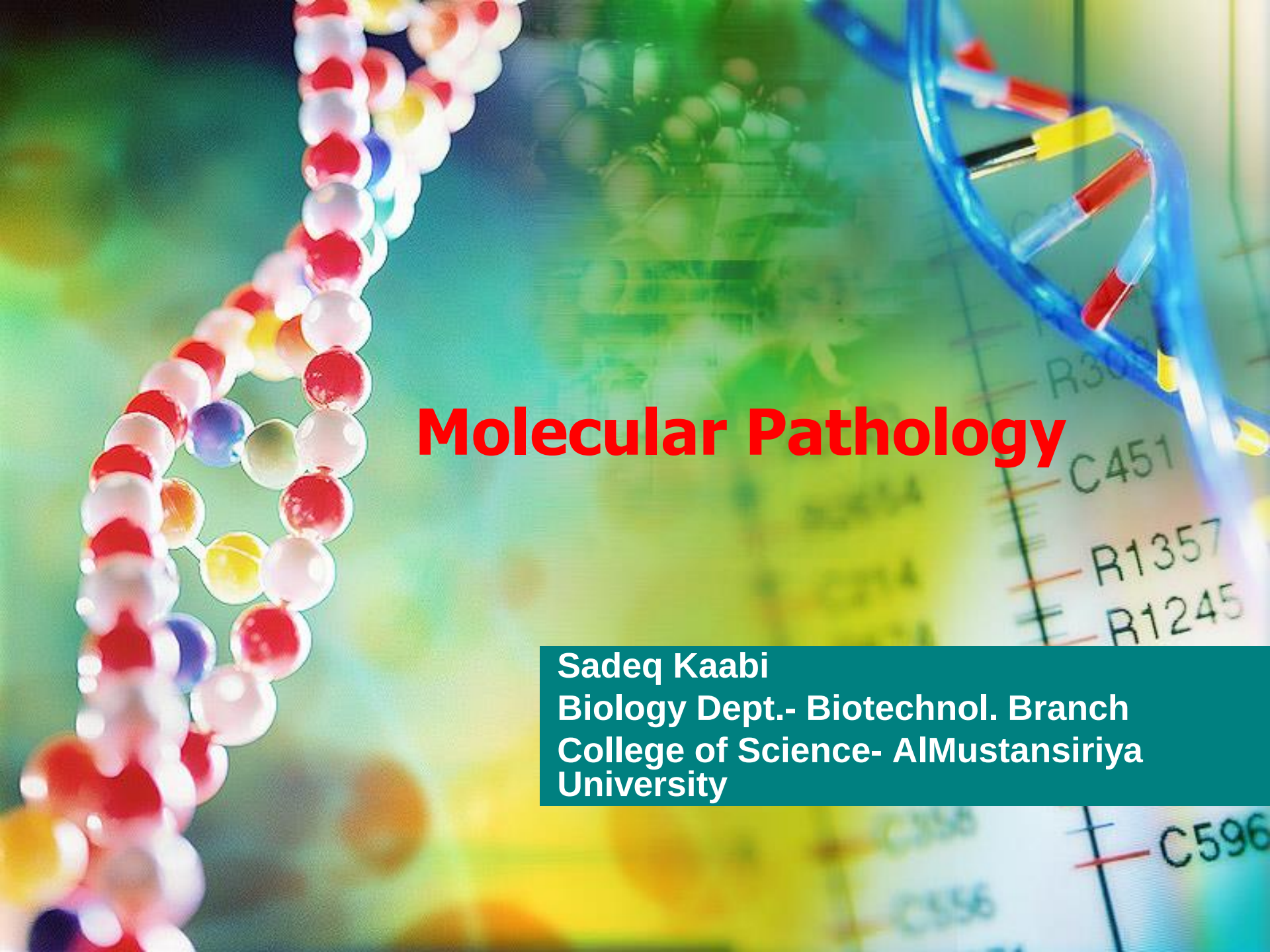


Molecular Pathology

Sadeq Kaabi
Biology Dept.- Biotechnol. Branch
College of Science- AlMustansiriya University

Basic molecular genetic techniques in Molecular pathology

Molecular pathology is an emerging discipline within pathology which is focused in the study and diagnosis of disease through the examination of molecules within organs, tissues or bodily fluids. Molecular pathology shares some aspects of practice with both anatomic pathology and clinical pathology, molecular biology, biochemistry, proteomics, and genetics, and is sometimes considered a "crossover" discipline. It is multi-disciplinary in nature and focuses mainly on the sub-microscopic aspects of disease. A key consideration is that more accurate diagnosis is possible when the diagnosis is based on both the morphologic changes in tissues (traditional anatomic pathology) and on molecular testing.[2]

It is a scientific discipline that encompasses the development of molecular and genetic approaches to the diagnosis and classification of human diseases, the design and validation of predictive biomarkers for treatment response and disease progression, the susceptibility of individuals of different genetic constitution to develop disorders.

Usage of Techniques of Molecular pathology

Molecular pathology is commonly used in diagnosis of cancer and infectious diseases. Techniques are numerous but include quantitative polymerase chain reaction (qPCR), multiplex PCR, DNA microarray, in situ hybridization, DNA sequencing, antibody based immunofluorescence tissue assays, molecular profiling of pathogens, and analysis of bacterial genes for antimicrobial resistance.

MPE

Integration of "molecular pathology" and “ epidemiology “ led to an interdisciplinary field, termed “ molecular pathological epidemiology " (MPE), which represents integrative molecular biologic and population health science.

AMP

ASSOCIATION FOR MOLECULAR PATHOLOGY

Current applications of molecular techniques in microbiology

- Diagnosis of non-culturable agents: e.g. Human papilloma virus, Hepatitis B virus etc.
- Fastidious, slow-growing agents: e.g. *Mycobacterium tuberculosis*, *Legionella* etc.
- Highly infectious agents that are dangerous to culture: e.g. *Francisella*, *Brucella*, *Coccidioidis immitis* etc.
- In situ detection of infectious agents: e.g. *H. pylori*, *Toxoplasma gondii* etc.
- Organisms when present in small volume in the specimen: e.g. detection of HIV - in antibody negative patients, intra-ocular fluid, forensic samples etc.
- For monitoring of viral load (for disease prognosis and treatment response): e.g. cytomegalovirus, Hepatitis B and C virus, HIV etc.
- Differentiation of antigenically similar agents: e.g. for detecting specific virus genotypes associated with human cancers (Papilloma viruses)
- Antiviral/ antibacterial drug susceptibility testing: e.g. HIV to assess drug resistance, Determination of resistant genes like Mec A gene, Van genes
- For the purpose of molecular epidemiology: e.g. Identify point sources for hospital and community-based outbreaks and to predict virulence
- For confirmation of culture

Molecular Methods are Broadly Classified as

1- **Hybridisation methods**- better for identification, not as sensitive as amplification methods

2- **Amplification methods**- improve the sensitivity due to amplification step.

3- **Sequencing and enzymatic digestion of nucleic acids**

Learning Objectives

- Describe three clinical molecular techniques (PCR, FISH, Southern, western, northern blotting and microarrays), and provide examples of their application in clinical medicine
- Specify the limitations of molecular techniques
- Understand the importance of tissue banking in Molecular Medicine and clinical research, and discuss its ethical implications

PCR … Technology evolution

1 - Traditional PCR

- - Reverse Transcription – PCR (RT-PCR

2 - Quantitative real-time PCR (Q-PCR)

the student is often get confused with these two types

Quantitative real-time PCR (qPCR) or (Q-PCR)

and

Reverse Transcription - PCR (RT-PCR)

which is

a technique that commonly used in molecular biology to detect mRNA expression(gene expression) *by the* complementary DNA(cDNA) transcripts from mRNA by (reverse transcriptas)

steps on RT-PCR

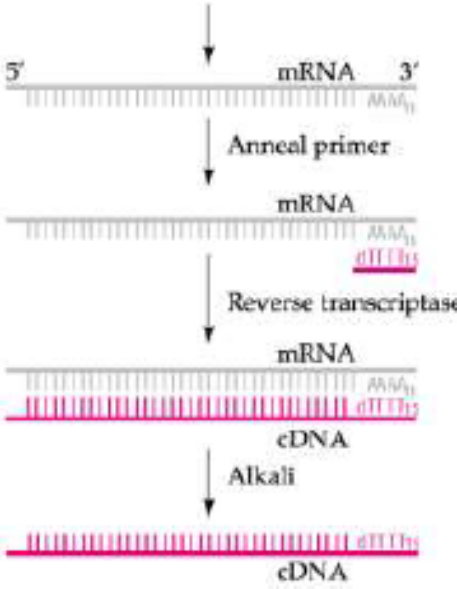
1 - **Isolate RNA**
2 - **cDNA synthesis**
3 - **PCR reaction**

do by traditional PCR

do by → traditional PCR

or → real time PCR

it will called RT-qPCR



Traditional PCR *vs.* real time PCR

Traditional PCR methods is use gel electrophoresis for the detection of PCR amplification in the final phase or at **end-point** of the PCR reaction.

---

real time PCR allows for the detection of PCR product (DNA)**during** the early phases of the reaction.

Polymerase chain reaction (PCR)

Polymerase chain reaction is an in vitro method of nucleic acid synthesis, in which a specific DNA-fragment (or fragments) is amplified. PCR involves the initial denaturation of the target DNA, followed by the annealing of target-specific oligonucleotide primers to each strand of DNA, and finally the extension of the primers by polymerase enzyme to produce a copy of the selected region. By repeating this cycle of denaturation, primer annealing and extension, there is an exponential amplification of the target.

- PCR was first conceived in 1983 by Kary Mullis, a molecular biologist who received a Nobel Prize for the discovery 10 years later
- A PCR (Polymerase Chain Reaction) is performed in order to make a large number of copies of a gene. Otherwise, the quantity of DNA is insufficient and cannot be used for other methods such as sequencing.

- A PCR is performed on an automated cycler, which heats and cools the tubes with the reaction mixture in a very short time.

- Performed for 30-40 cycles, in three major steps: 1)denaturation, 2)annealing, and 3)extension.

Applications of PCR

- 1) Diagnosis of Disease: Linkage analysis, detection of mutant alleles, diagnosing infectious agents, epidemiological studies
- 2) Forensics: paternity testing, DNA typing for identification, criminal investigations.
- 3)Recombinat DNA engineering
- 4) DNA sequence determination
- 5) new gene isolation
- 6) Anthropological studies: population genetics, migration studies.
- 7) Evolution studies
- If you need to look at 100 genes is PCR a good approach?

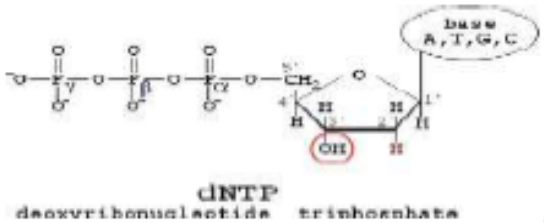
1 - Traditional PCR (Material)

Sterile deionized water

Free Nucleotides (A, T, C, G)

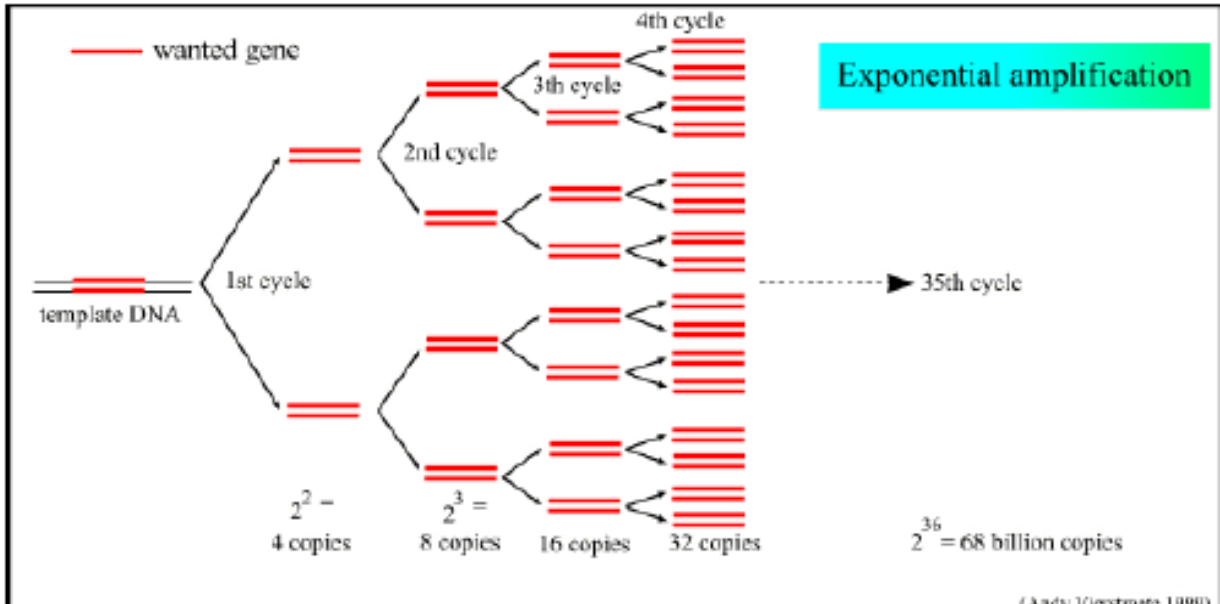
(*De.oxy nucleotide triphosphate*)

dATP, dCTP, dGTP and dTTP



Components	Volume Per sample	Concentration reaction
D.DW	38.8 µl	-
Buffer	5.00 µl	1X
dNTP's	1.00 µl (0.25 µl each)	200 µM each
Primer F	0.04 µl	5-10 p moles
Primer R	0.04 µl	5-10 p moles
Taq Polymerase	0.04 µl	1.5 U
$MgCl_2$	2.00 µl	-
Total Volume	48.00 µl	-

cofactor that DNA Polymerase III needs to work



Steps of ***Traditional PCR***

Steps	Temperature (°C)	Time	Cycle
Initial denaturation	95°	120 sec	1 Cycle
Denaturation	94°	30 sec	35 Cycle
Annealing	54°	30 sec	
Extension	72°	60 sec	
Elongated extended	72°	600 sec	1 cycle
Storage	4°	Forever	-

Denaturation:

- This step is the first regular cycling event and consists of heating the reaction to 94–98 °C

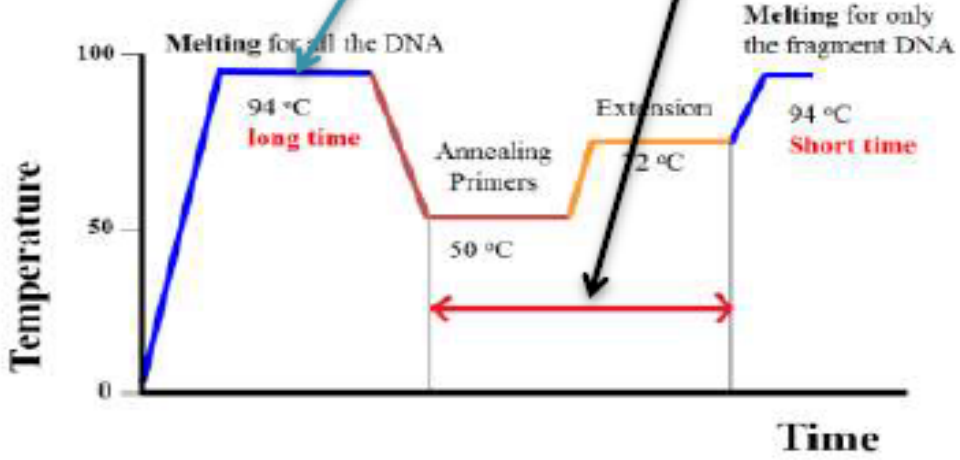
Annealing

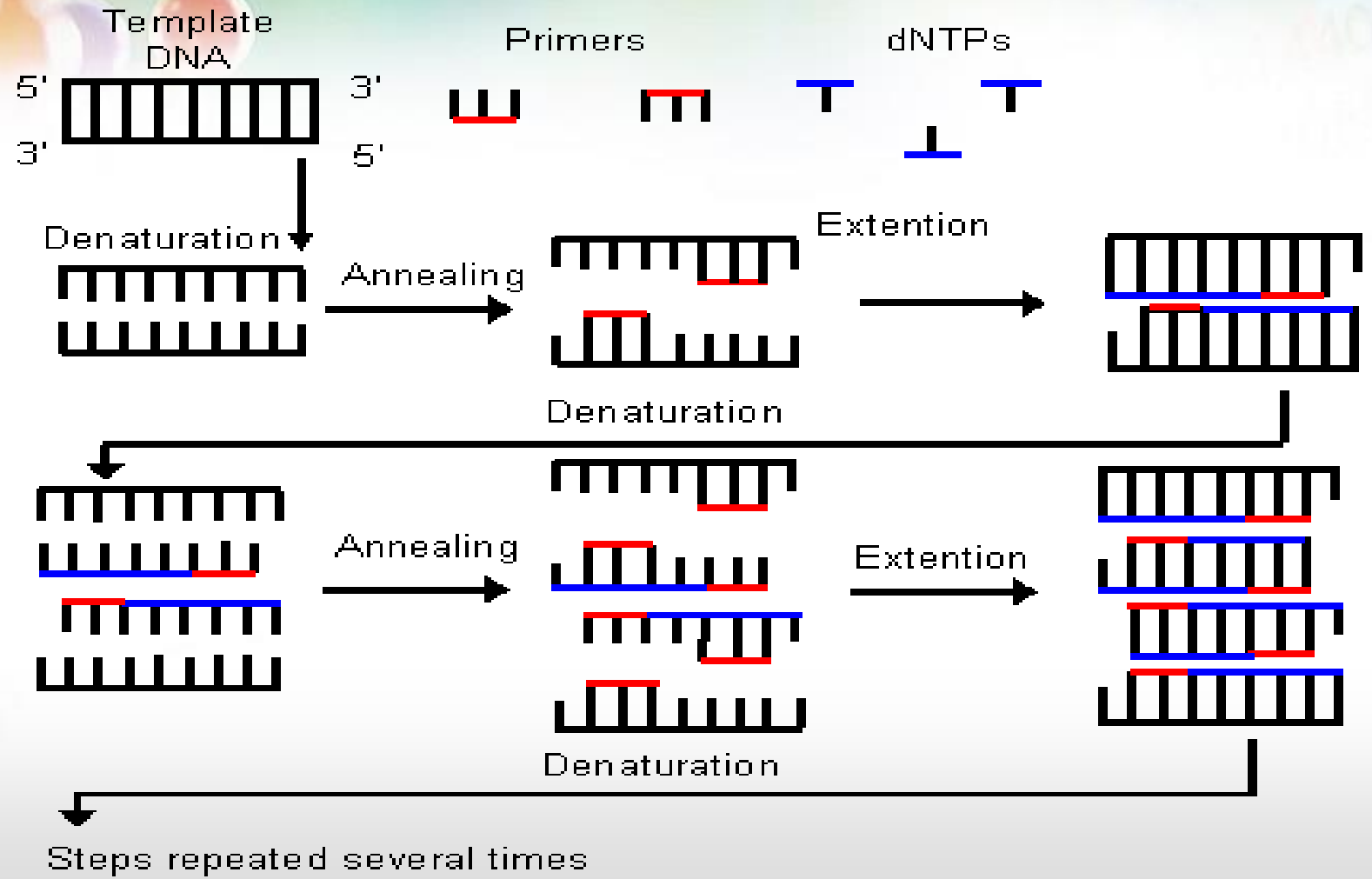
- The reaction temperature is lowered to 45–60 °C for primer attachment

Extension/elongation:

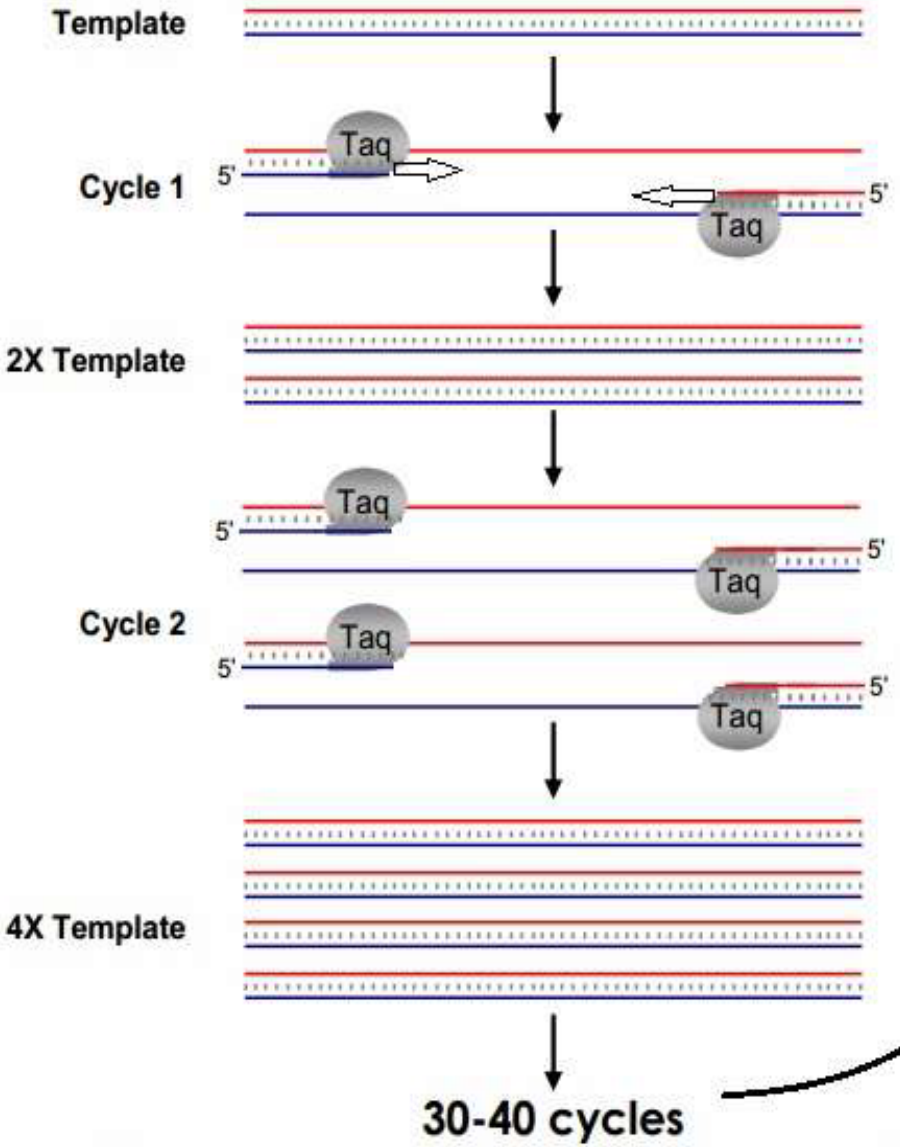
- At 72 °C for taq plymerase

Final elongation:

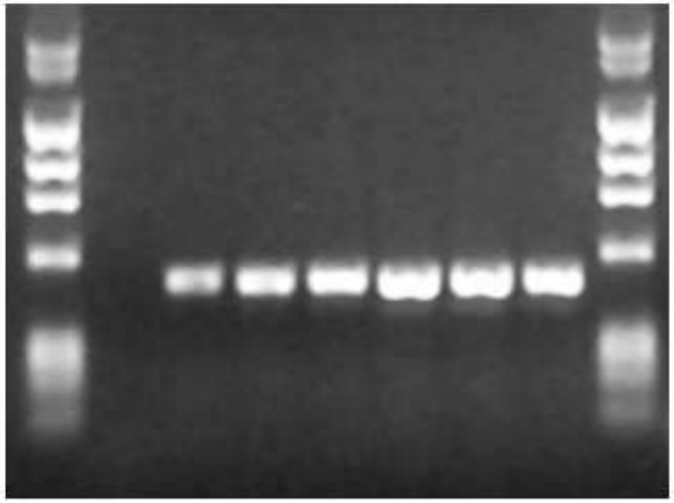


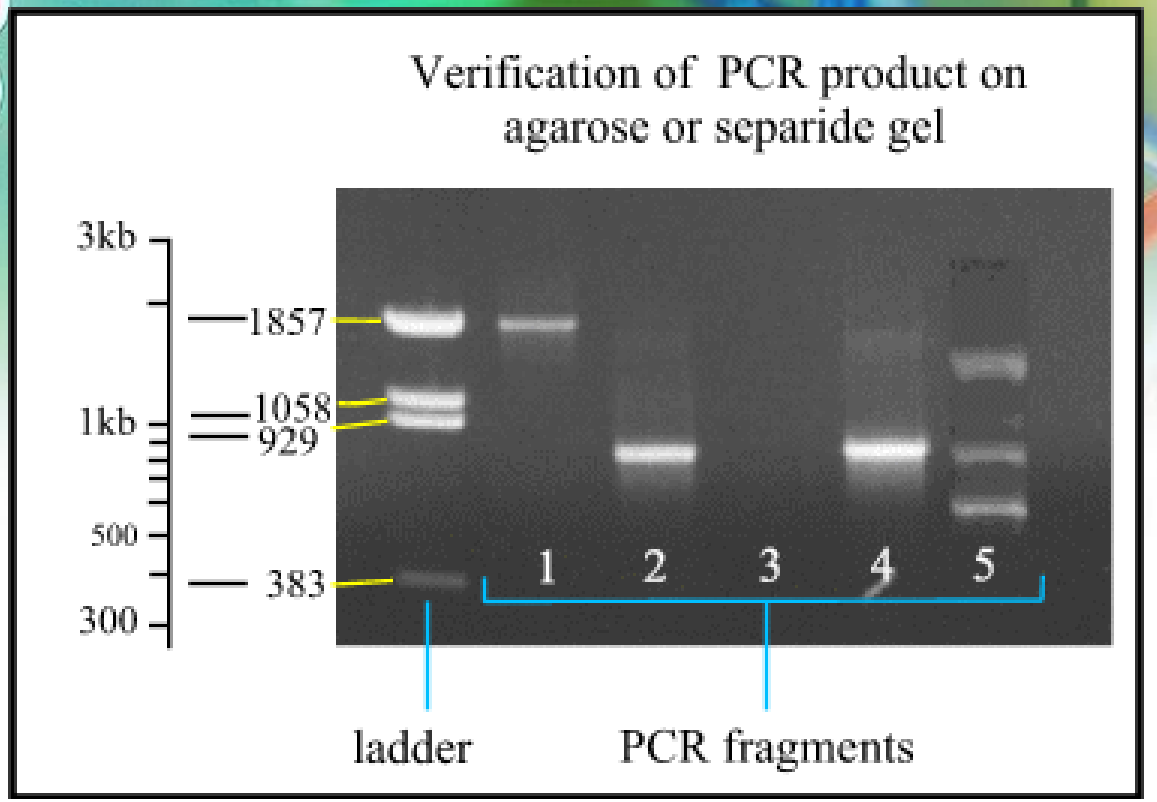

Template DNA
5'
3'
3'
5'
Primers
dNTPs
Denaturation
Annealing
Extention
Denaturation
Annealing
Extention
Denaturation
Steps repeated several times

Traditional PCR



End-point analysis of
DNA amplification product





The ladder is a mixture of fragments with known size to compare with the PCR fragments. Notice that the distance between the different fragments of the ladder is logarithmic. Lane 1 : PCR fragment is approximately 1850 bases long. Lane 2 and 4 : the fragments are approximately 800 bases long. Lane 3 : no product is formed, so the PCR failed. Lane 5 : multiple bands are formed because one of the primers fits on different places.

Primer design

Imagine you have the 5'-3' sequence of a gene and you wish to amplify a fragment of it.

5'-NNNNNNAGAGACA GTGGGACCGT CTG -------- TGGA CTTGAGGATT CTAGAGNNNNNN-3'

A and G are the start and end of the fragment you wish to amplify.

--------- : **means** the fragment is big enough so can't write the whole sequence.

(SENSE template)
5'-NNNNNNAGAGACAGTGGGACCGTCTG--------TGGACTTGAGGATTCTAGAGNNNNNN-3'

(ANTISENSE template)
3'-NNNNNNTCTCTGTCACCCTGGCAGAC---------ACCTGAACTCCTAAGATCTCNNNNNN-5'

(ANTISENSE IN 5'-3' MODE):
5'-NNNNNNCTCTAGAATCCTCAAGTCCA---------CAGACGGTCCCACTGTCTCTNNNNNN-3'

complement-reverse

Some software like **DNassist** and **WinGene** can do "reverse-complement" in one click.

Thus, complement –reverse means: to have the sequence of the antisense in 5'-3' direction.

Thus, complement –reverse means: to have the sequence of the antisense in 5'-3' direction.

Now to design the primers using the 5'-3' (sense) strand:

One of the primers (the forward primer) will be directed from 5'-3' and anneal with the antisense template. This primer is written as same as the sense template nucleotide sequence.

Forward primer: **5'-NNNNNNAGAGACAGTGGGACCGTCTG-3'**

NNNNN: the restriction sequence, GC clamp, …etc.

The second primer (backward/antisense) will anneal with the sense template and thus it is 3'-5'.

Backward primer: **3'-ACCTGAACTCCTAAGATCTCNNNNNN-5'**

Usually both primers are written in 5'-3' formula. Thus, the backward primer can be written as: **5'-NNNNNNCTCTAGAATCCTCAAGTCCA-3'**

where NNNNN are the restriction sequence, extra nucleotides or GC clamps.

Summary:

If you got the 5'-3' strand sequence of a gene fragment that you wish to design primers to amplify it by PCR, e.g.:

5'- AGAGACAGTGGGACCGTCTG---------TGGACTTGAGGATTCTAGAG-3'

do the following:

(1) Select the nucleotides in the same sequence to the sense template and add at the 5' end any extra sequence of restriction enzyme, GC clamp, ..etc. This serves as a sense (forward) primer.

Thus, a forward primer can be as: 5'-NNNNNNNNNNNNAGAGACAGTGGGACCGTC-3'

For a reverse primer: write the complement sequence of the 3' end of the sense template, reverse it, so it can be read as 5'-3' and add any extra sequence at the 5'end of this primer.

Thus, for the example given above, the 5'-3' mode of the reverse primer will be: 5'- NNNNNNNNNN-CTCTAGAATCCTCAA-3'.

It's easy, isn't it?

* **The 3'end of the primers is critical for the specificity and sensitivity of PCR.**

* **Since 5'end of the primer is less critical for annealing, add the restriction sequence at this end.**

Melting temperature (T_m): the temperature at which half of the primer is annealed to the template. T_m is important for primer specificity.

T_m for both primers should be (1) similar and (2) >60C.

Rules for primer design:

(1) Length: 17-30 nt (shorter primers lead to nonspecific amplification of PCR products).

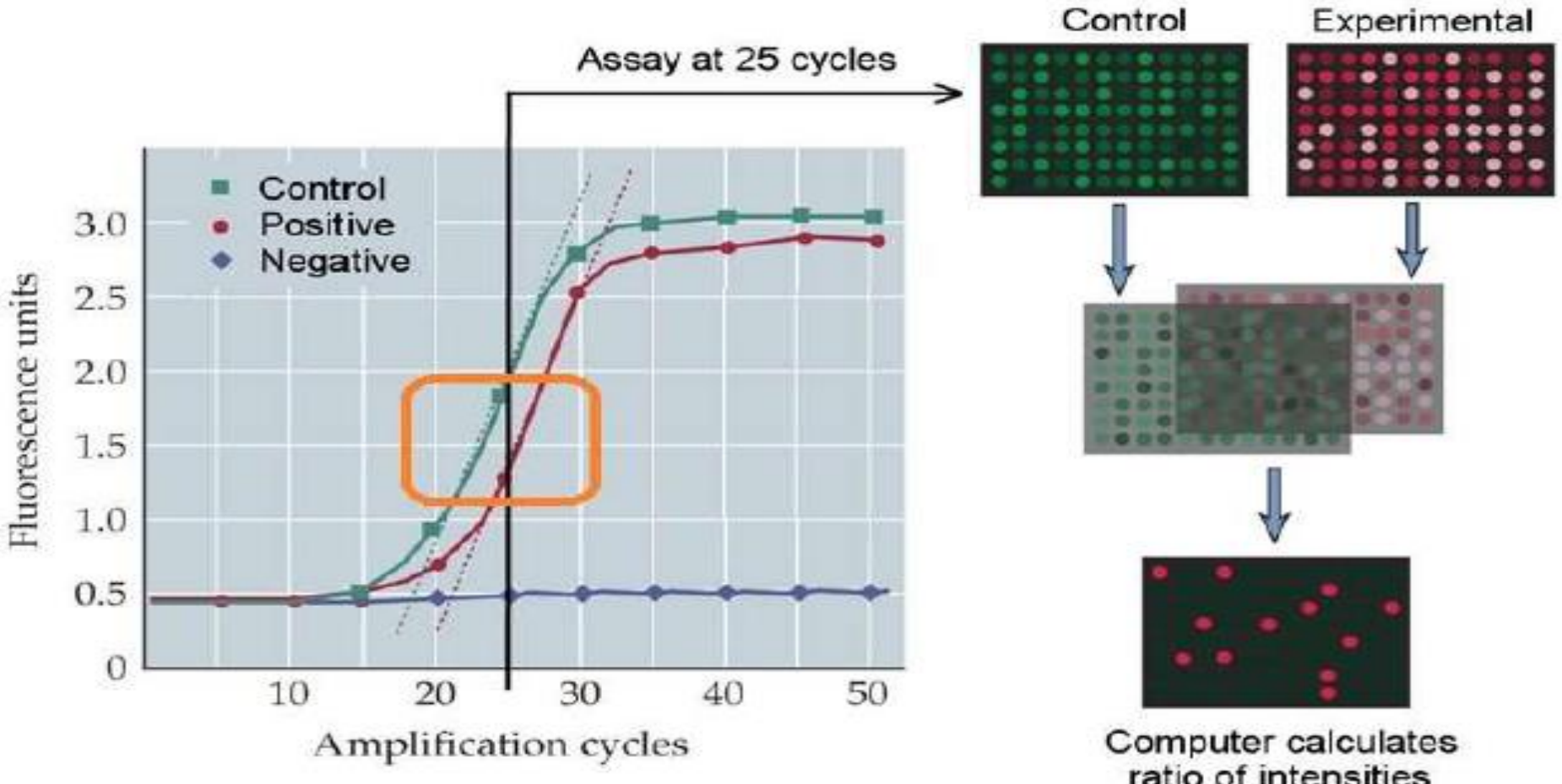
(2) 50-60% (G+C).

(3) the 3'end should:
(a) Should end with C, G, CC or GG (for more efficient priming as this avoid breathing).

2 - Quantitative real-time PCR (Q-PCR)

real-time PCR instruments consist of ***two main*** components:

1. Thermal Cycler (**PCR** machine)
2. ***Optical*** Module (to **detect the fluorescence** in the tubes during the run of DNA amplification)



Types of real-time PCR (Q-PCR)

dependent on types of the **Dye**

1 - Hydrolyzation based Assays

- **TaqMan** ✓

Beacons

2 - DNA-binding agents

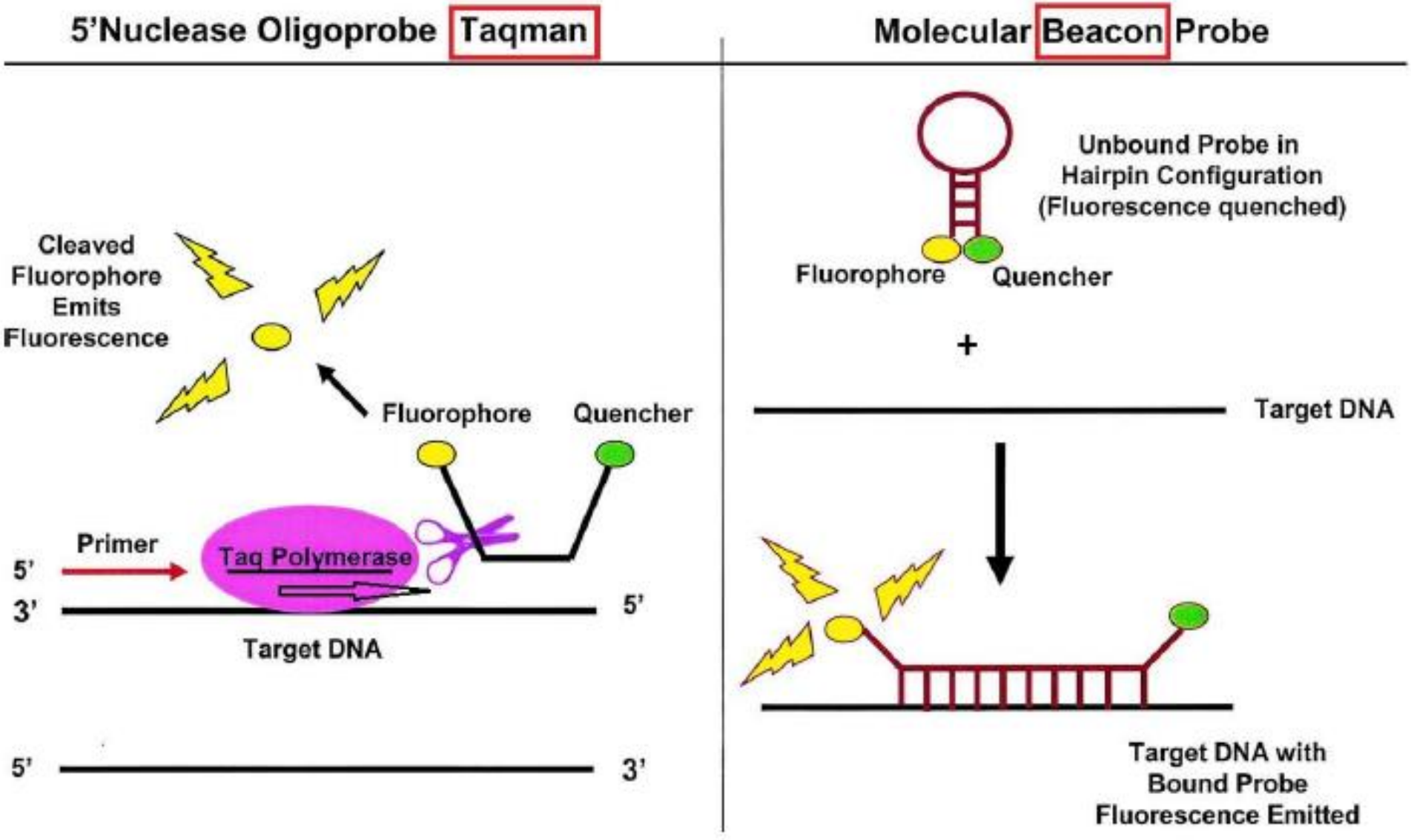
* **SYBR Green** ✓

Application of Q-PCR

real-time PCR, is used for many applications,,, **including**

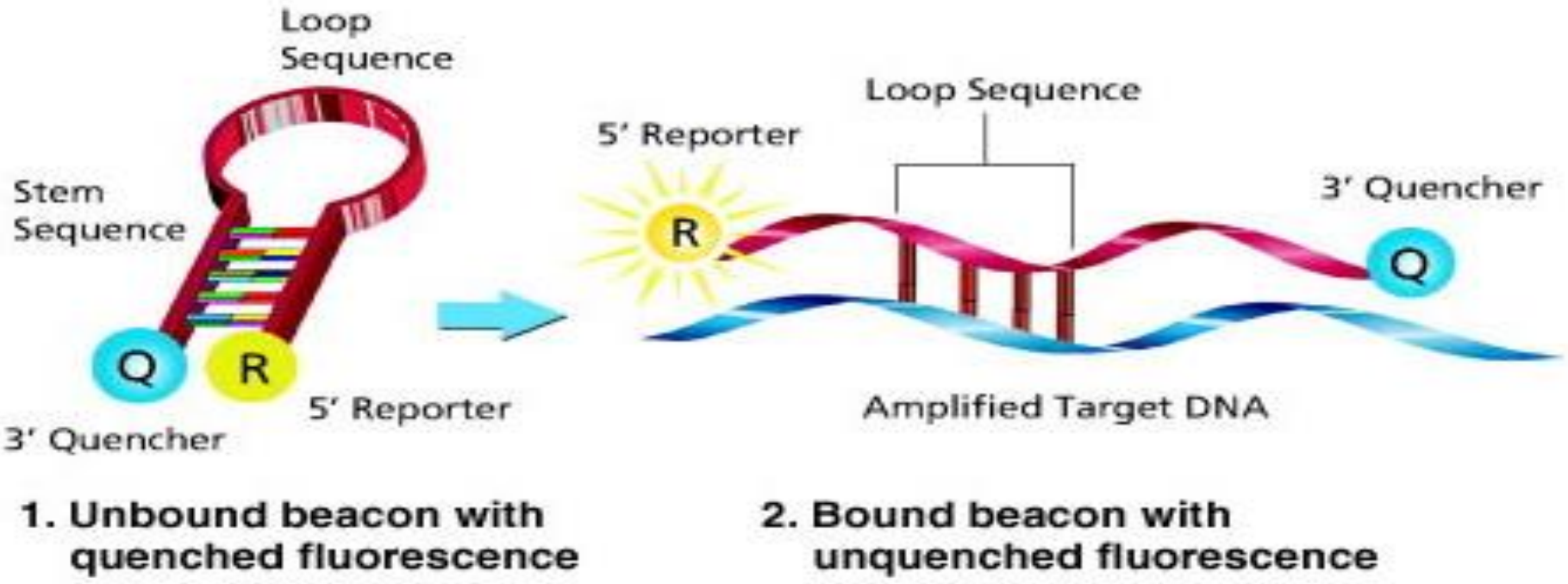
-- gene expression analysis,
-- microRNA analysis,
-- single nucleotide polymorphism (SNP) genotyping,
-- copy number variation (CNV) analysis,
-- and even protein analysis.

Hydrolyzation based Assays



Molecular Beacon

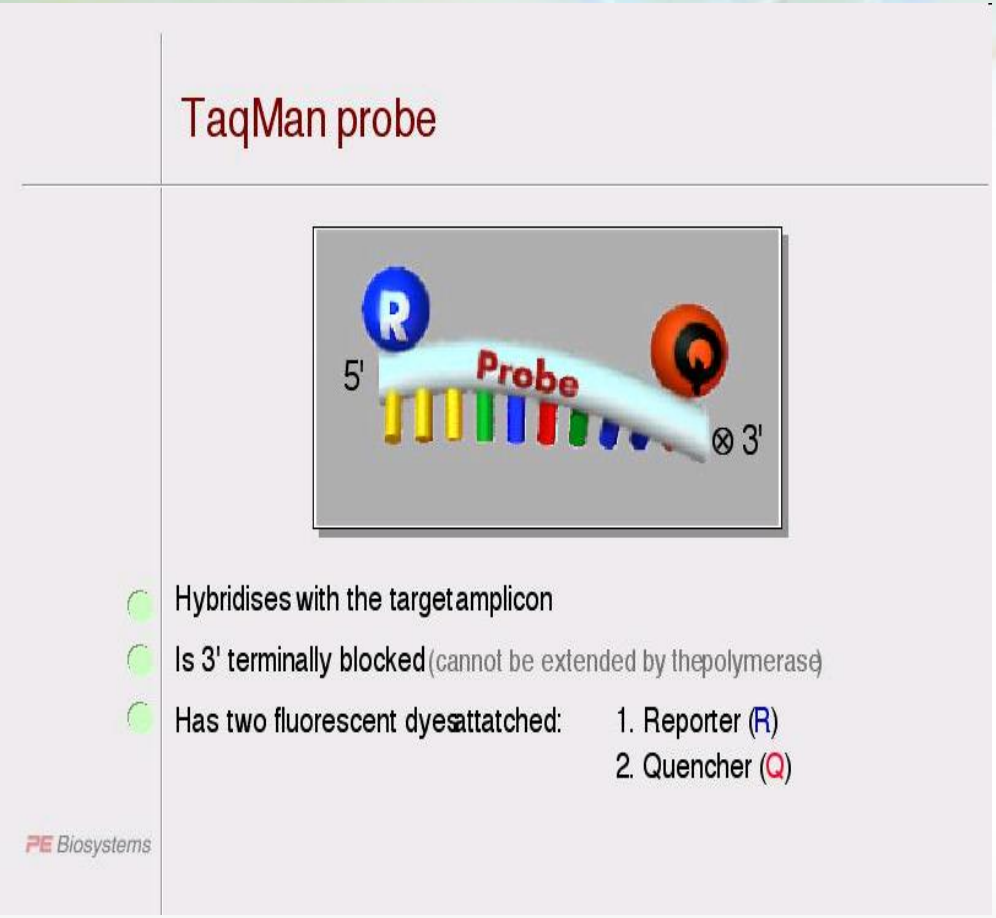
A Molecular Beacon is a single-stranded bi-labeled fluorescent probe held in a hairpin-loop conformation (around 20 to 25 nt) by complementary stem sequences (around 4 to 6 nt) at both ends of the probe. The 5' and 3' ends of the probe contain a reporter and a quencher molecule, respectively. The loop is a single-stranded DNA sequence complementary to the target sequence. The close proximity of the reporter and quencher causes the quenching of the natural fluorescence emission of the reporter. The structure and mechanism of a Molecular Beacon is shown below.



Molecular Beacons hybridize to their specific target sequence causing the hairpin-loop structure to open and separate the 5' end reporter from the 3' end quencher. As the quencher is no longer in proximity to the reporter, fluorescence emission takes place. The measured fluorescence signal is directly proportional to the amount of target DNA.

FRET probes

- Two most popular alternatives to SYBR green are **TaqMan®** and **Molecular Beacons**.
- Both technologies depend on hybridization probes relying on fluorescence resonance energy transfer.(FRET) and quantization

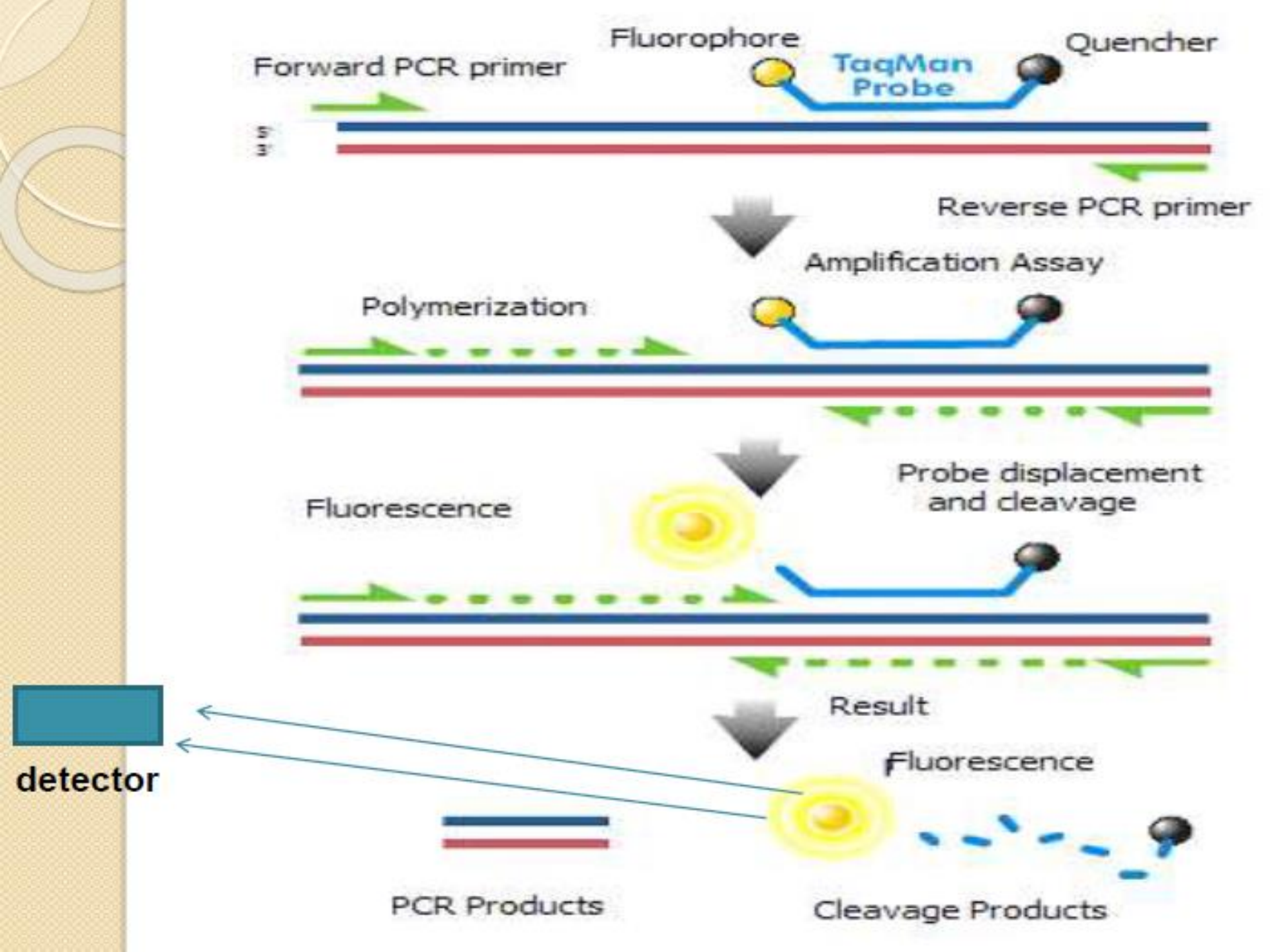


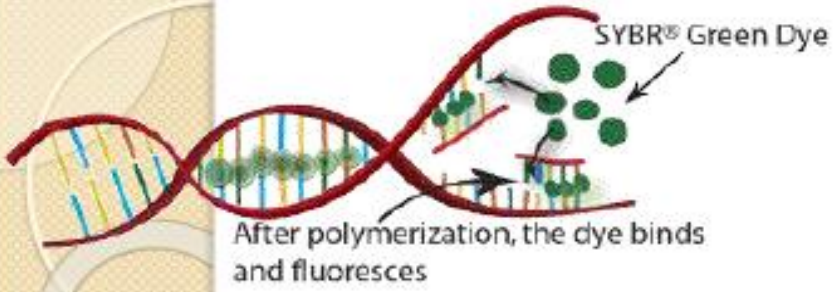
Hydrolyzation Probes

- **Double- Dye** Oligonucleotides -**TaqMan**
or **Dual labeled** probes- **Beacon**

- Consists of a ssDNA probe that is complemenatry to one of the amplicon strands

- A **fluorophore** is attached to one end of the probe and a **quencher** to the other end.

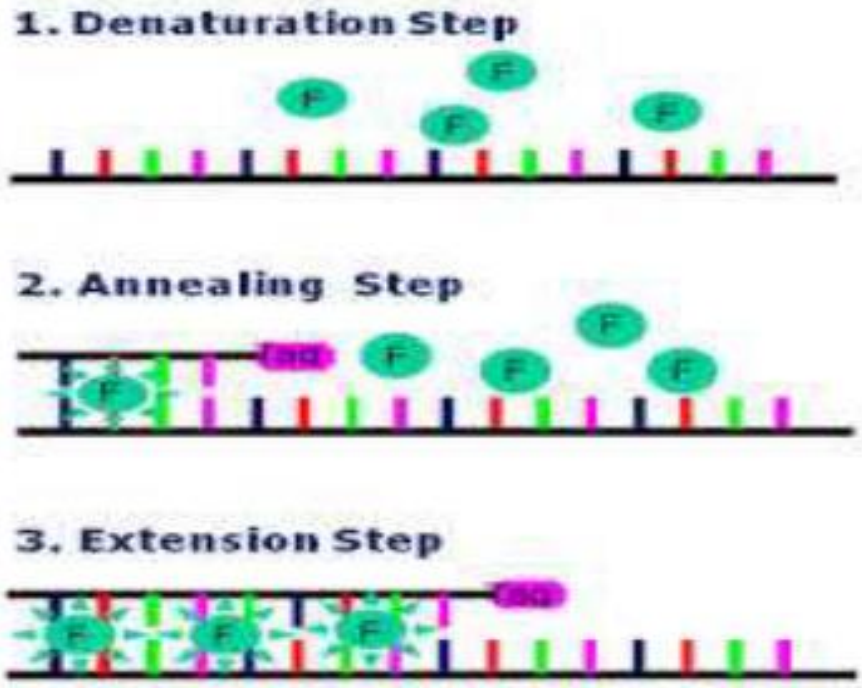

Fluorophore
Quencher
Forward PCR primer
TaqMan Probe
5'
3'
Reverse PCR primer
Amplification Assay
Polymerization
Probe displacement and cleavage
Fluorescence
Result
Fluorescence
detector
PCR Products
Cleavage Products



SYBR green

DNA-binding agents

- It is used as a dye for the ***quantification*** of double stranded DNA in some methods of quantitative PCR
- It is also used to visualise DNA in gel electrophore



detector

TaqMan vs. SYBR Green

TaqMan Probe

Advantages:

- **Increased specificity**
- Use when the most accurate quantitation of PCR product accumulation is desired.
- Option of detecting multiple genes in the same well (multiplexing).
- ----------

Disadvantages:

- Relative **high cost** of labeled probe.

SYBR Green

Advantages:

- Relative **low cost** of primers.
- No fluorescent-labeled probes required.
- ----------

Disadvantages:

- **Less specific**
- Not possible to multiplex multiple gene targets.

How Real-Time PCR Works

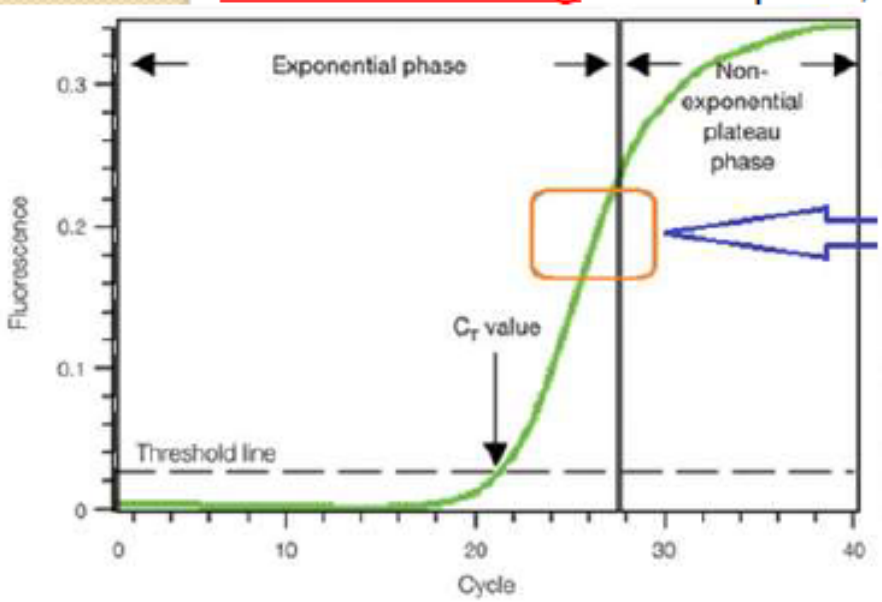
To understand how real-time PCR works, let's start by examining a sample amplification plot

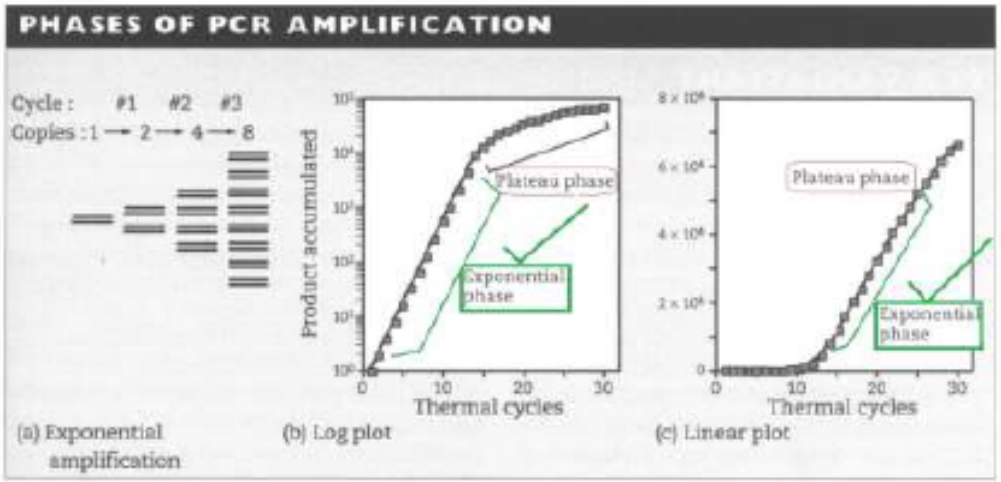
-- **In this plot**, the PCR **cycle number** is shown on the **x-axis,** and the **fluorescence from the amplification reaction,**is shown on the **y-axis**.

… *which is proportional to the amount of amplified product in the tube*,

The amplification plot shows two phases, an **exponential phase** followed by a **nonexponential plateau phase**.

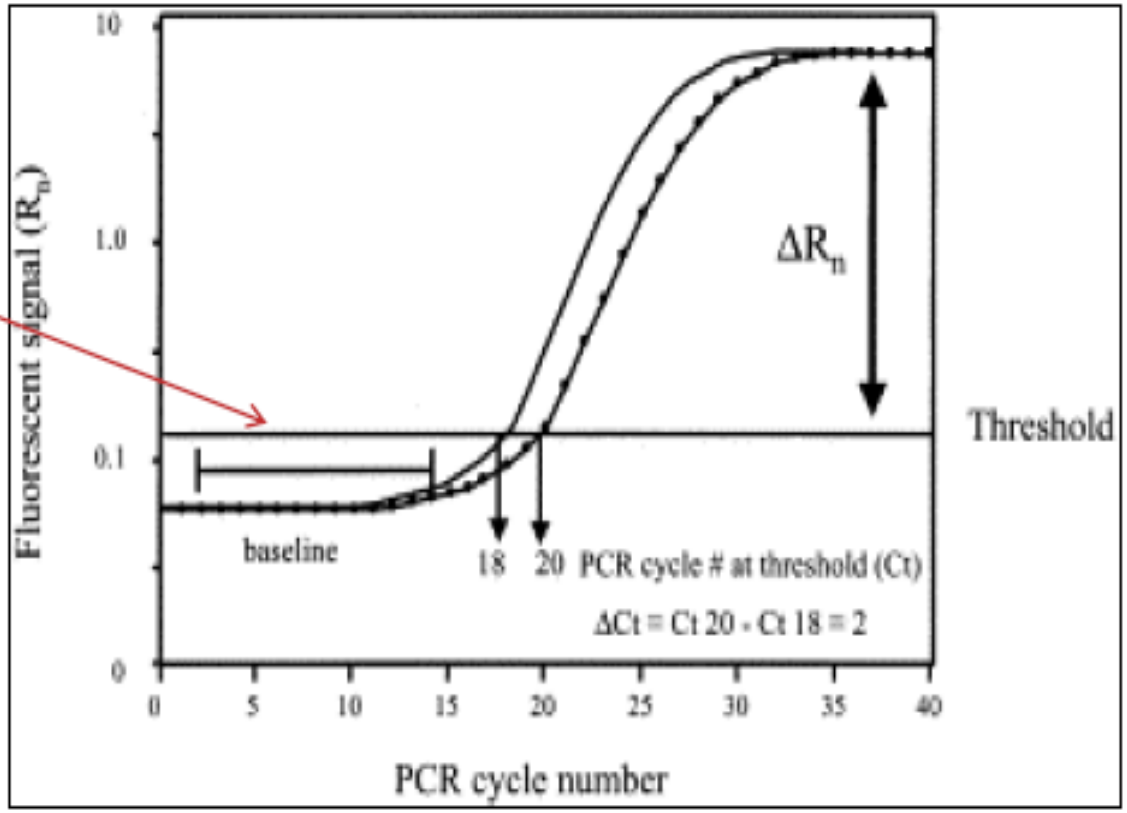
- During the exponential phase, the amount of PCR product approximately doubles in each cycle. **As the reaction proceeds**, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau





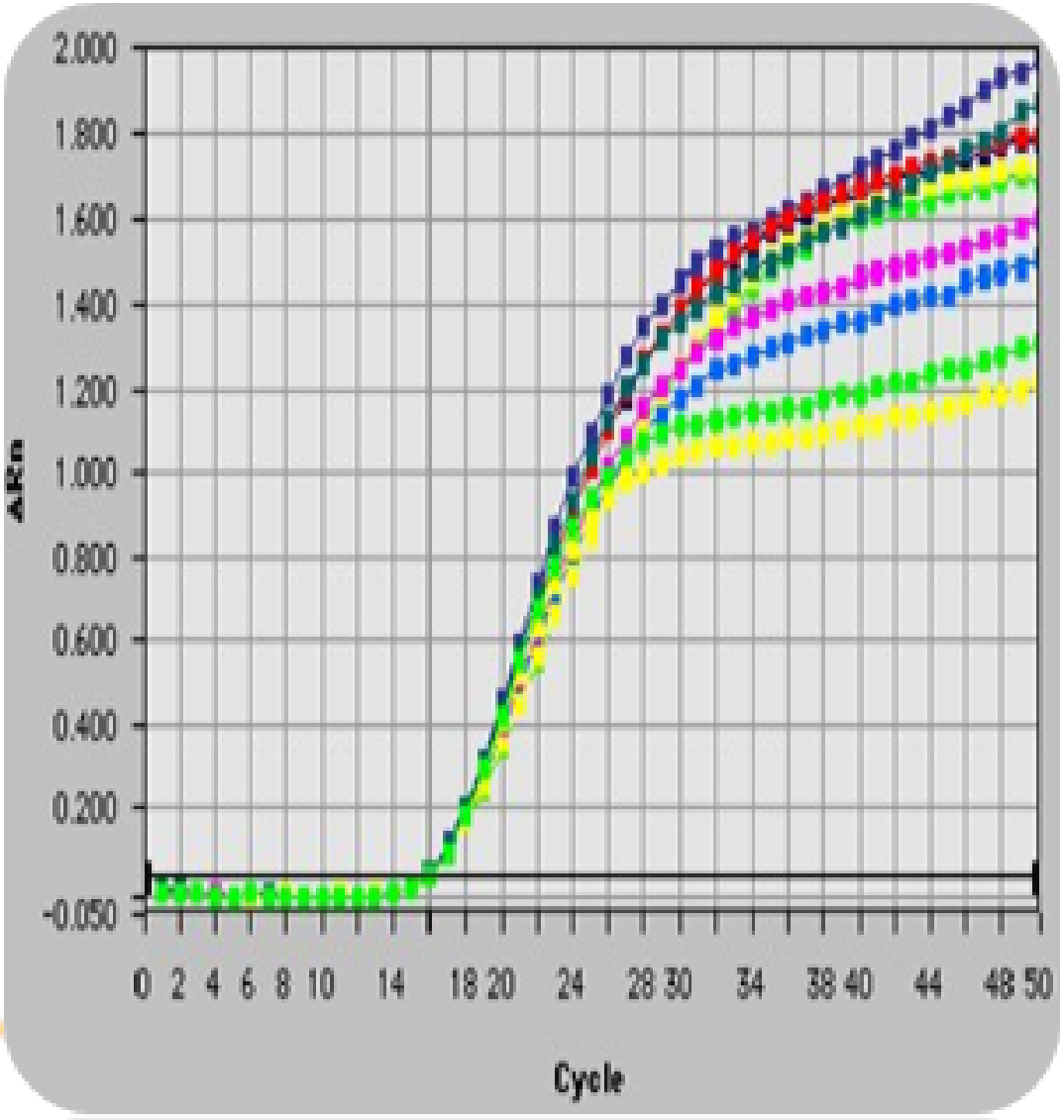
How the signal of Real time PCR is quantified

1. The signal measured during these PCR cycles is used to plot the threshold.
2. The threshold is calculated as 10 times the standard deviation of the average signal of the baseline fluorescent signal.
3. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample.
4. The Ct is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level.



MULTIPLEX PCR IN REAL TIME

Multiplex real time quantitative RT-PCR assays have been developed for simultaneous detection identification and quantification of HBV, HCV and HIV-! In plasma and Serum samples.



RT-PCR

- An **RT-PCR** (Reverse transcriptase-polymerase chain reaction) is a highly sensitive technique for the detection and quantitation of mRNA (messenger RNA).
- The technique consists of two parts:

  1) **The synthesis of cDNA (complementary DNA) from RNA by reverse transcription (RT)**

  2) **The amplification of a specific cDNA by PCR.**

  Compared to Northern blot analysis and RNase protection assay used to quantify mRNA, RT-PCR can be used from much smaller samples. It is sensitive enough to enable quantitation of RNA from a single cell.
- Real-time RT-PCR is the method of choice for quantitating changes in gene expression. Furthermore, real-time RT-PCR is the preferred method for validating results obtained from array analyses and other techniques that evaluate gene expression changes.

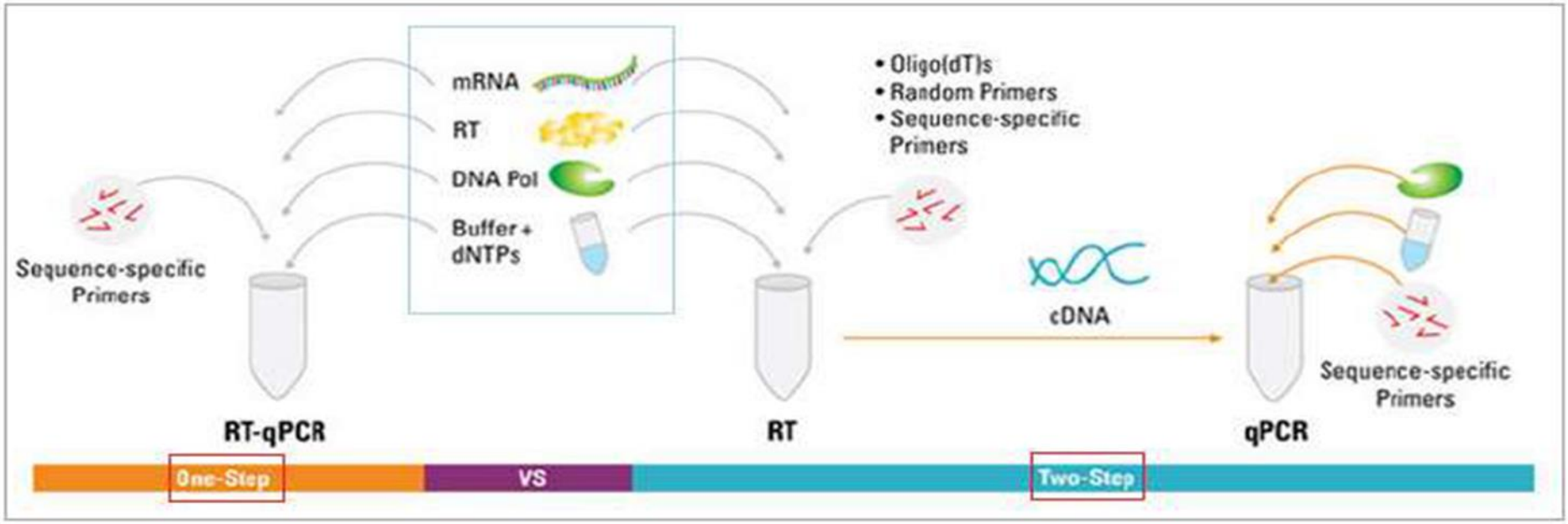
Introduction to RT-qPCR

RNA as the Starting Material

Quantitative reverse transcription PCR (RT-qPCR) is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction. RT-qPCR is used in a variety of applications including gene expression analysis, RNAi validation, microarray validation, pathogen detection, genetic testing, and disease research.

One-step vs. Two-step RT-qPCR

RT-qPCR can be performed in a one-step or a two-step assay (Figure1, Table 1). One-step assays combine reverse transcription and PCR in a single tube and buffer, using a reverse transcriptase along with a DNA polymerase. One-step RT-qPCR only utilizes sequence-specific primers. In two-step assays, the reverse transcription and PCR steps are performed in separate tubes, with different optimized buffers, reaction conditions, and priming strategies.



Techniques: Southern Blot

- **Southern Blotting** (named after Ed Southern, the inventor) is the detection of specific sequences of DNA on a gel by hybridisation with a labelled DNA probe.

- DNA is first transferred out of a gel by capilliarity (the "blot") to a thin membrane which can be incubated with a probe and washed.

- By hybridising at different temperatures, and washing to different ionic strengths ("stringencies") it is possible to tune the process to pick up sequences that are either similar, or exactly identical, to the probe.

Techniques: Southern Blot

- **Applications**:

- 1) To confirm the presence of a gene, often in conjunction with PCR.
- 2) To test for the presence of a specific allele of a gene (i.e. human disease genetics).
- 3) To estimate gene complexity, before you have the gene sequence.
- 4) To detect Restriction Fragment Length Polymorphism (RFLP) and Variable Number of Tandem Repeat Polymorphism (VNTR). The latter is the basis of DNA fingerprinting.

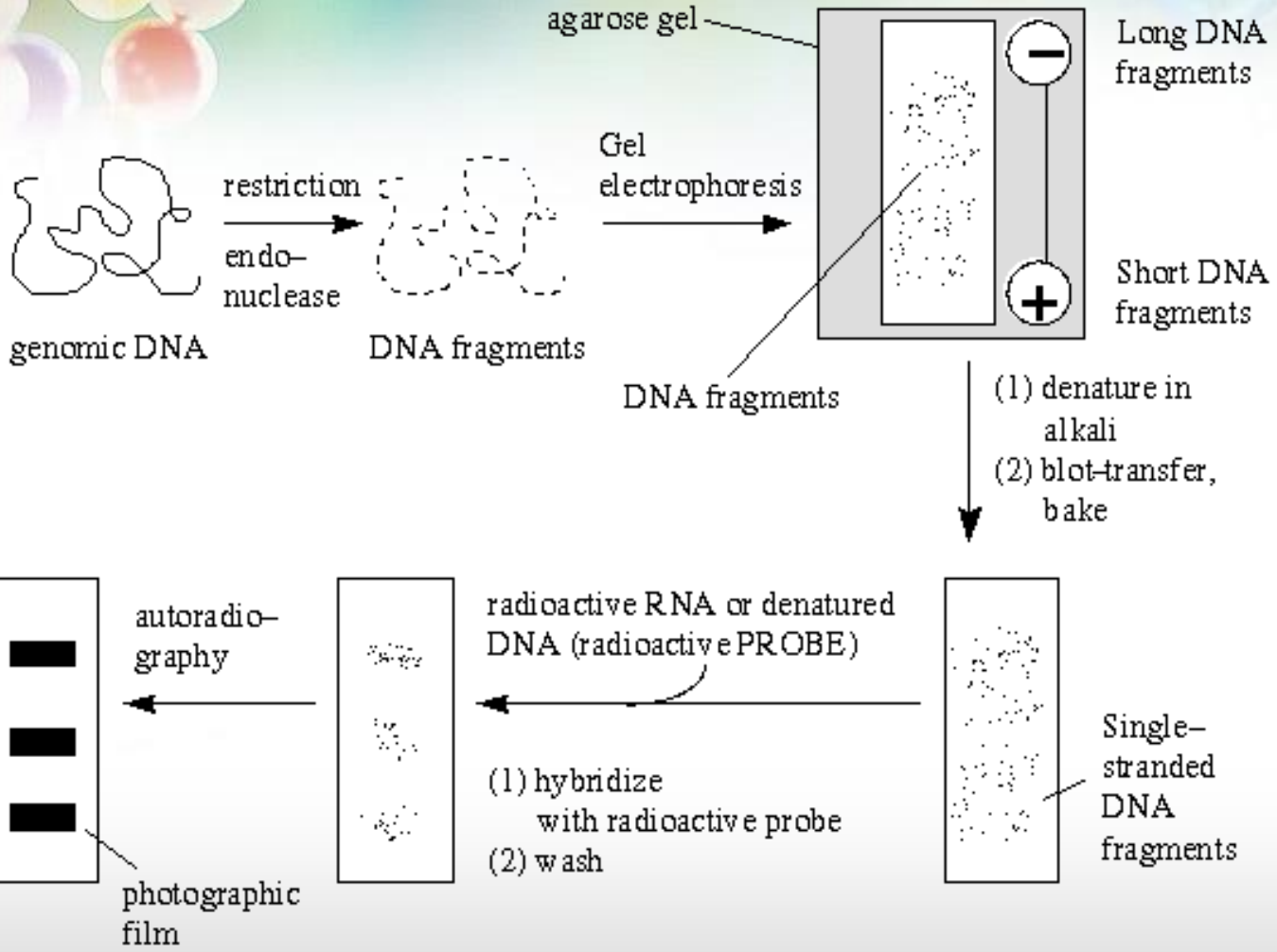
Techniques: Southern Blot

- **Other uses for Southern blotting:**

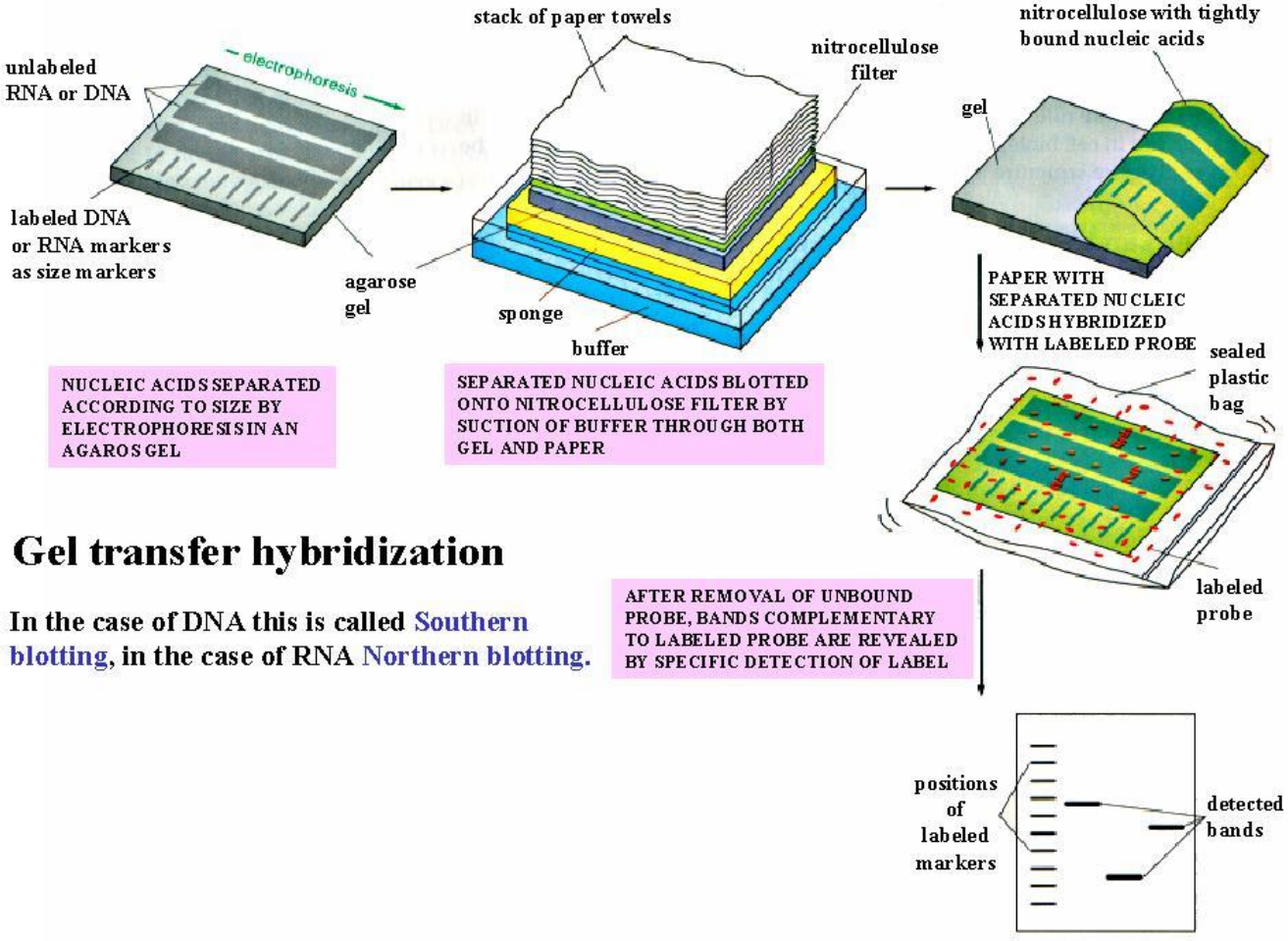
- It is the standard way to screen either a genomic or cDNA library ("plaque lifts"). Similarly, it can be used to identify a bacterial colony carrying a desired plasmid / insert ("colony lifts").

- If genomic DNA is cut with several restriction enzymes, and the gel probed for a specific gene, the number of bands in each lane gives an indication as to whether there are single or multiple copies of the gene in the genome.

Techniques: Southern Blot



Techniques: Southern Blot



Gel transfer hybridization

In the case of DNA this is called Southern blotting, in the case of RNA Northern blotting.

Techniques: Northern Blots

- Northern blots are similar to Southern, except that RNA from different tissues is run out on a gel, and probed with a DNA or RNA probe corresponding to a particular gene.

- Northern blotting is used for detecting and quantitation of **RNA** fragments, instead of DNA fragments. The technique is exactly like Southern Blotting. It is called "Northern" simply because it is similar to "Southern", not because it was invented by a person named "Northern".
- RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing conditions. The RNA is then transferred to a membrane, crosslinked and hybridized with a labeled probe.

Techniques: Western Blot

- Western blot analysis can detect **one** protein in a mixture of any number of proteins while giving you information about the size of the protein.
- Allows investigators to determine with a specific primary antibody, the relative amounts of the protein present in different samples.
- Western blots are analogous to Northern and Southern, except that proteins are run out in an SDS polyacrylamide gel, and are detected with specific antibodies.
- In clinical settings, Western Blotting is routinely used to confirm serious diagnosis suggested by ELISA such as HIV seroconversion

DNA sequencing

In DNA sequencing the nucleotide sequence of DNA is determined. In sequencing, appropriate treatments are used to generate DNA fragments that end at the four bases: adenine (A), guanine (G), cytosine (C) and thymine (T). Then the fragments are subjected to polyacrylamide gel electrophoresis so that molecules with one nucleotide difference in length are separated on the gel. Two different procedures have been developed to accomplish sequencing, called the Maxam-Gilbert and the Sanger dideoxy procedures. In the **Maxam-Gilbert procedure** (Figure 1) the DNA fragment is labelled with ^{32}P at its 5' end. Then chemicals are used that break the DNA preferentially at each of the four nucleotide bases under conditions in which only one break per chain is made. Thus four separate test tubes are prepared, one for each base (A, T, G, C). After gel electrophoresis and autoradiography only the fragments possessing 5'-terminal ^{32}P-phosphate group show up on the gel (Figure 2). This chemical method is only seldom used because it is time-consuming and requires handling of toxic chemicals.

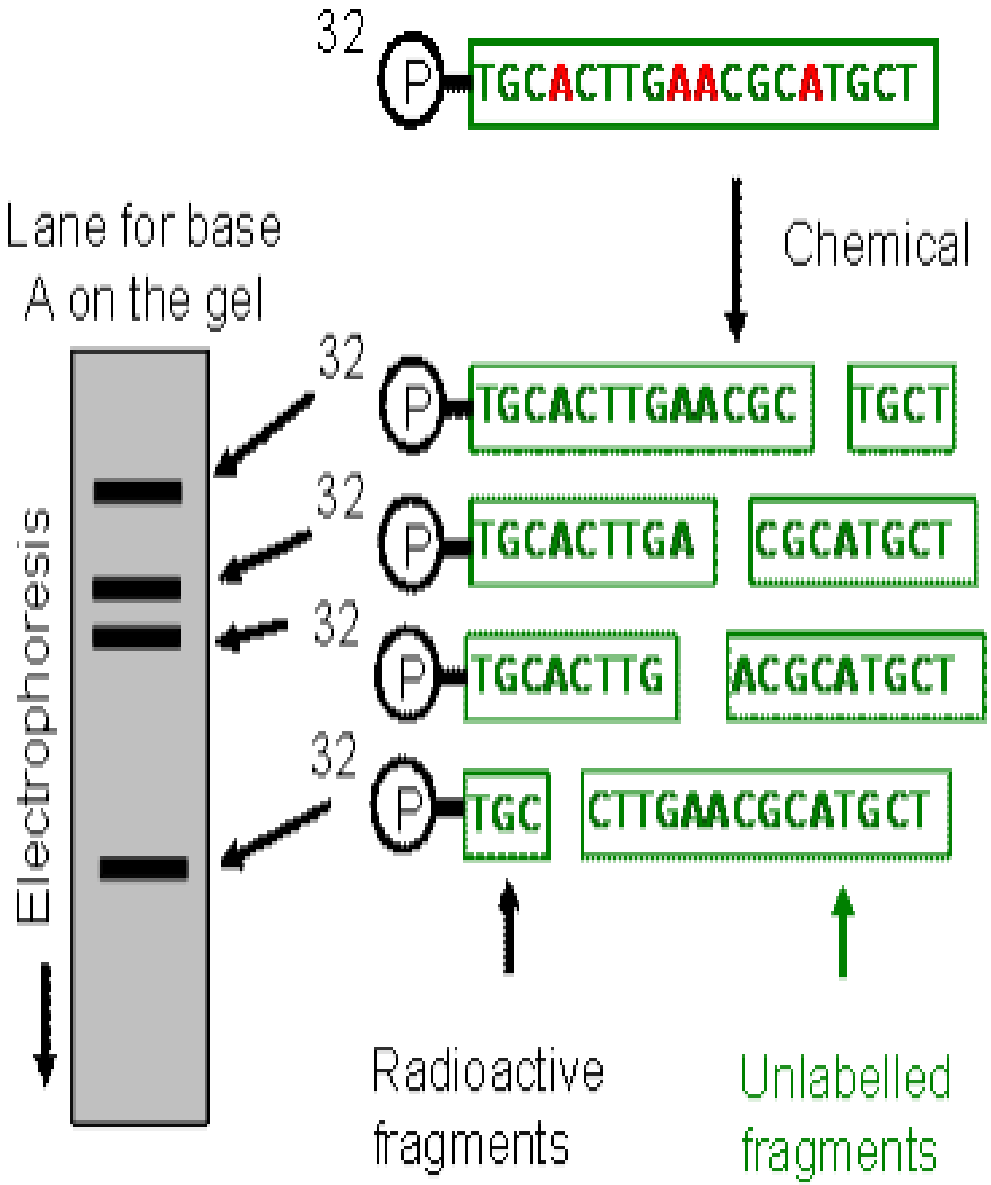


Figure 1. Principle of Maxam-Gilbert procedure. The figure shows an example for only one base (A).

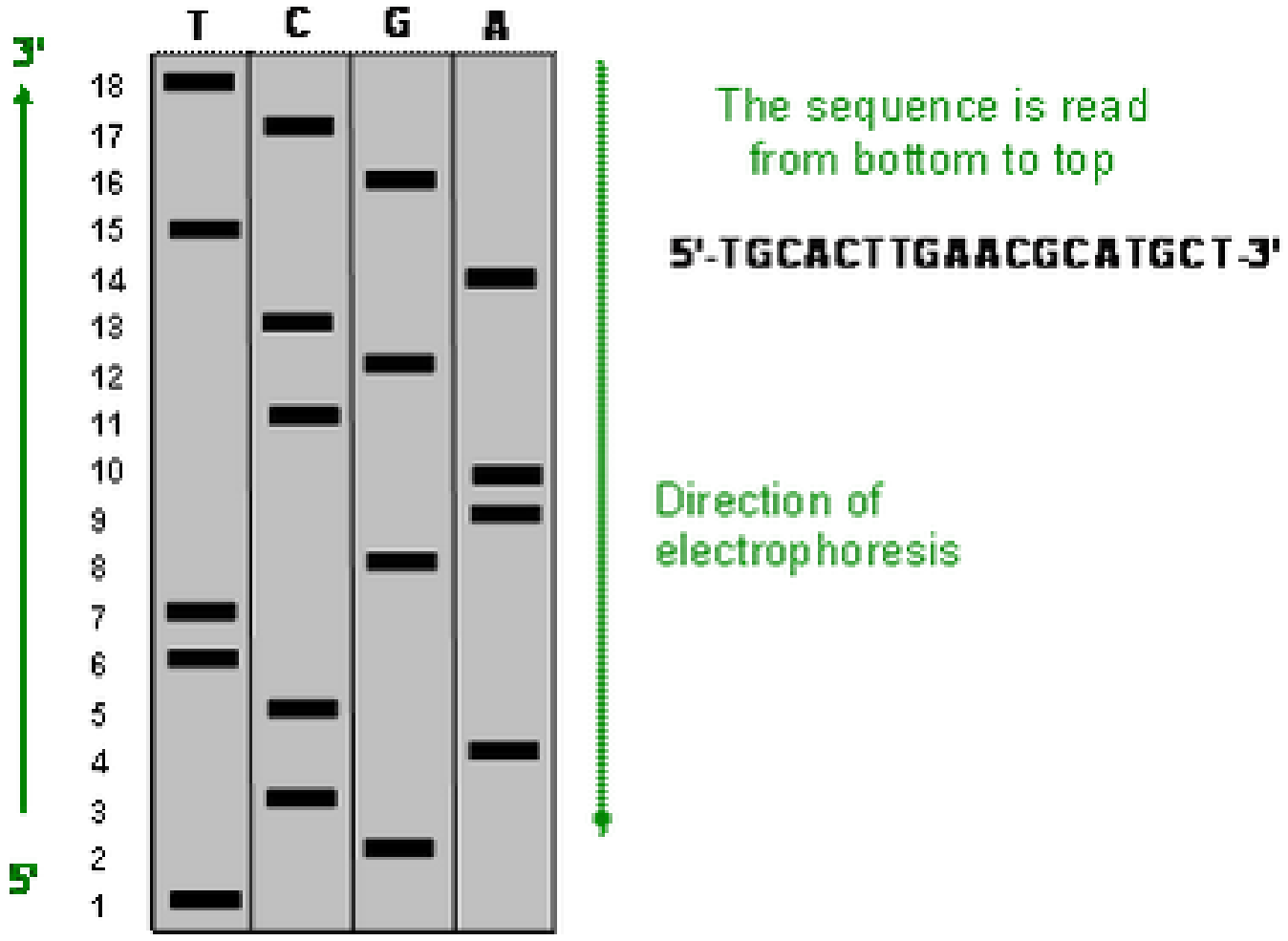


Figure 2. Reading the sequence from the gel.

Sanger dideoxy procedure

In the enzymatic **Sanger dideoxy procedure** (Figure 3) the sequence is determined by making a copy of the single-stranded DNA, using DNA polymerase. This enzyme uses deoxyribonucleoside triphosphates (dNTPs) as substrates and adds them to a primer. The primer is hydrogen bonded to the 3' end of the DNA to be sequenced. The DNA with the primer is divided into four separate reaction mixtures. Each reaction mixture contains all four dNTPs and in addition, one of the four dideoxy analogs (dideoxyribonucleoside triphosphates ddNTPs) of the deoxyribonucleoside triphosphates. Because in the dideoxy sugar the 3'-hydroxyl has been replaced by a hydrogen, continued extension of the chain cannot occur. The dideoxy analog thus acts as specific chain-termination reagent. Fragments of variable length are obtained depending on the ddNTP in the mixture. The formed nucleic acid fragments are visualizes by using either a labelled (radioactive or fluorescent) primer or dNTPs.

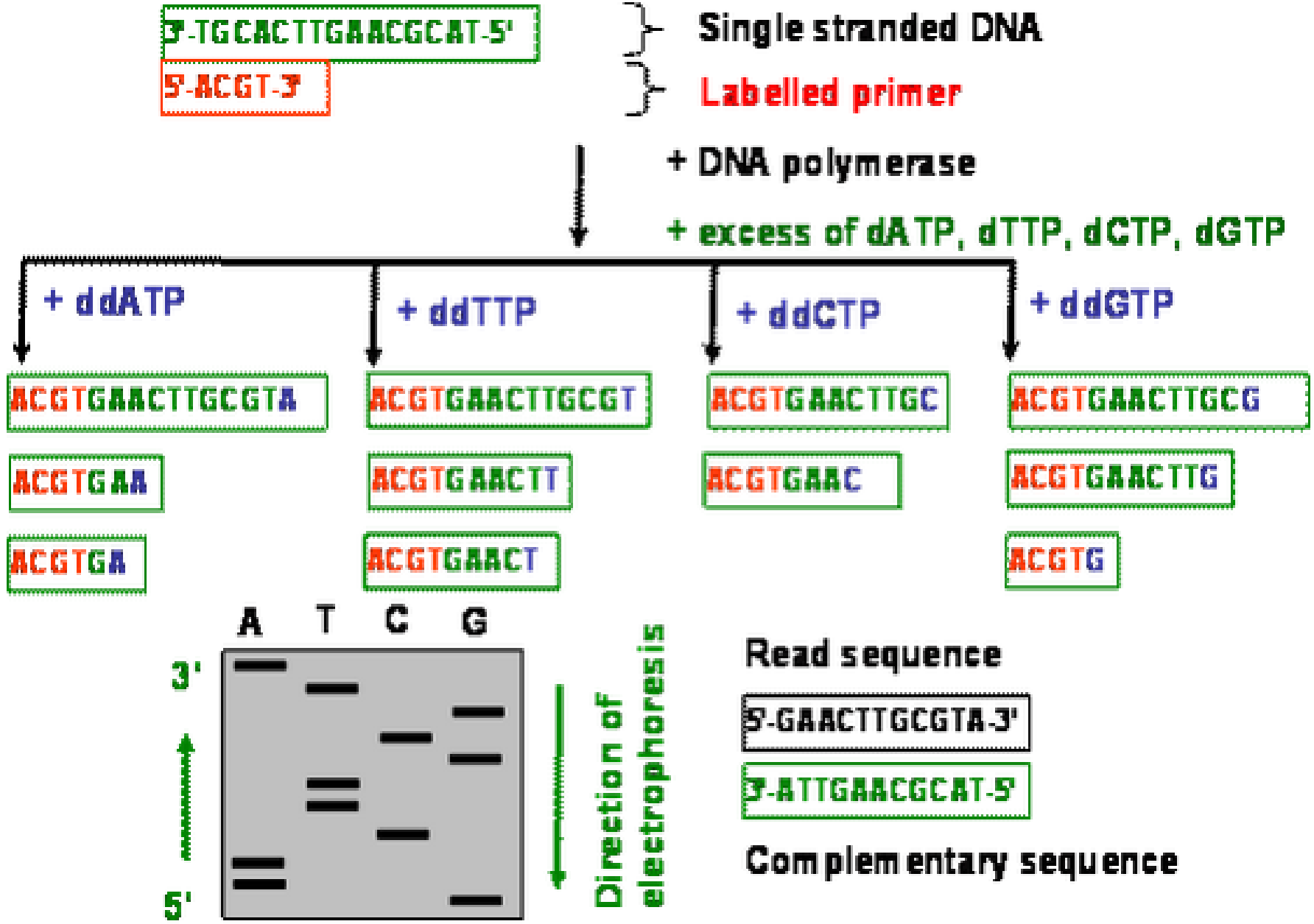


Figure 3. Principle of Sanger dideoxy sequencing.

A major advantage of the Sanger method is that it can be used to sequence RNA as well as DNA. To sequence RNA, a single-stranded DNA copy is made (using the RNA as the template) by the enzyme reverse transcriptase. By making the single stranded DNA by the presence of ddNTPs, various-sized DNA fragments are generated suitable for Sanger-type sequencing.

Automated Sequencing

Automated sequencing is based on the dideoxy methodology, but four different fluorescent dye-labelled ddNTPs are used. Thus each fluorescent label can be detected by its characteristic spectrum. The products are separated by automated electrophoresis and the bands detected by fluorescence spectroscopy.

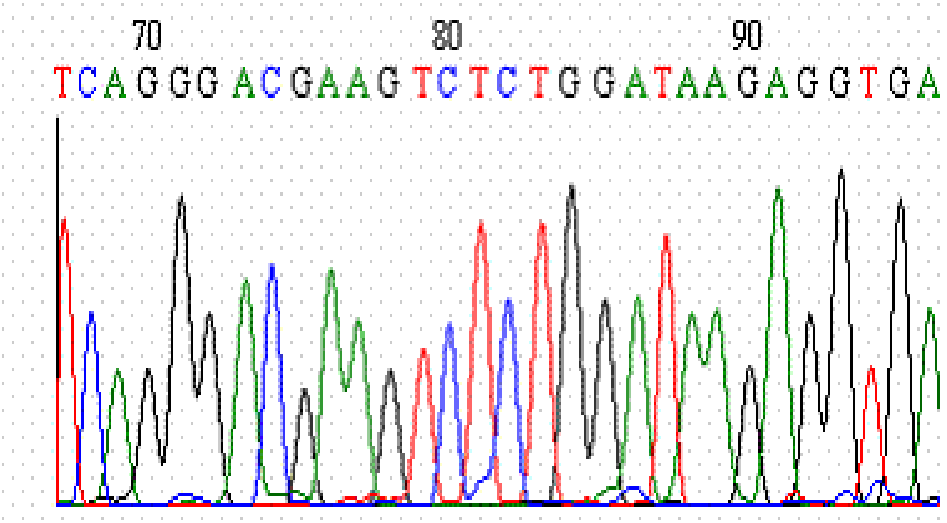
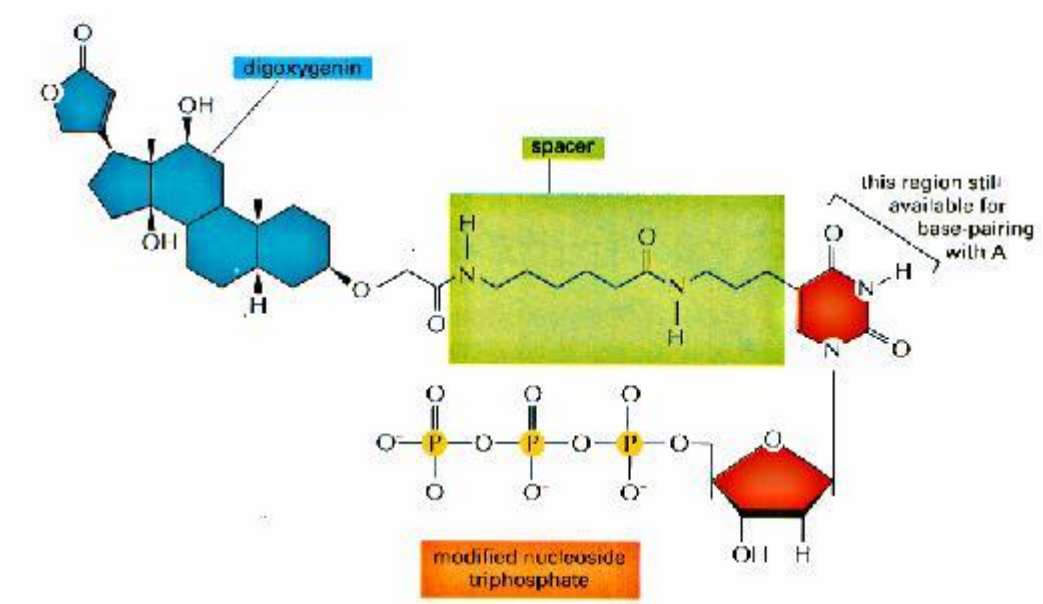


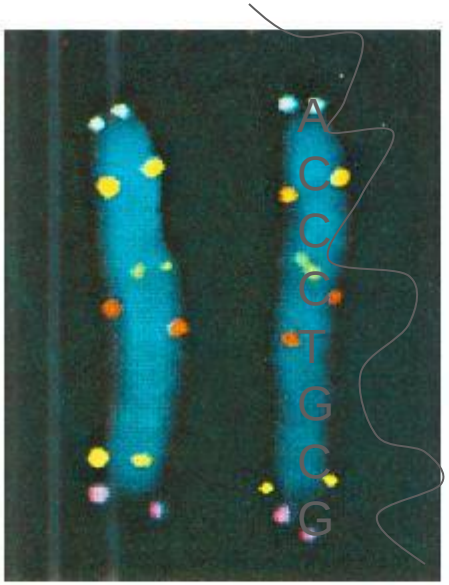
Figure 4. An example of a portion of a chromatogram from automated sequencing.

For determining the DNA sequence of a long molecule, such as a whole gene, it is necessary to proceed in stages. First, the DNA is broken into small overlapping fragments and the sequence of each fragment is determined. Using the overlaps as a guide, the sequence of the whole molecule can be deduced.

In situ hybridization to locate specific genes on chromosomes



A chemical label like digoxygenin, a plant steroid, is attached via a spacer to the 3'-end of the DNA probe. The digoxygenin-tagged probe is then hybridized to metaphase chromosomes which have been briefly treated with alkali to denature the DNA. The binding of the probe can be visualized with fluorescent antibodies against digoxygenin. Different probes can be used on the same chromosome. Note that each probe produces two dots on a chromosome since a metaphase chromosome has replicated its DNA and therefore contains two identical DNA helices (sister chromatids).



FISH

- Fluorescence In-Situ Hybridization is a method used to identify specific parts of a chromosome. For example, if you know the sequence of a certain gene, but you don't know on which chromosome the gene is located, you can use FISH to identify the chromosome in question and the exact location of the gene.
- If you suspect that there has been a translocation in a chromosome, you can use a probe that spans the site of breakage/translocation. If there has been no translocation at that point, you will see one signal, since the probe hybridizes to one place on the chromosome. If, however, there has been a translocation, you will see two signals, since the probe can hybridize to both ends of the translocation point.
- To use FISH efficiently, you have to know what you're looking for, i.e. you usually suspect a particular defect, based on the appearance of certain chromosomes, etc.

FISH

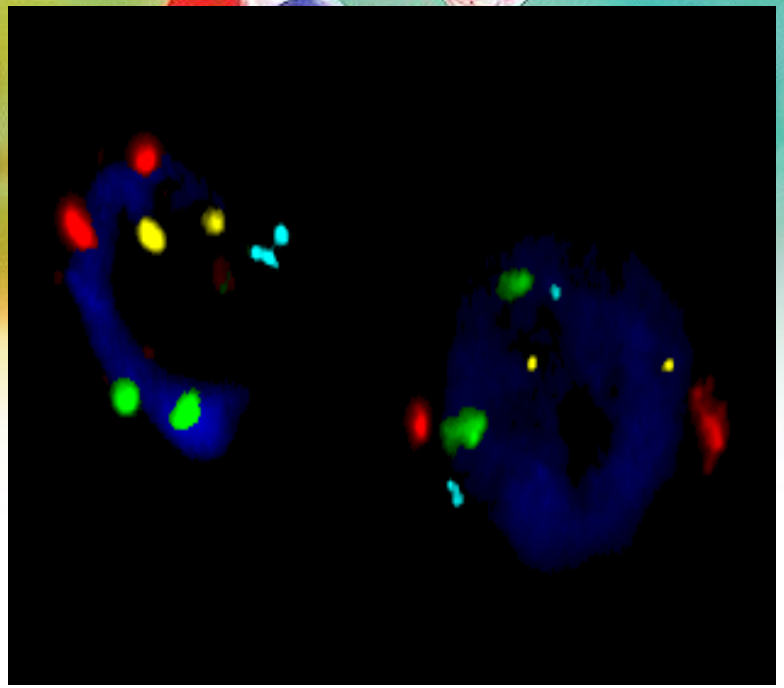
Method

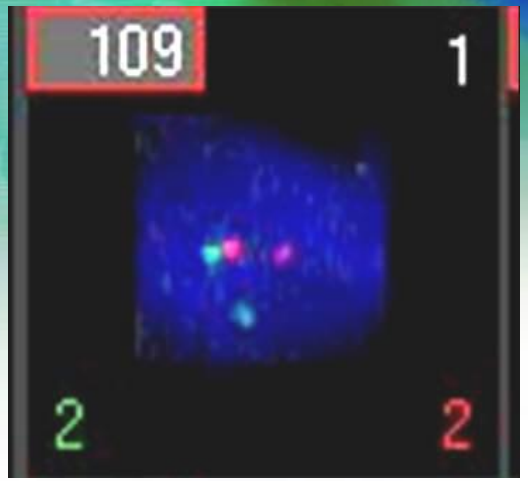
- Make a probe complementary to the known sequence. When making the probe, label it with a fluorescent marker, e.g. digoxigenin, by incorporating nucleotides that have the marker attached to them.
- Put the chromosomes on a microscope slide and denature them.
- Denature the probe and add it to the microscope slide, letting the probe hybridize to its complementary site.
- Wash off the excess probe and look at the chromosomes in a fluorescence microscope. The probe will show as one or more fluorescent signals in the microscope, depending on how many sites it can hybridize to.

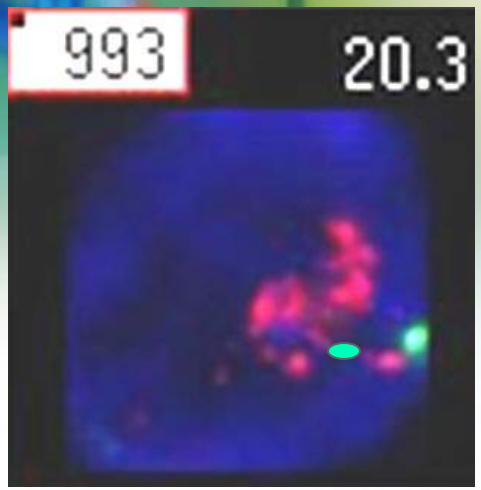
FISH

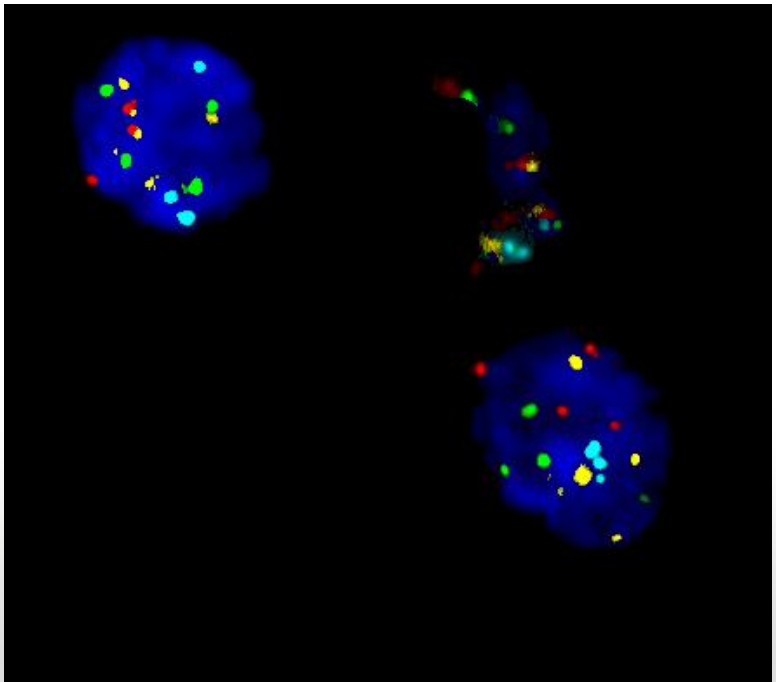
Applications

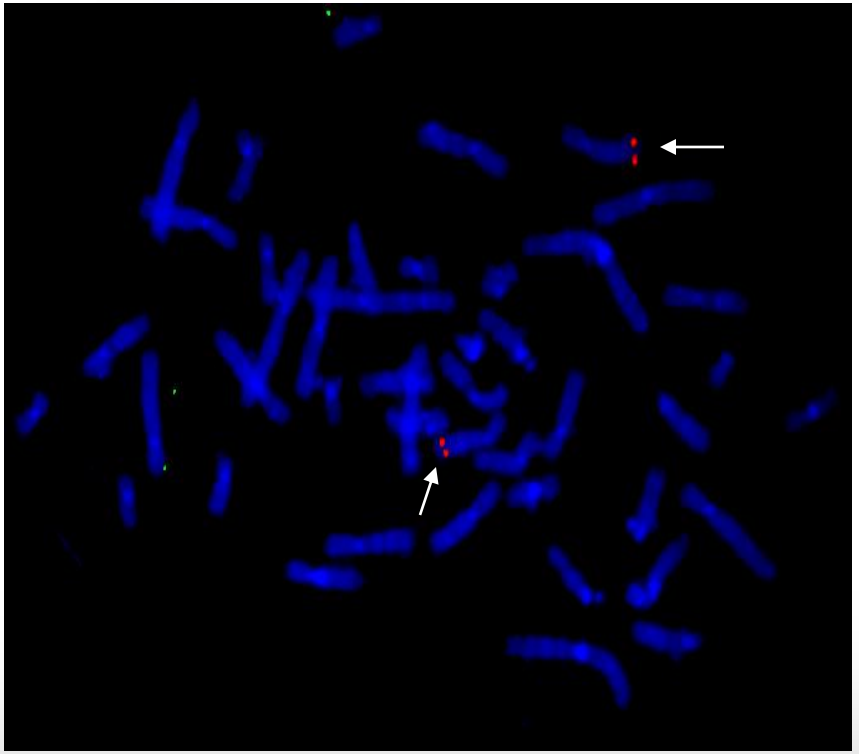
- Diagnosis in clinical and cancer cytogenetics.
- Interspecies studies of evolutionary divergence.
- Analysis of aberrations in animal models of human diseases.
- Many more applications. THINK





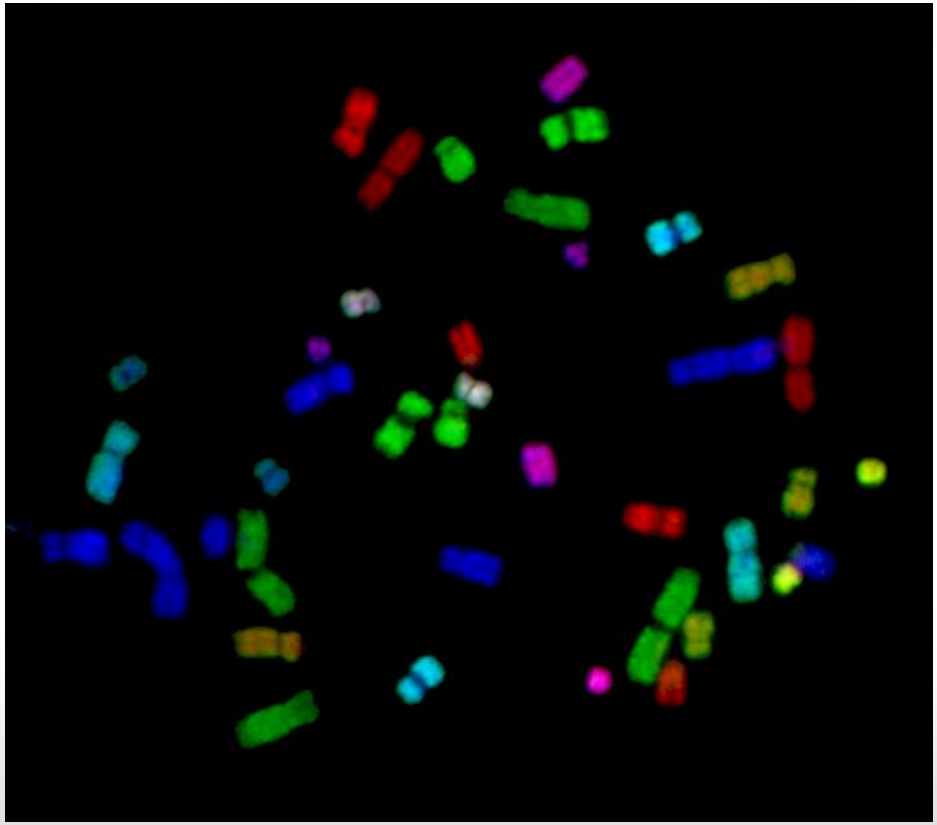




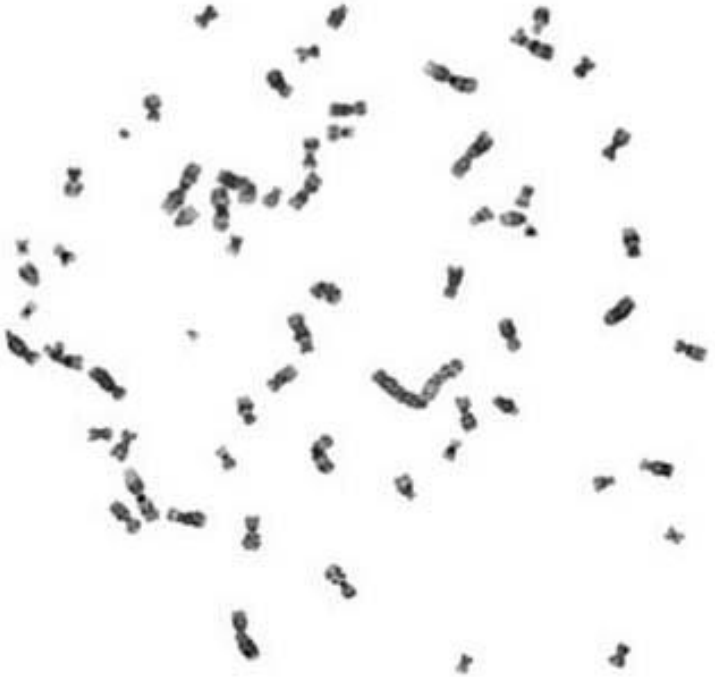


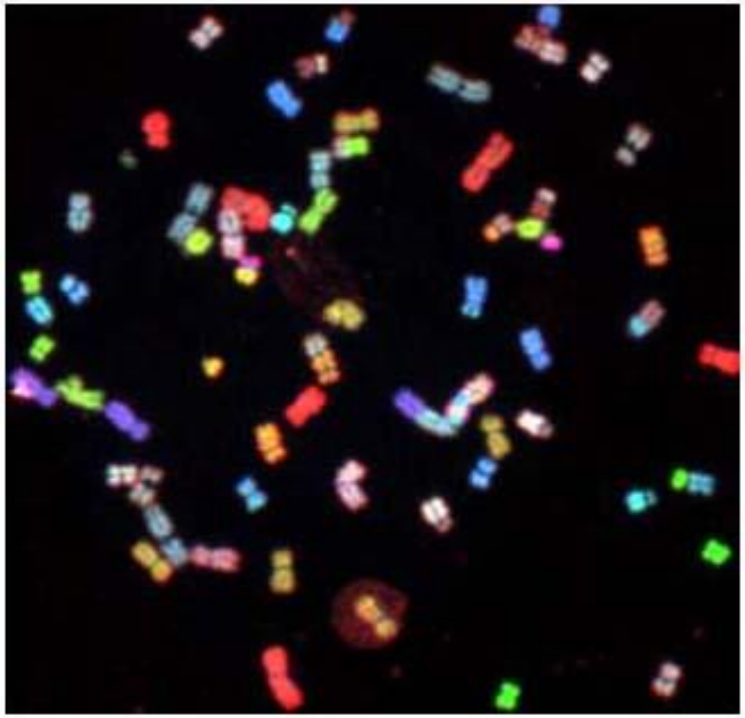
Interphase/Metaphase FISH

Multicolour-FISH, chromosome paints



FISH

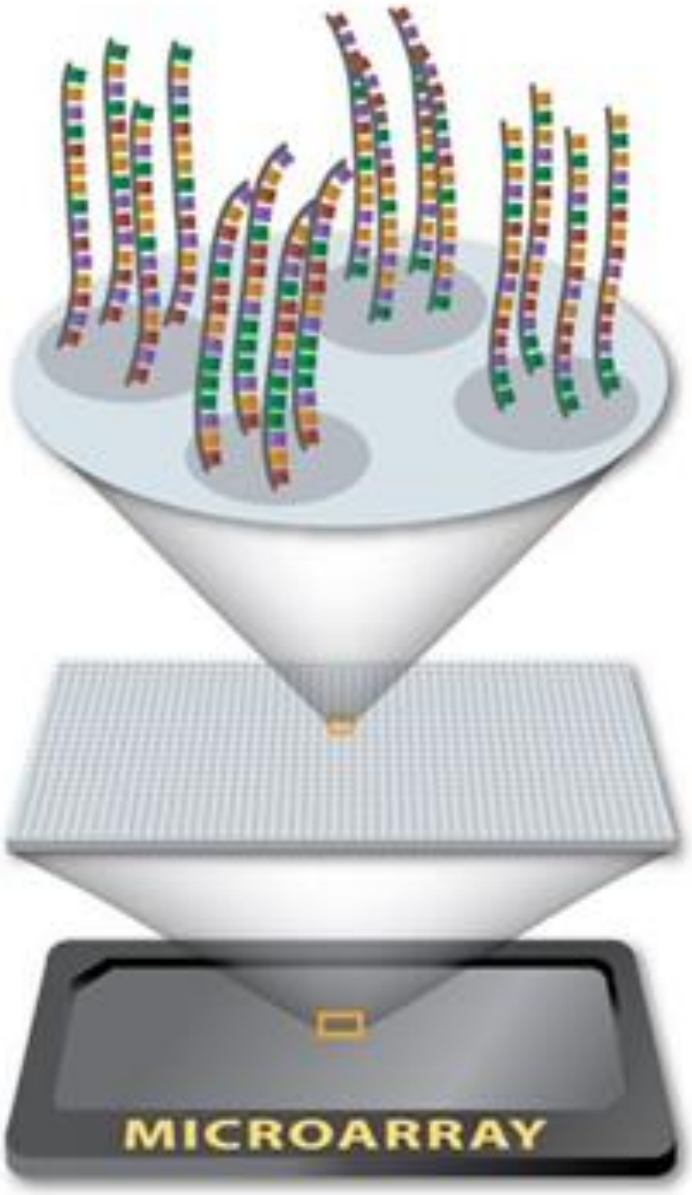




DNA Microarray: Definition

- DNA microarrays are solid substrates on which thousands of DNA molecules corresponding to the genes under investigation are deposited in an ordered arrangement.

- DNA Microarrays consist of 100 - 1 million DNA probes attached to a surface of 1 cm by 1 cm(chip).



DNA Microarray Steps (Procedure) and Applications

DNA Microarray Procedure

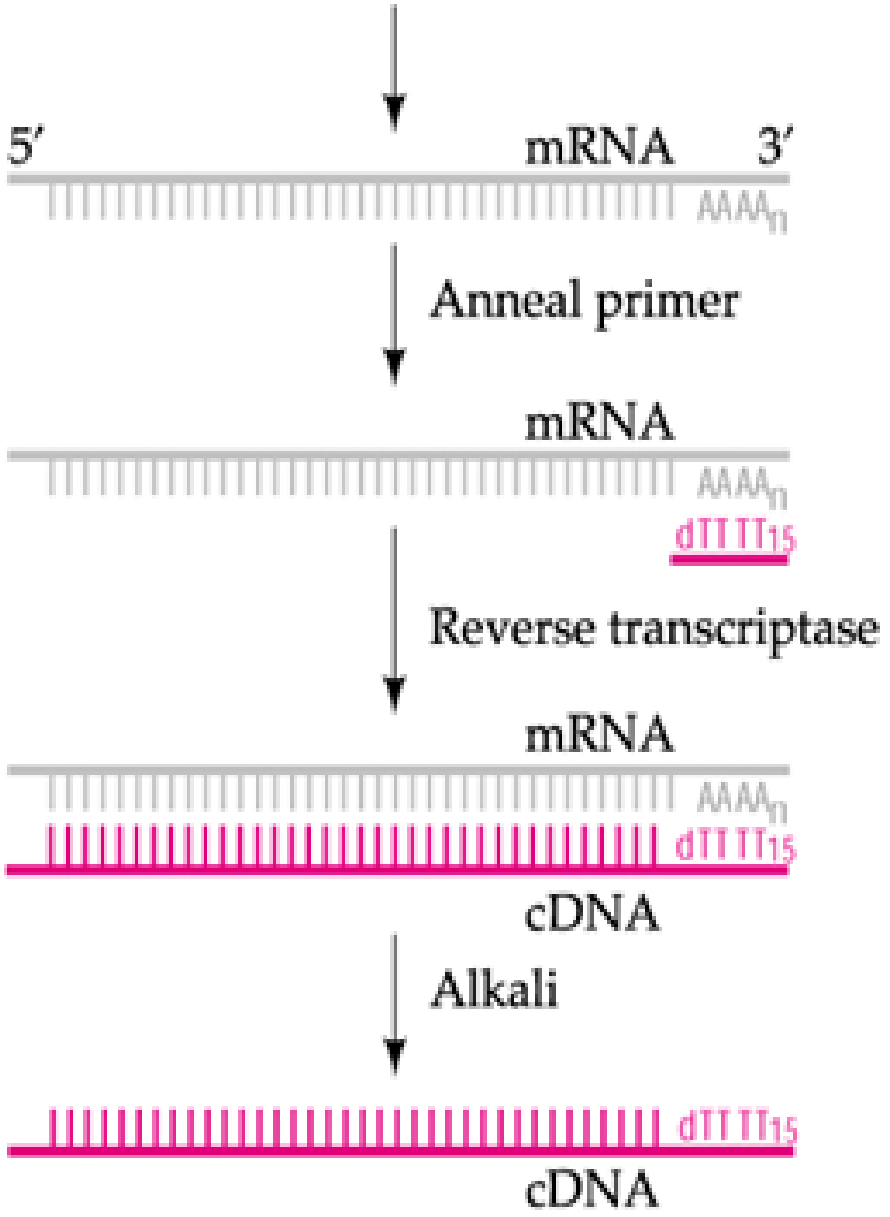
1) **Collect Samples:** This can be from a variety of organisms. Two samples cancerous human skin tissue & healthy human skin tissue

2) **Isolate mRNA:**

- Extract the RNA from the samples. Using either a column, or a solvent such as phenol-chloroform.
- After isolating the RNA, we need to isolate the mRNA from the rRNA and tRNA. mRNA has a poly-A tail, so we can use a column containing beads with poly-T tails to bind the mRNA.
- Rinse with buffer to release the mRNA from the beads. The buffer disrupts the pH, disrupting the hybrid bonds.

3) **Create labelled cDNA:**

- Add a labelling mix to the RNA. The labelling mix contains poly-T (oligo dT) primers, reverse transcriptase (to make cDNA), and fluorescently dyed nucleotides.
- We will add cyanine 3 (cy3-fluoresces green) to the healthy cells and cyanine 5 (cy5-fluoresces red) to the cancerous cells.
- The primer and RT bind to the mRNA first, then add the fluorescently dyed nucleotides, creating a complementary strand of DNA



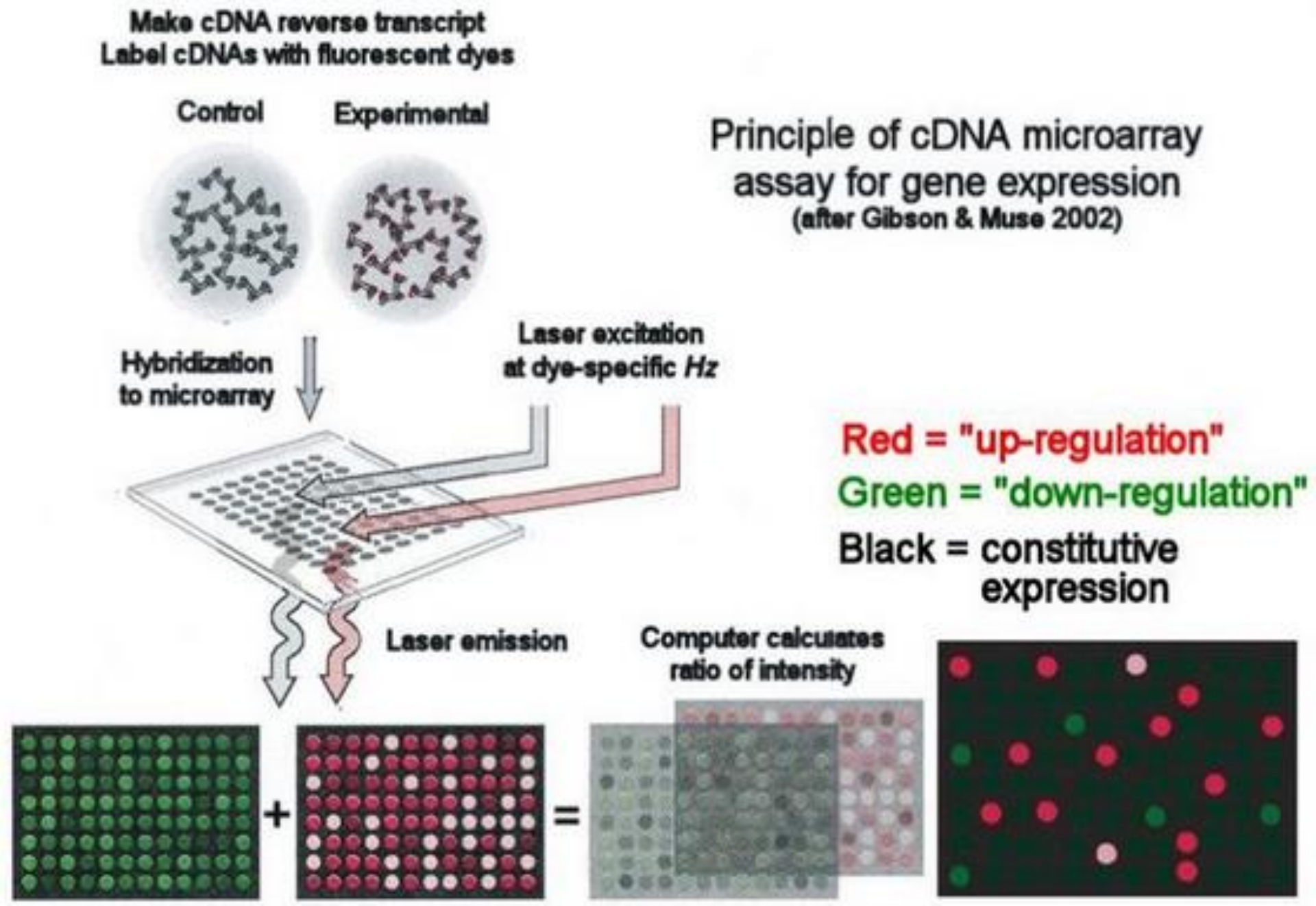
4) **Hybridization**

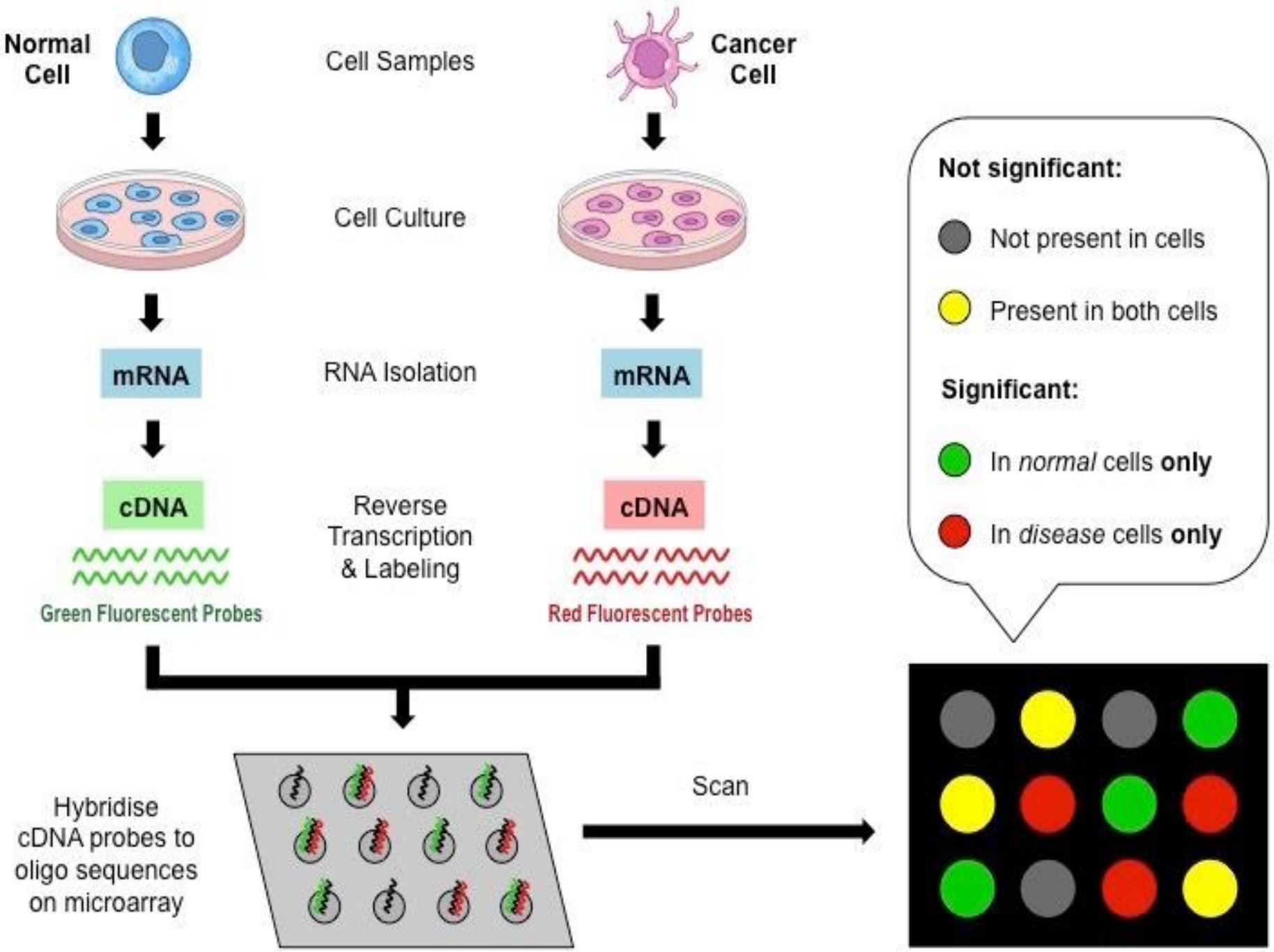
- Apply the cDNA we have just created to a microarray plate.
- When comparing two samples, apply both samples to the same plate.
- The ssDNA will bind to the cDNA already present on the plate.

5)**Detect the relative intensities of fluorescence under Microarray Scanner**

- The scanner has a laser, a computer, and a camera.
- The laser causes the hybrid bonds to fluoresce.
- The camera records the images produced when the laser scans the plate.
- The computer allows us to immediately view our results and it also stores our data.

6) Analyze Data




Normal Cell
Cell Samples
Cancer Cell
Cell Culture
mRNA
RNA Isolation
mRNA
cDNA
Reverse Transcription & Labeling
cDNA
Green Fluorescent Probes
Red Fluorescent Probes
Not significant:
Not present in cells
Present in both cells
Significant:
In normal cells only
In disease cells only
Hybridise cDNA probes to oligo sequences on microarray
Scan

Application of DNA microarray

- It is used in the analysis of transcriptomes and proteomes.
- Gene chips are available to diagnose several pathogenic and genetic diseases in man.
- With the help of species specific probes, DNA microarray is used to identify microbes in the environment.
- It is employed in genotyping of genomes through single nucleotide polymorphism (SNP) analysis.
- DNA microarray is used to detect gene expression by analyzing cDNAs produced from mRNAs of a cell type at different times.
- To measure changes in gene expression levels – two samples' gene expression can be compared from different samples, such as from cells of different stages of mitosis.
- To observe genomic gains and losses. Microarray Comparative Genomic Hybridization (CGH)
- To observe mutations in DNA.

Limitations of Techniques

- False positives/negatives
- Expense
- Complicated, require high expertise and standardization
- Can’t do them without tissues. Thus clinicians have to collect and make databases. “tissue banking”
- Remember consent forms. Ethical issues raised by testing.

Thank you

- Concentrate on the basic information
- Any questions?