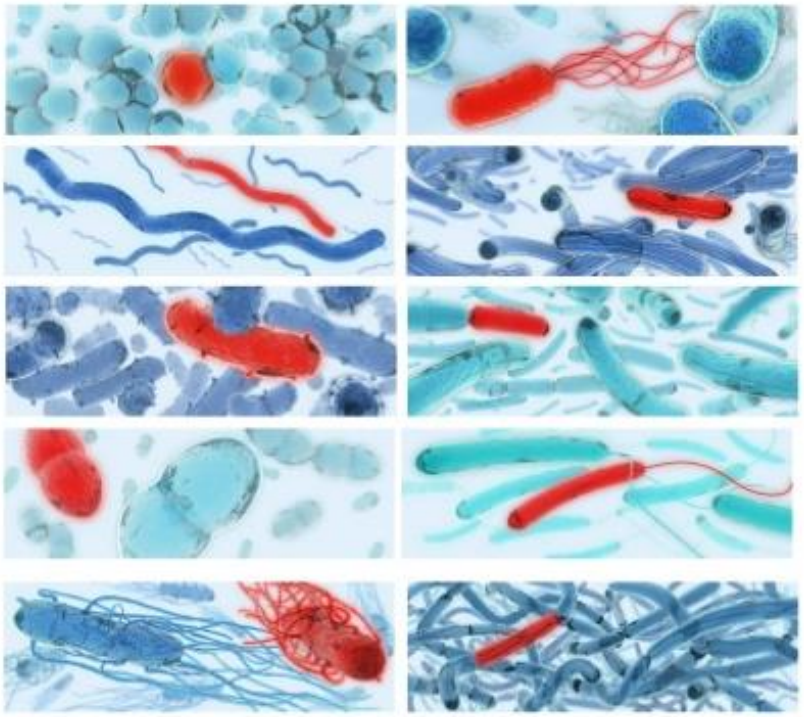
There are three types of Quantitation Assays

- DNA/cDNA quantitation
- RNA quantitation using one-step reverse transcription polymerase chain reaction (RT-PCR)
- RNA quantitation using two-step RT-PCR

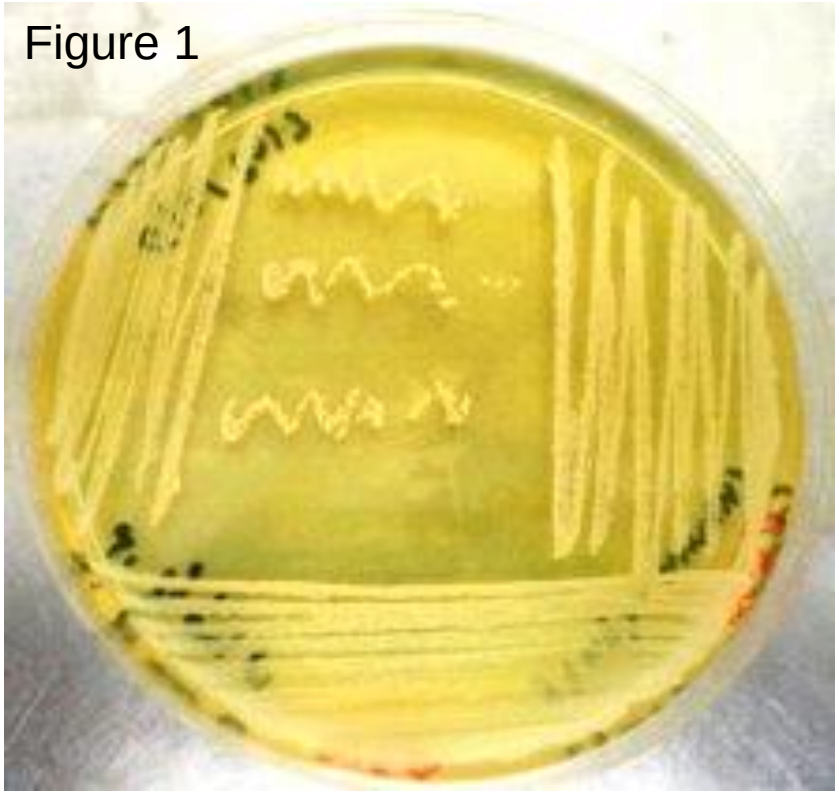


Antibiotic resistance

Conventional PCR



Antibiotic resistance



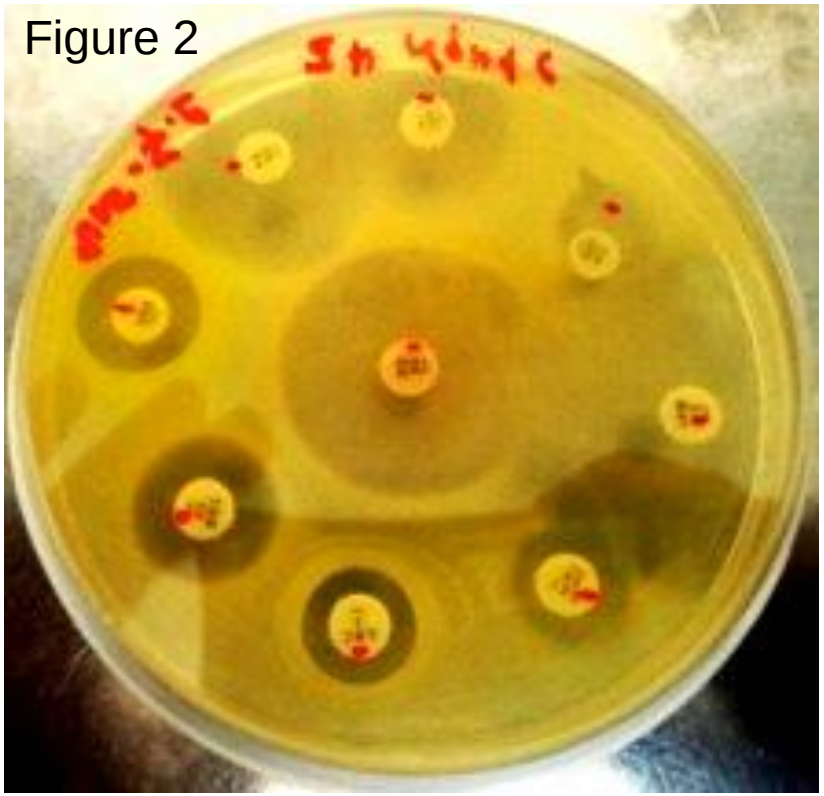


Figure 1

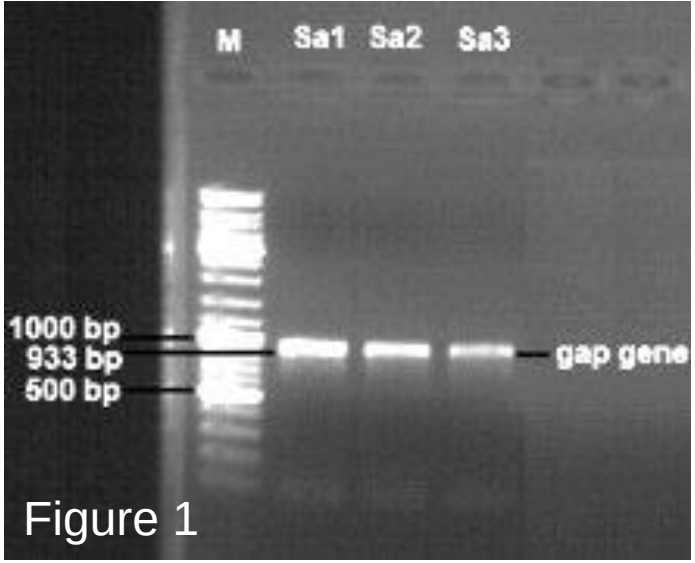
S. aureus on LB Agar.

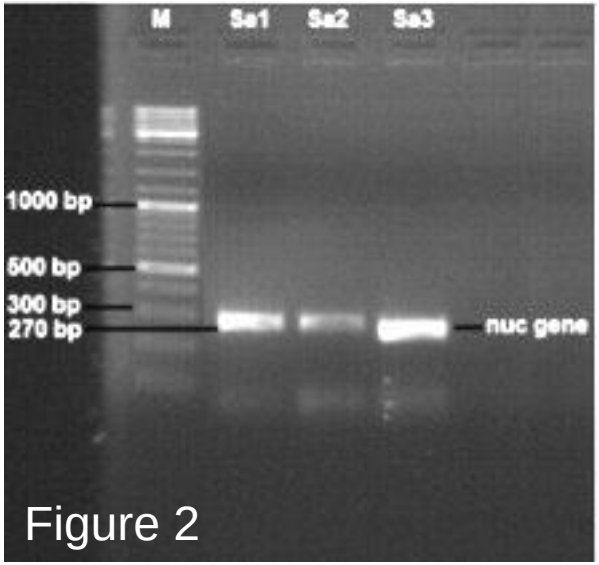
Figure 2

Disc diffusion method with inhibition zones for some antibiotics against S. aureus.

Role of Polymerase Chain Reaction (PCR) in the detection of antibiotic-resistant Staphylococcus aureus

http://dx.doi.org/10.1016/j.ejmhg.2014.05.003





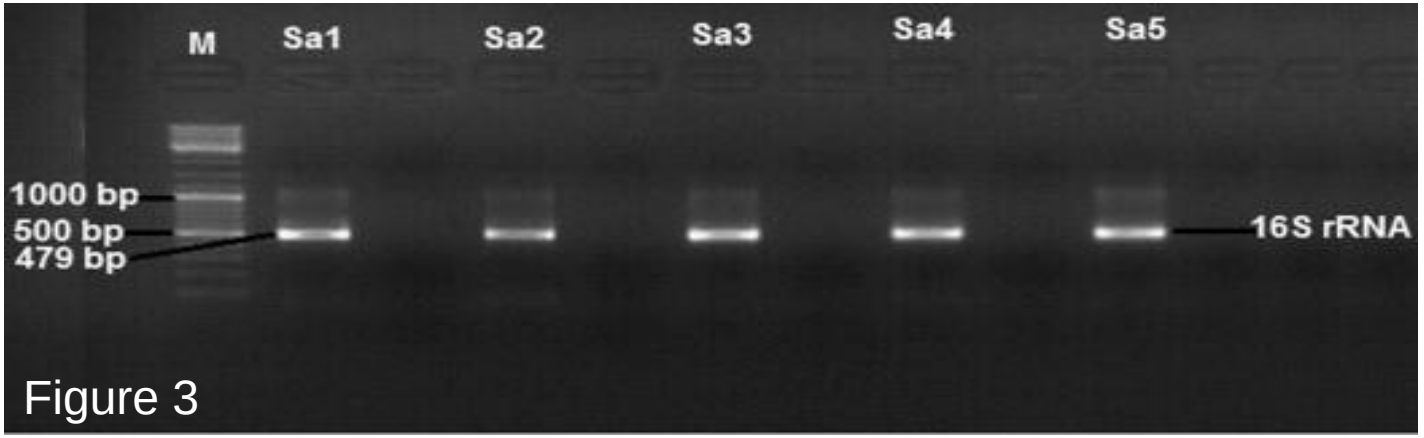


Figure 1. Gel electrophoresis shows the gap gene fragments of S. aureus, M: DNA marker, Sa(1-3): S. aureus.

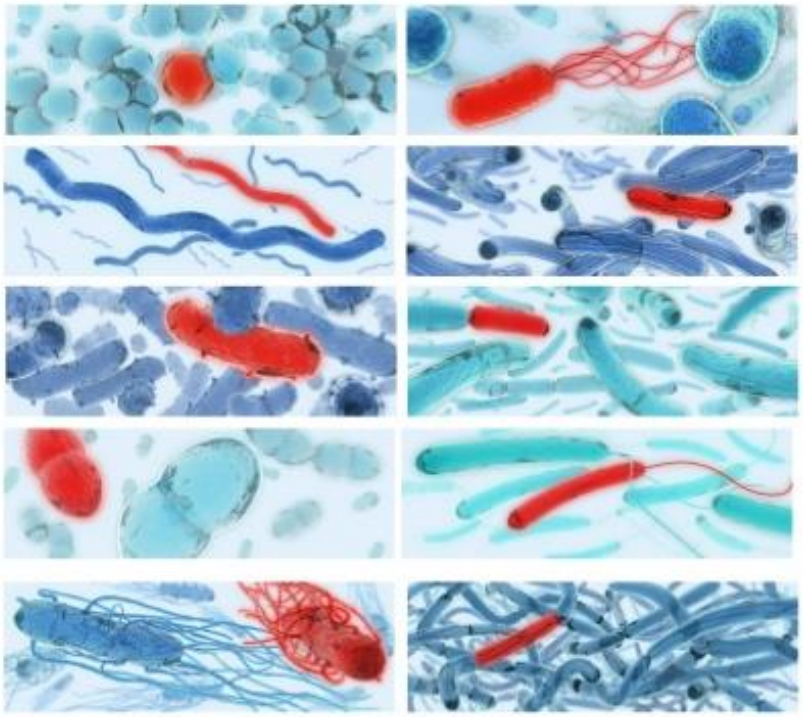
Figure 2. Gel electrophoresis shows the nuc gene fragments of S. aureus, M: DNA marker, Sa(1-3): S. aureus.

Figure 3. Gel electrophoresis shows the 16S rRNA fragments of S. aureus, M: DNA marker, Sa(1-5): S. aureus.

Role of Polymerase Chain Reaction (PCR) in the detection of antibiotic-resistant Staphylococcus aureus

http://dx.doi.org/10.1016/j.ejmhg.2014.05.003

Real time PCR



Antibiotic resistance

Food testing

Intestinal infections

QIAGEN's Microbial DNA qPCR assay pipeline

Develop an assay pipeline to support microbiome research

Over 500 assays that target species-specific or gene-specific microbial DNA

- >300 Bacteria identification assay
- 8 Fungi identification assay
- 1 Protist identification assay
- 87 Antibiotic resistance genes
- 87 Virulence factor genes

Microbial DNA qPCR Array content

14 Arrays

- Antibiotic Resistance Genes
- Bacterial Vaginosis
- Biodefense
- Food testing: Dairy
- Food testing: Meat
- Food testing: Poultry
- Food testing: Seafood
- Food testing: Vegetable
- Intestinal infections
- Respiratory Infections
- Sepsis
- Urinary Tract Infections
- Vaginal Flora
- Water Analysis

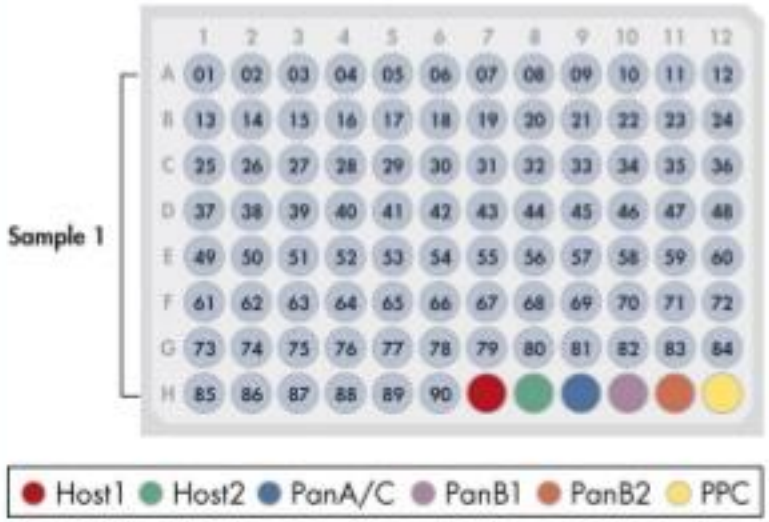
Antibiotic resistance genes in our food supply?

Genes	Resistance classification	Sewage
aacC1	Aminoglycoside resistance	+
aadA1	Aminoglycoside resistance	+
GES	Class A beta-lactamase	+
SHV	Class A beta-lactamase	+/-
SHV (156G)	Class A beta-lactamase	+/-
SHV (238G240E)	Class A beta-lactamase	+/-
TLA-1	Class A beta-lactamase	+
VEB	Class A beta-lactamase	+
ACT-1 group	Class C beta-lactamase	+/-
LAT	Class C beta-lactamase	+/-
MIR	Class C beta-lactamase	+/-
MOX	Class C beta-lactamase	+
OXA-10 group	Class D beta-lactamase	+
OXA-2 group	Class D beta-lactamase	+
AAC(6)-Ib-cr	Aminoglycoside resistance	+
QnrB-5 group	Fluoroquinolone resistance	+
QnrS	Fluoroquinolone resistance	+
ermB	Macrolide lincosamide streptogramin B	+
mefA	Macrolide lincosamide streptogramin B	+
tetA	Tetracycline efflux pump	+

Microbial DNA qPCR Array

Pre-printed assays profile up to 90 different species/genes

DNA Isolation → Detection by qPCR → Data Analysis



PCR plates (either 96-well or 384-well) are pre-printed with primers and probes.

Each numbered well is a separate assay that tests the same sample.

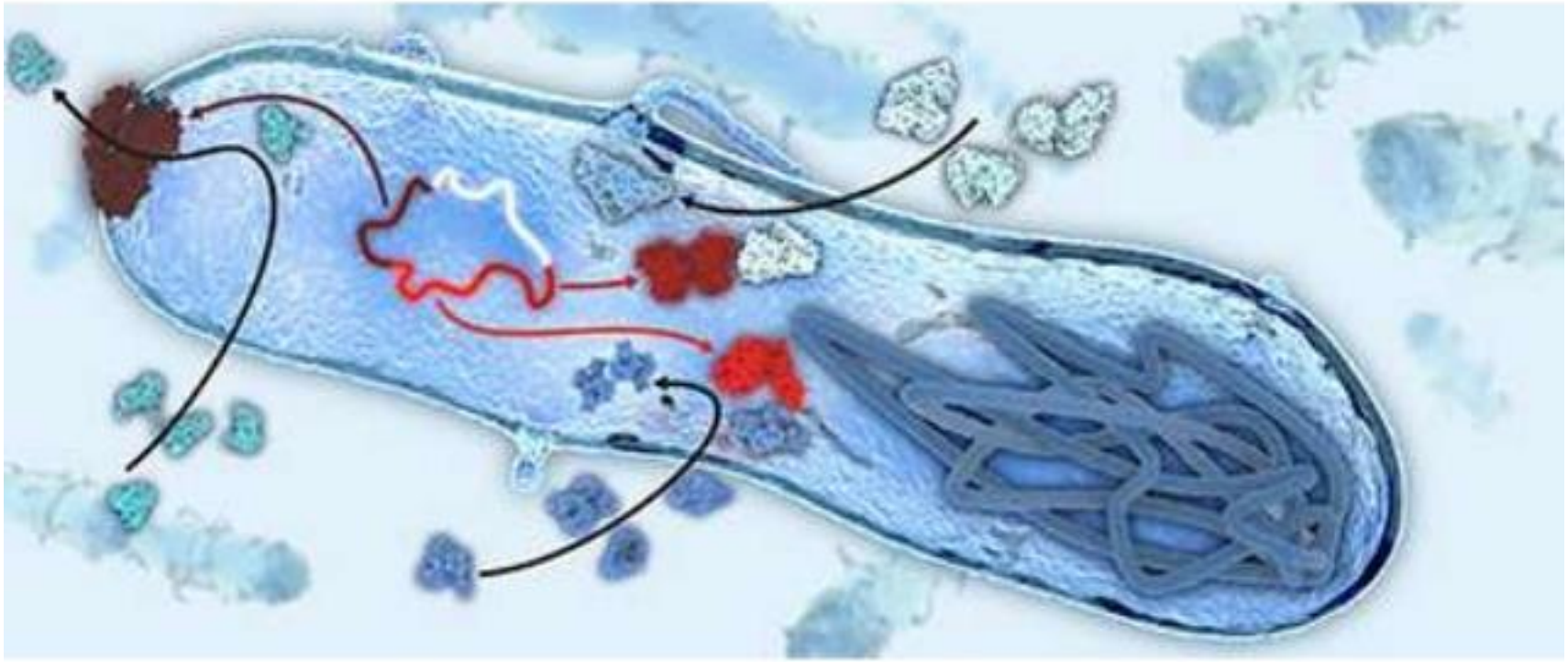
Integrated control assays:

Host assays detect genomic DNA to test sample collection

Pan A/C is a pan-fungal assay that detects the presence of fungal 16S rRNA

PanB1 and PanB2 detect bacterial 16S rRNA to determine bacterial load in the sample

PPC is a positive PCR control reaction that tests if the PCR reactions failed from PCR inhibitors from the sample, etc.



Identification of antibiotic resistance genes in *Klebsiella pneumoniae* isolates and metagenomic samples using real-time PCR arrays

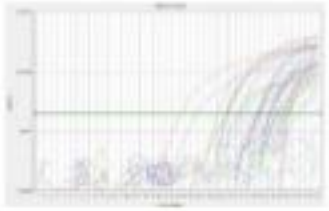
Matthew Fosbrink[1], Geoffrey Wilt[1], Liang Chen[2], Barry Kreiswirth[2], Vikram Devgan[1]

[1]QIAGEN Sciences Inc., Frederick, MD, [2]Public Health Research Institute Center, New Jersey Medical School, UMDNJ Newark, NJ

Microbial PCR array method

Genomic DNA from *Klebsiella pneumoniae* isolates were extracted using a Promega Wizard® Genomic DNA Purification Kit. Genomic DNA from stool samples were extracted using a QIAGEN QIAamp® DNA Stool Mini Kit.

Each sample comprised 250 ng of genomic DNA from *Klebsiella pneumoniae* isolates or 500 ng of genomic DNA from stool samples. Samples were mixed with microbial qPCR probe mastermix and microbe-free water, and this mixture was then uniformly dispensed into a 96-well PCR plate containing dried-down primers and 5'-hydrolysis probes for each of the antibiotic resistance genes tested.



Each PCR plate was run on a Roche LightCycler 480 using the following cycling conditions:

Step	Time	Temperature	Number of cycles
Initial PCR activation step	10 min	95° C	1
2-step cycling:			
Denaturation	15 sec	95° C	45
Annealing and extension	2 min	60° C	

After the PCR run, raw C_T values were exported to the microbial qPCR analysis software to detect the presence of antibiotic resistance genes. The identification criteria were as follows: $C_T<32$ was identified as positive, $C_T>35$ was identified as negative and a $32<C_T<35$ was inconclusive. In addition, the control assay PPC (Positive PCR Control) had to have a $C_T=22\pm2$ to show that the PCR instrument and mastermix performed properly and there were no PCR inhibitors in the sample.

Antibiotic resistance gene screening microbial qPCR array

	1	2	3	4	5	6	7	8	9	10	11	12
A	KPC	GES	IMI & NMC-A	SME	IMP-1 group	IMP-2 group	IMP-5 group	IMP-12 group	SFC-1	SHV	SHV (156G)	SHV (156D)
B	SHV (238G240E)	SHV (238S240K)	SHV (238S240E)	SHV (238G240K)	BIC-1	ereB	ermA	ermB	ermC	mefA	msrA	tetA
C	tetB	ccrA	vanB	vanC	CTX-M-1 Group	CTX-M-2 Group	CTX-M-8 Group	CTX-M-9 Group	OXA-2 Group	OXA-10 Group	OXA-18	OXA-45
D	OXA-48 Group	OXA-23 Group	OXA-24 Group	OXA-51 Group	OXA-58 Group	OXA-50 Group	OXA-54	OXA-55	OXA-60	OXA-62	CMY-2 Goup	CMY-10 Group
E	DHA	FOX	MOX	ACT-1 group	ACT 5/7 group	ACC-1 group	ACC-3	MIR	LAT	CFE-1	VIM-1 group	VIM-7
F	VIM-13	NDM	Per-1 group	Per-2 group	VEB	QnrA	QnrB-1 group	QnrB-4 group	QnrB-5 group	QnrB-8 group	QnrB-31 group	QnrC
G	QnrD	QnrS	QepA	AAC(6)-Ib-cr	aphA1	aphA6	aacC1	aacC2	aacC4	aadB	aadA1	BES-1
H	SFO-1	TLA-1	oprj	Pan Bacteria 1	Pan Bacteria 1	Pan Bacteria 1	Pan Bacteria 2	Pan Bacteria 2	Pan Bacteria 2	PPC	PPC	PPC

Figure 1. Layout of antibiotic resistance gene screening microbial qPCR array. The antibiotic resistance gene screening microbial qPCR array allows identification of different antibiotic resistance genes in a single PCR run. Each array contains controls such as Pan-Bacteria 1 and Pan-Bacteria 2 to detect total bacteria and ensure bacterial genomic DNA was added to the array. The control PPC (Positive PCR Control) confirms a positive PCR run and the absence of PCR inhibitors in the sample.

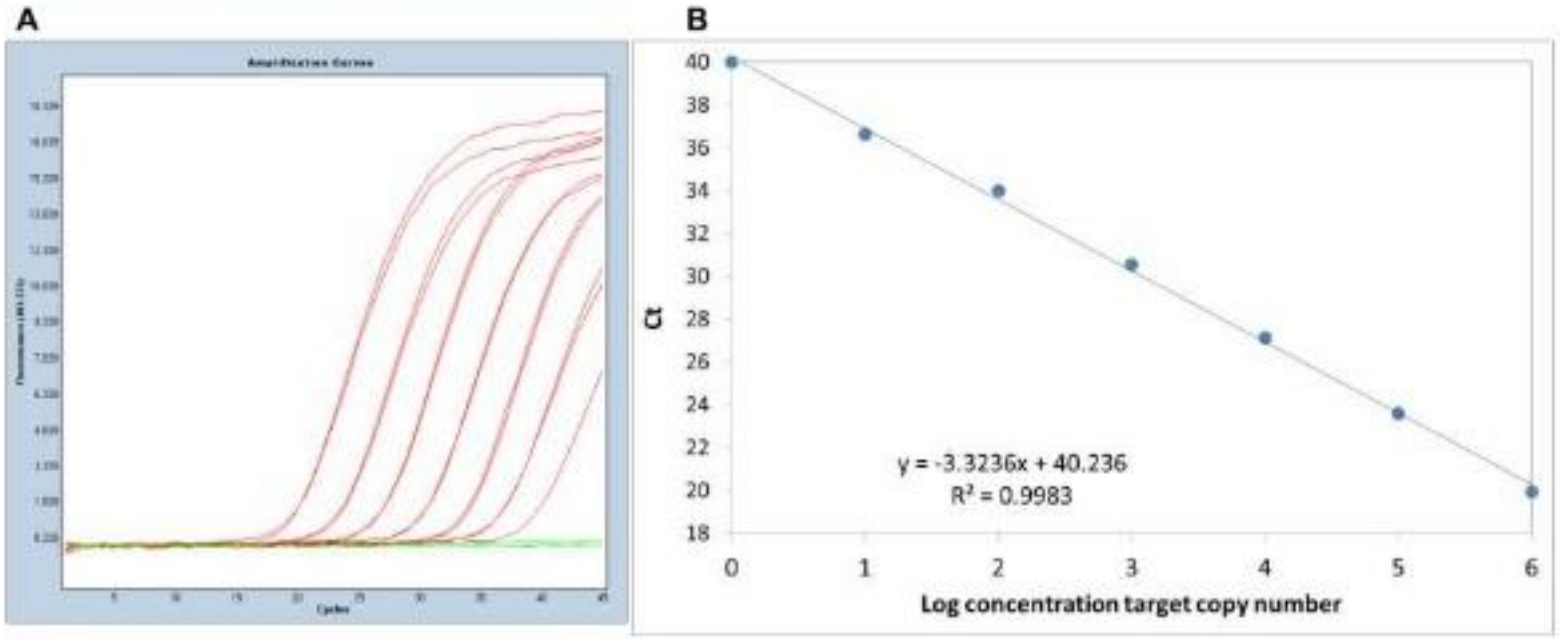


Figure 2. Linearity and sensitivity for each assay on the microbial antibiotic resistance gene qPCR array. Linearity and sensitivity were determined using synthetic templates over a 6-log serial dilution ranging from 1 copy to 1 million copies. The following are representative results for all the assays on the array. [A] Real-time amplification curves of the KPC antibiotic resistance gene qPCR assay. [B] A standard curve shows the primer efficiency equals 103% (calculated from slope=-3.3236) with a correlation coefficient of 0.9983, indicating optimum performance for the KPC qPCR assay.

Identification of multi-drug antibiotic resistance in *Klebsiella pneumoniae* isolates

Genes	Resistance classification	1	2	3	4	5	6	7	8	9	10	11	12	13	14
AAC(6)-Ib-cr	Aminoglycoside-resistance					+	+	+	+	+	+	+	+	+	+
aacC2	Aminoglycoside-resistance			+		+		+	+	+	+	+	+		+
aacC4	Aminoglycoside-resistance							+							
aadA1	Aminoglycoside-resistance				+	+	+	+			+		+	+	+
aadB	Aminoglycoside-resistance						+								
aphA1	Aminoglycoside-resistance										+	+/-	+	+	
CTX-M-1 Group	Class A beta-lactamase					+	+	+			+				+
CTX-M-2 Group	Class A beta-lactamase								+	+		+/-			
KPC	Class A beta-lactamase	+						+						+	
SHV	Class A beta-lactamase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SHV(156D)	Class A beta-lactamase				+										
SHV(156G)	Class A beta-lactamase	+	+	+		+	+	+	+	+	+	+	+	+	+
SHV(238G240E)	Class A beta-lactamase	+	+		+	+	+	+	+	+	+			+	+
SHV(238S240E)	Class A beta-lactamase			+								+	+		
IMP-2 group	Class B beta-lactamase						+								
IMP-5 group	Class B beta-lactamase						+								
OXA-2 Group	Class D beta-lactamase								+	+		+			
QnrB-1 group	Fluoroquinolone resistance					+				+/-	+				
QnrB-5 group	Fluoroquinolone resistance							+							
QnrS	Fluoroquinolone resistance			+											
tetA	Tetracycline efflux pump							+						+	
tetB	Tetracycline efflux pump				+										

Figure 3. Screening of *Klebsiella pneumoniae* isolates using antibiotic resistance gene microbial qPCR array. 250 ng of genomic DNA, isolated from *Klebsiella pneumoniae* isolates, was loaded onto an antibiotic resistance gene microbial qPCR Array. PCR was performed and raw CT values were imported into the microbial qPCR array analysis spreadsheet. Positive + /negative - / inconclusive +/- result for each antibiotic resistance gene was determined by the analysis software using the identification criteria. The results show that different classes of antibiotic resistance genes may be present in the same *Klebsiella pneumoniae* isolate.

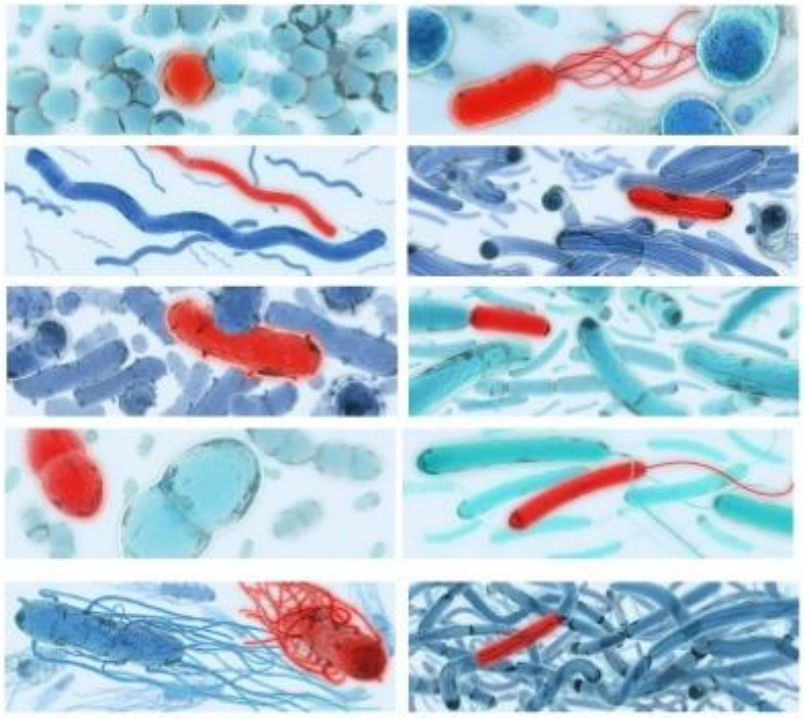
Screening of gut microbiota for presence of antibiotic resistance genes

Genes	Resistance Classification	1	2	3	4	5
aacC2	Aminoglycoside-resistance					+
aadA1	Aminoglycoside-resistance	+/-				
SFO-1	Class A beta-lactamase	+/-	+/-	+/-	+/-	+/-
ACT-1 group	Class C beta-lactamase		+/-			
ACT 5/7 group	Class C beta-lactamase		+			+/-
MIR	Class C beta-lactamase		+/-			
ermB	Macrolide Lincosamide Streptogramin_b	+	+	+	+	+
mefA	Macrolide Lincosamide Streptogramin_b	+	+	+	+	+
tetA	Tetracycline efflux pump	+	+	+		
tetB	Tetracycline efflux pump	+/-				+

Figure 5. Several antibiotic resistance genes are prevalent in stool samples. Stool samples from five healthy adults were collected and genomic DNA was isolated. 500 ng of genomic DNA from each stool sample were analyzed for presence of antibiotic resistance genes using the antibiotic resistance gene microbial qPCR array. *ErmB*, *mefA*, and *tetA* were found in all or most of the stool samples tested, showing that they may be highly prevalent in the gut. These antibiotic resistance genes have been reported to be isolated from bacterial strains originating from food, suggesting a possible source of origin. This highlights the importance of increased surveillance of antibiotic resistance reservoirs to identify potential sources of antibiotic resistant bacteria.

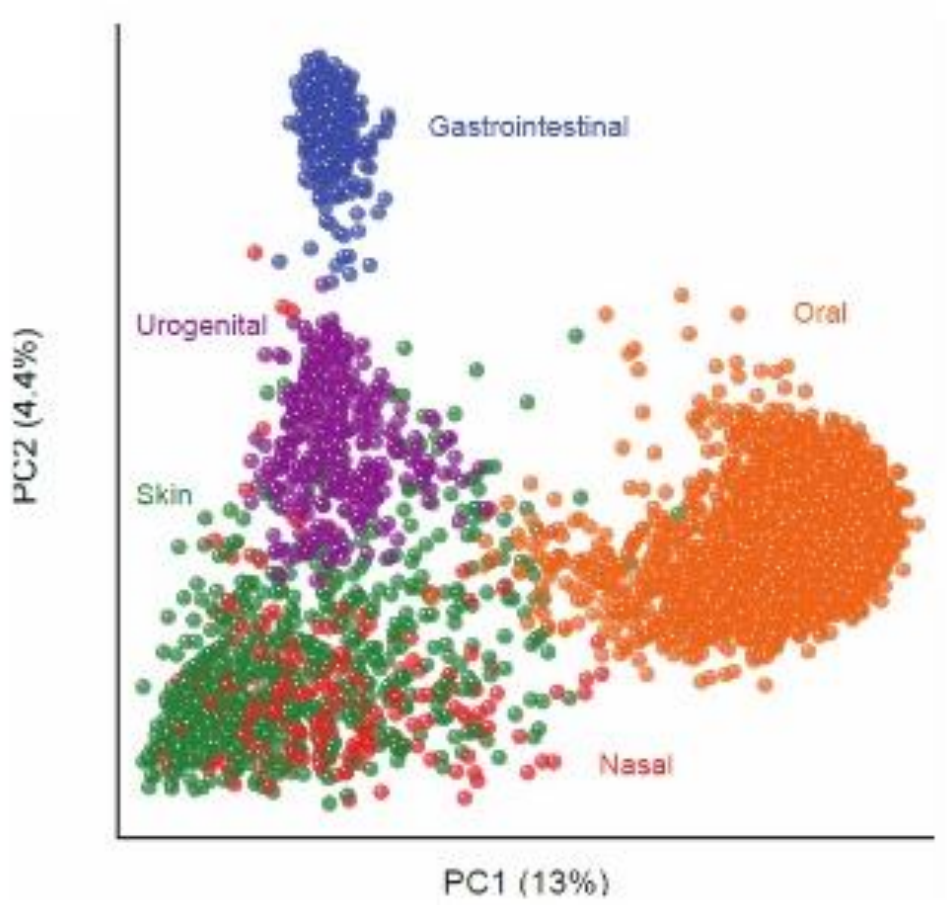
- NGS

Gut Microbiome and antibiotic resistance



The human microbiome as a reservoir of antimicrobial resistance

Microorganisms cluster by body site



<u>Cataloguing efforts by the NIH Human microbiome project suggest:</u>

~10,000 organisms live with us

~ 8×10^6 genes in this "second genome"

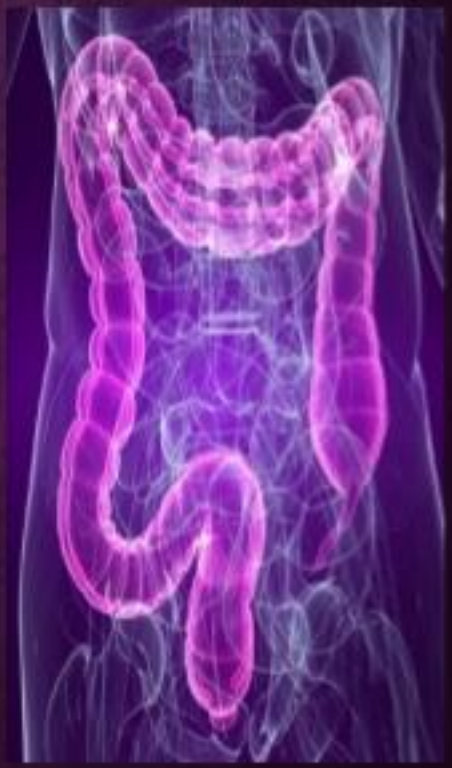
<u>Identifying microbiota in healthy individuals revealed:</u>

Different body sites have unique communities

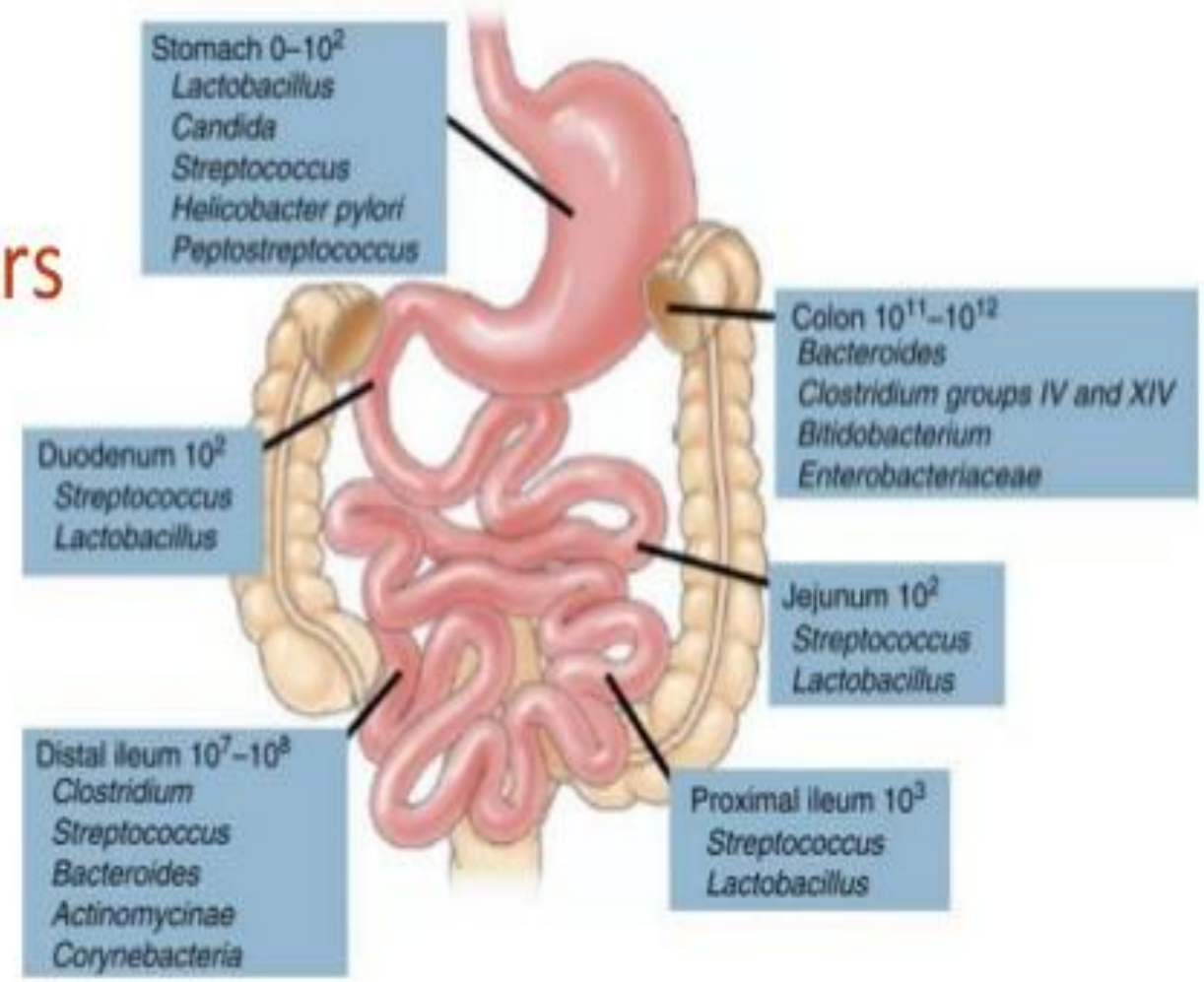
Race, Age, Gender, Weight or Ethnicity have no effect

INTESTINAL MICROBIOME

- >1,000 species but most in adults are from 2 phyla: Firmicutes and Bacteroidetes
- Outnumber human somatic cells by factor of 10^2
- Total Weight: 1-2 kg
- 60% of total fecal content
- Concentration: ~1012/gram in colon
- •Total #: ~1014



Location Matters



DOI: 10.1038/ajgsup.2012.4

How did we get started?

First report of the composition of human body site…

Science

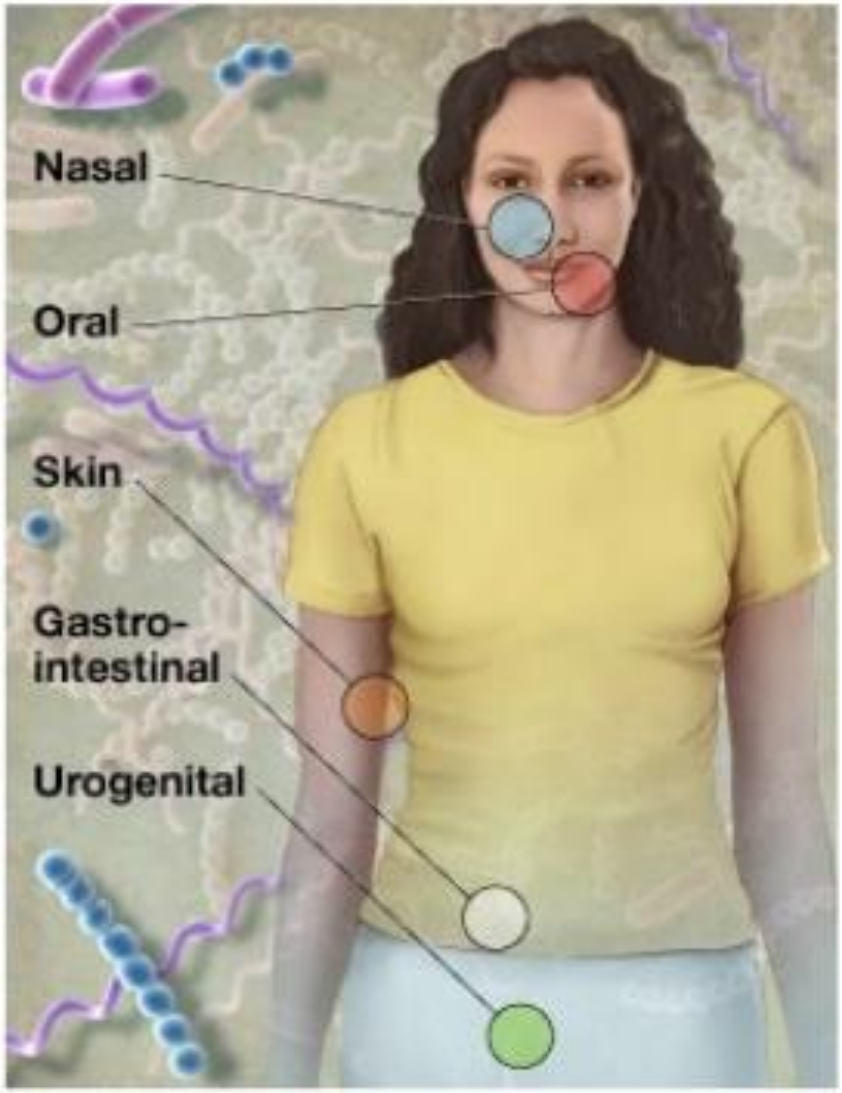
VOL 312 2 JUNE 2006

Metagenomic Analysis of the Human Distal Gut Microbiome

Steven R. Gill,[1]*‡ Mihai Pop,[1]† Robert T. DeBoy,[1] Paul B. Eckburg,[2,3,4] Peter J. Turnbaugh,[5] Buck S. Samuel,[5] Jeffrey I. Gordon,[5] David A. Relman,[2,3,4] Claire M. Fraser-Liggett,[1,6] Karen E. Nelson[1]

If we can do one….can we do them all….

NIH Human Microbiome Project



Profiling 5 body sites

- Nasal
- Mouth
- Skin
- Gastrointestinal system
- Urogential

Compare between individuals:

- Healthy vs. Disease
- Treated vs. Untreated
- Twin studies
- Diet
- …

- **Gut**
  - Intestinal infections
  - Obesity
  - Inflammatory Bowel Disease
- **Airway**
  - Pneumonia and other respiratory infections
  - Chronic Obstructive Pulmonary Disease
  - Cystic Fibrosis
- **Urogenital**
  - Bacterial Vaginosis
  - Urinary Tract Infections
  - Sexually Transmitted Disease
- **Blood**
  - Sepsis/Blood-stream infections
- **Cancer**
- **Heart disease**
- **Neurological disorders**
- **Oral**
  - Periodontitis
  - Gingivitis