Biotechnology course

Polymerase Chain Reaction (PCR)

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ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
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Central Dogma of Molecular Biology

by
FRANCIS CRICK
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The central dogma of molecular biology deals with the detailed residue-by-residue transfer of sequential information. It states that such information cannot be transferred from protein to either protein or nucleic acid.

"The central dogma, enunciated by Crick in 1958 and the keystone of molecular biology ever since, is likely to prove a considerable over-simplification."

THIS quotation is taken from the beginning of an unsigned article[1] headed "Central dogma reversed", recounting the very important work of Dr Howard Temin[2] and others[3] showing that an RNA tumour virus can use viral RNA as a template for DNA synthesis. This is not the first

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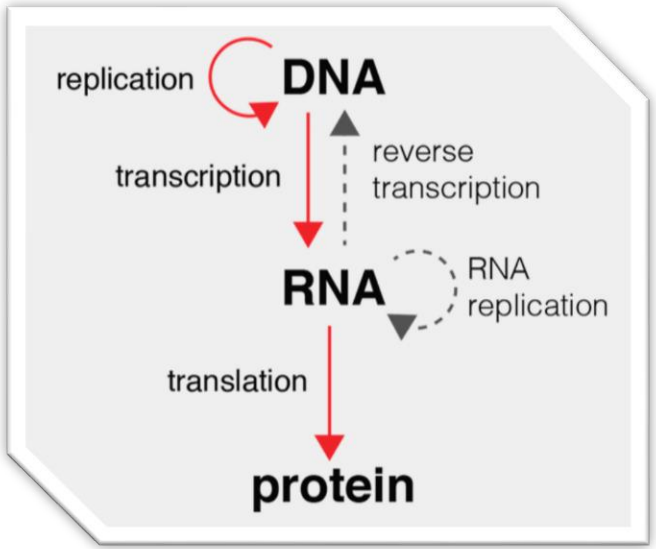
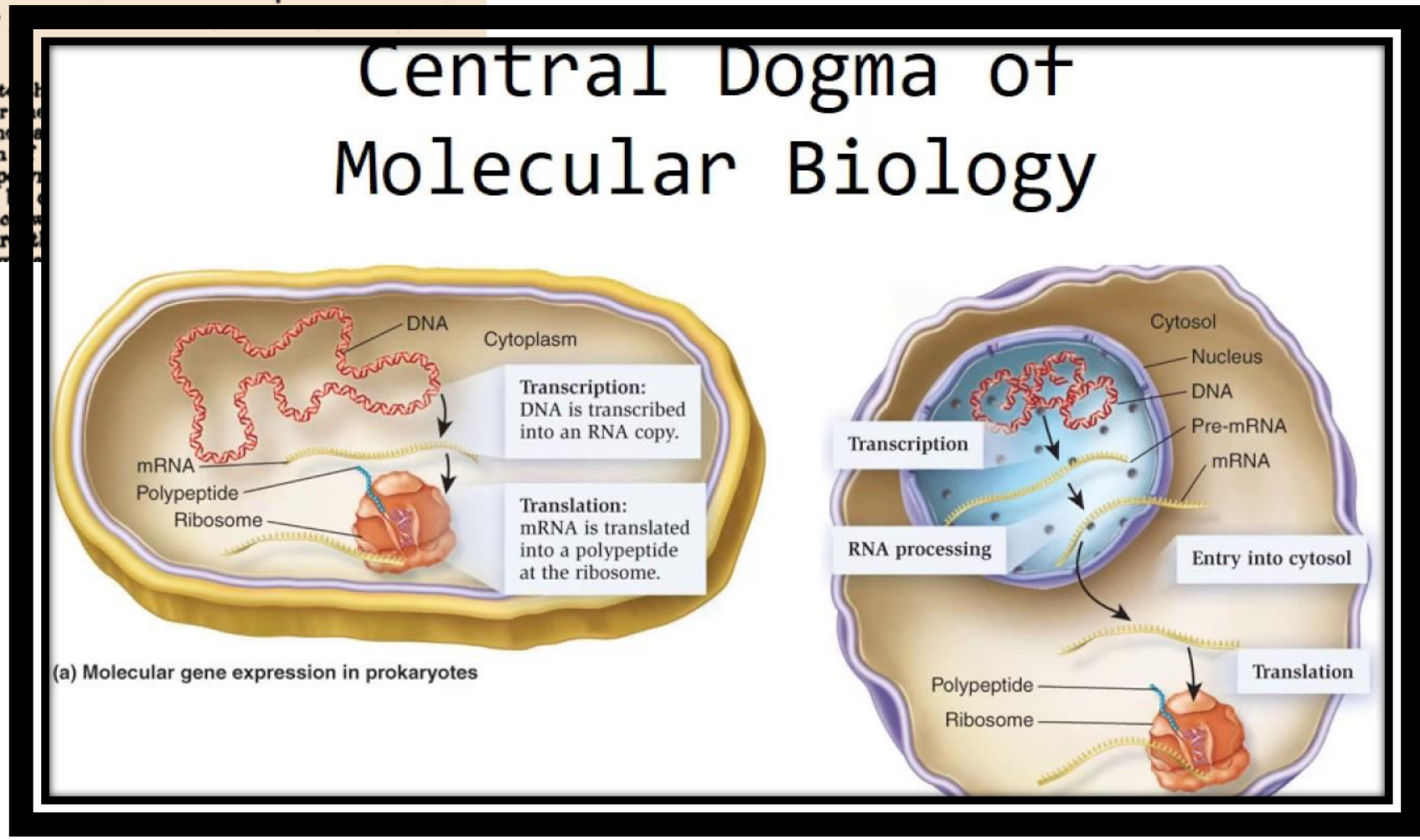
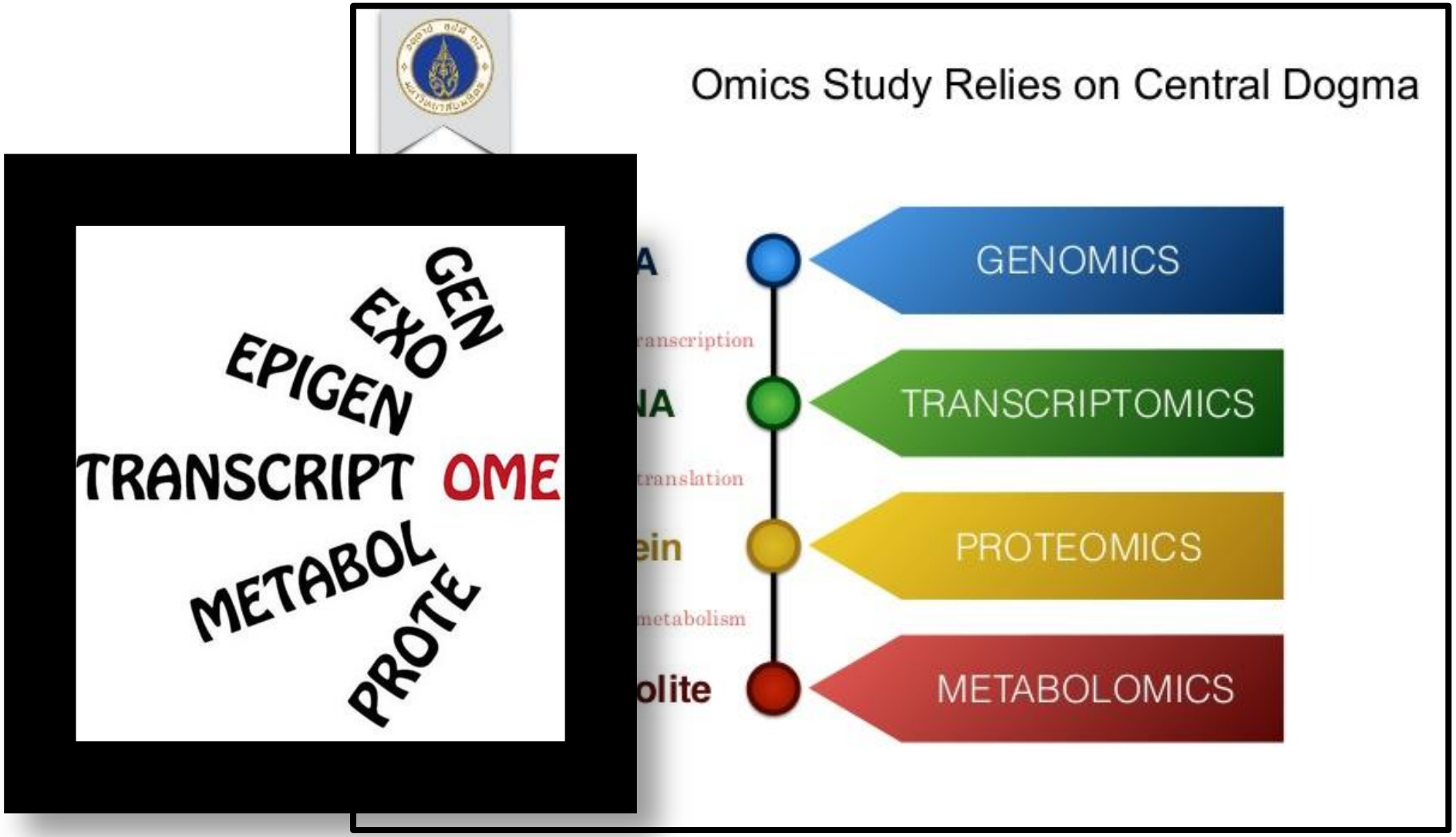




multi-OMICS approach



multi-OMICS approach

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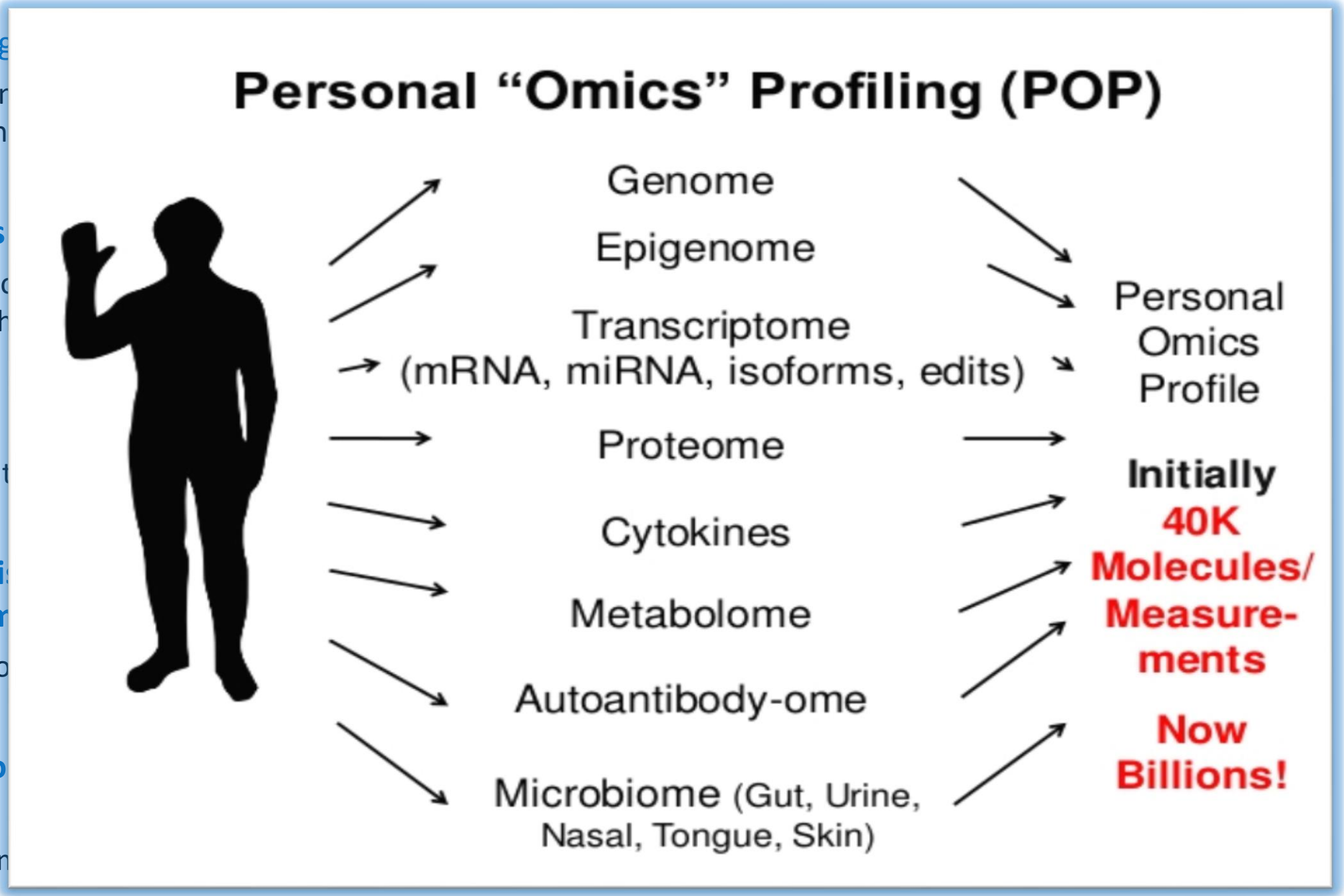
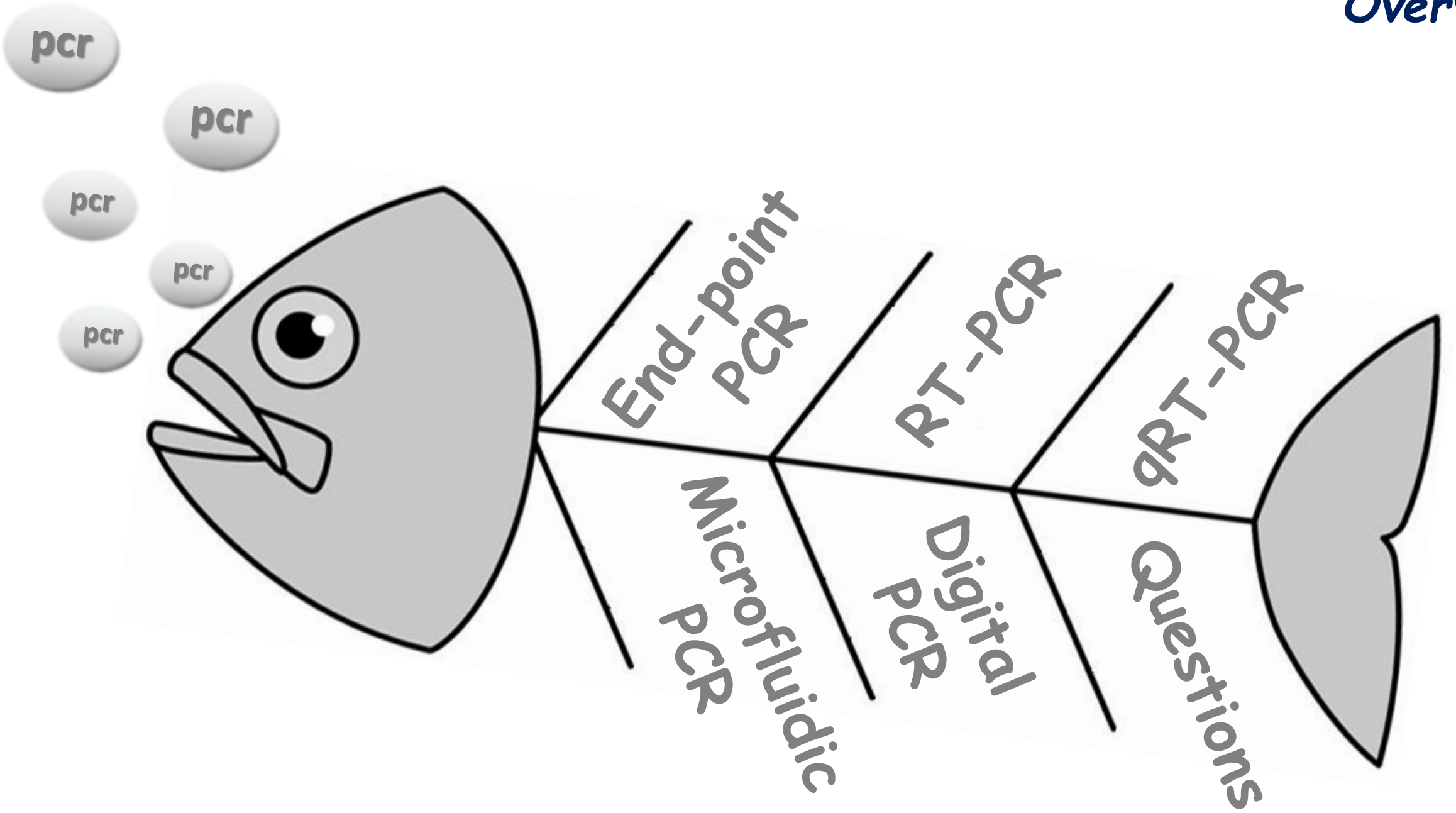


Overview


pcr
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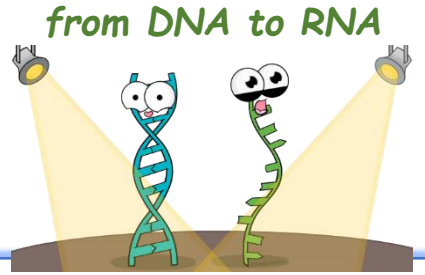
End-point PCR
RT-PCR
qRT-PCR
Microfluidic PCR
Digital PCR
Questions

What is the goal of the experiment?

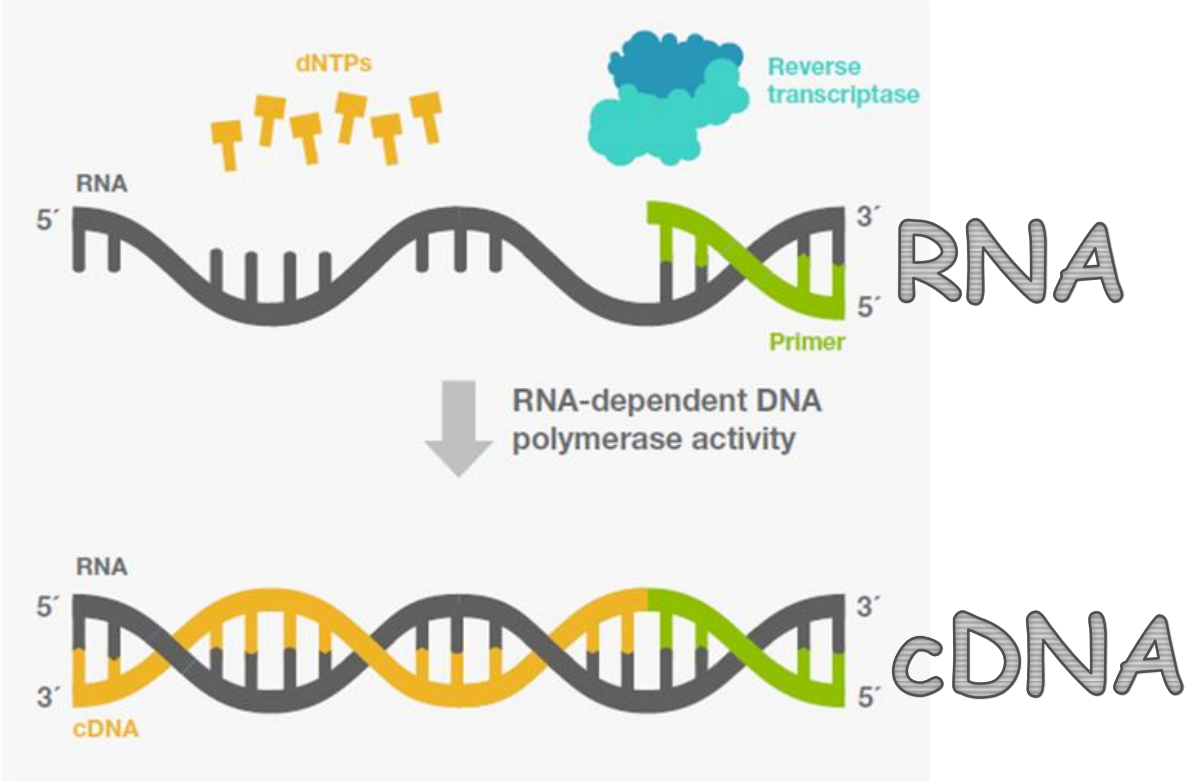
- What **biological question** is to be answered?
- What is the total number of **genes** to be analyzed?
- What **control samples and genes** will be used to measure the changes in expression levels?
- Are there any **limitations** to the amount of target material available?
- What is the **sensitivity** required to obtain the data necessary to answer?

When and why to check for RNA expression?



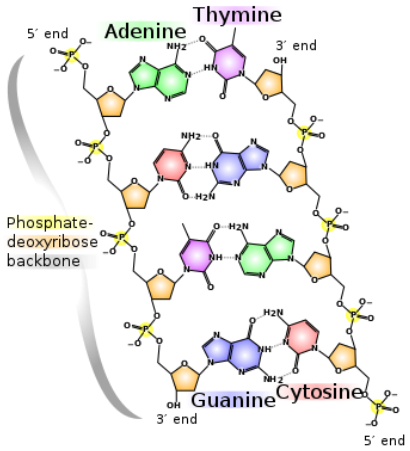
- Gene expression changes between different cell types, developmental changes, treatments, diseases/infections/cancer, stress, alternative splicing, etc…

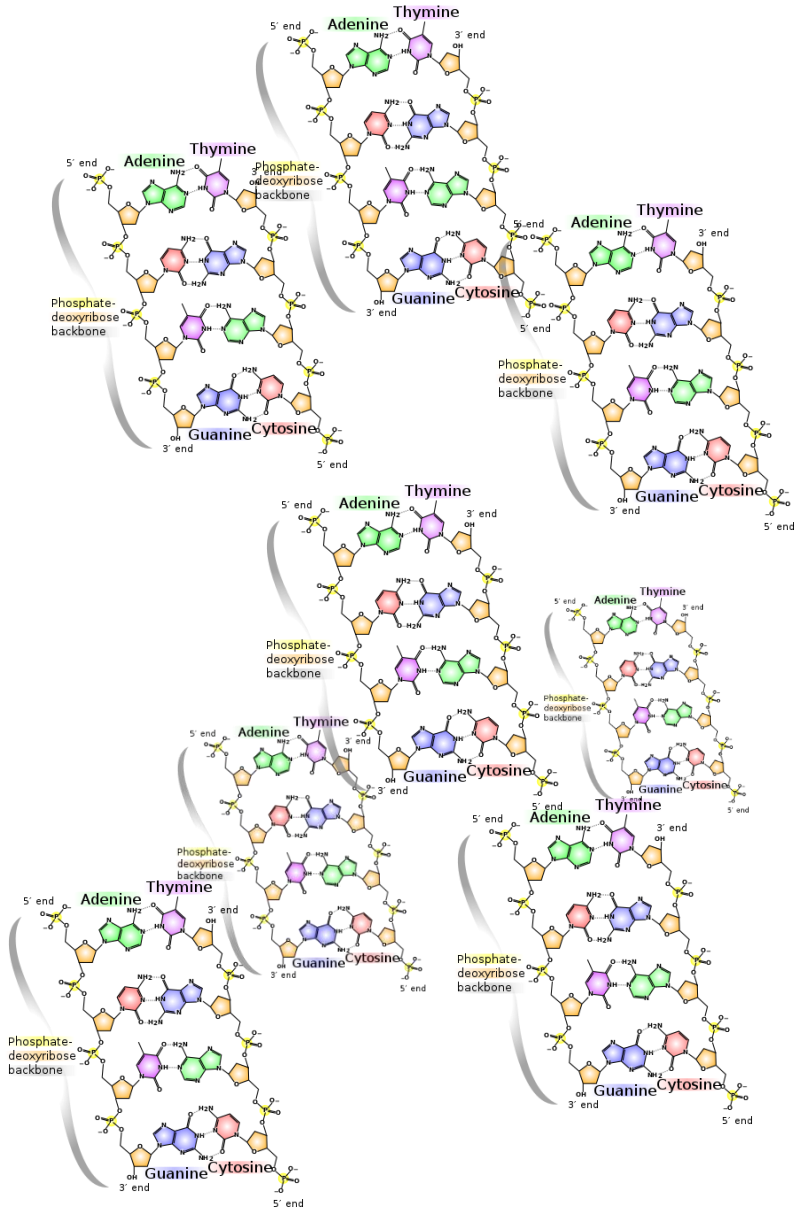
Reverse transcription:



How do we make copies of DNA?

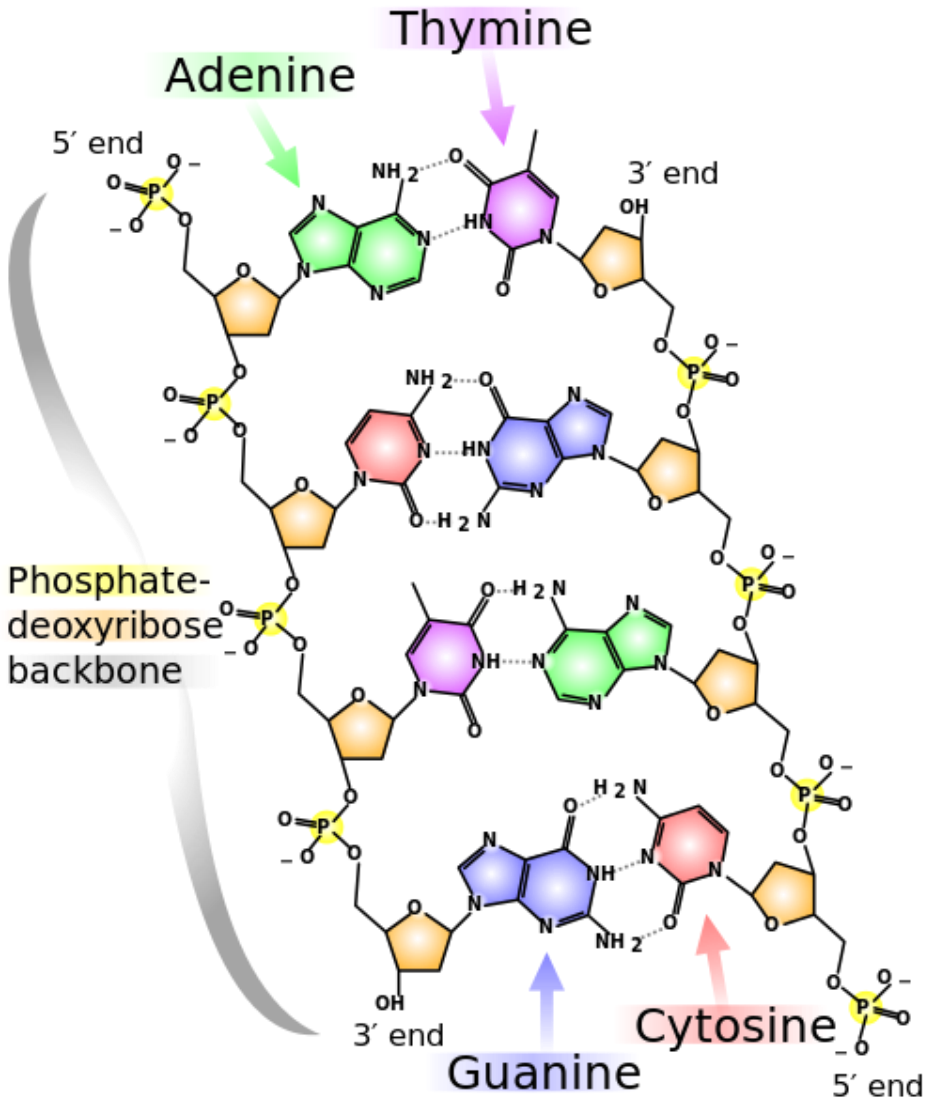
Polymerase Chain Reaction (PCR)





Polymerase Chain Reaction (PCR)

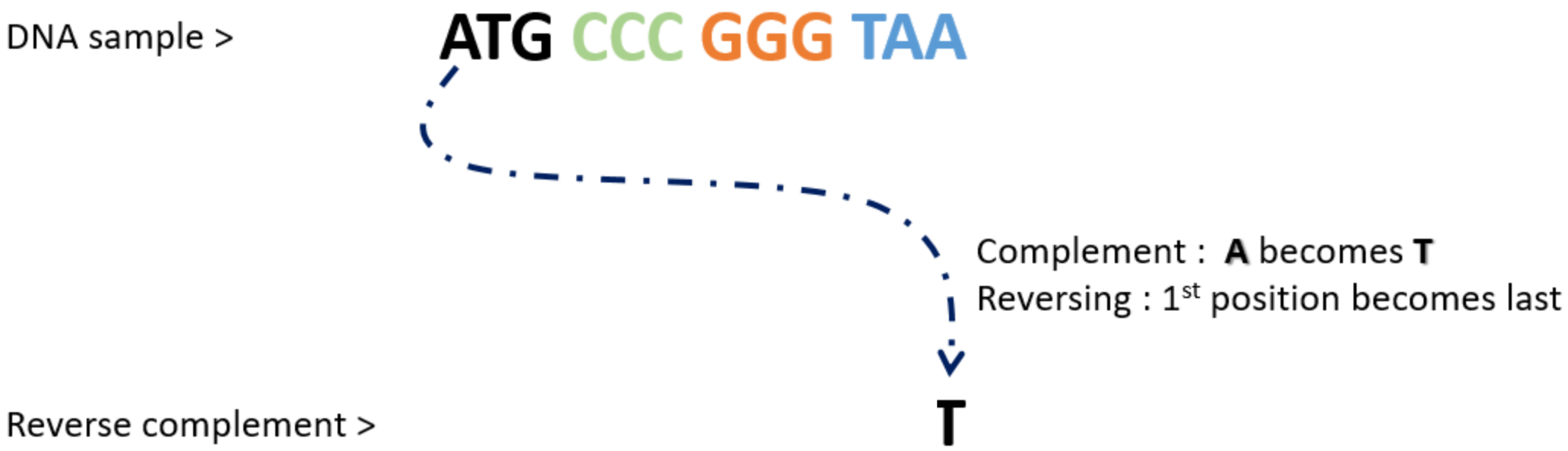
PCR relies on the complementary base pairing of the DNA



PCR uses DNA hybridization

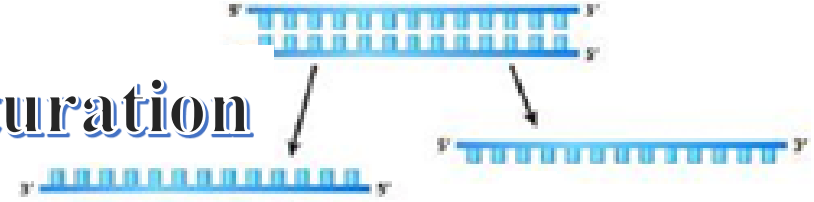
- DNA hybridization involves the formation of a double-stranded nucleic acid
- DNA occurs as a double strand where each *A* is paired with a T and vice versa, and each *C* is paired with a *G* and vice versa

The reverse complement of a DNA sequence is formed by reversing the letters, interchanging *A* and T and interchanging *C* and *G*

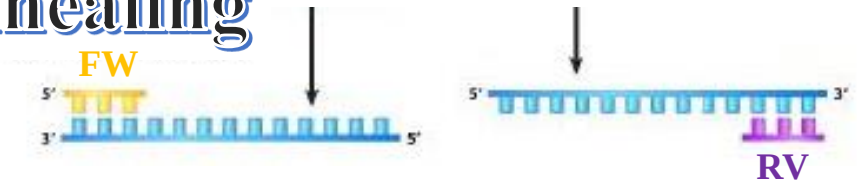


PCR steps

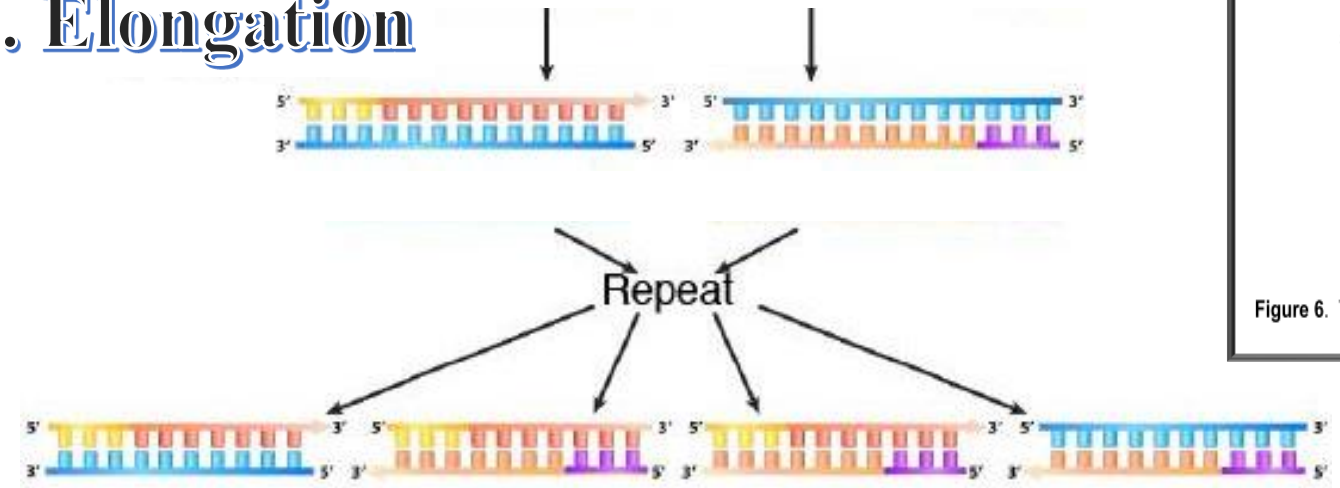
1. Denaturation



2. Annealing



3. Elongation



Fold expression= 2^n

n = number of cycles

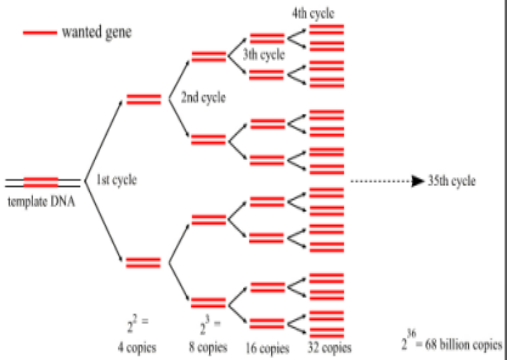
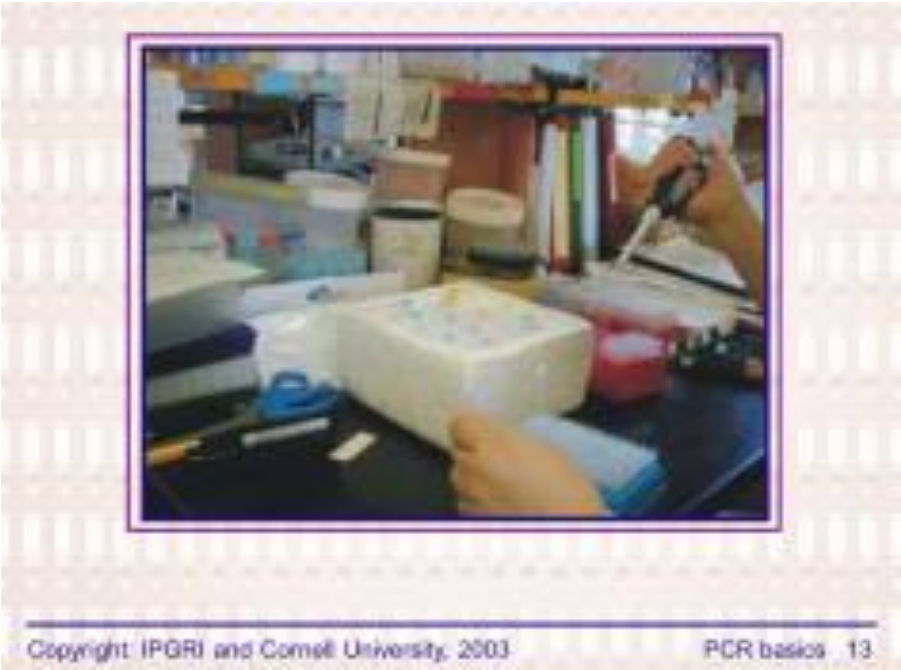


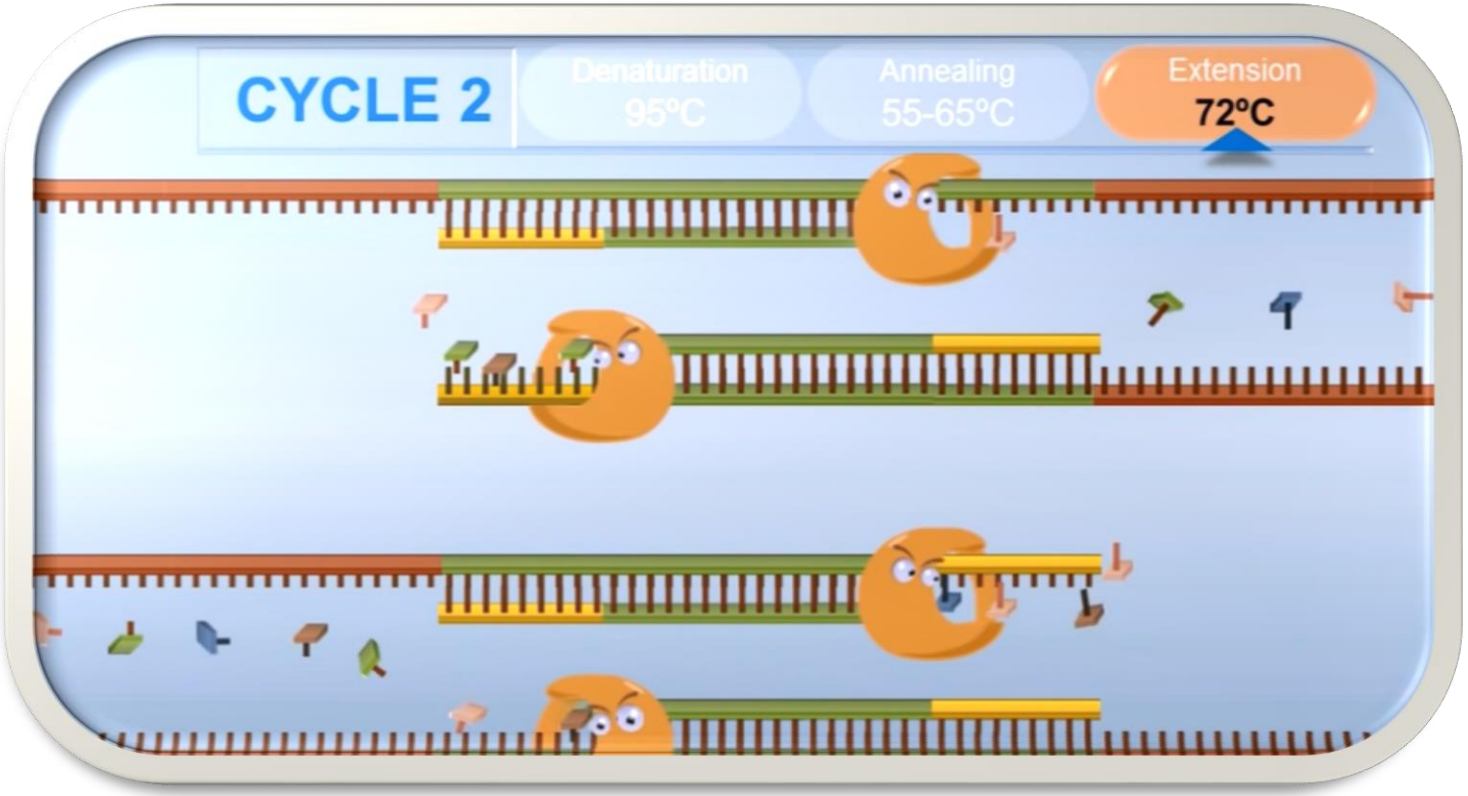
Figure 6. The exponential amplification of DNA in PCR

PCR experiment

What really happens..

How everything starts..



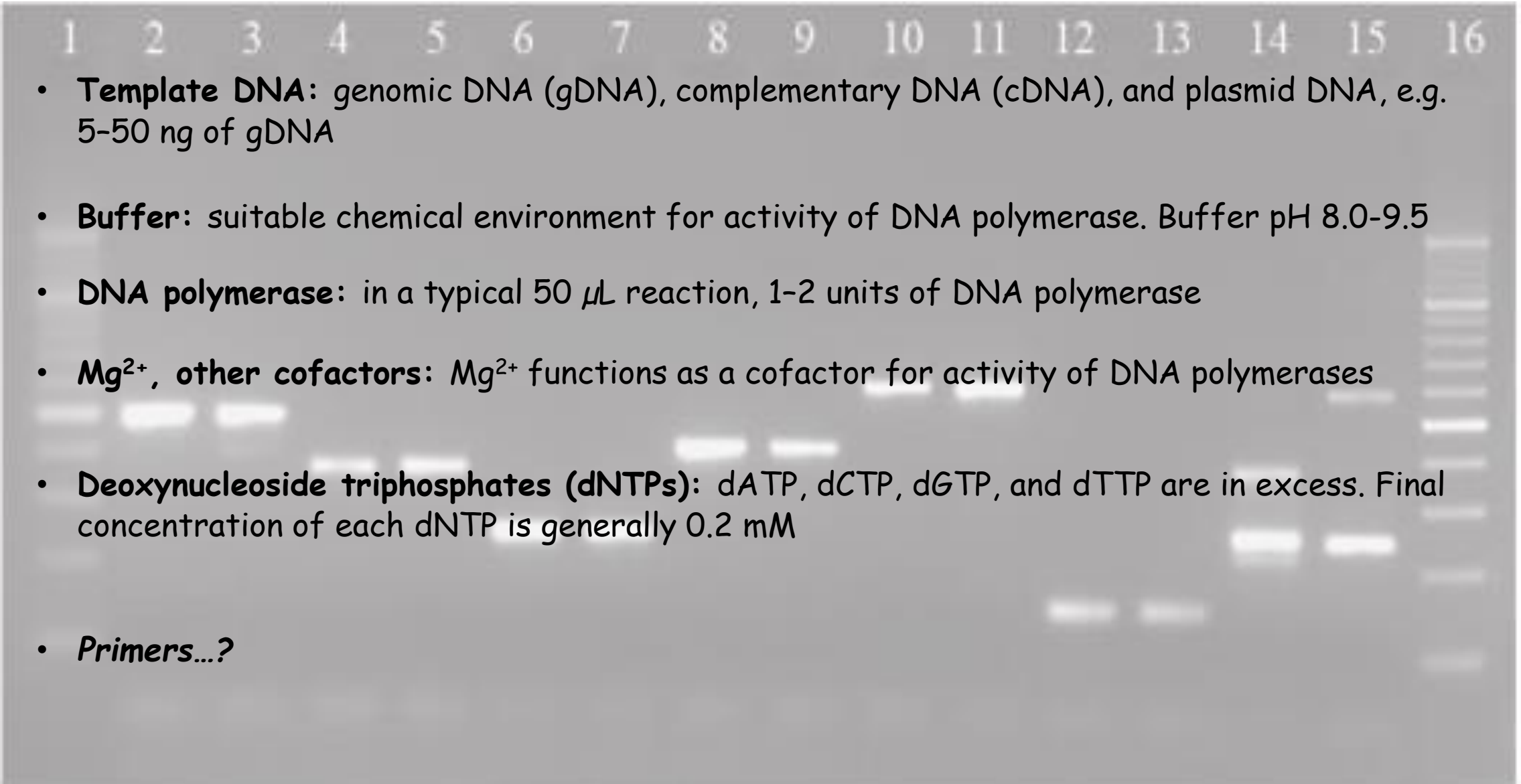


PCR experiment

what usually happens in the lab..

PCR experiment

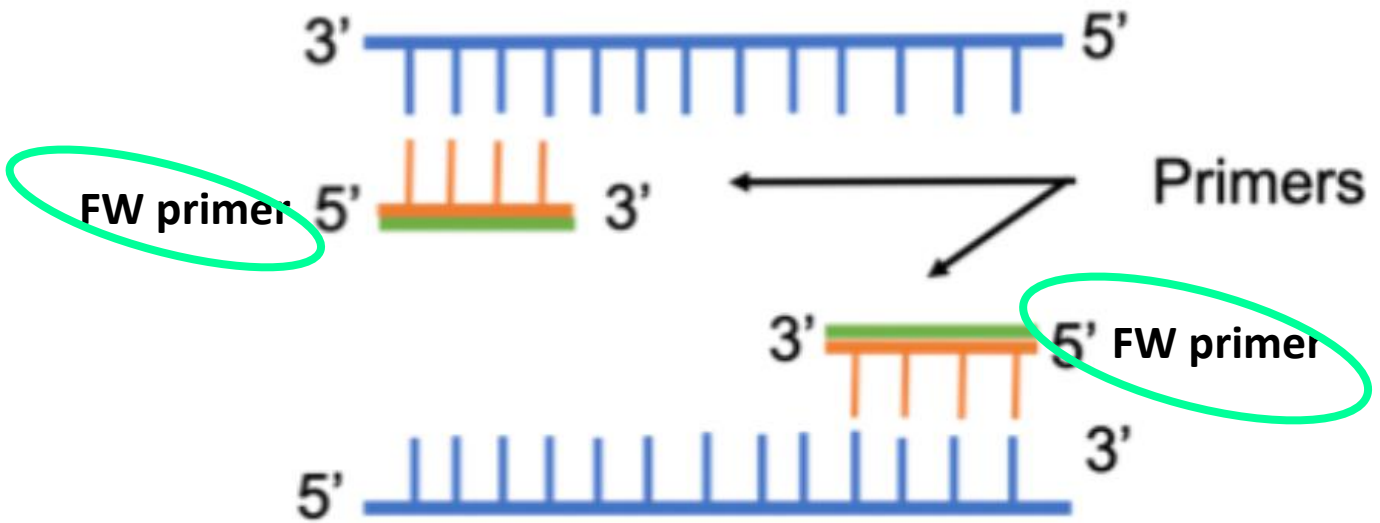
Successful PCR: everything is optimized



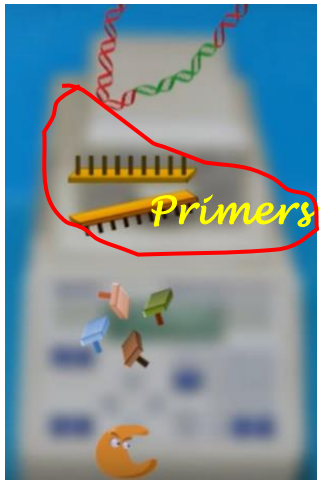
- **Template DNA:** genomic DNA (gDNA), complementary DNA (cDNA), and plasmid DNA, e.g. 5–50 ng of gDNA
- **Buffer:** suitable chemical environment for activity of DNA polymerase. Buffer pH 8.0-9.5
- **DNA polymerase:** in a typical 50 µL reaction, 1–2 units of DNA polymerase
- **Mg^{2+}, other cofactors:** Mg^{2+} functions as a cofactor for activity of DNA polymerases
- **Deoxynucleoside triphosphates (dNTPs):** dATP, dCTP, dGTP, and dTTP are in excess. Final concentration of each dNTP is generally 0.2 mM
- ***Primers…?***

PCR FW and RV primers

- The forward (FW) primer will anneal with 3'-5' DNA strand
- The reverse (RV) primer will anneal with the 5´-3' DNA strand



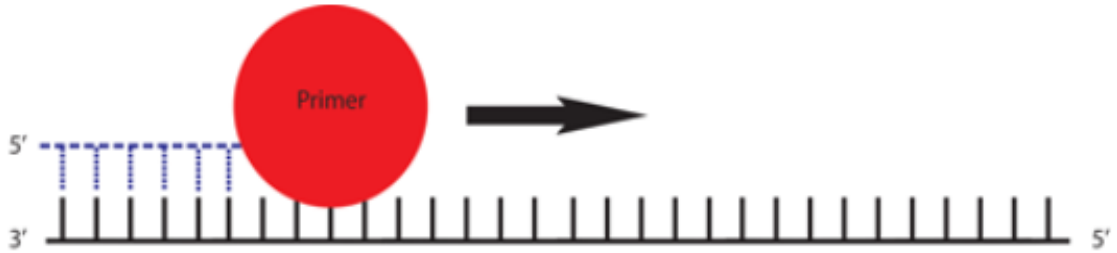
How to choose FW & RV primers?



primers should generally have the following properties:

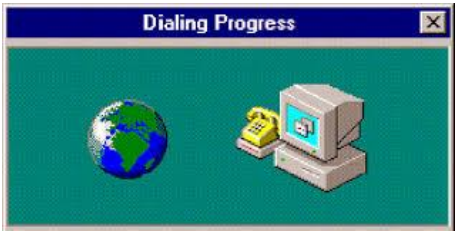
- Length of 18-24 bases
- 40-60% G/C content
- Start and end with 1-2 G/C pairs
- Melting temperature (Tm) of 50-60°C
- Primer pairs should have a Tm within 5°C of each other
- Primer pairs should not have complementary regions

Note: *If you will be including a restriction site at the 5' end of your primer, note that a 3-6 base pair "clamp" should be added upstream in order for the enzyme to cleave efficiently (e.g. GCGGCG-restriction site-your sequence).*



Important for the analysis

Gene-specific!
Or transcript-specific



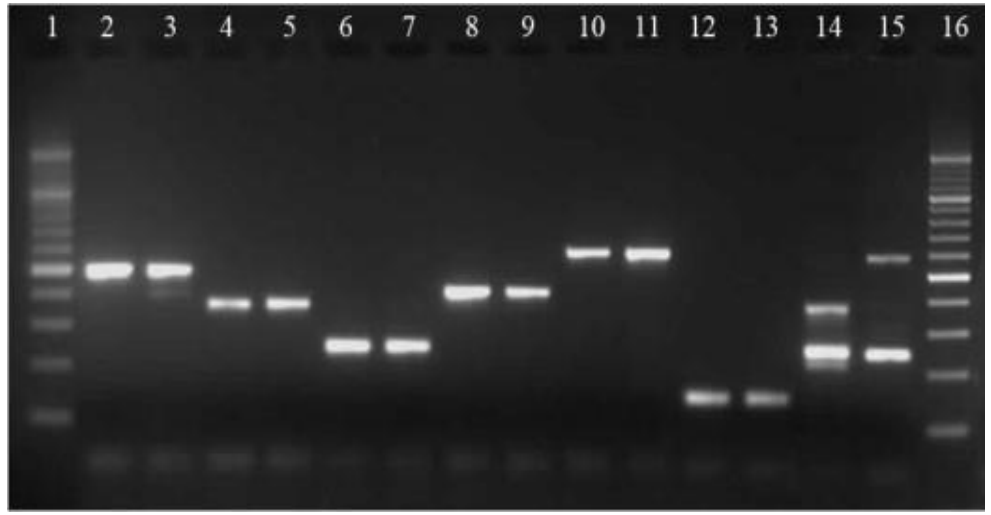
PCR products visualization

- **In agarose gels the samples are separated by their size**
- Electrophoresis is the motion of charged particles relative to a fluid caused by an electric field
- As particle velocities depend on particle net charge and size, it can be used for separation

Old times:
Ethidium bromide

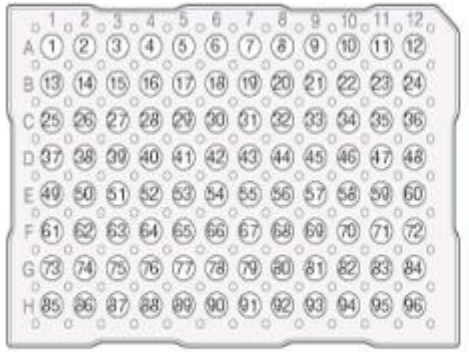
\+ *cost-effective*

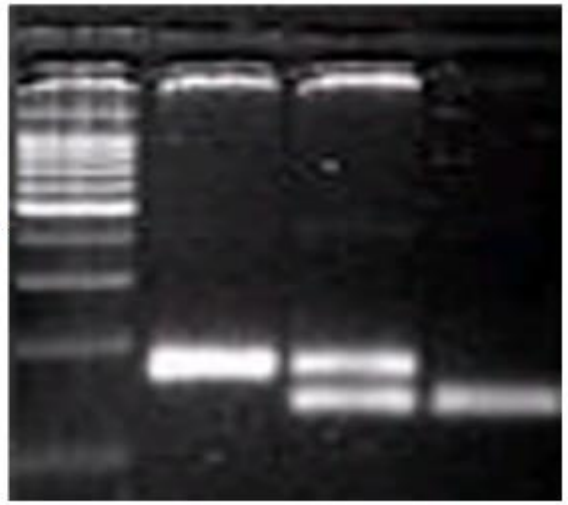
mutagenic



Traditional PCR

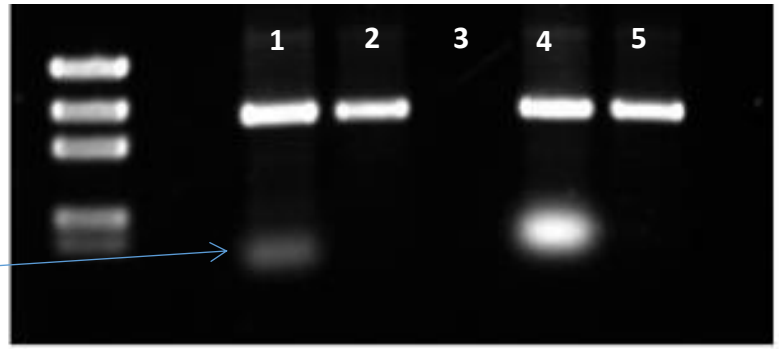
In traditional PCR the product is detected only after amplification is completed, using methods as gel electrophoresis



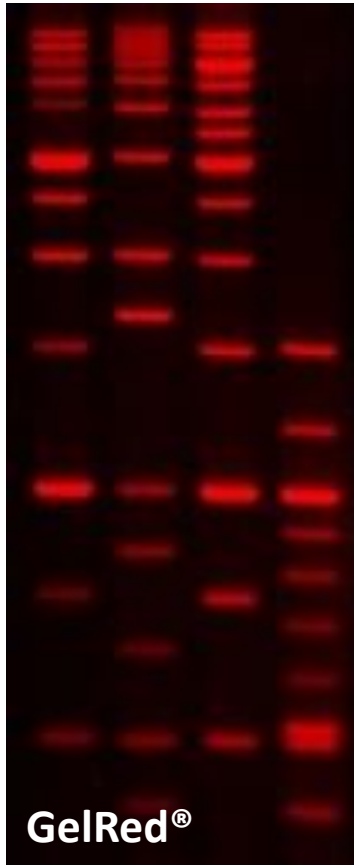


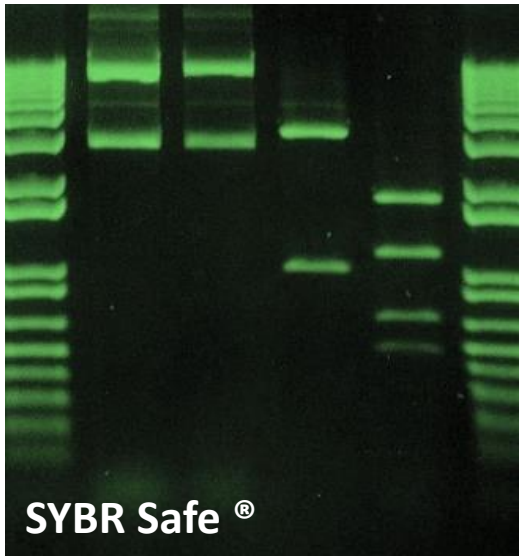
Gel electrophoresis separates non-target products from target based on size difference

Non-target amplification is commonly observed in gel-based PCR



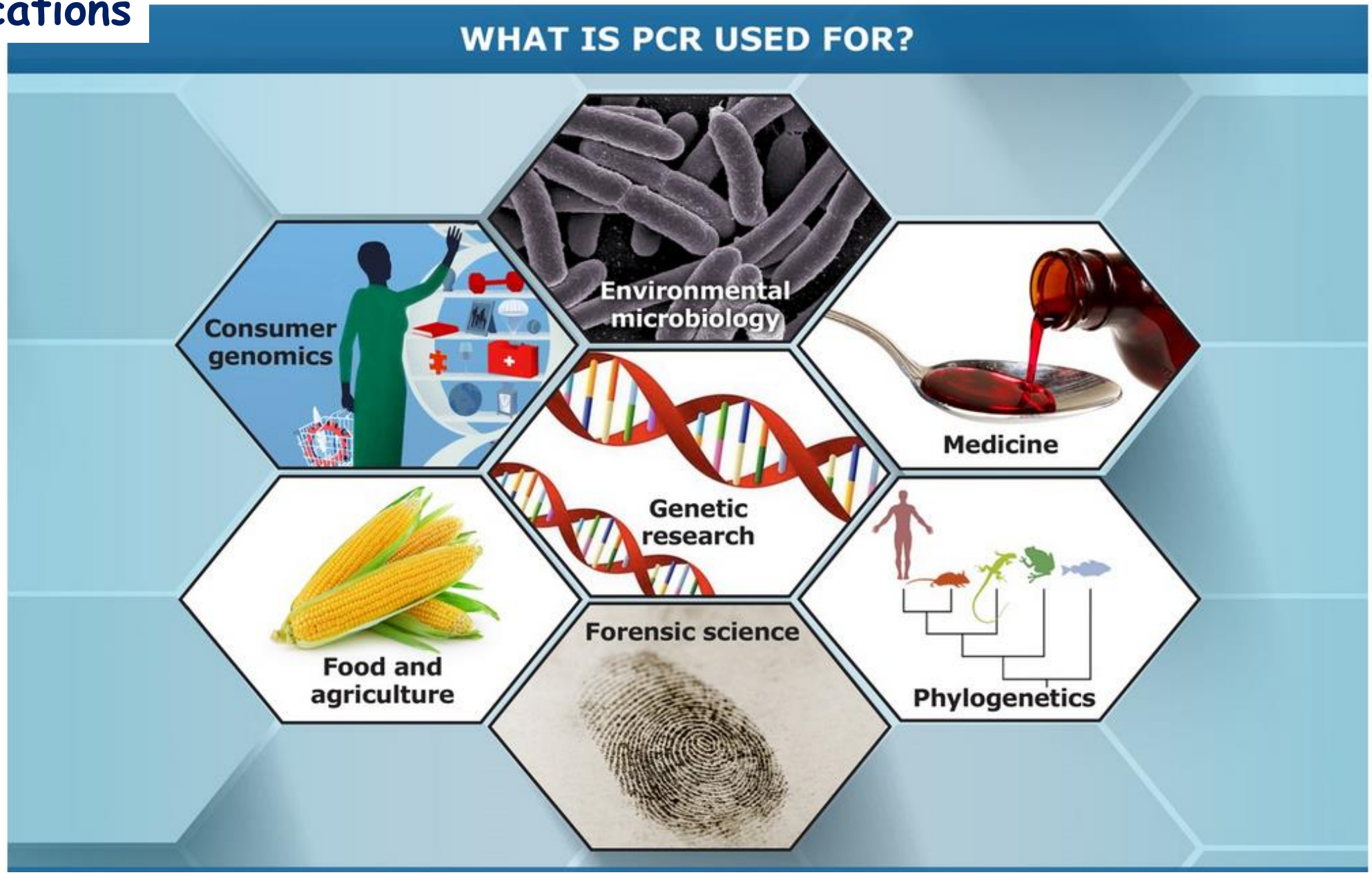
PCR products visualization





Modern times:
Non-toxic dyes, such as GelRed, RedTaq, etc

PCR applications



PCR applications

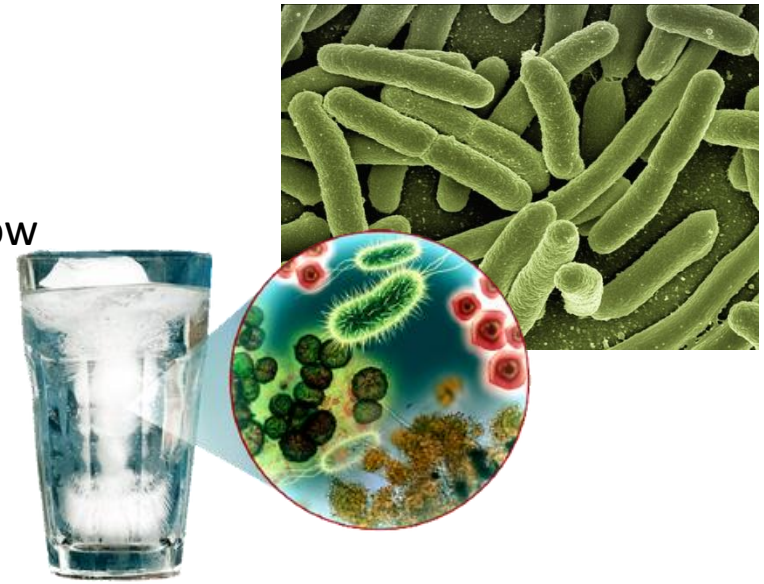
WHAT IS PCR USED FOR?

Environmental biology:

Detection of microorganisms in the environment:

- PCR allows specific target species to be identified and quantified, even when very low numbers exist

e.g. searching for pathogens or indicator species such as coliforms in water supplies



Consumer genomics:

PCR has enabled personalized genome testing. An industry has sprung up offering consumers tailor-made products and services based on information in their genome. e.g. nutrigenomics

- **Nutrigenomics** is concerned with the impact of dietary components on the genome, the proteome (the sum total of all proteins), and the metabolome (the sum of all metabolites)

- Much of the nutrigenomic focus has been on single-nucleotide polymorphisms (SNPs): Dietary factors may differentially alter the effect of one or more SNPs to increase or decrease disease risk

SNPs: DNA sequence variations that account for 90% of all human genetic variation. SNPs that alter the function of "housekeeping genes" involved in the basic maintenance of the cell are assumed to alter the risk of developing a disease.

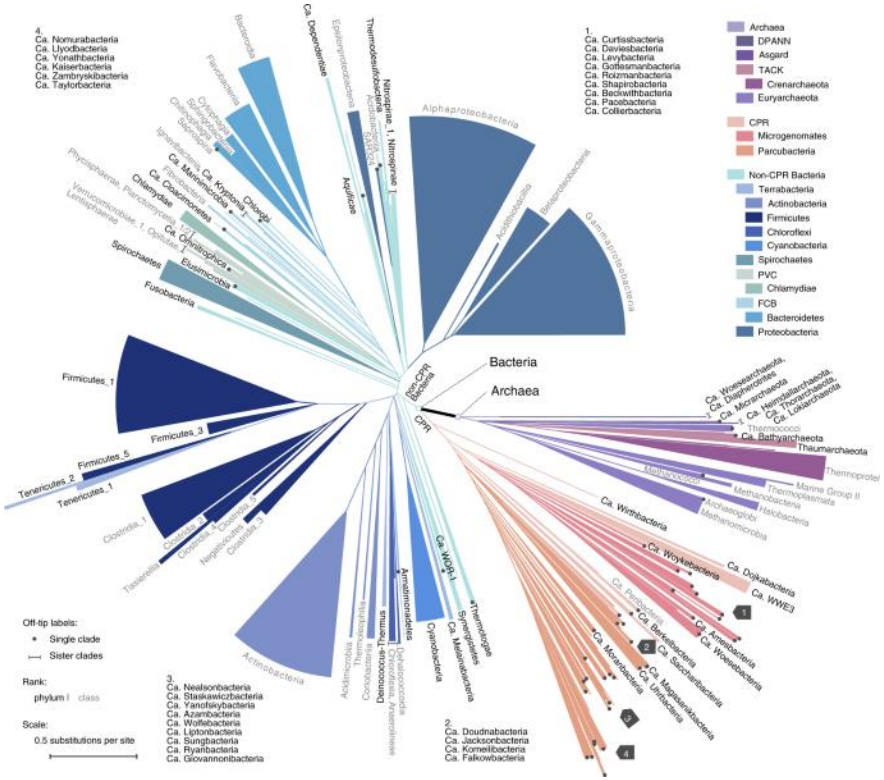
PCR applications

WHAT IS PCR USED FOR?

Phylogenetics

Minute quantities of DNA, including ancient DNA, from sources such as hair, bones and other tissues can be amplified using PCR. The DNA can then be identified and analysed, and genomes can be sequenced.

- understand evolution, paleontology and organisms' evolutionary relationships to each other
- support conservation efforts and understanding species unique adaptations



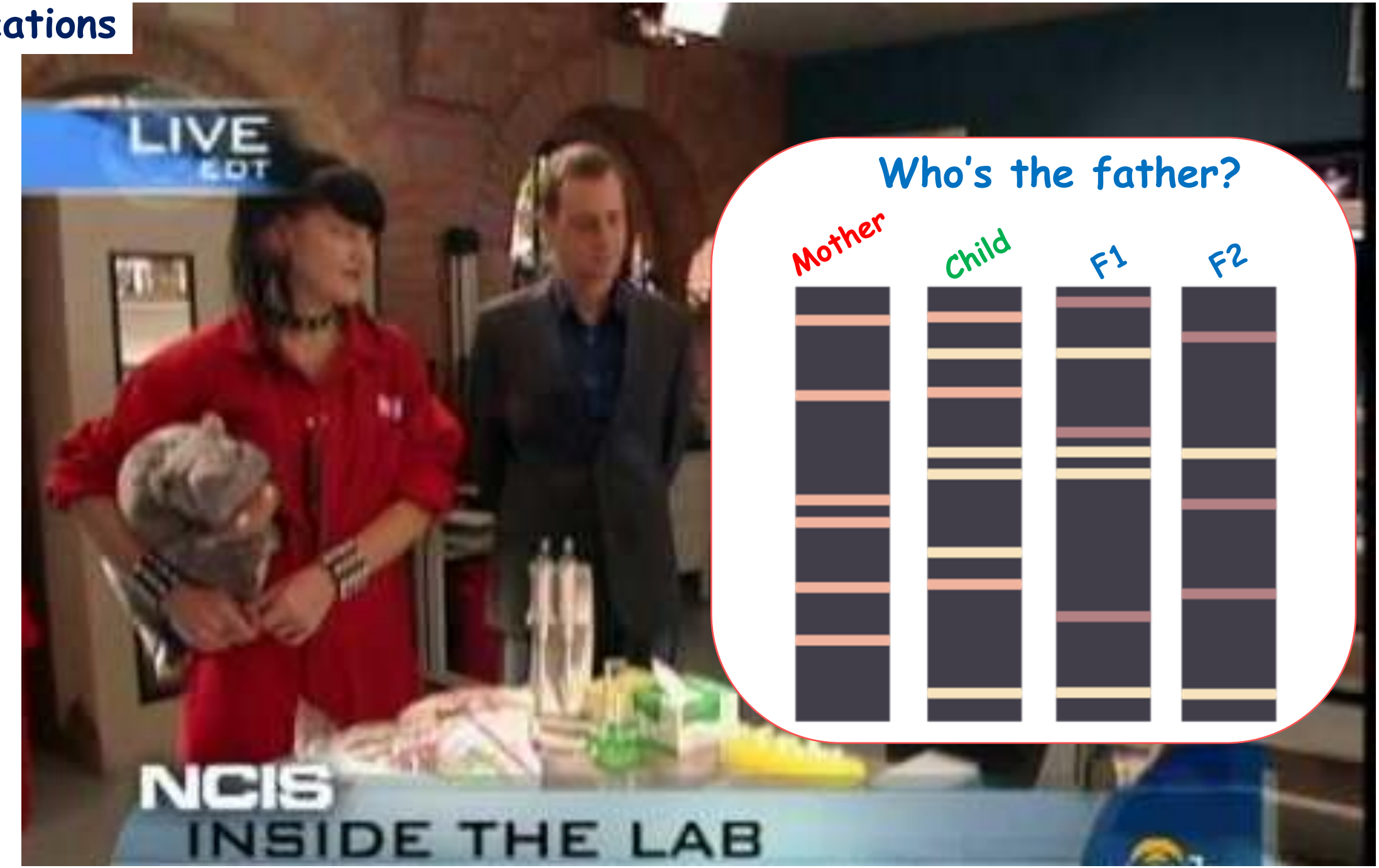
Forensic science

PCR is very important for the collection and the identification of organic crime scene evidence such as blood, hair, pollen, semen and soil

DNA fingerprints identification of familial relationships, genomic DNA isolation and other molecular diagnostics and biochemical analyses can be undertaken forensically through the use of PCR.

PCR allows DNA to be identified from tiny samples – a single molecule of DNA can be enough for PCR amplification.

PCR applications



PCR applications

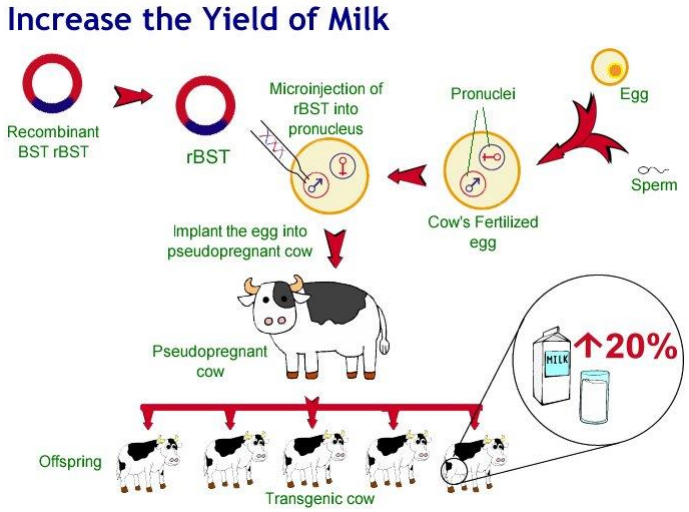
WHAT IS PCR USED FOR?

Food and agriculture

Genetic technologies include a range of techniques that enable the modification of existing organisms for the purpose of improving foods and food production.

Although selective breeding has been around for centuries, with the advent of technology genetic manipulation can be more targeted and new crops produced more quickly.

e.g. transgenic cows: The transgene is present in every cell in the transgenic cow. However, it's only expressed in mammary tissue. This means that the transgene's protein will only be found in the cow's milk.



Genetic research

There are many fields of genetic research that use PCR as an essential tool. The majority of these technologies have multidisciplinary applications. These include the creation, detection and monitoring of genetically modified organisms (GMOs), genetic engineering, gene modification, transgenics, cloning, synthetic biology and directed evolution.

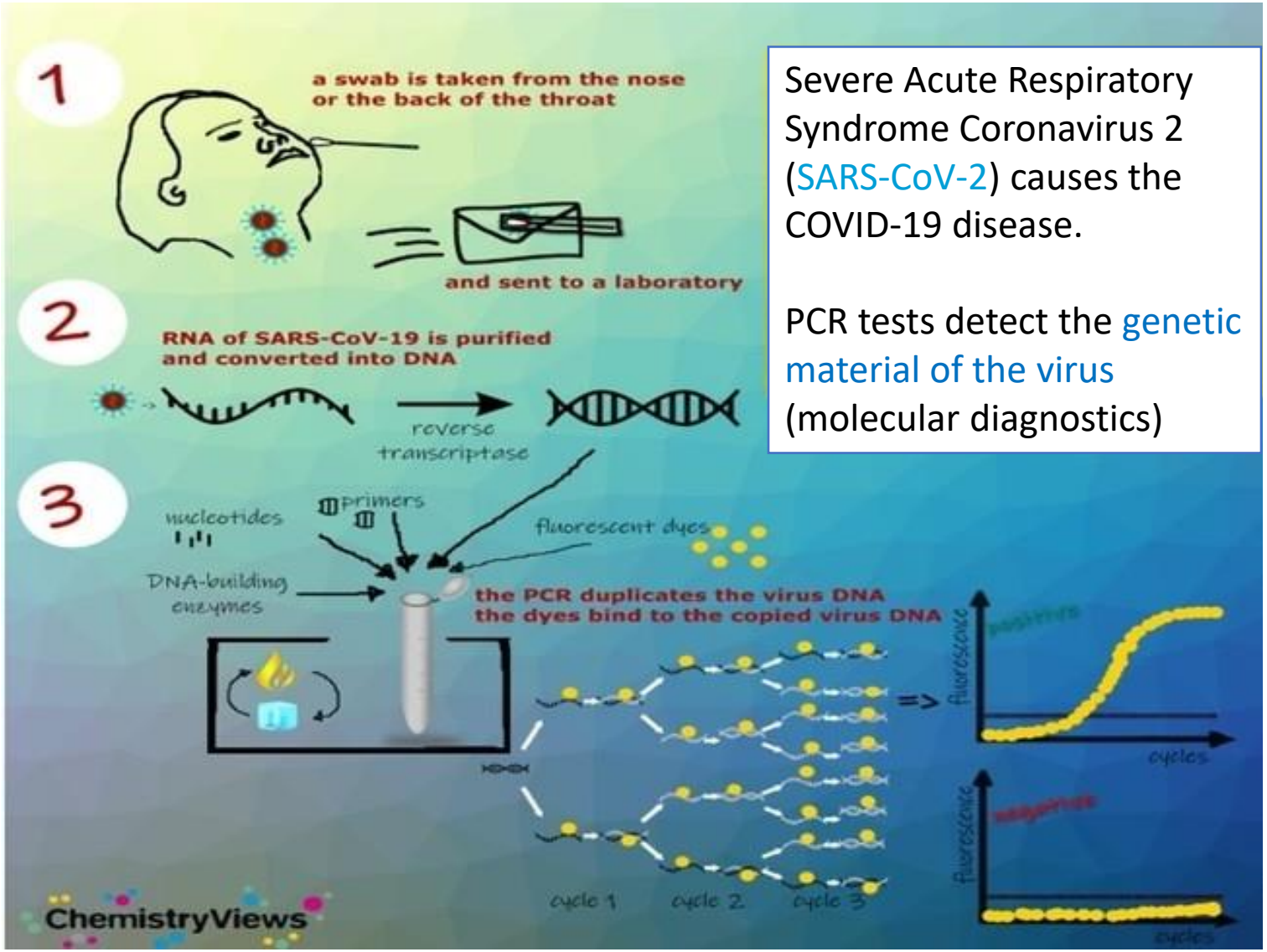
PCR applications

WHAT IS PCR USED FOR?

PCR in medicine:

- Genetic disorders: for the identification of many genes related to certain phenotypes, including genetic disorders. Genetic disorders are caused by mutations that range from simple changes in the base sequence of the DNA double helix through to changes in large DNA sequences and even whole chromosomes.
- The Human Genome Project for identifying and mapping all of the genes of the human genome
- Genotyping for sex determination of embryos as well as detecting chromosomal and genetic disorders in the fetus
- Personalized medicine: to tailor therapy with the best response and highest safety margin to ensure better patient care
- Pharmaceutical production: some medicinal products are produced by genetic modification (GM) of existing organisms. This includes GM microorganisms to mass produce pharmaceuticals such as insulin and some antibiotics.
- Transgenics: involves transfer of genes from one organism to another via genetic modification
- Transplant testing: technologies can use PCR to improve the accuracy of tissue compatibility, thereby reducing the likelihood of transplant rejection
- Pathogenic microorganisms, including some viruses, bacteria, parasites and fungi, cause infectious diseases and can be identified using PCR, aiding efficient diagnosis and treatment. e.g testing for Legionnaires' disease, tracing zoonoses and SARS-CoV-2

PCR applications

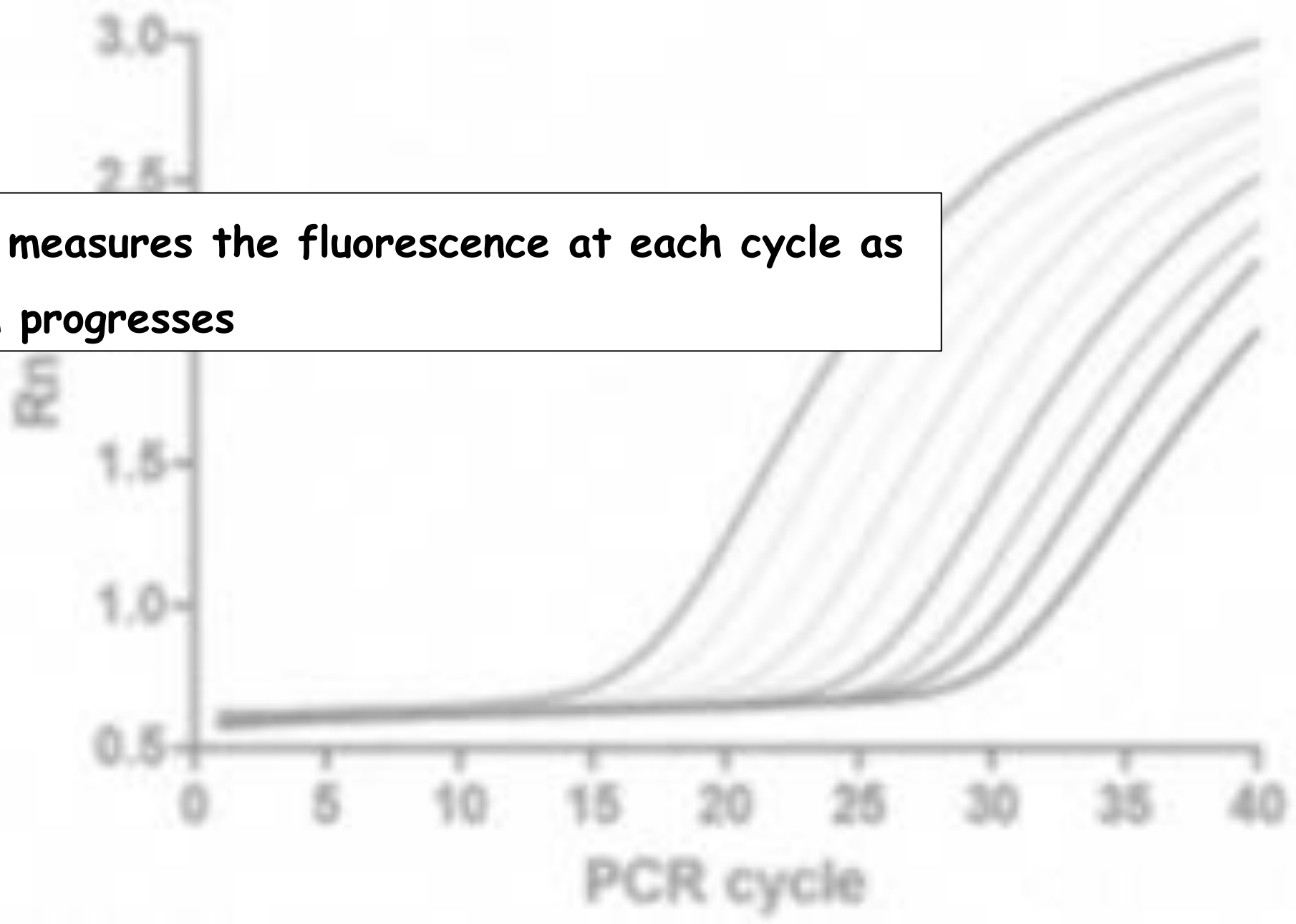


Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causes the COVID-19 disease.

PCR tests detect the genetic material of the virus (molecular diagnostics)

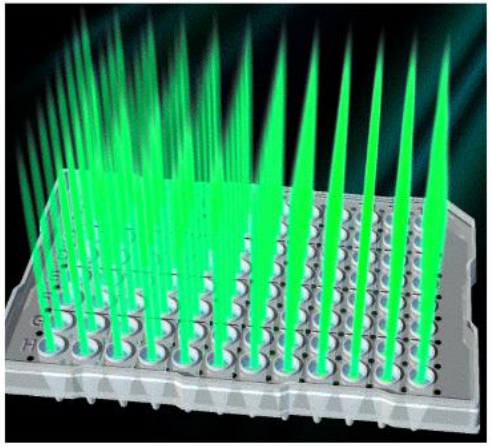
How to monitor the levels in gene expression?

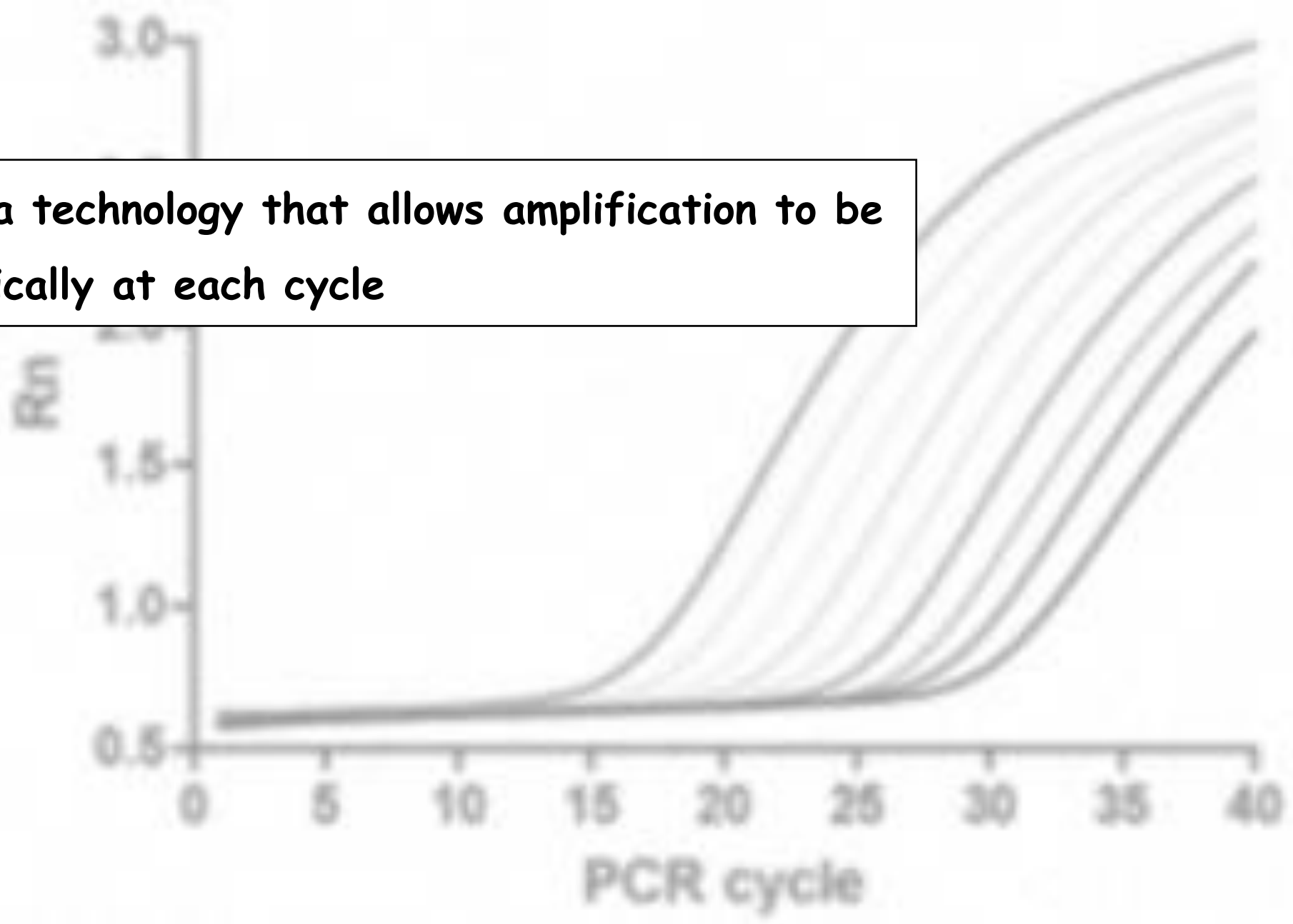
Real-time QPCR measures the fluorescence at each cycle as the amplification progresses



Real-time PCR

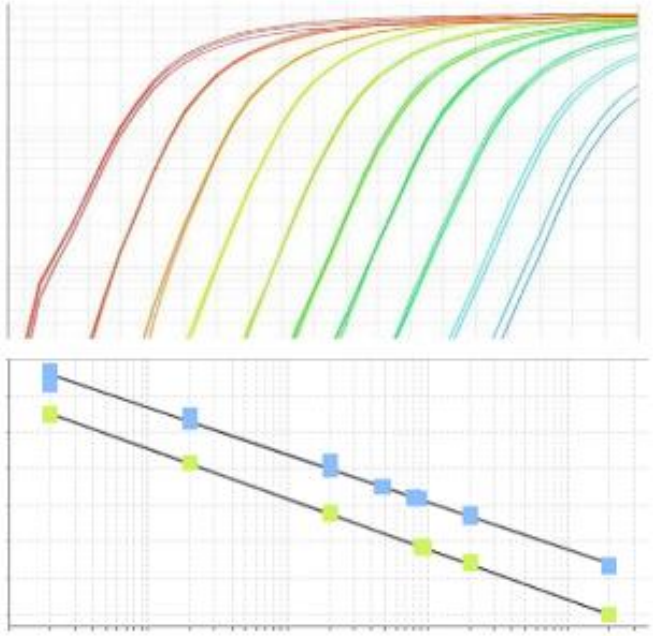
Real-time PCR is a technology that allows amplification to be detected automatically at each cycle





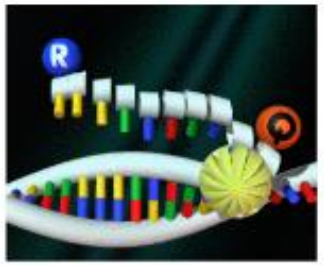
Real-time Quantitative PCR

Real-time Quantitative PCR is a method to measure gene concentrations in samples



Required components for Real-Time PCR

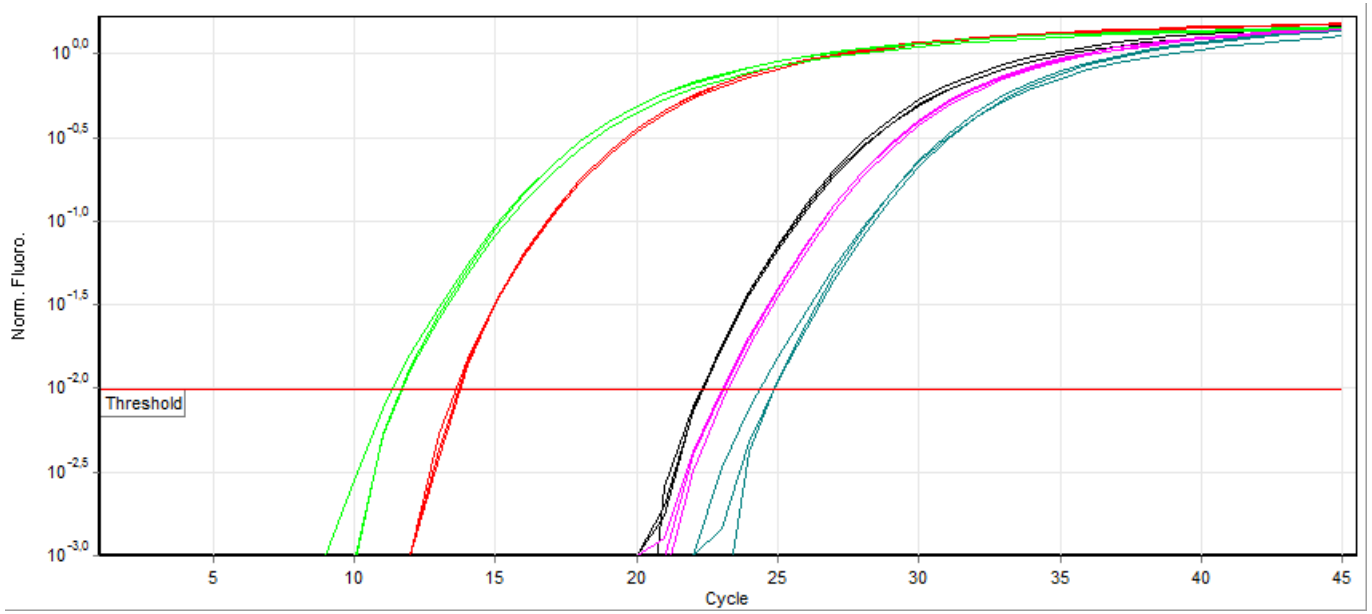
Real-Time PCR Instrument:
Thermal cycler & optical detector



Real-Time PCR Chemistry:
Fluorescent signal automatically emitted each cycle

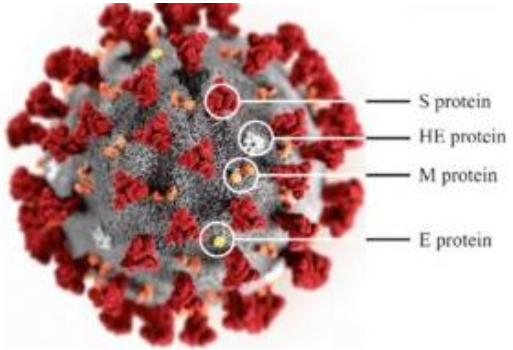
Benefits of Real-Time PCR

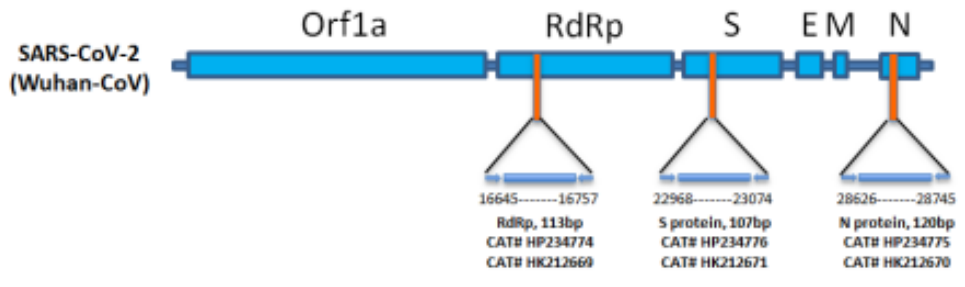
- No laboratory procedures post-PCR
- More information than gels
- **Gene quantification with accuracy, precision and throughput**



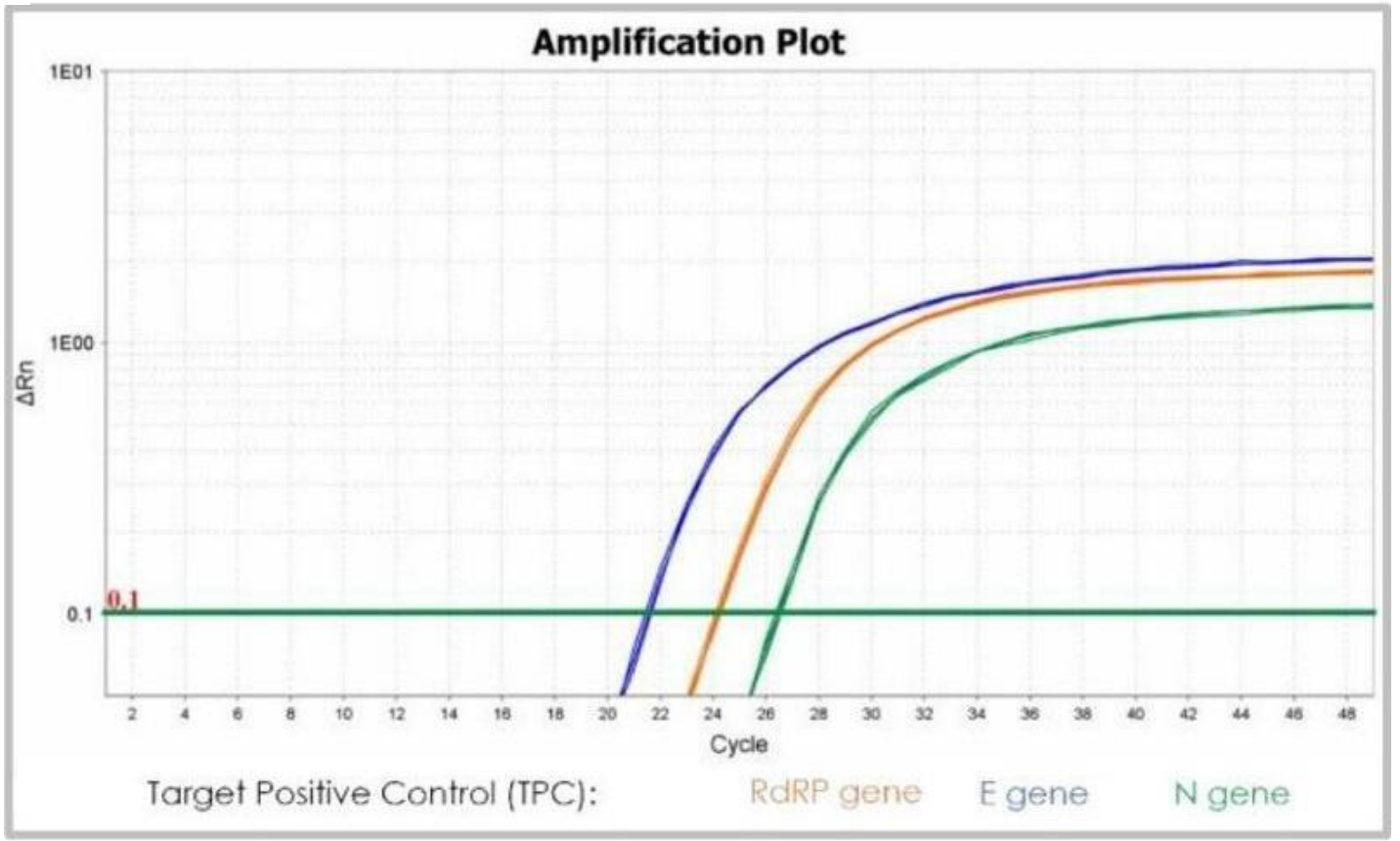
PCR applications

WHAT IS PCR USED FOR?





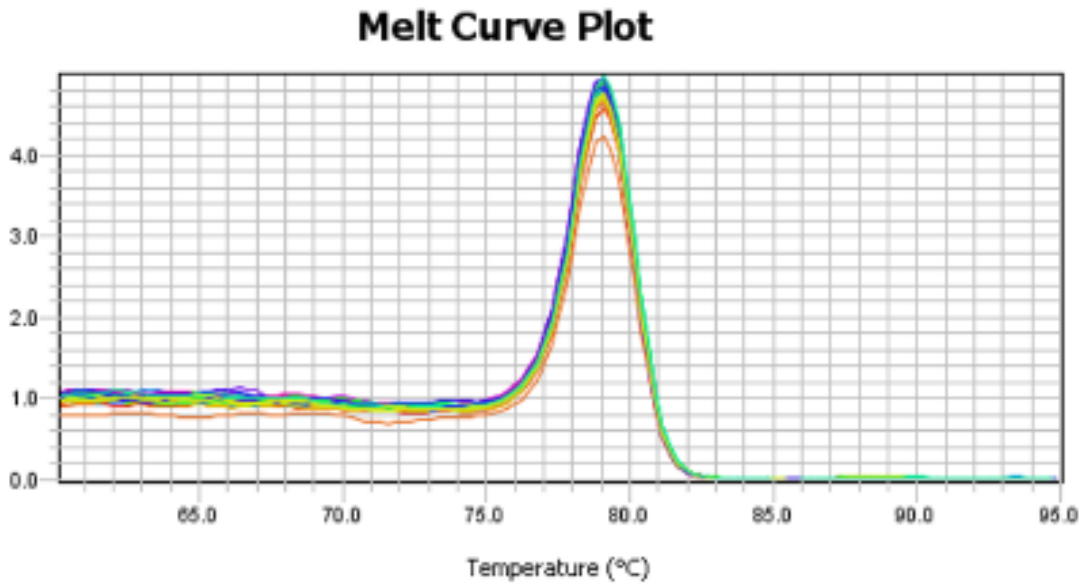
Relative positions of amplicon targets on SARS-CoV-2 (previous Wuhan-CoV) genome. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase; S: surface glycoprotein; N: nucleocapsid. Numbers below amplicon are genome locations corresponding to Wuhan-CoV genome, [MN908947].



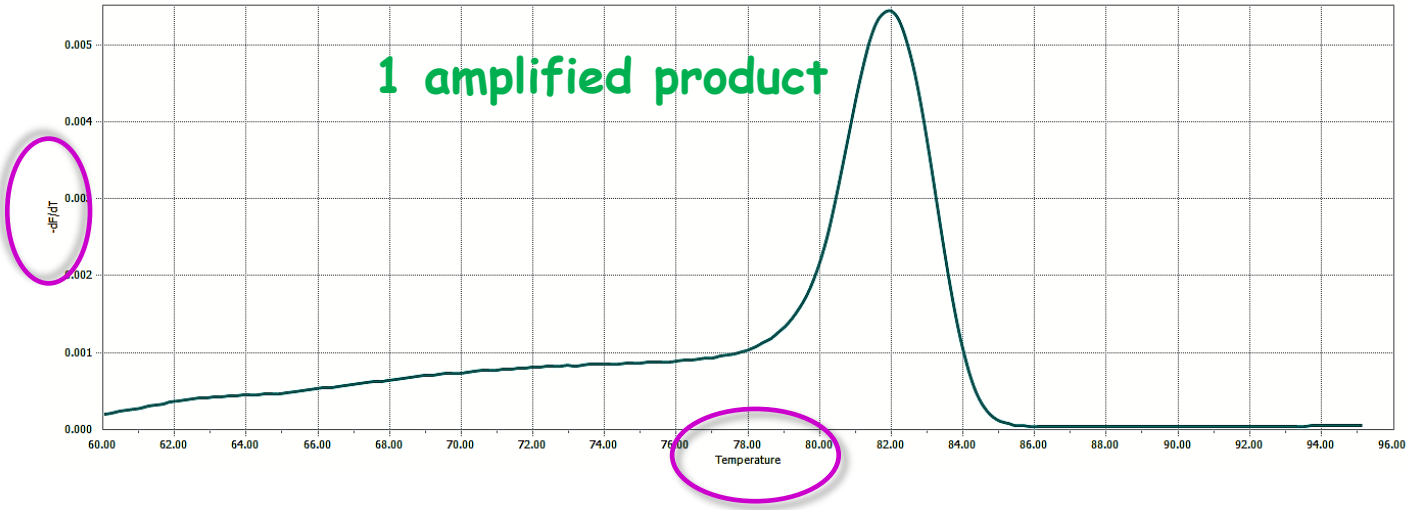
Real-time PCR: SYBR Green Melting Curve

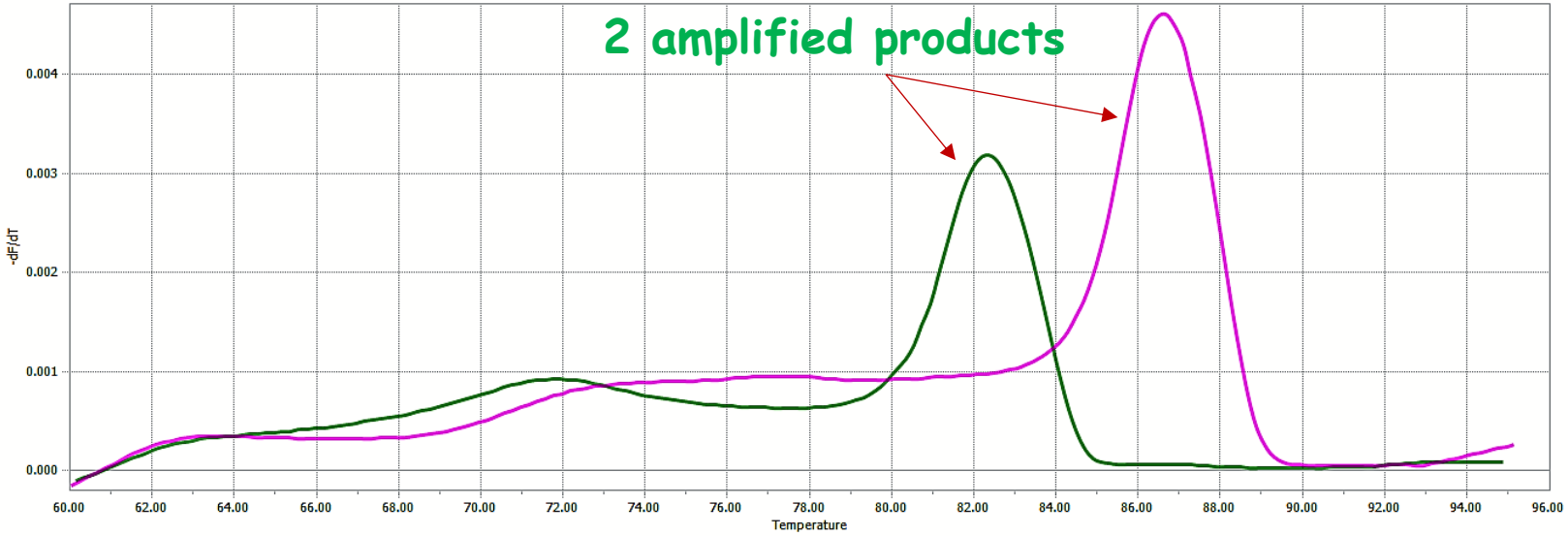
For SYBR Green assays where specificity is important, a melting curve should be performed after each amplification and the melting curve data evaluated

For example, a narrow symmetrical melting peak that overlays with a known pure target melting peak is consistent with single target amplification

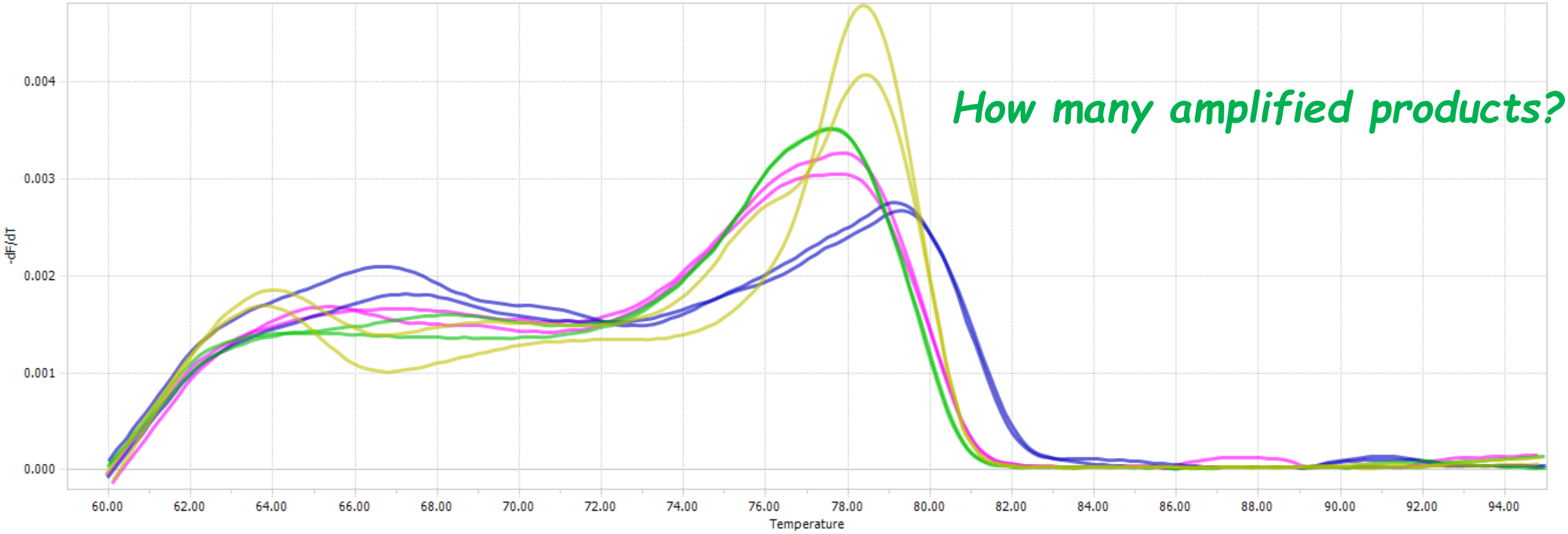


Real-time PCR: SYBR Green Melting Curve (or Dissociation curve)



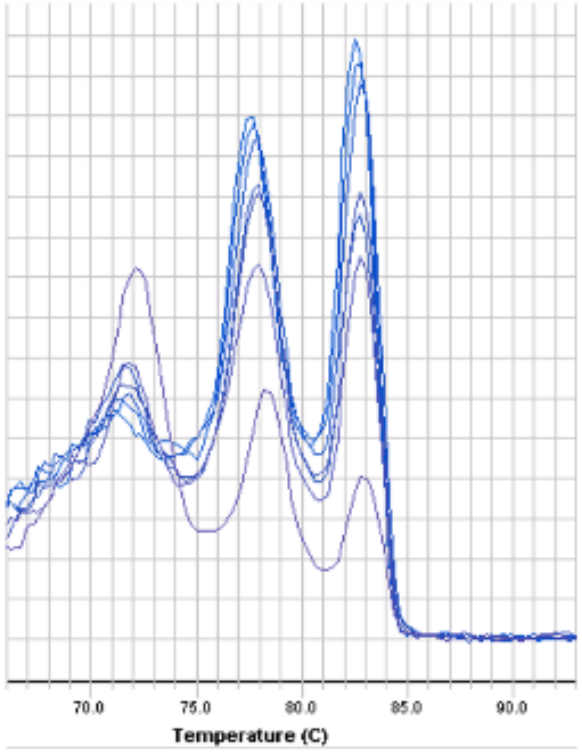


Real-time PCR: SYBR Green Melting Curve (or Dissociation curve)

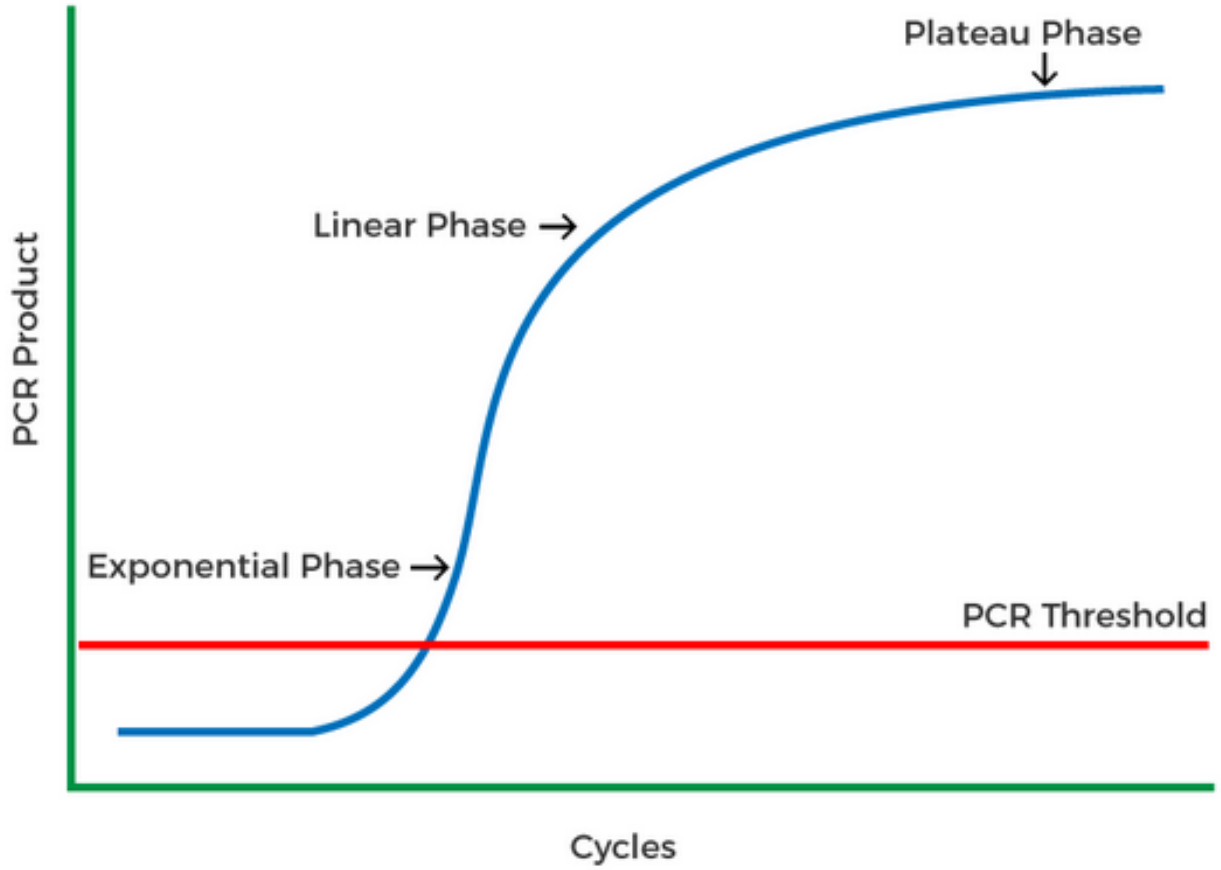


Real-time PCR: SYBR Green Melting Curve

For SYBR Green assays intended to amplify one target, data from wells with multiple melt peaks should be considered compromised

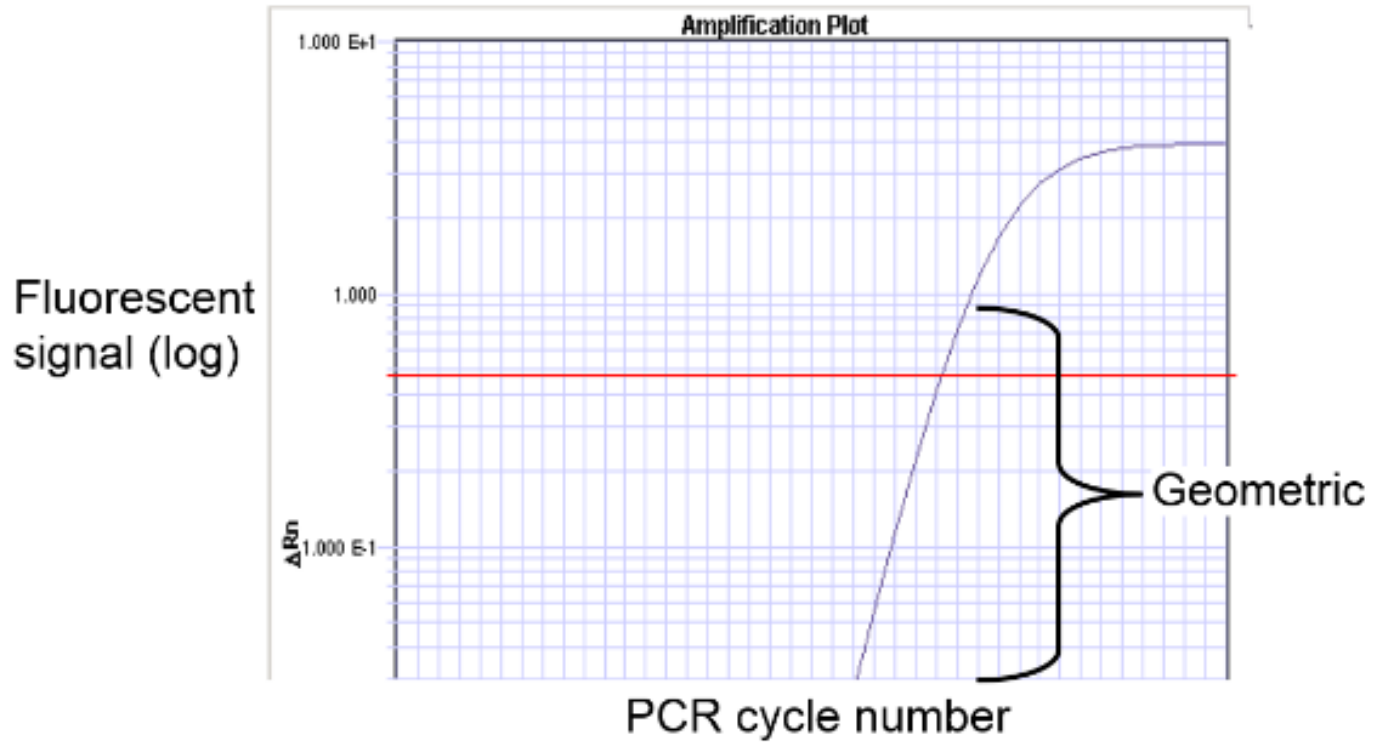


Real-time PCR: 3 phases



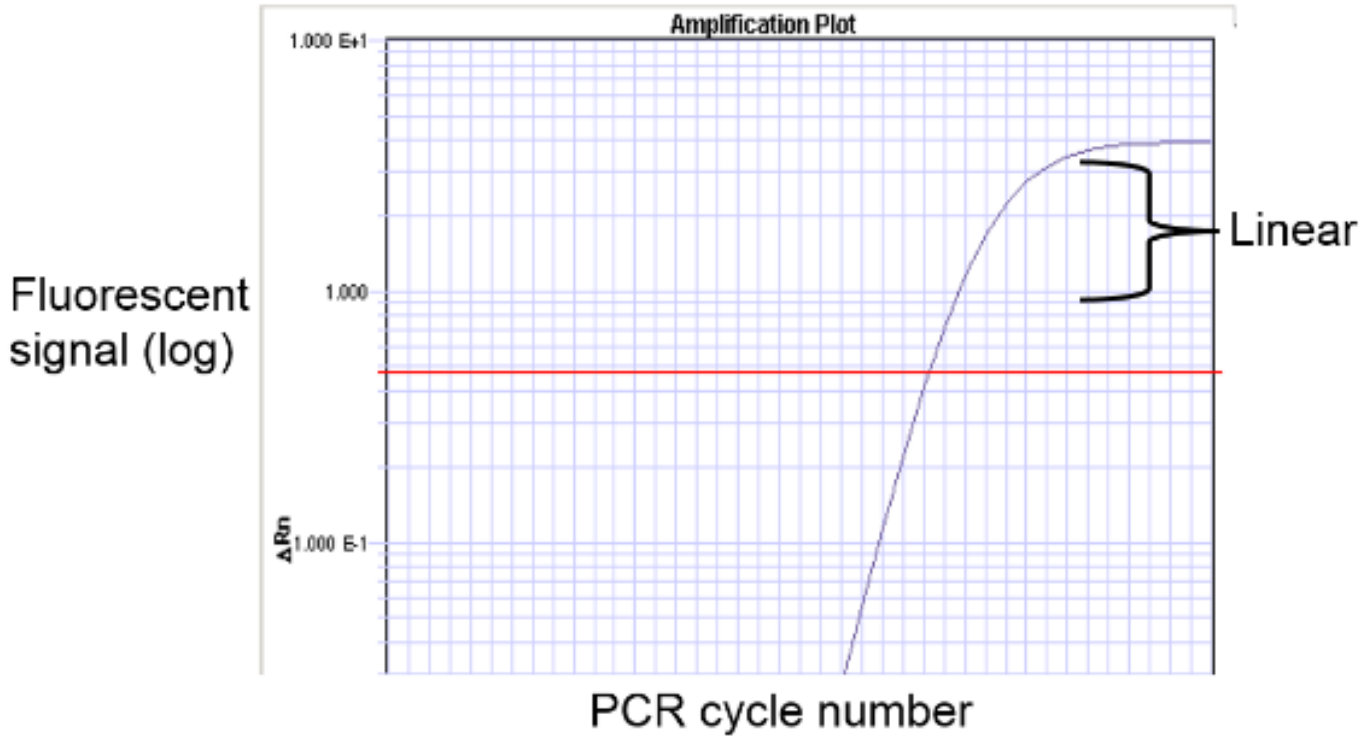
Real-time PCR: Exponential phase

- Exponential (or geometric or logarithmic) phase is the first phase of real-time PCR where the efficiency is constant
- Quantitative data are derived from this phase



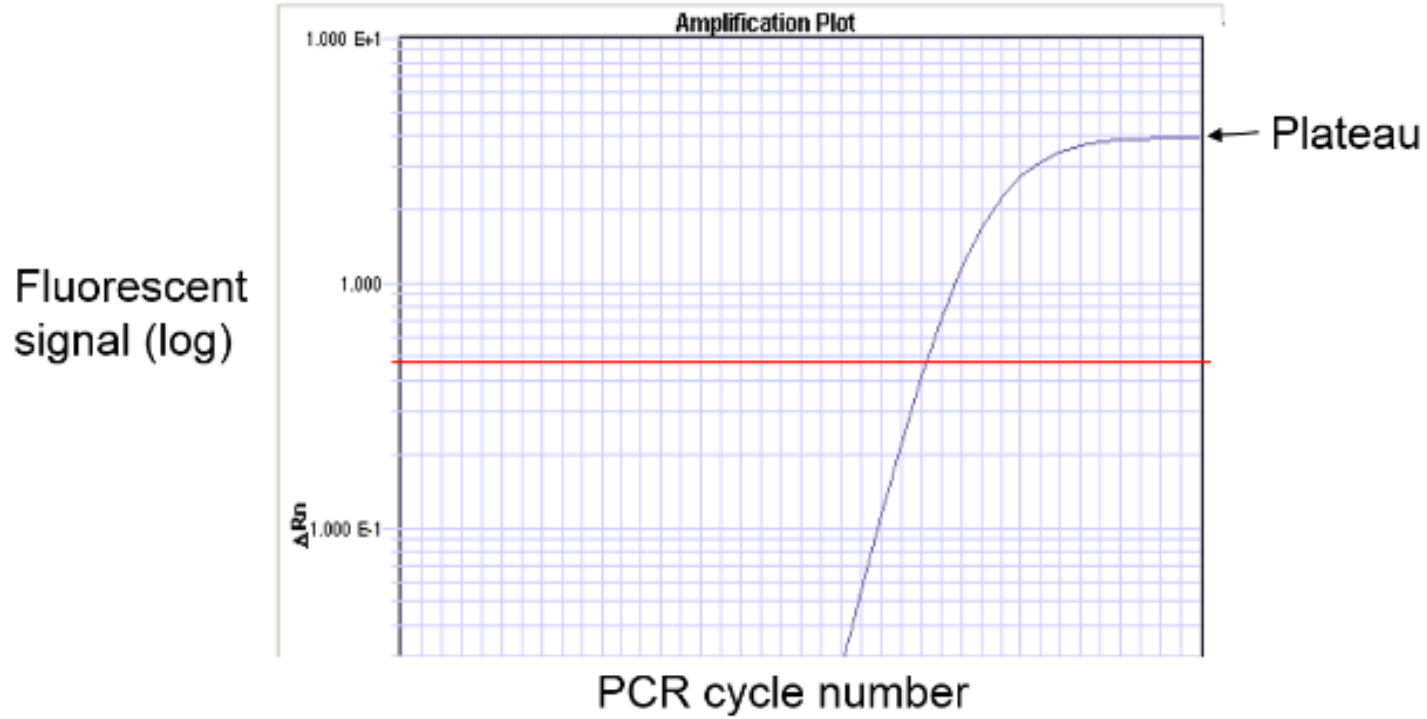
Real-time PCR: Linear phase

- Linear is the second phase where the efficiency continually declines, cycle to cycle



Real-time PCR: Plateau phase

- Plateau is the last phase where there is no significant amplification

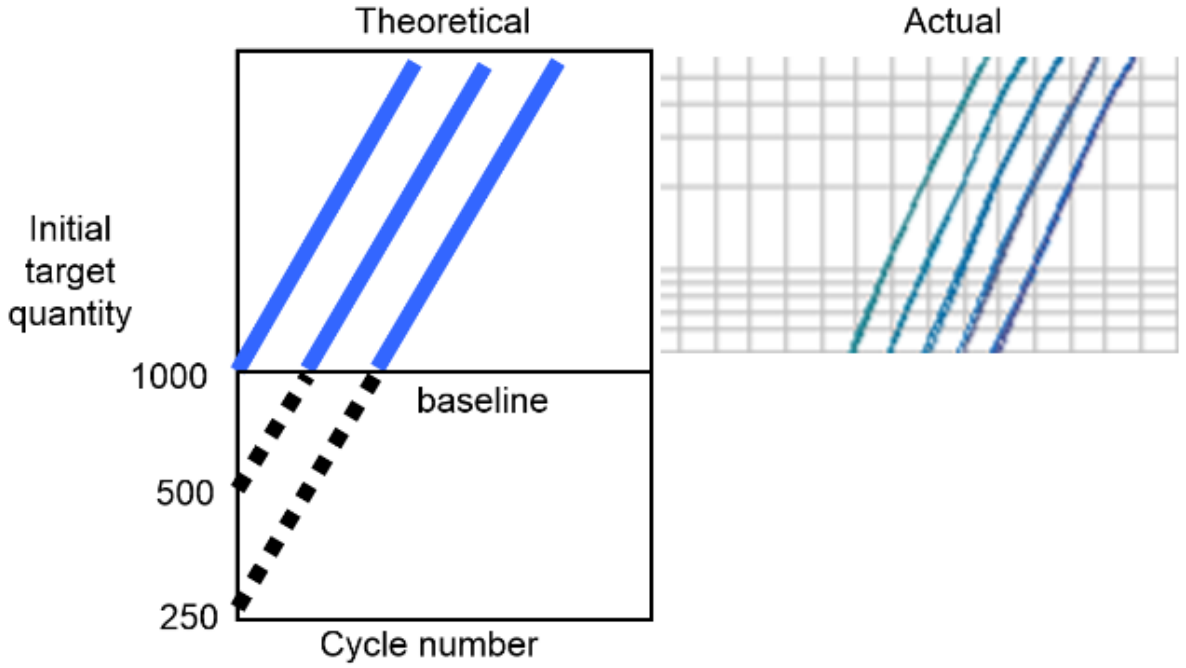


Real-time PCR: reaction efficiency

The exponential phase has a unique behavior:

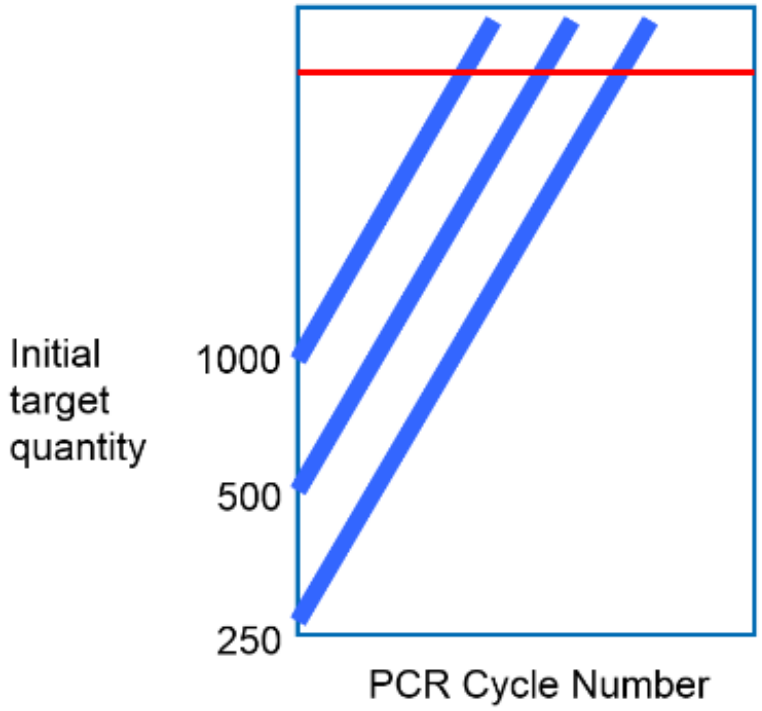
efficiency is not affected by initial target quantity

Reaction Efficiency is measured in the Exponential phase

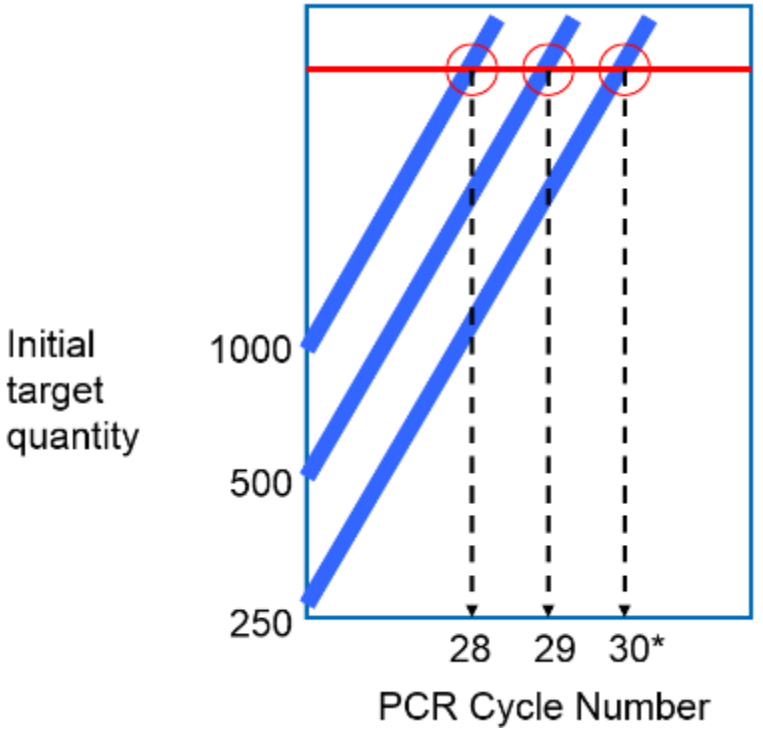


Real-time PCR: threshold

Threshold is a horizontal line drawn through the exponential phase data

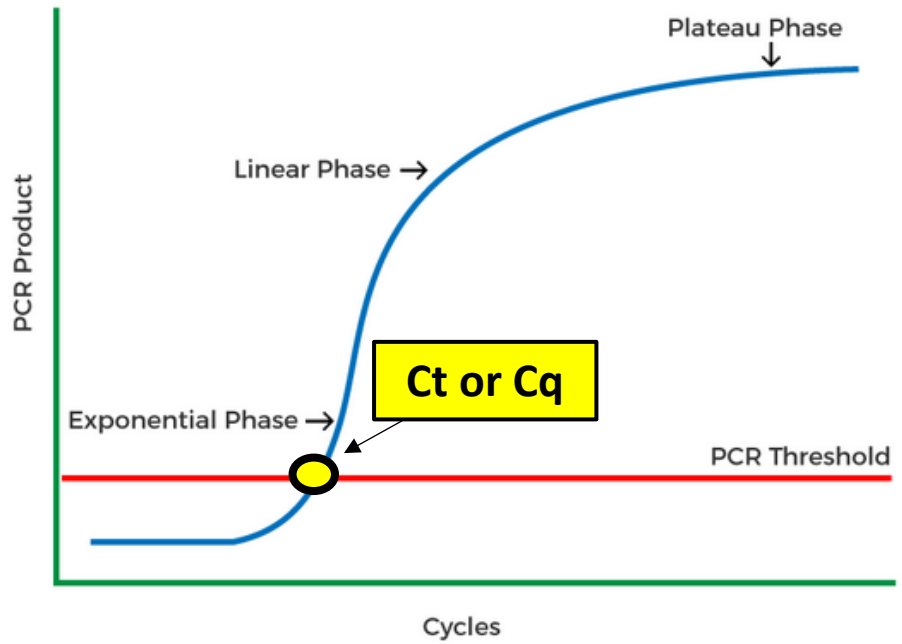


Threshold cycle (Ct or Cq) is the PCR cycle where the exponential data and threshold intersect



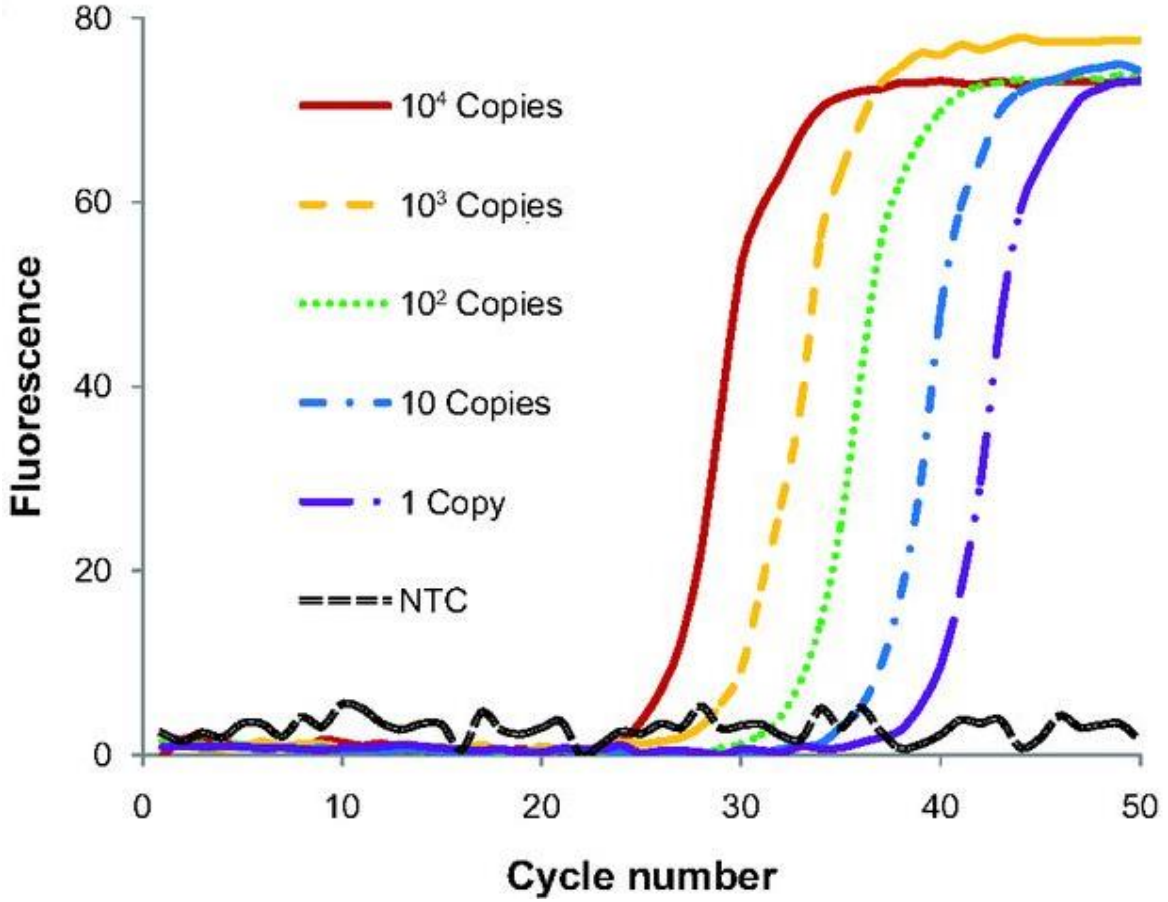
*Ct values are usually not whole numbers.

principles of real-time PCR: Ct & amplification plot



Ct: The **first cycle** at which the instrument can **distinguish the amplification generated fluorescence** as being **above the ambient background**

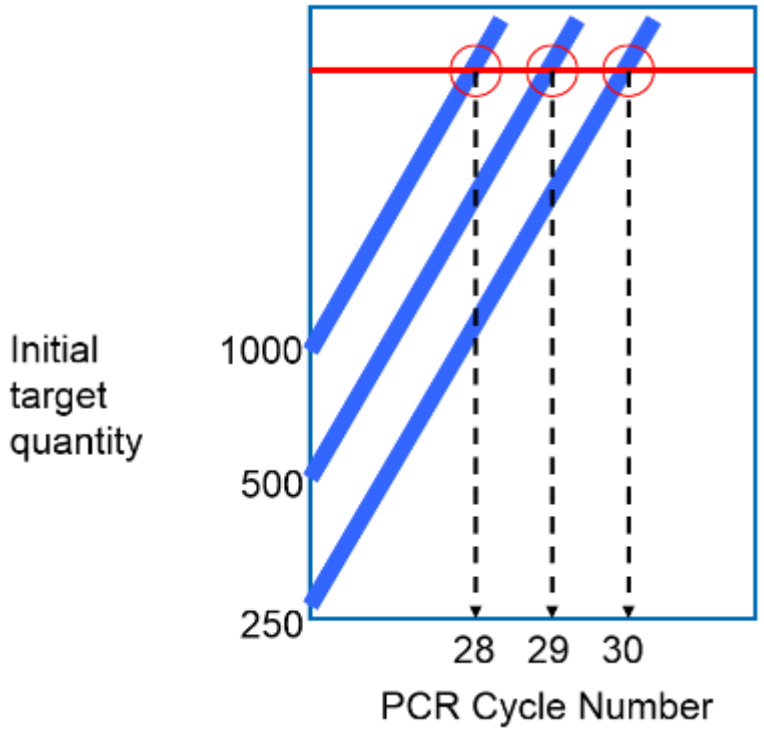
*The **greater the amount of initial DNA** template in the sample, the **earlier the Ct** value for that sample*



Real-time PCR: Ct & target quantity

Ct marks initial target quantity

A mathematical relationship exists between Ct and initial target quantity



Quantity ~ e^{-Ct}

e = geometric efficiency
e = 2 in this example.

Do I need a standard curve?

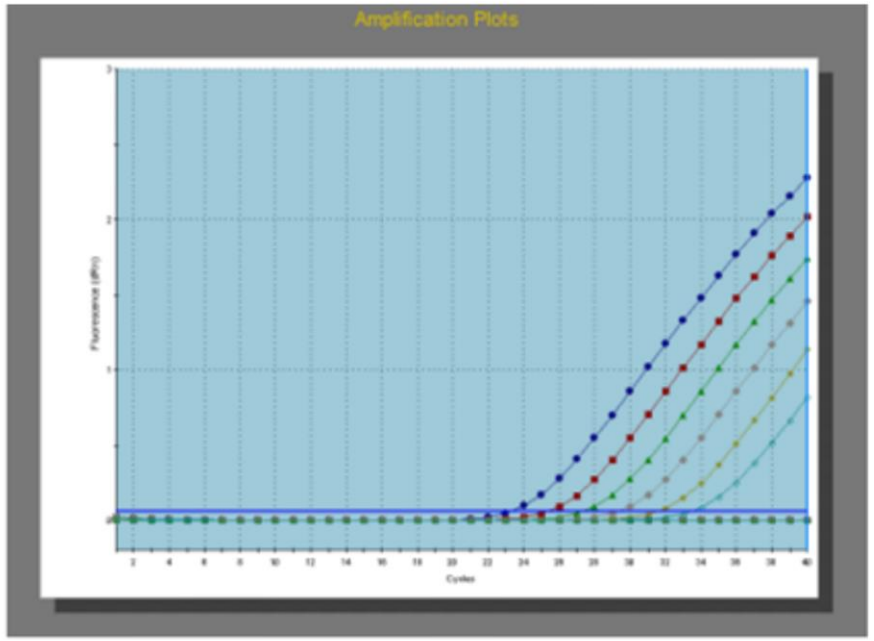


Figure 20
Amplification plots of standards in a four-fold dilution series over three orders of magnitude.

A standard curve enables you to identify the linear working range and the efficiency of your assay

- Perform a serial dilution series over a wide range of concentrations using a positive sample/control
- Use replicates for your standard curve
  → enables outlier detection and statistics

Properties of a good standard curve:

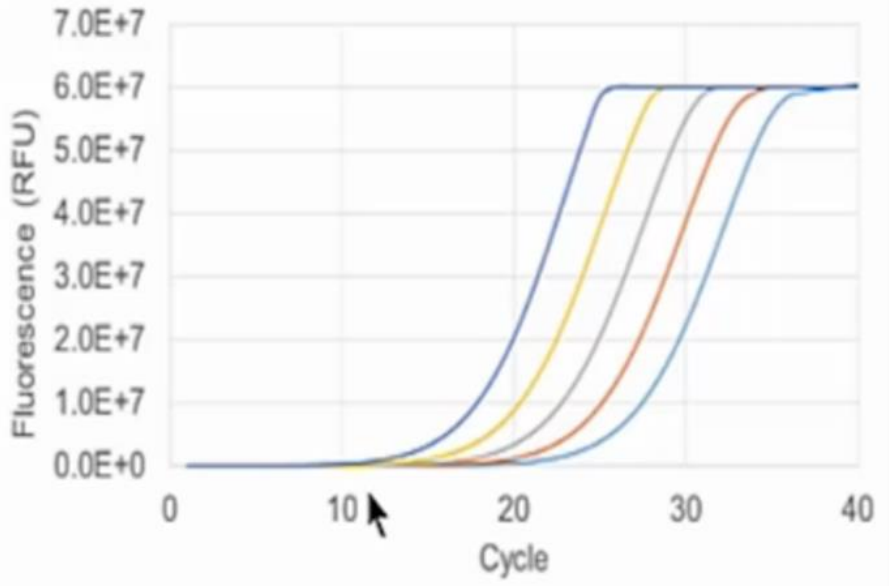
- high efficiency (90%-110%)
- good R^2 (>0.98)
- low replicate variability for individual standards

? *Since there is* ***a theoretical doubling*** *of the starting amount in each cycle what is the* ***efficiency?*** **E ~ 2**

Real-time PCR: target concentration

- Quantification of gene targets can be defined as the concentration of the target in a biological sample

- Biological events in cells and tissues are based on changes in the concentration of molecules



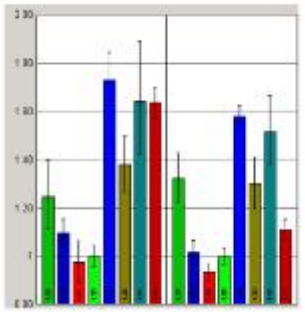
Real-time PCR: absolute or relative quantification

- Real-time PCR experiments can be used to produce either relative or absolute data -> semi-quantitative or quantitative real-time PCR

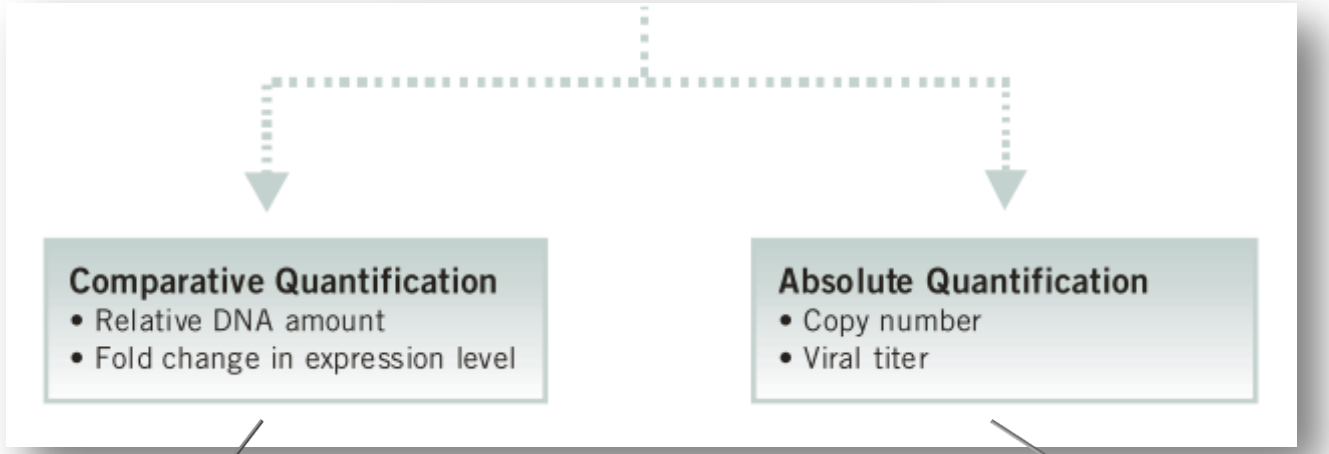
Absolute quantification: molecular concentration of the target is calculated

Relative quantification: concentration of the target is calculated using a non-molecular or arbitrary unit

Application	Measurement Goal	Type
Gene Expression	Changes in mRNA concentration	Relative
Virus quantification	Molecular concentration of virus	Absolute
Transgenic copy #	Copies transgene/genome	Absolute



What Is Your Goal?



Comparative or Relative quantitation:

- comparing the levels of a target gene in test samples relative to a sample of reference

Quantitative as Absolute quantitation:

- a standard curve to quantitate the amount of target present in an Unknown sample
- or a series of Standard samples, containing dilutions of a known amount of target

The standard curve is then used to **derive the initial template quantity in Unknown wells based on their Ct values**

Real-time PCR: absolute or relative normalization

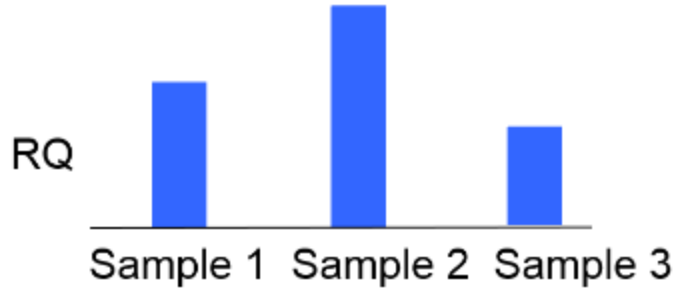
Absolute quantity (AQ) values are expressed as molecules of target in the numerator per normalization unit in the denominator

1300 molecules target/ng total RNA

Examples are:

- ng of total DNA
- copies of genomic DNA
- cell number

Relative quantity (RQ) values are expressed as numbers without a denominator



For example, a RQ of 2 means that the sample has twice the concentration of the target compared to a sample with a RQ of 1

Real-time PCR: normalizers

- A normalizer (or housekeeping or reference or control) is a gene used in real-time quantitative PCR experiment to normalize sample mass

Examples

18S rRNA
Acidic ribosomal protein
Beta-actin
Cyclophilin
Glyceraldehyde-3-phosphate dehydrogenase
Phosphoglycerokinase
β_2-Microglobulin
β-Glucronidase
Hypoxanthine ribosyl transferase
Transcription factor IID, TATA binding protein
Transferrin receptor

The expression levels of reference genes should remain constant between the cells of different tissues, the developmental stages, under different experimental conditions, etc.

- In order to compare samples within a group, normalization should be based on the same reference gene

Real-time PCR: endogenous and exogenous normalizers

Normalizers are categorized into two types:

- ✓ Endogenous
- ✓ Exogenous

- Endogenous normalizers are genes naturally present in each sample at a concentration relevant for normalization
- Endogenous normalizers are commonly used for samples derived from cells or tissues

Examples include 18S ribosomal protein to measure eukaryotic mass

- Exogenous normalizers are genes that are introduced to the sample at a concentration relevant for normalization
- Exogenous normalizers are typically used for liquid samples, such as blood, plasma, etc
- The exogenous normalizers are genes not naturally found in the samples being studied

Real-time PCR: normalizers' functions

Additional functions of the normalizer genes

- Correct for extraction efficiency of liquid samples
- Assist assay assessments and troubleshooting
- Amplification control
- Reverse transcription control
- Correct for pipette calibration error

Real-time PCR: normalization

- Normalization using quantity values is a division process
- Normalizing using Ct values is a subtraction process. It is used in the ΔΔCt method

$$\frac{\text{Target Quantity}}{\text{Normalizer Quantity}}$$

$$\text{Target Ct} - \text{Normalizer Ct} = \Delta\text{Ct}$$

Real-time PCR: normalization

- Normalization using quantity values is a division process
- Normalizing using Ct values is a subtraction process. It is used in the ΔΔCt method

$$\frac{\text{Target Quantity}}{\text{Normalizer Quantity}}$$

$$\text{Target Ct} - \text{Normalizer Ct} = \Delta\text{Ct}$$

Real-time PCR: normalization using the ΔΔCt method

- ΔΔCt is the recommended method to calculate real-time data
- ΔΔCt is based on the equation: $\text{Quantity} = 2^{-\Delta\Delta Ct}$

Sample	Detector	Task	Ct	ΔCt	Avg ΔCt	ΔCt SD	ΔΔCt	RQ
Sample 2	B	Target	34.034378		1.07405205E1	1.7314075E-1	8.1801796E-1	5.6722069E-1
Sample 2	B	Target	34.711212		1.07405205E1	1.7314075E-1	8.1801796E-1	5.6722069E-1
Sample 2	H	Target	30.378319		6.514831543	1.9364803E-1	-1.3458252E-2	1.0093722343
Sample 2	H	Target	29.62249		6.514831543	1.9364803E-1	-1.3458252E-2	1.0093722343
Sample 2	V	Target	27.189629		3.0258388519	2.184637E-1	1.1824417E-1	9.2130822E-1
Sample 2	V	Target	26.394093		3.0258388519	2.184637E-1	1.1824417E-1	9.2130822E-1

Real-time PCR: normalization using the ΔΔCt method

Normalisation according to an internal reference gene

***"delta-delta Ct method"* for comparing relative expression results between treatments in real-time PCR**

ABI Prism Sequence detection System User Bulletin #2 (2001)

Relative quantification of gene expression

$$\Delta CP = CP_{\text{target gene}} - CP_{\text{refence gene}}$$

$$\text{expression ratio} = 2^{-[\Delta CP\ \text{treatment} - \Delta CP\ \text{control}]}$$

$$\text{expression ratio} = 2^{-\Delta\Delta CP}$$

Livak KJ, Schmittgen TD. (2001)
Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2 [- delta deltaC(T)] method.
Methods, 2001 ***25(4):*** *402-408.*

Important for the analysis:
Include a negative control & a positive control

PCR controls

Positive controls

- Necessary for initial validation

- help to generate performance data: linear range, sensitivity

Examples:
PCR products, linearized plasmids, positive samples

Negative controls

- Necessary for initial validation and troubleshooting

- address specificity

Examples:
No Template Control (NTC)
→ Primer dimers, probe degradation

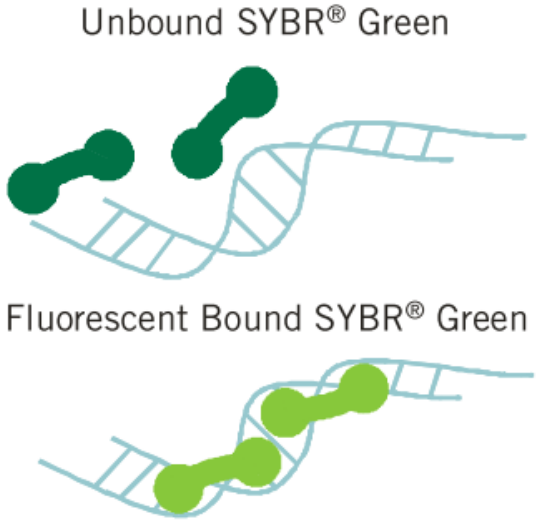
No RT Control (NoRT)
→ Genomic amplification

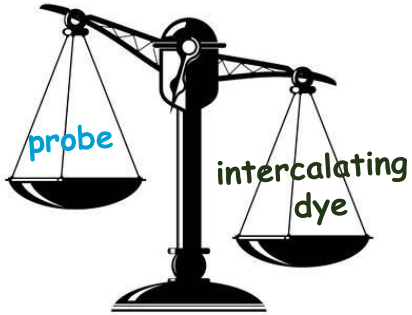
Controls should identify which data is good or bad!

How can the products be spotted?

SYBR-Green chemistry:

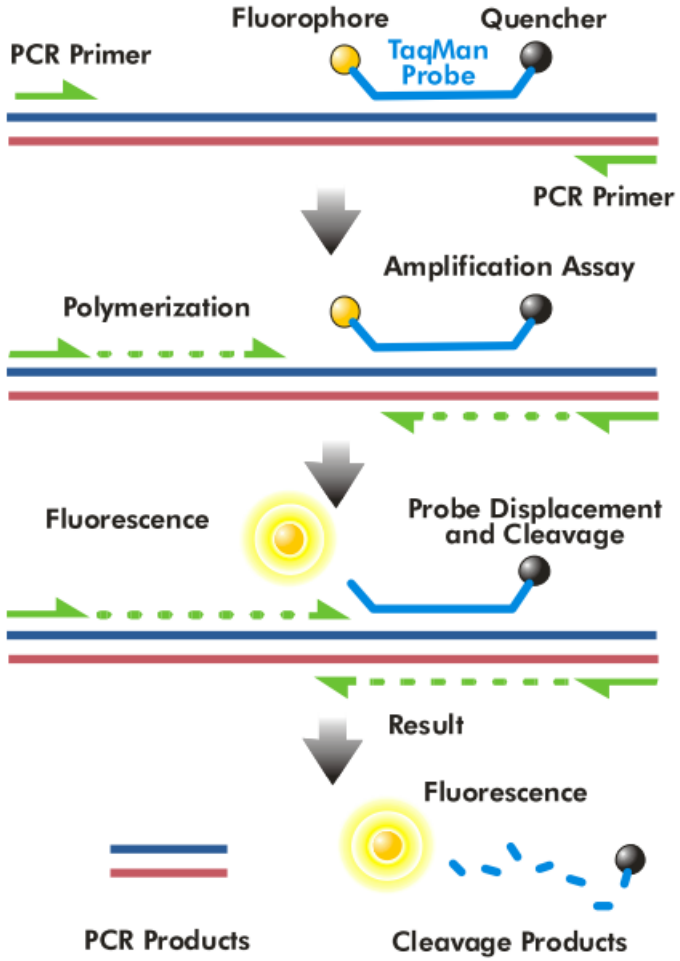
- cost—effective
- easy to use





Probe-Based chemistries (e.g. Taqman)

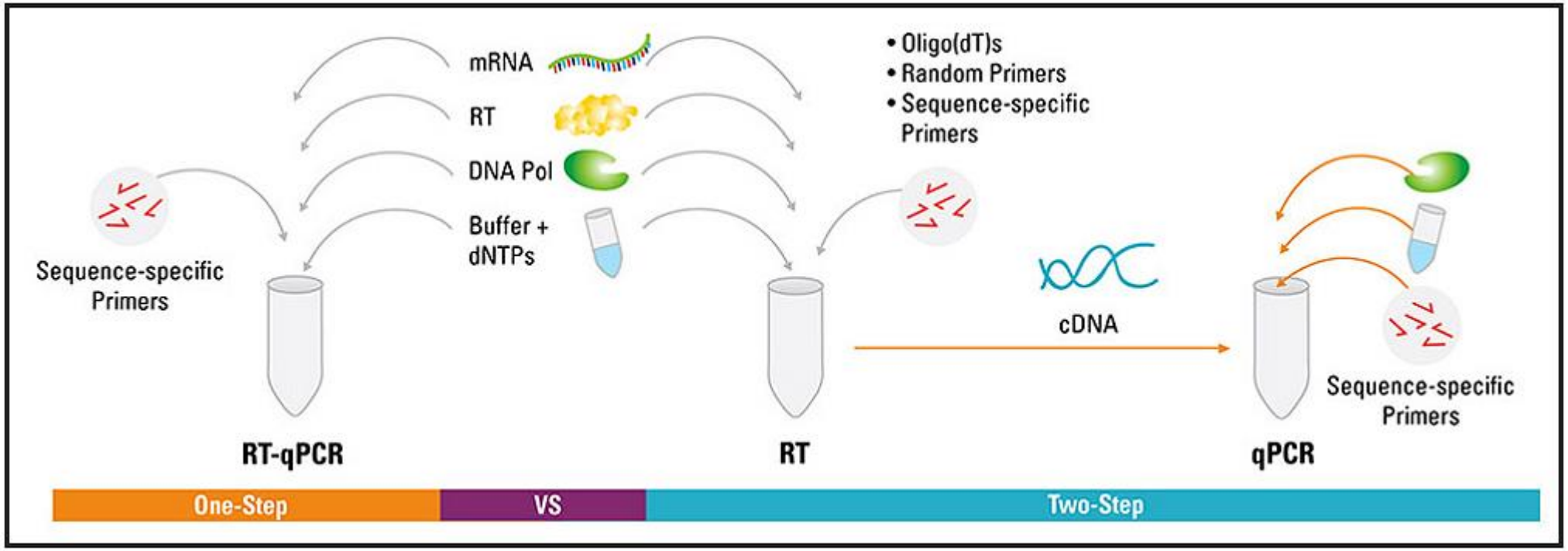
- **higher detection specificity**
- multiple probes labeled with different reporter dyes allow detection of more than one target in a single reaction (**multiplex QPCR**)



The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration

Important for the analysis:
Sequences of primers or probes should be removed!

qRT-PCR reactions: One-step vs Two-step: limiting sample amount?



Design an experiment!

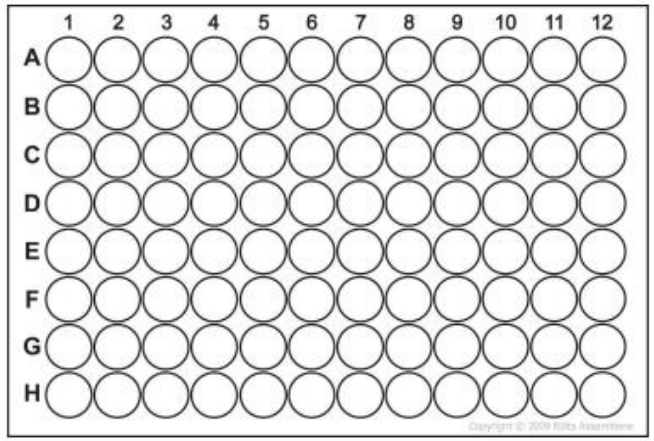
To considerate:

1. is this an absolute or relative quantification?
2. what is the target gene and what is the reference gene?
3. how many samples
4. how many replicates? Monoplicates / duplicates / triplicates, etc for each sample?
5. positive / negative controls? If yes, how many?
6. how will you choose the appropriate cycling conditions? What will they be?

INVESTIGATOR:

RUN No:

DATE:



Samples:

3 patients with breast cancer: P1, P2, P3

Reagents:

Reaction volumes per well:

ciDNA	3uL
SYBR Green Mix	17uL
Forward primer (10mM)	0.24uL
Reverse primer (10mM)	0.24uL
ddH_2O	*up to 20mL*

mRNA targets:

1) BRAF
2) GADPH

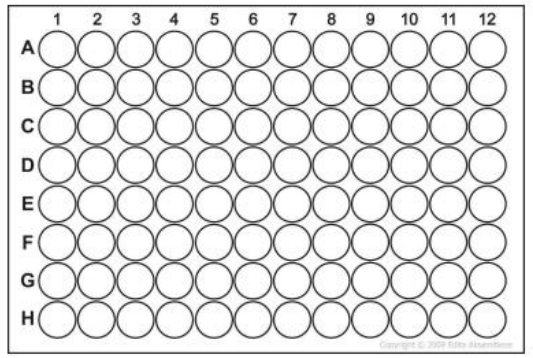
Results:

Design an experiment!

INVESTIGATOR:

RUN No:

DATE:



Samples:

3 patients with breast cancer: P1, P2, P3

Reagents:

Reaction volumes per well:

cDNA	3uL
SYBR Green Mix	17uL
Forward primer (10mM)	0.24uL
Reverse primer (10mM)	0.24uL
ddH_2O	*up to 20mL*

mRNA targets:

1) BRAF
2) GADPH

Results:

To considerate:

is this an absolute or relative quantification?

relative quantification

what is the target gene and what is the reference gene?

target gene: BRAF, reference gene: GADPH

how many samples & how many replicates? Monoplicates / duplicates / triplicates, etc. for each sample?

it depends!

positive / negative controls? If yes, how many?

negative controls: NTC, NoRT, positive controls: any gene that you know it is stably expressed

how will you choose the appropriate cycling conditions? What will they be?

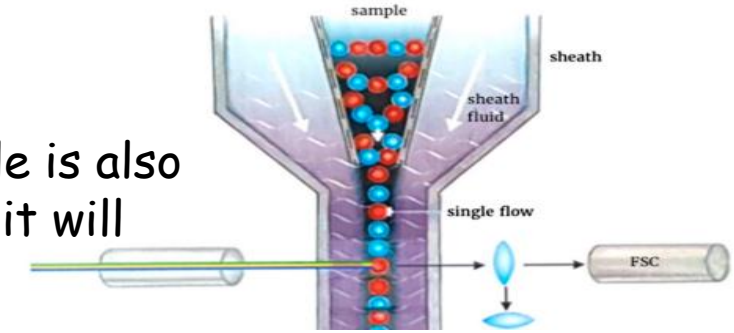
that depends on SYBR Green qPCR Kit Master Mix

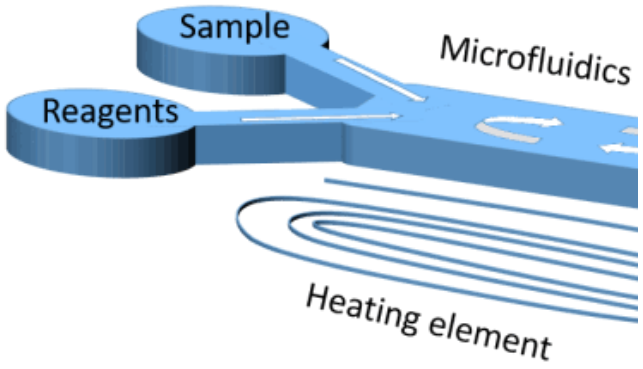
microfluidics

The principle

When a particle passes through a laser beam, it scatters light according to its physical properties.

e.g. A larger particle will cause higher deviation of the light. If this particle is also fluorescent, after being excited with light of the appropriate wavelength, it will emit light at another (usually higher) wavelength.





For cells

- Cells should be in single-cell suspension
- **Cells suspended in a liquid (fluidics system) pass through a laser beam causing light scatter that can be sensed by optical-to-electronic coupling system detectors**
- The electronics system process the signal from the detectors giving data that represent the features (size and granularity) of the cells

Jalali, M., Saldanha, F. Y. L., & Jalali, M. (Eds.). (2017). Basic science methods for clinical researchers. London: Academic Press.

microfluidic PCR

Microfluidic devices offer small thermal mass, low thermal inertia, and rapid heat transfer:

- The small volumes reduce sample and reagent consumption, leading to inexpensive operation of the system
- More efficient mass transport and heat conduction in microfluidic devices: while in macroscale systems diffusion and heat conduction are slow for efficient transport, the smaller scales of microfluidic devices allow for utilization of these effects

Microfluidics have been used for PCR, qPCR, RT-PCR

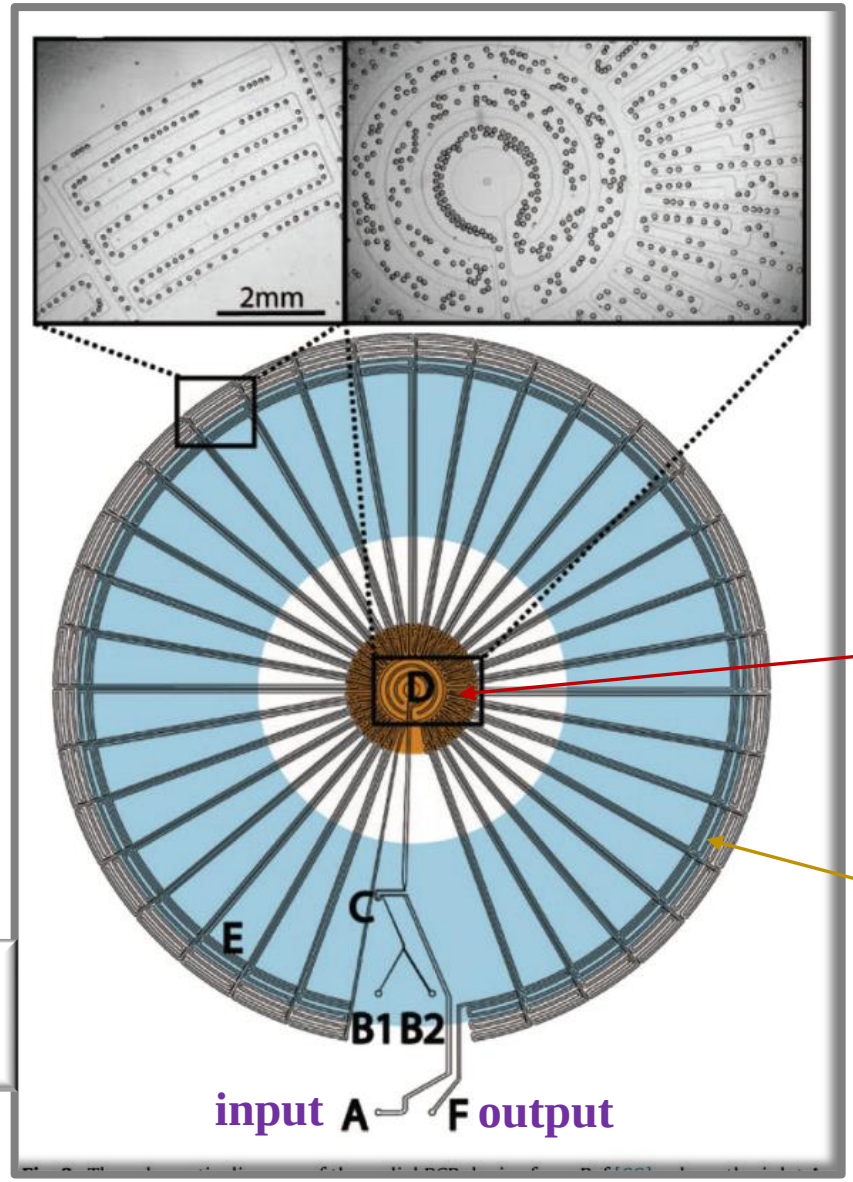
Lab-on-a-Chip

microfluidics benefits: **large number of samples, speed, parallelization, sensitivity, multiplexity, reduction of reagents cost**

microfluidics drawbacks: PCR inhibition and carryover contamination

microfluidic PCR examples

Various designs and approaches of microfluidic PCR devices



85bp DNA are amplified in 17min within 34 cycles

denaturation

annealing extension

Radial PCR
water-in-oil emulsion

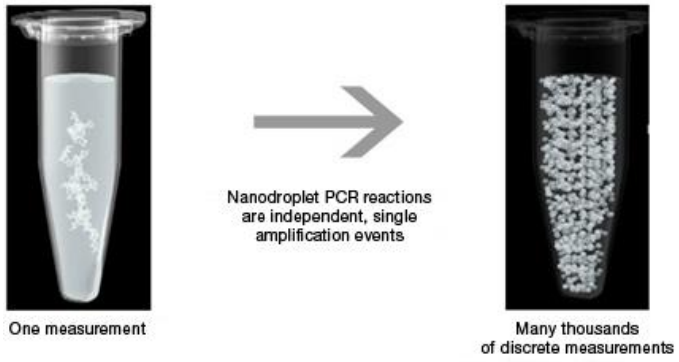
microfluidic PCR: Droplet Digital PCR (ddPCR)

Endless Frontier of 'Divide and Conquer'

- Droplet Digital PCR (ddPCR) is a method for performing PCR that is based on water-oil emulsion droplet technology
- ddPCR technology uses a combination of microfluidics and proprietary surfactant chemistries to divide PCR samples into water-in-oil droplets. **A sample is fractionated into thousands of nanoliter-sized droplets**, and **PCR amplification of the template molecules occurs in each individual droplet**
- This partitioning enables the measurement of thousands of independent amplification events within a single sample
- The droplets serve essentially the same function as individual test tubes or wells in a plate in which the PCR reaction takes place, albeit in a much smaller format

microfluidic PCR: Droplet Digital PCR (ddPCR)

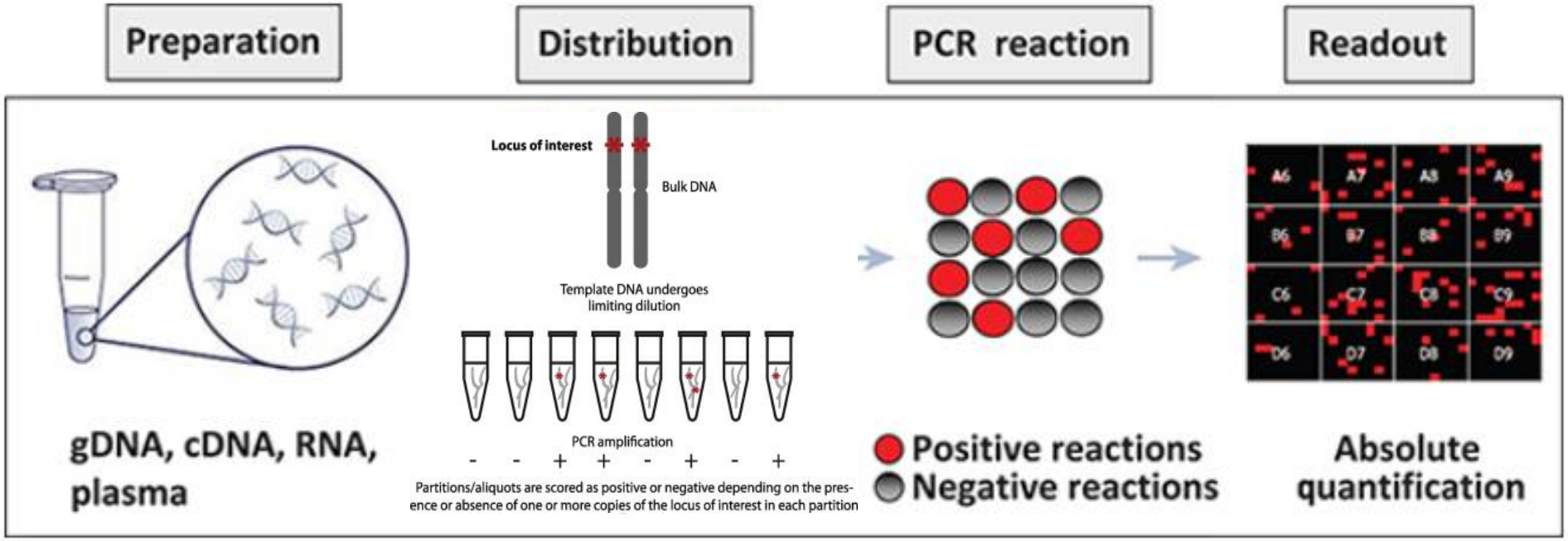
- Following PCR, each droplet is analyzed or read to determine the fraction of PCR-positive droplets in the original sample. These data are then analyzed using Poisson statistics to determine the target DNA template concentration in the original sample
- ddPCR technology uses reagents and workflows similar to those used for most standard TaqMan probe-based assays. This technique has **a smaller sample requirement** than other commercially available digital PCR systems, reducing cost and preserving precious samples



microfluidic PCR: Droplet Digital PCR (ddPCR)

DNA undergoes limiting dilution

- partition volumes: >5 pL
- the number of partitions: >100.000 for a single experiment



QX100™ Droplet Digital™ PCR

1. Isolate nucleic acid and quantitate
2. Mix samples with real-time PCR reagents and prepare dilutions
3. Split reaction mixture into many partitions
4. Amplify and collect data
5. Analyze data to identify copies/reaction

microfluidic PCR: Droplet Digital PCR (ddPCR)

ddPCR should be performed when

☺ **rare events must be quantified**

☺ **individual mutations must be detected**

ADVANTAGES of ddPCR:

- No need to rely on references or standards
- Absolute quantification
- Increased precision
- Removal of PCR bias
- Capability to analyze complex mixtures
- Linear detection of small-fold changes

ddPCR APPLICATIONS:

✓ viral detection

✓ diagnostic monitoring

✓ fetal screening

✓ *research applications* such as phage-host interactions and intracellular profiling

ddPCR data output

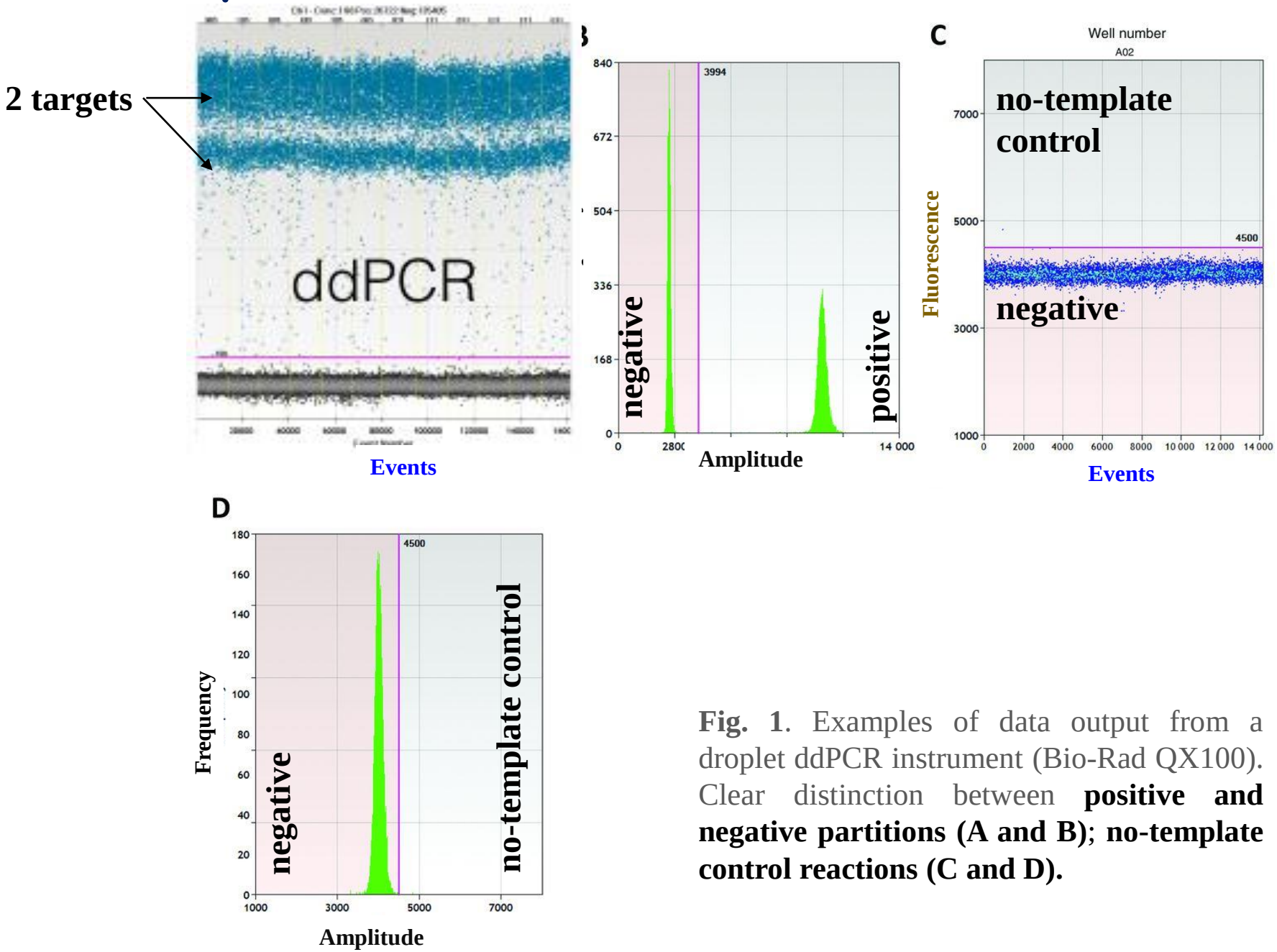


Fig. 1. Examples of data output from a droplet ddPCR instrument (Bio-Rad QX100). Clear distinction between **positive and negative partitions (A and B)**; **no-template control reactions (C and D).**

organ-on-a-chip

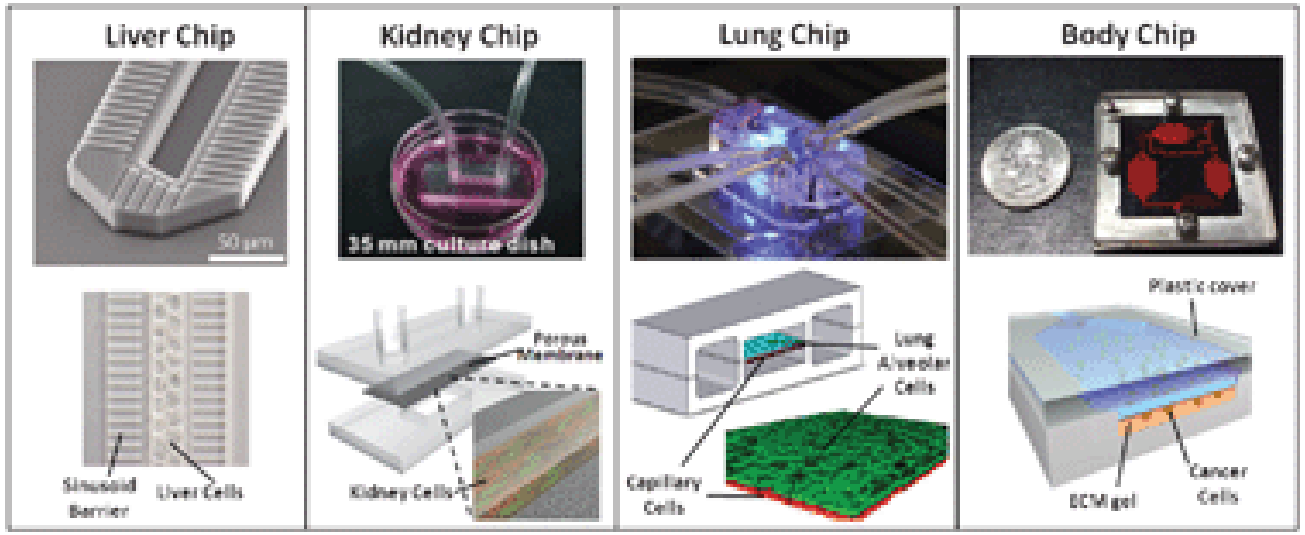
An **organ-on-a-chip is a microfluidic cell culture device** created with microchip manufacturing methods that contains continuously perfused chambers inhabited by living cells arranged to simulate tissue- and organ-level physiology.

By *recapitulating the multicellular architectures, tissue-tissue interfaces, physicochemical microenvironments and vascular perfusion of the body*, these devices produce levels of tissue and organ functionality not possible with conventional 2D or 3D culture systems.

They also enable high-resolution, real-time imaging and *in vitro* analysis of biochemical, genetic and metabolic activities of living cells in a functional tissue and organ context.

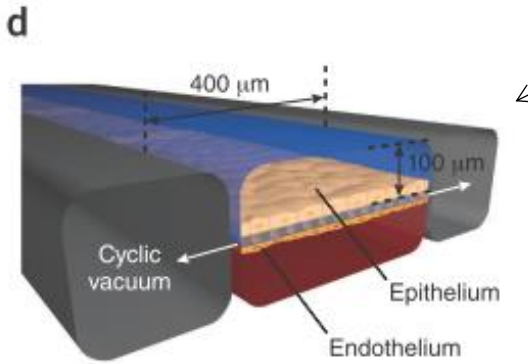
Applications

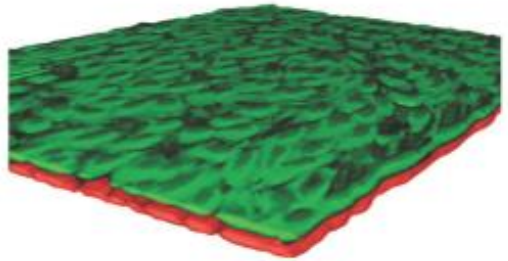
- Study tissue development, organ physiology and disease etiology
- Study molecular mechanisms of action, toxicity testing and biomarker identification in the area of drug discovery and development

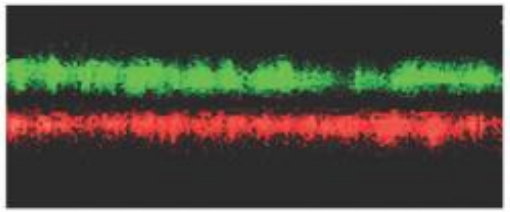


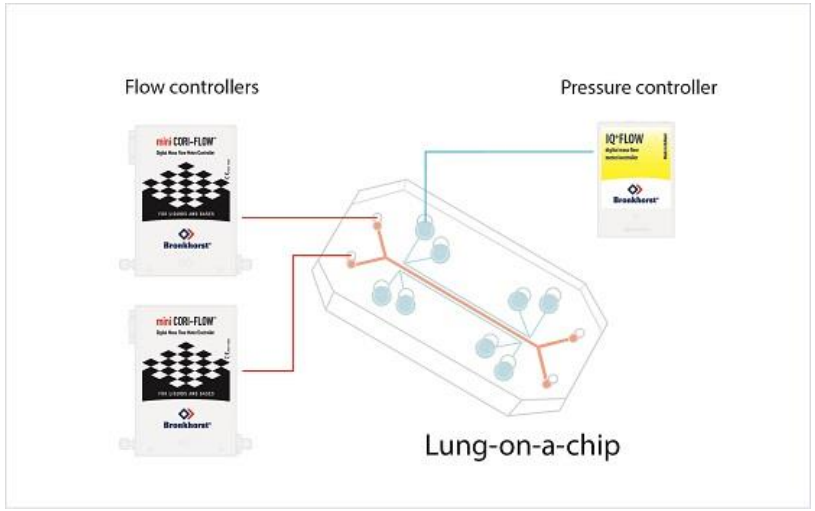
lung-on-a-chip

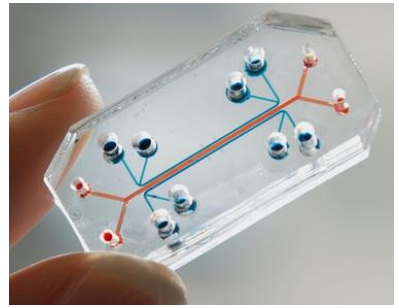
A microfluidic chip is suitable for growing lung-on-a-chip cell cultures, with an upper channel used for air flow, a lower channel used for liquid flow, and side chambers to apply different pressures











(d) A 'breathing' lung-on-a-chip that recapitulates the alveolar-capillary interface. Human alveolar epithelial cells are cultured on top of a flexible, porous, ECM-coated membrane and human capillary endothelial cells on the bottom. Air is passed through the upper channel to create an air-liquid interface with the alveolar epithelium, and culture medium is flowed through the vascular channel, with or without human immune cells (top). Breathing motions are mimicked by applying cyclic suction to full-height side chambers that rhythmically distort and relax the flexible side walls and attached porous membrane. The fluorescence confocal 3D reconstruction at the bottom and the cross-sectional view shown at higher magnification in the inset show the tissue-tissue interface formed between the alveolar epithelium (green) and the endothelium (red).

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PCR algorithms, packages…

for housekeeping genes:

geNorm, NormFinder, BestFinder, BestKeeper, Genenvestigator, RefGenes, REST

R packages:

SLqPCR, EasyqpcR, ddCt, NormqPCR, ReadqPCR, FPK-PCR, HTqPCR

For extra info

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Polymerase Chain Reaction (PCR)

key-notes

PCR steps: 1. denaturation, 2. annealing, 3. extension

PCR
- **Absolute** with a standard curve
- **Relative** with reference genes

endpoint PCR
digital PCR
microfluidic PCR

VS.

Real-time PCR


Polymerase Chain Reaction (PCR)
questions?

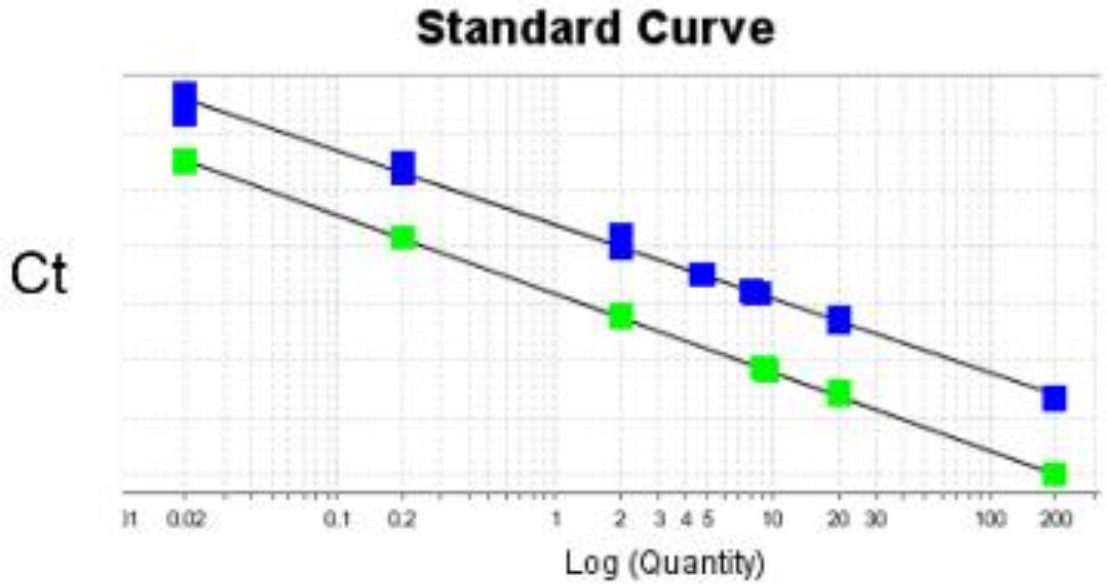

ТНАПК


hands on!
Learn
Aa
Focus on

At the bench side..

Real-time PCR: Standard curve for dynamic range testing

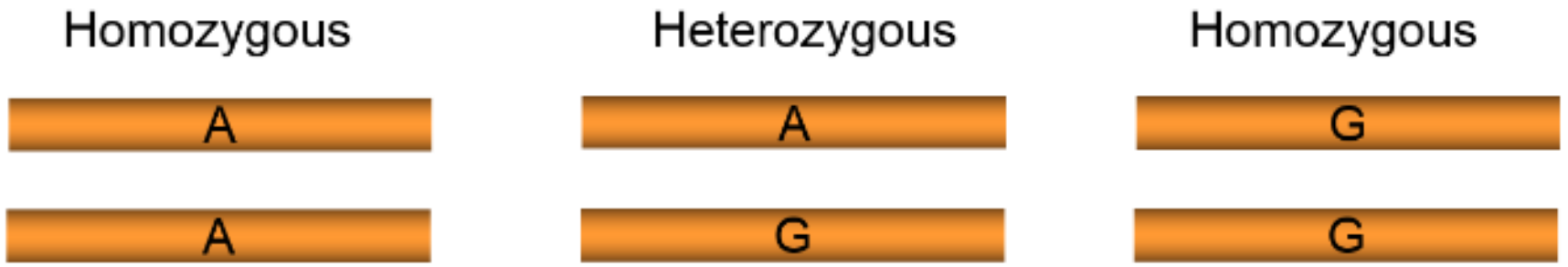
- Dynamic range is the minimum and maximum sample amount that may be run in a quantitative assay and produce accurate and precise data
- Before starting a new quantitative experiment, dynamic range should be tested
- Dynamic range testing involves standard curves



Real-Time PCR applications -> SNP genotyping

- **Single Nucleotide Polymorphisms (SNPs) are single base changes in genomic DNA**
- **SNPs may cause disease or regulate metabolic traits**
- **SNPs have consisted prominent biomarkers**, *e.g.* in patients with localized renal cell cancer shorter overall survival (VEGFR2: rs10013228; VEGFR3: rs6877011) ($p<0.05$)

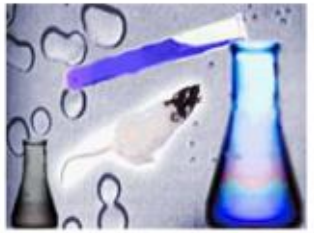
SNPs in diploid loci have 3 genotypes:



Real-Time PCR applications -> Quantitative applications

Quantitative applications:

- Gene expression
- Transgenic copy number
- Cancer
- Forencics
- GMO testing
- Quality control
- Pathogen quantification
- Chimerism
- Next Gen library quantification



Real-time PCR -> Allelic discrimination

- Allelic discrimination is a real-time PCR chemistry for SNP genotyping
- Allelic discrimination uses two probes: one for each allele
- Analysis is performed on post-PCR read results