Biotechnologie Moléculaire

3ème année

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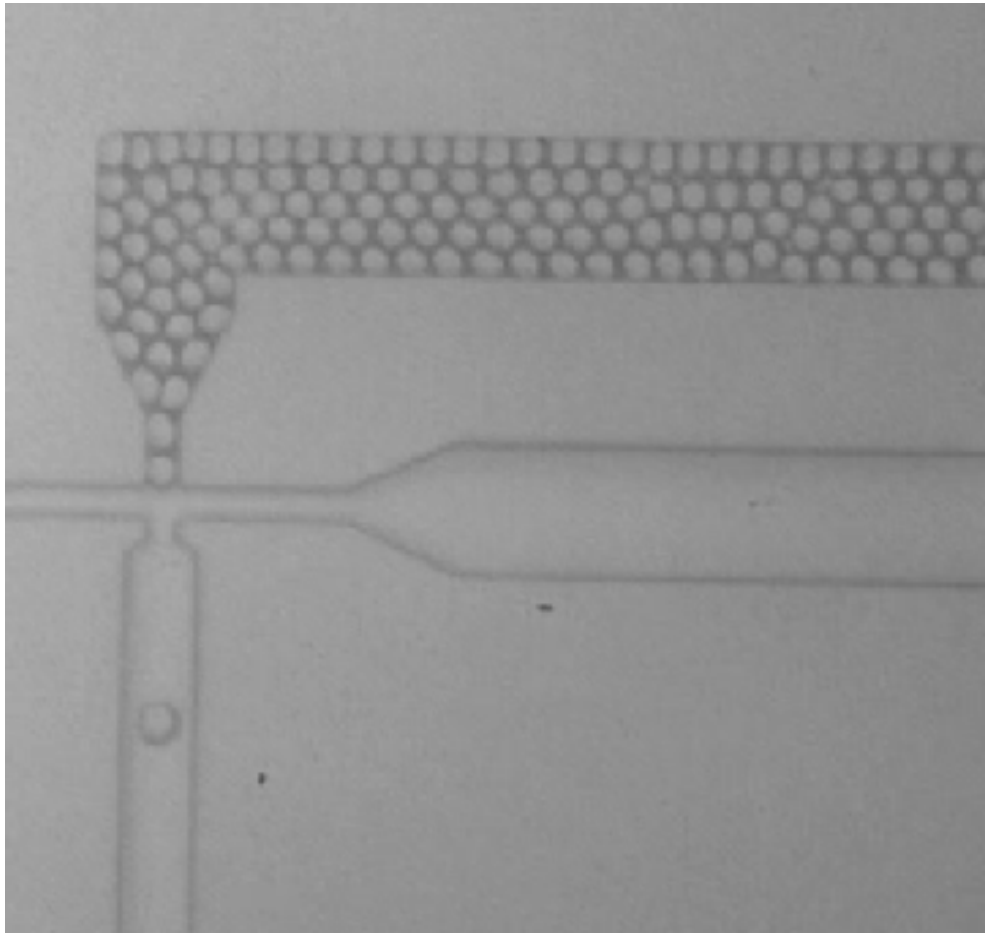


Molecular Diagnostics

Methods to Diagnose Parasitic Infection

A comparison

Method	Advantages	Disadvantages
Microscopic examination	Simple Direct detection of parasite Differentiates morphologically distinct organisms	Slow, laborious, and tedious Low sensitivity Cannot discriminate between similar organisms Requires a high skill level
In vitro culture and mouse inoculation	Detects only viable parasites Measures virulence and infectivity	Slow and expensive Different strains show a range of responses Parasite may lose its viability in the specimen Uses animals
Detection of antibodies in serum	Simple and fast Automatable Can be used to screen a large number of samples	Not always specific Does not distinguish between active and latent infections
DNA hybridization and PCR	Fast, sensitive, and specific Detects parasite directly Can distinguish different species Independent of previous infections Parasites need not be viable Automatable	Expensive and multistep Does not distinguish between live and dead organisms Possible false positives and false negatives

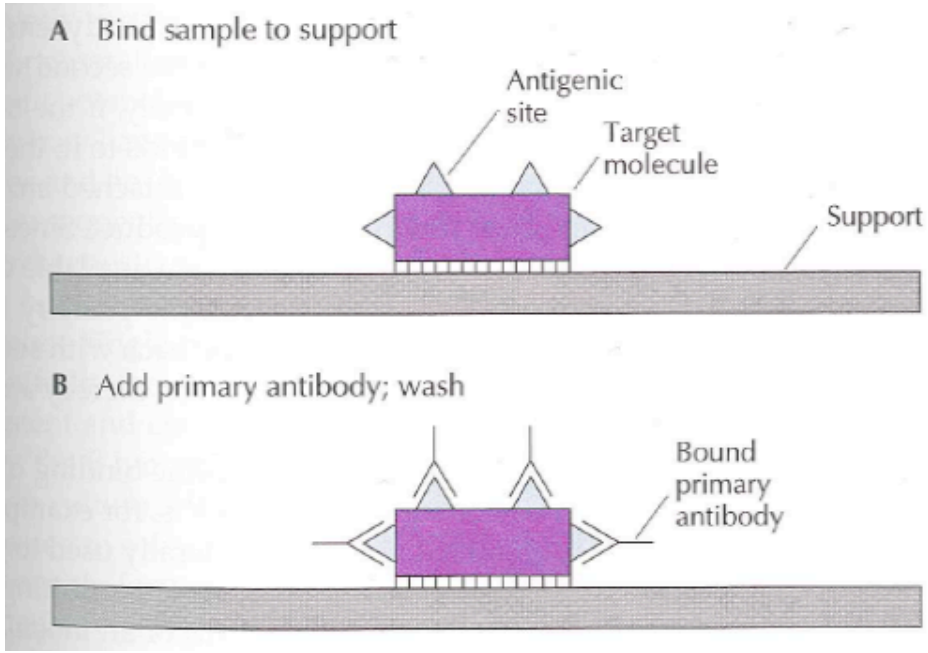
Adapted from Weiss, *Clin. Microbiol. Rev.* 8:113–130, 1995.

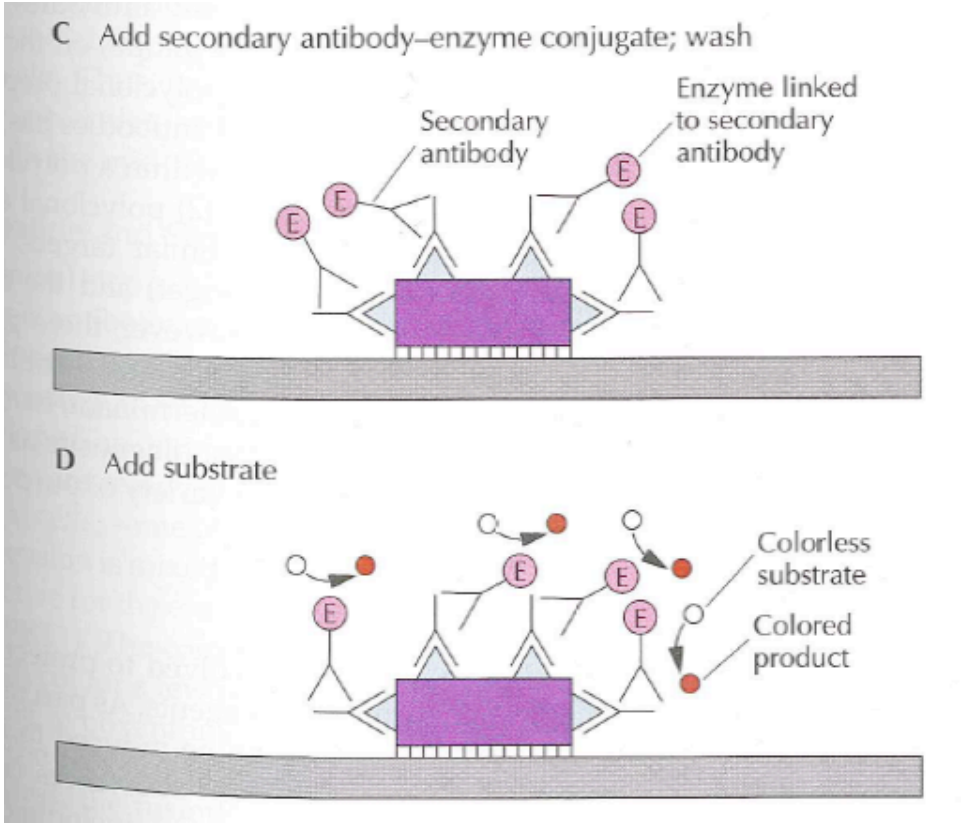
Techniques based on culture are slow (several days)
Techniques based on antibodies or DNA hybridization and PCR are fast

Immunological Diagnostic Procedures Using Antibodies

ELISA

Enzyme-linked immunosorbent assay

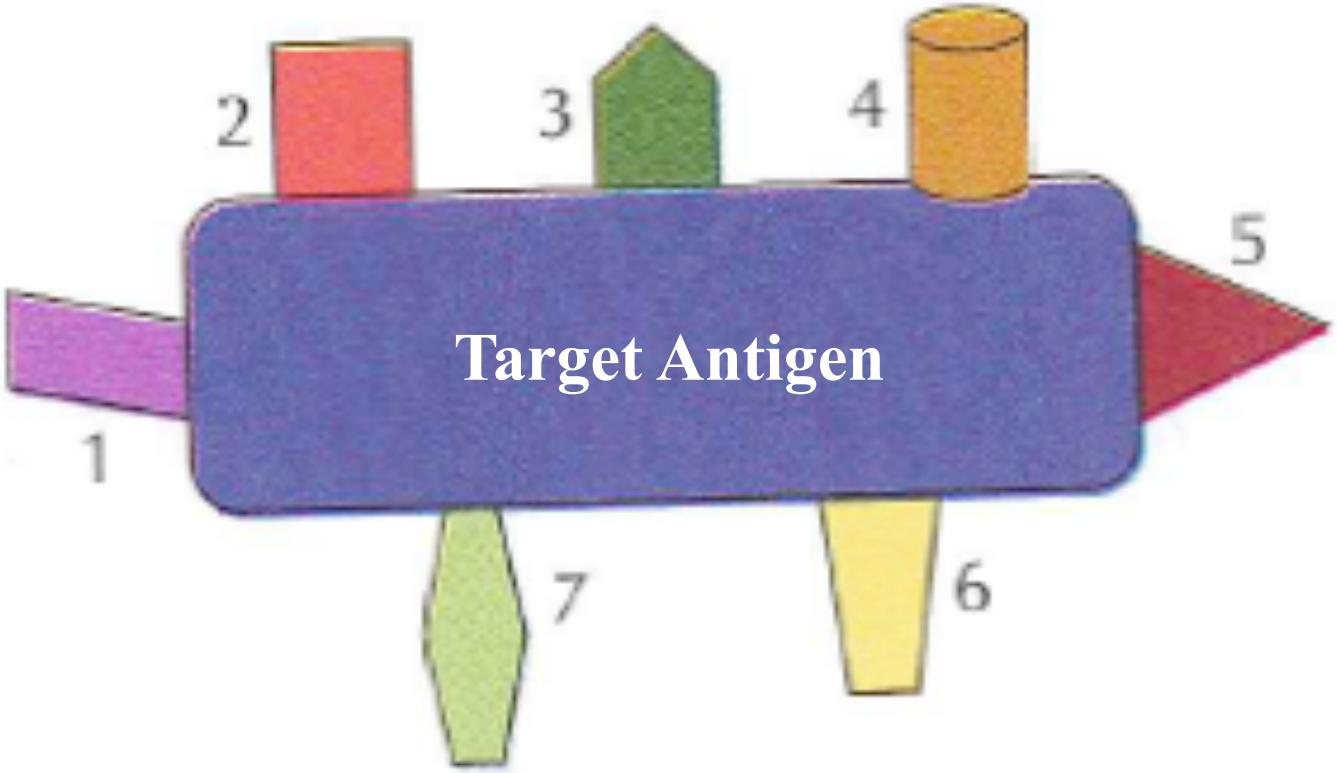




Polyclonal Antibodies

Polyclonal Antibodies

A collection of antibodies all targeted against the same antigen



Polyclonal antibodies are isolated from immunized animals (often rabbits)
The antiserum is polyclonal- it contains multiple antibodies against different sites (epitopes) on the target antigen

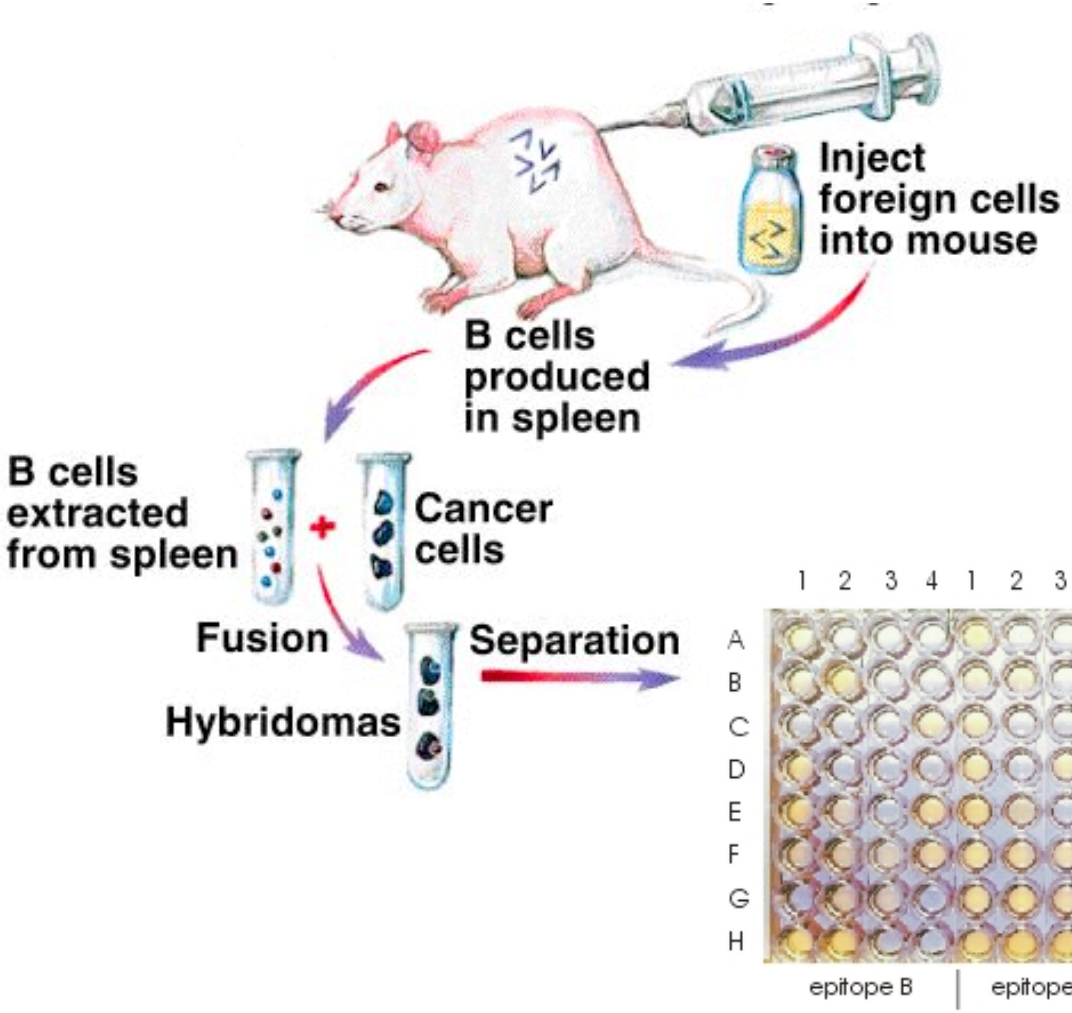
Disadvantages:

1. The amounts of different antibodies in the polyclonal preparation can vary from batch to batch
2. Polyclonal antibodies can often not distinguish between two similar targets (e.g. pathogenic (target) and nonpathogenic (nontarget)

Monoclonal Antibodies

Mouse Monoclonal Antibodies

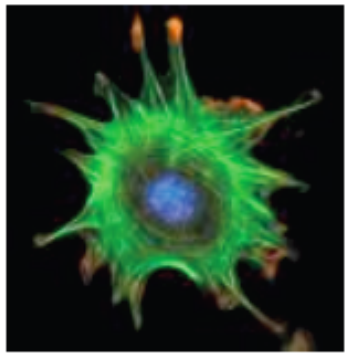
Köhler and Milstein 1975

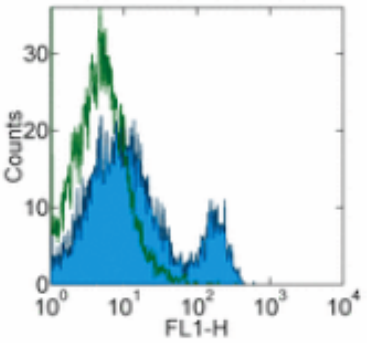


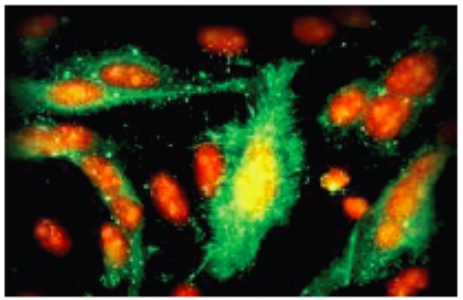
Enabled large scale production of single pure antibody species from immortalised B-cells

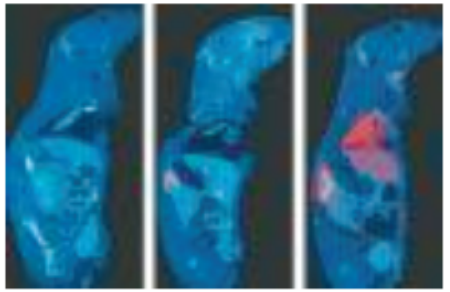
Monoclonal Antibodies

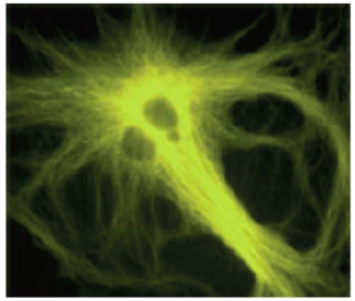
Amazingly useful for research and diagnostics

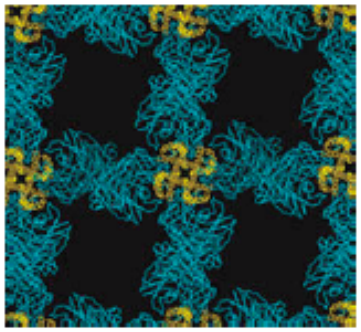


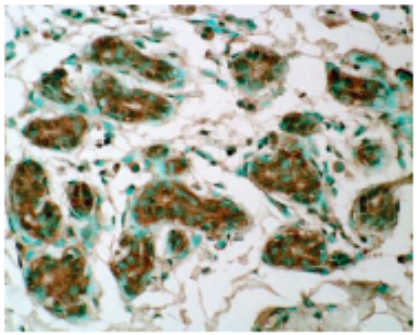


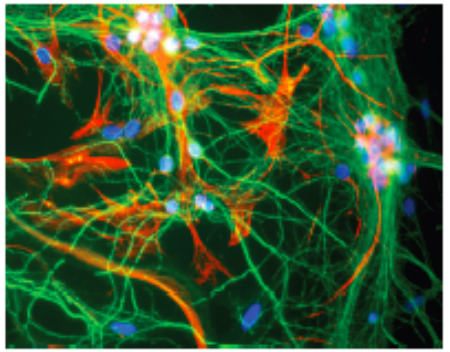






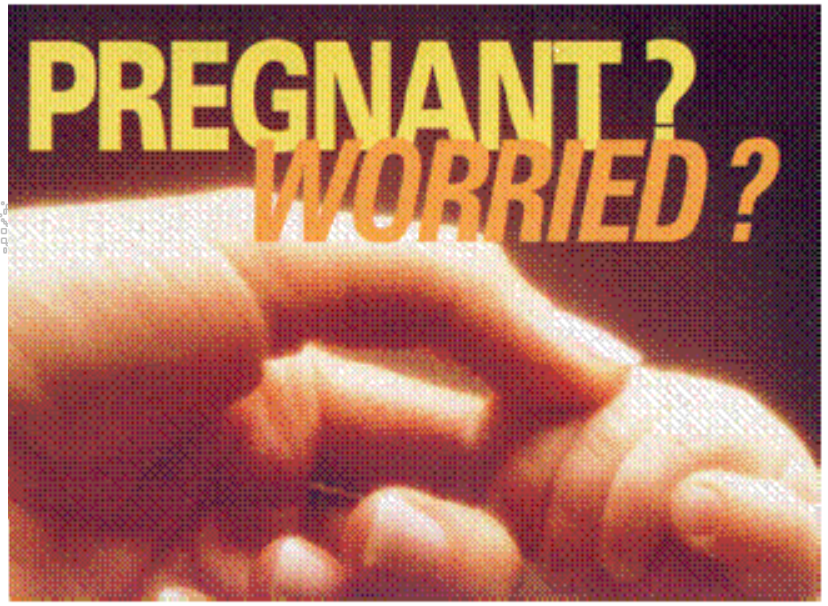




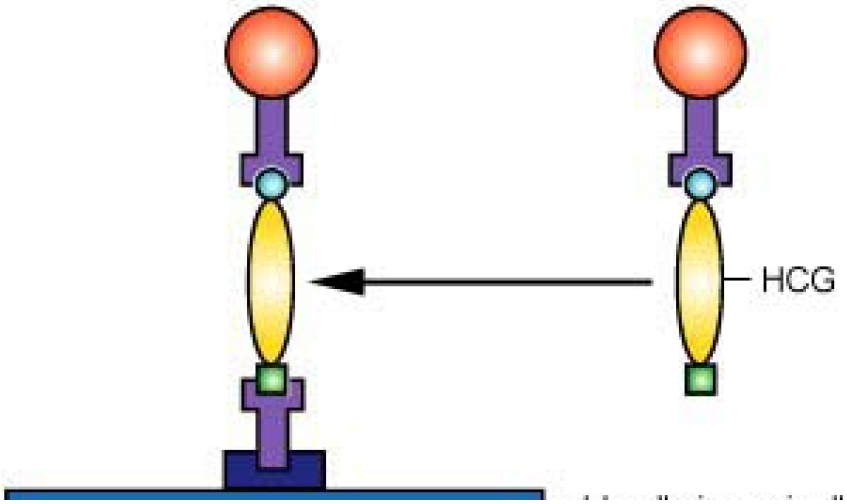


Monoclonal Antibodies

Pregancy Testing



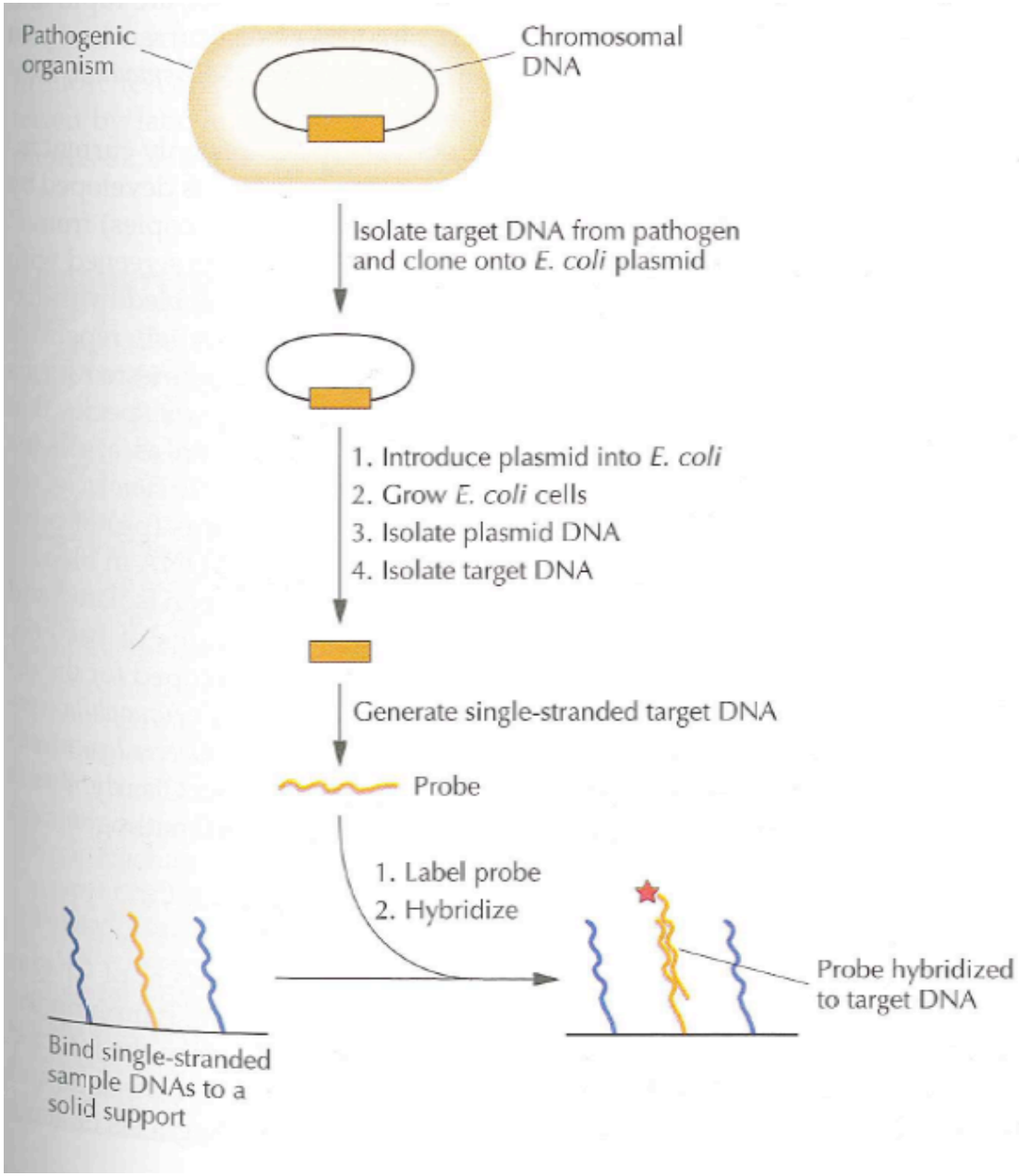
mAb against α-chain HCG



mAb against β-chain HCG

DNA Diagnostic Systems

Development of DNA Hybridization Probes



General scheme:

1. Bind single-stranded DNA (the target) to a membrane support

2. Add single-stranded DNA (the probe) at a temperature and ionic strength allowing hybridisation with target DNA

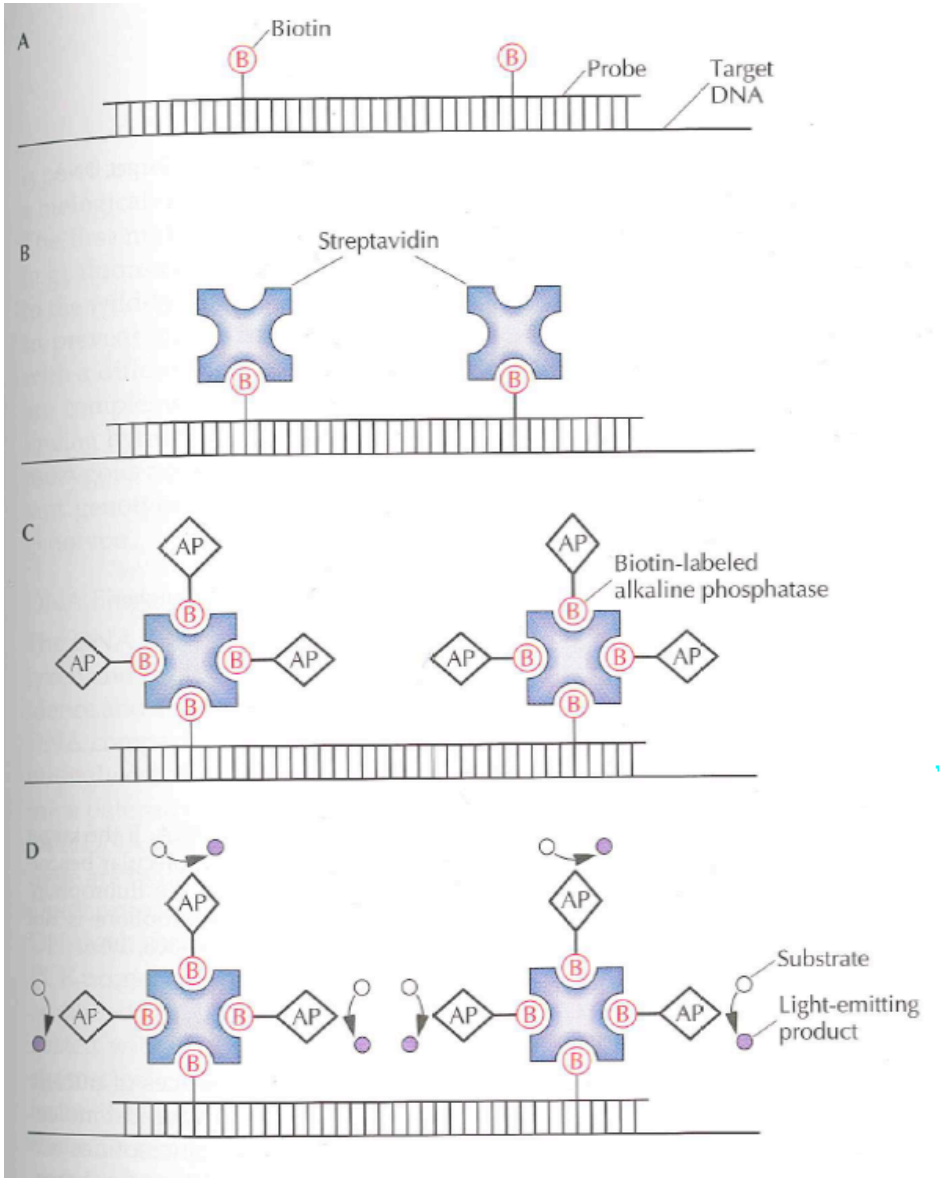
3. Wash to remove unbound probe DNA

4. Detect the hybrid sequences that form between the probe and target DNA (e.g. by using a radioactive probe DNA and autoradiography)

This type of system is **highly specific** (few false positives) and **highly sensitive** (few false negatives)

Non-Radioactive Detection

Chemiluminescent detection of hybridized DNA probes

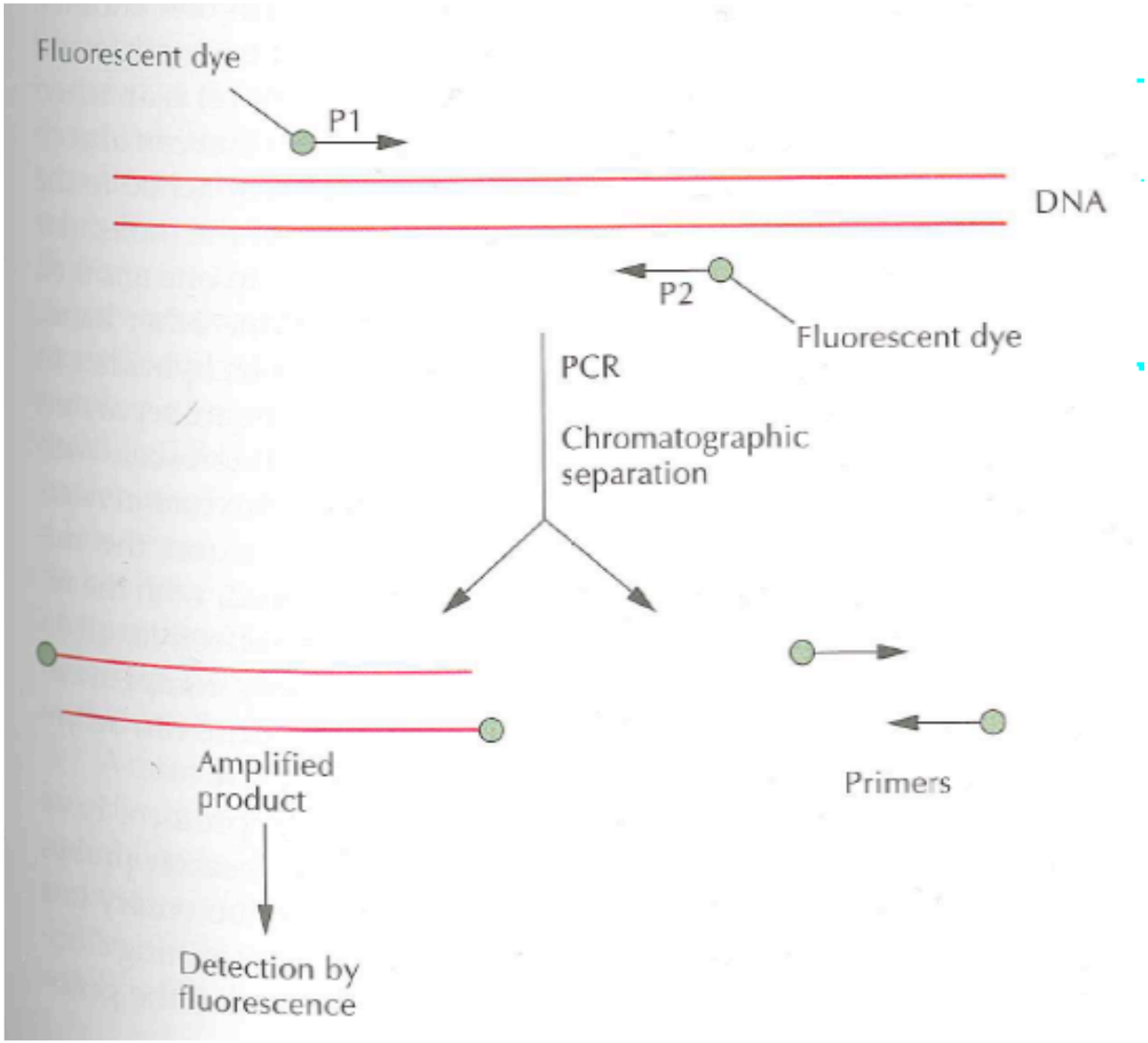


Advantages:

1. Safer than using radioisotopes
2. Cheaper due to absence of costs associated with disposing of radioactive material
3. Long shelf-life

Non-Radioactive Detection of Amplified DNA

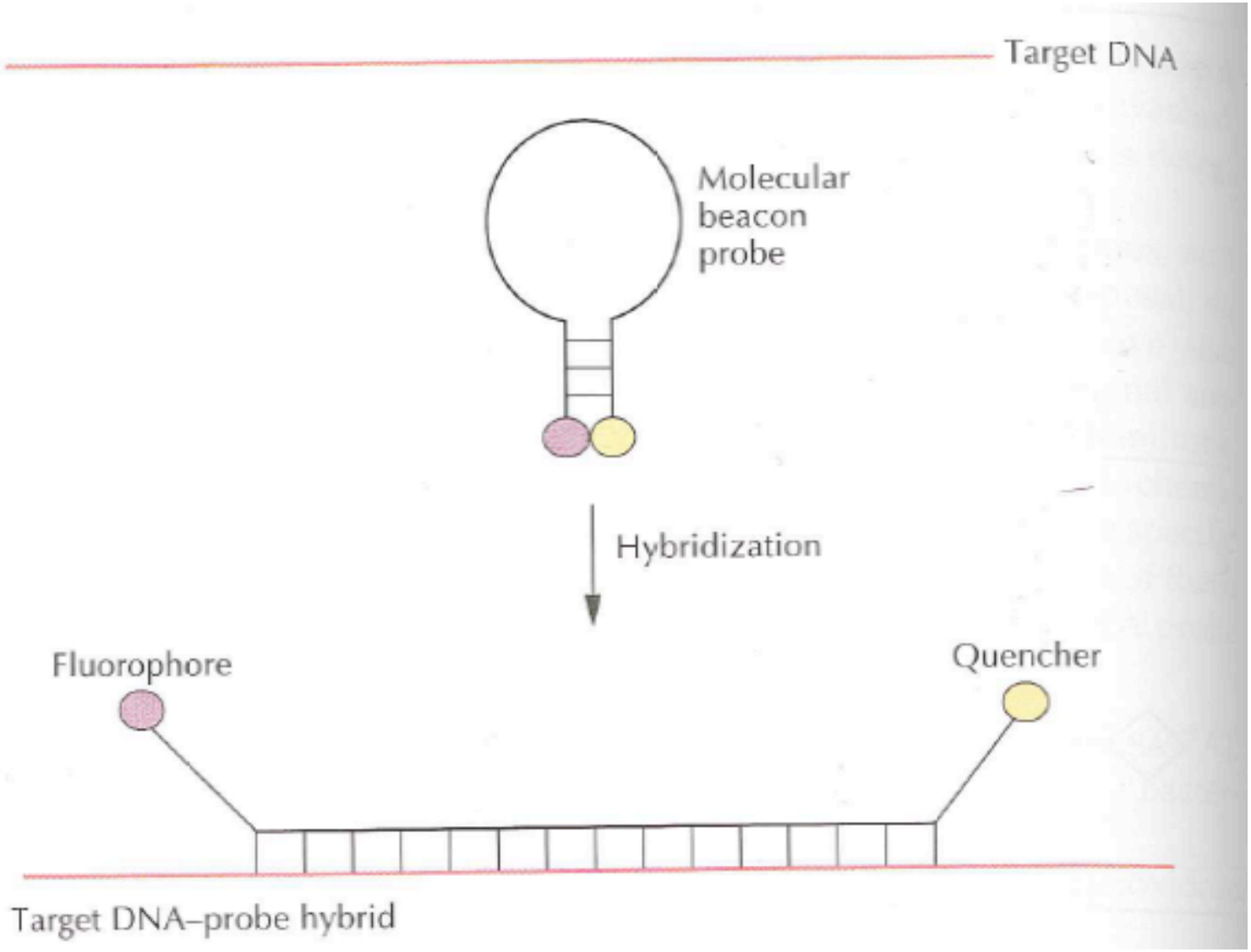
Detecting amplified PCR products using fluorescent primers



Alternatively, amplified DNA can be quantified directly using fluorogenic dyes which become brightly fluorescent when intercalated into double-stranded DNA (e.g. Cyber Green and Pico Green)

Molecular Beacon Probes

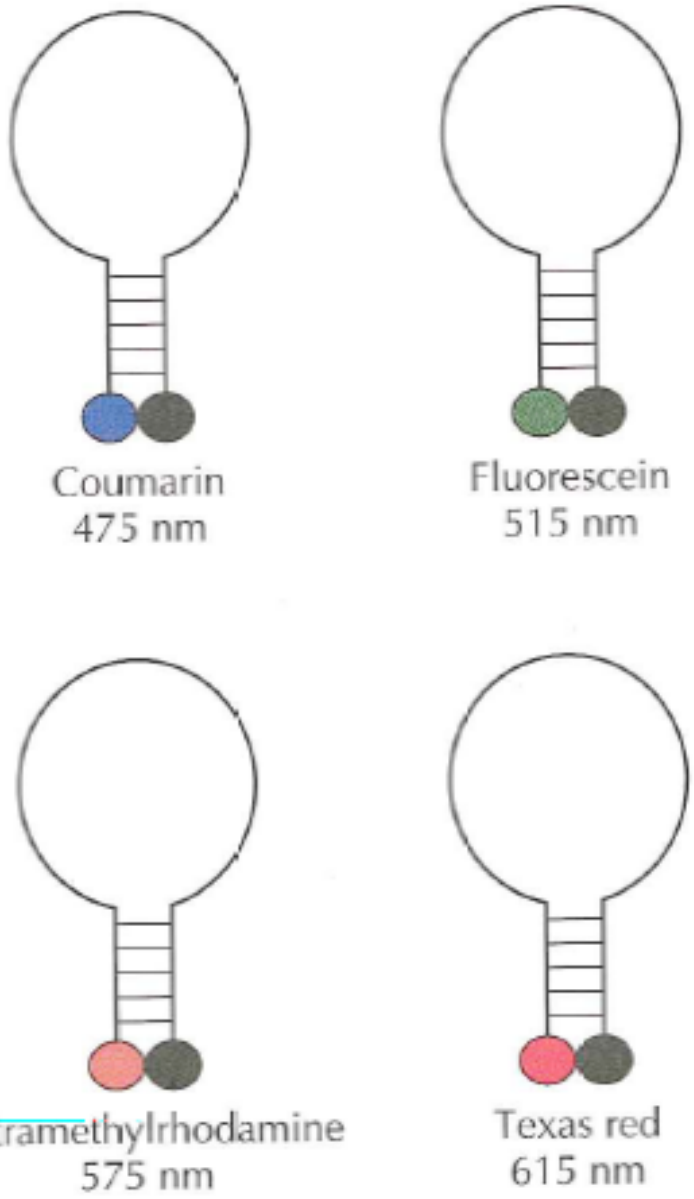
Detecting hybridization using fluorescence



Typically, the probe contains 15 nucleotides in the middle which are complementary to the target DNA, and 5 nucleotides at each end which are complementary to each other and not to the target DNA

Molecular Beacon Probes

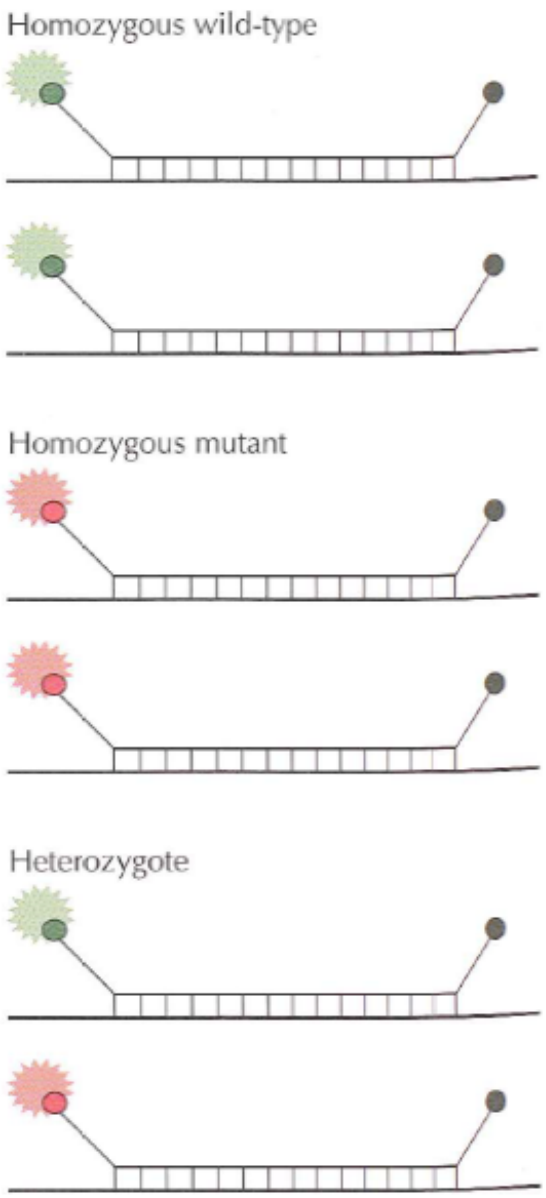
Detecting hybridization using fluorescence



Molecular beacons can be created with different coloured fluorophors

Molecular Beacon Probes

Detecting hybridization using fluorescence

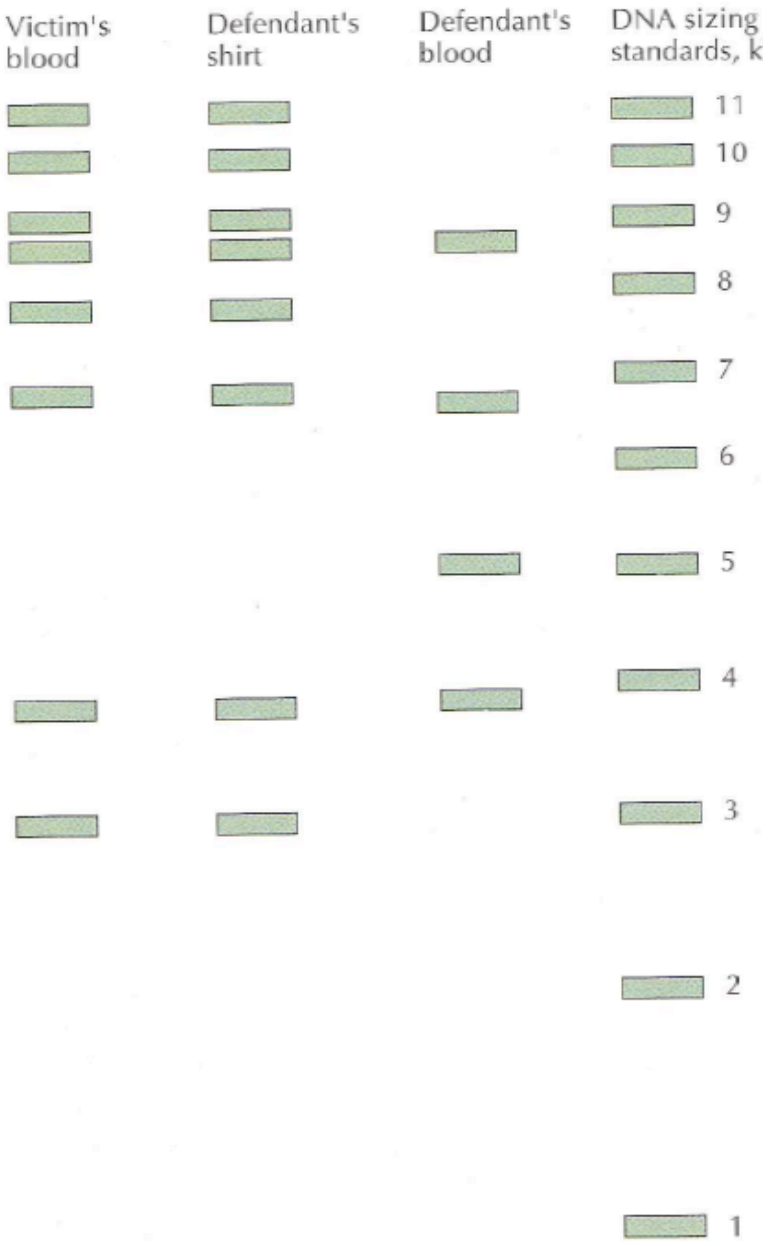


Molecular beacons with with different coloured fluorophors can be used to distinguish between different genotypes

Once the probe is bound to the target DNA, the fluorophor is no longer quenched

DNA Fingerprinting

Forensic analysis



Southern blot of a forensic DNA sample.

1. DNA samples from the victim' blood, defendant's blood and the defendant's shirt are cut with the same restriction enzymes

2. The fragments are separated on an agarose gel

3. The DNA is transferred to a nylon membrane

4. The membrane is hybridized with 4 or 5 separate DNA probes (one after the other) that each recognize a distinct DNA sequence

The probes are typically for **minisatellite DNA**

DNA Fingerprinting

Human minisatellite DNA

Minisatellite DNA regions occur throughout the human genome

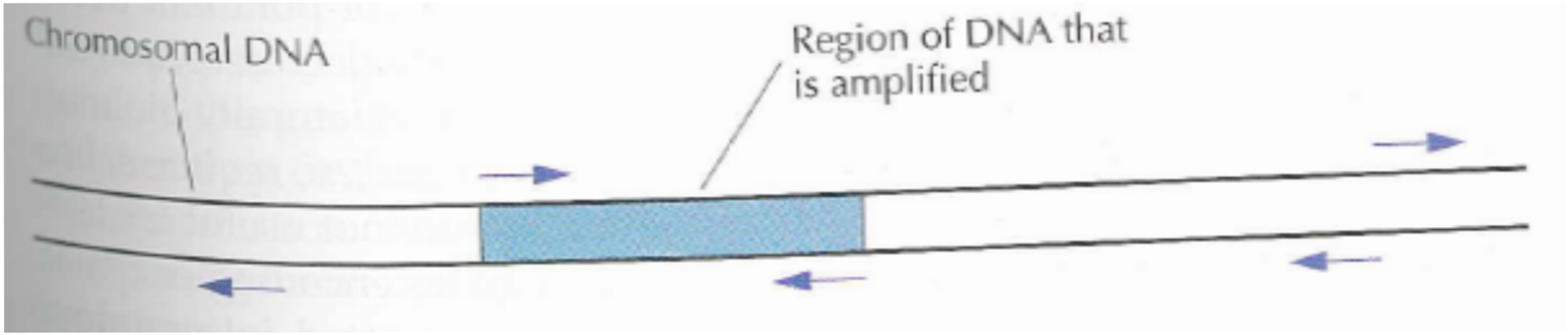
They are non-coding regions consisting of 10 to 30 tandemly repeated sequences each of 9 to 40 base Pairs

The lengths of the minisatellites vary from individual to individual and they are inherited from the parents. The chance of finding two unrelated individuals with the same DNA fingerprint is one in 10^5 to 10^8

In this exmple, the repeating unit is 9 bp and there are 5, 6 and 7 repeating units per cluster (only one DNA strand is shown).

Random Amplified Polymorphic DNA (RAPD)

Using random oligonucleotide primers

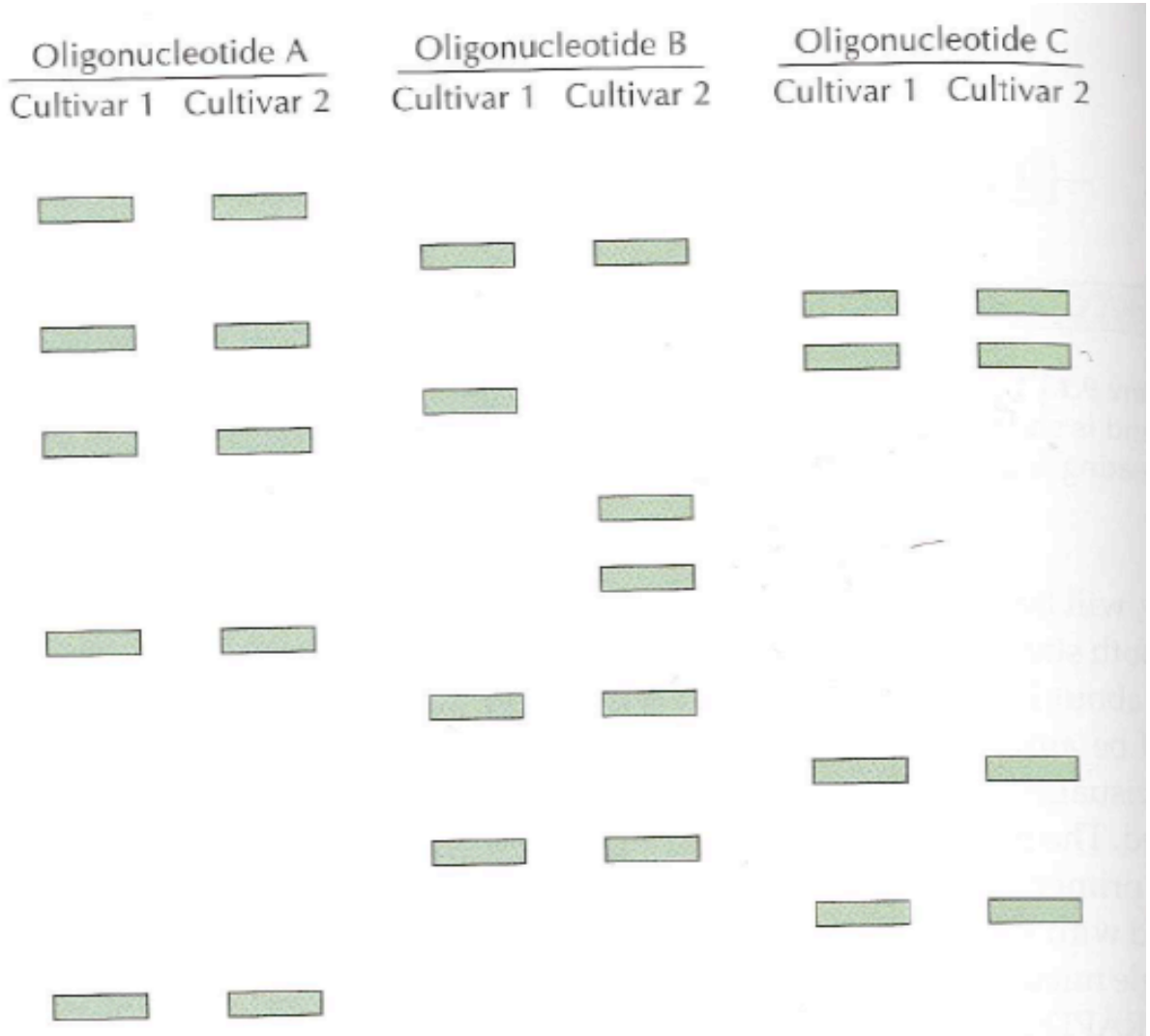


A single random oligonucleotide (usually 9-10 base pairs long and G+C content of 50 to 80%) is bound to the chromomal DNA of an animal, plant or microbe

When two of the oligonucleotides on oposite strands are facing each other and are 100 to 3000 base pairs apart, the intervening DNA is amplified by PCR

Random Amplified Polymorphic DNA (RAPD)

Using random oligonucleotide primers to identify different plant cultivars

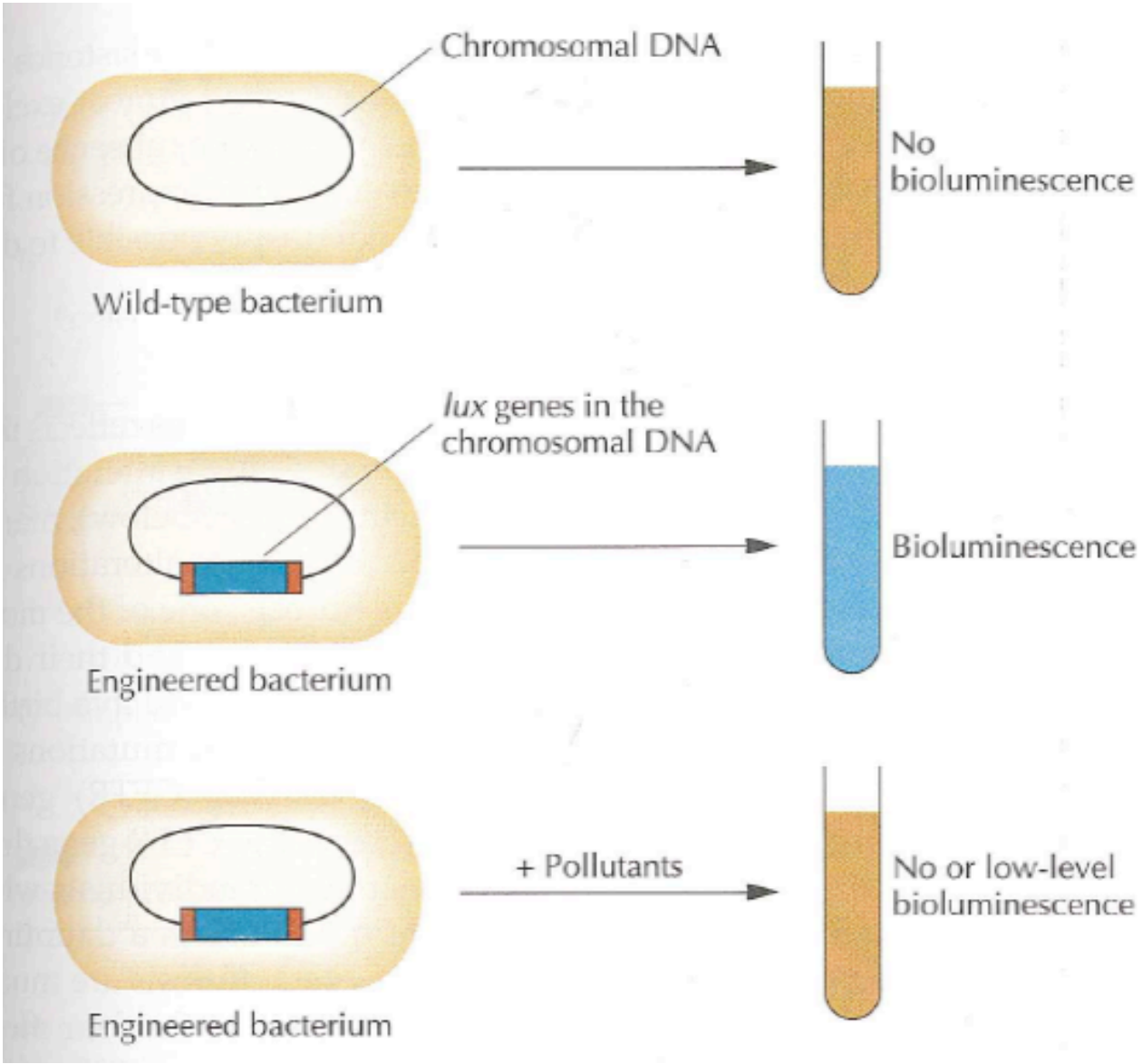


Ethidium-bromide stained bands following polyacrylamide gel electrophoresis

Oligonucleotide B can be used to distinguish cultivars 1 and 2

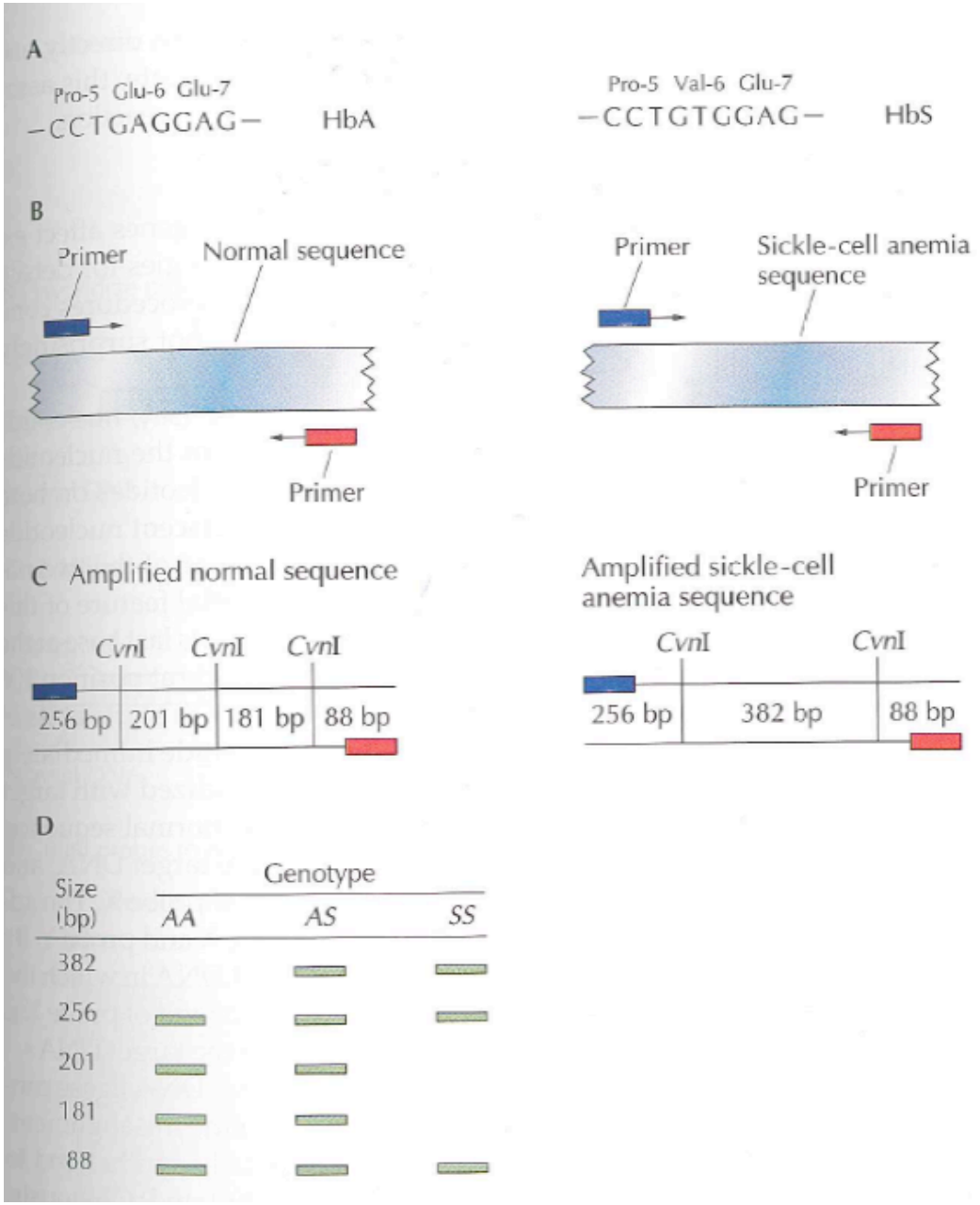
Bacterial Biosensors

Assaying for the presence of pollutants with genetically engineered bioluminescent *Pseudomonas fluorescens*



Molecular Diagnosis of Genetic Disease

Detection of the sickle-cell anemia gene at the DNA level



Sickle-cell anemia is an extremely serios disease in individuals homozygous (*SS*) for a single point mutation in the β-chain of hemoglobin

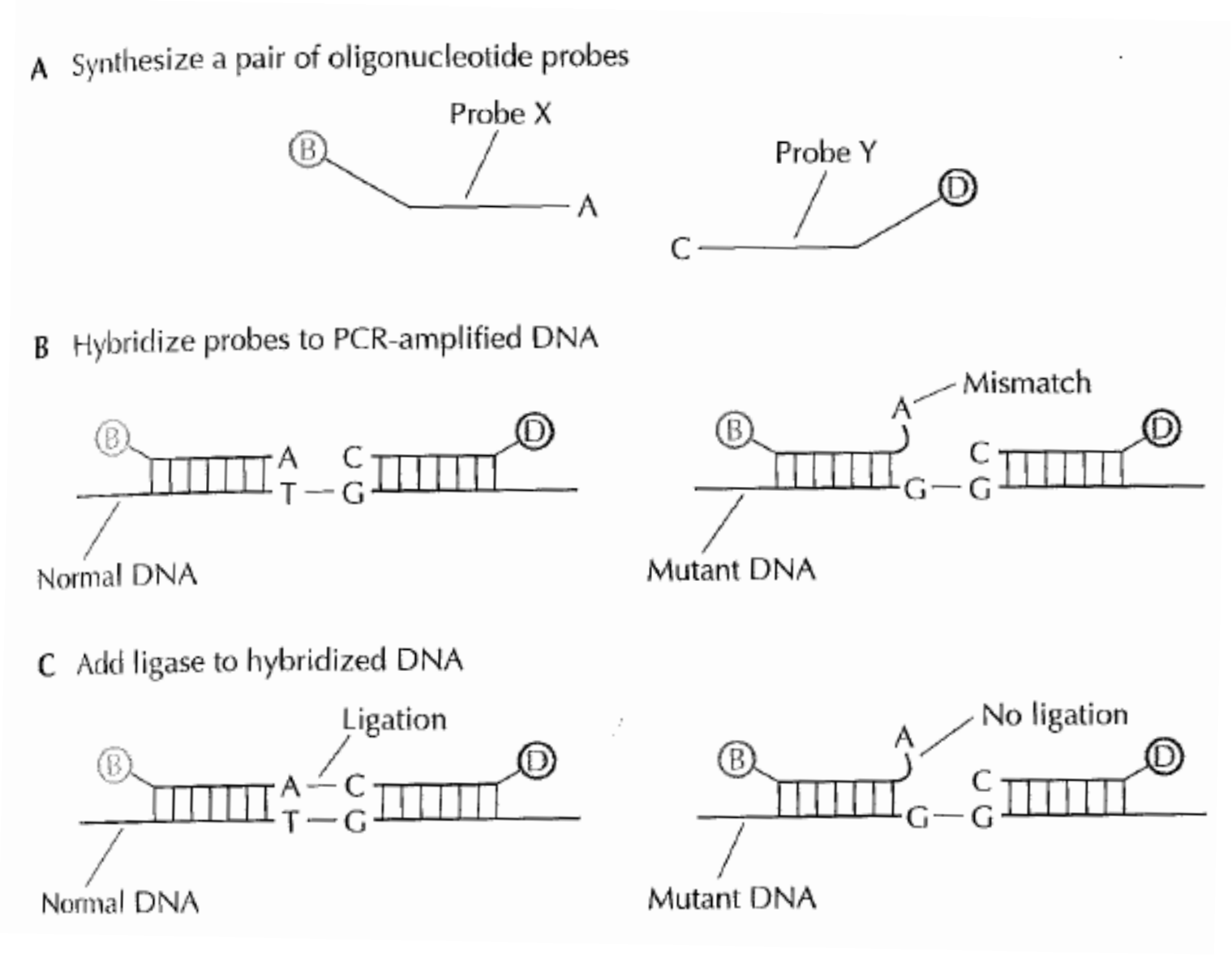
Genetic carriers who are heterozygous, (*AS*) are routinely screened in the USA

The sickle-cell point mutation changes a Glu to a Valine and (by chance) removes a *Cvn*I restriction site

After PCR of the gene for the β-chain of hemoglobin, and digestion with *Cvn*I, individuals homozygous from the wild type gene (*AA*) can be distinguished from heterozygous carriers (*AS*) and homozygous sickle-cell anemics (*SS*)

Molecular Diagnosis of Genetic Disease

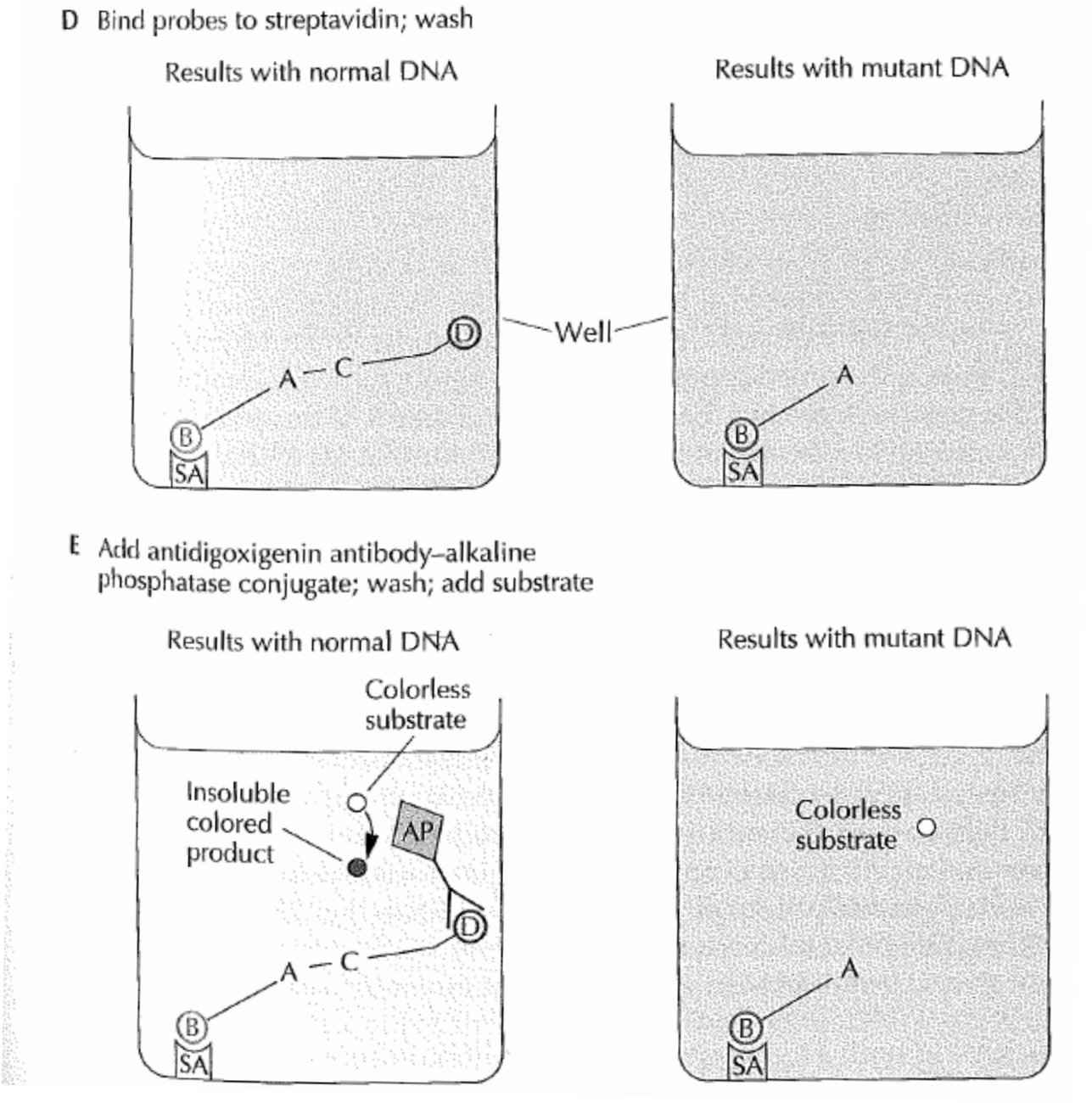
A combined PCR oligonucleotide ligation assay (PCR/OLA)



B = biotin; D = digoxigenin

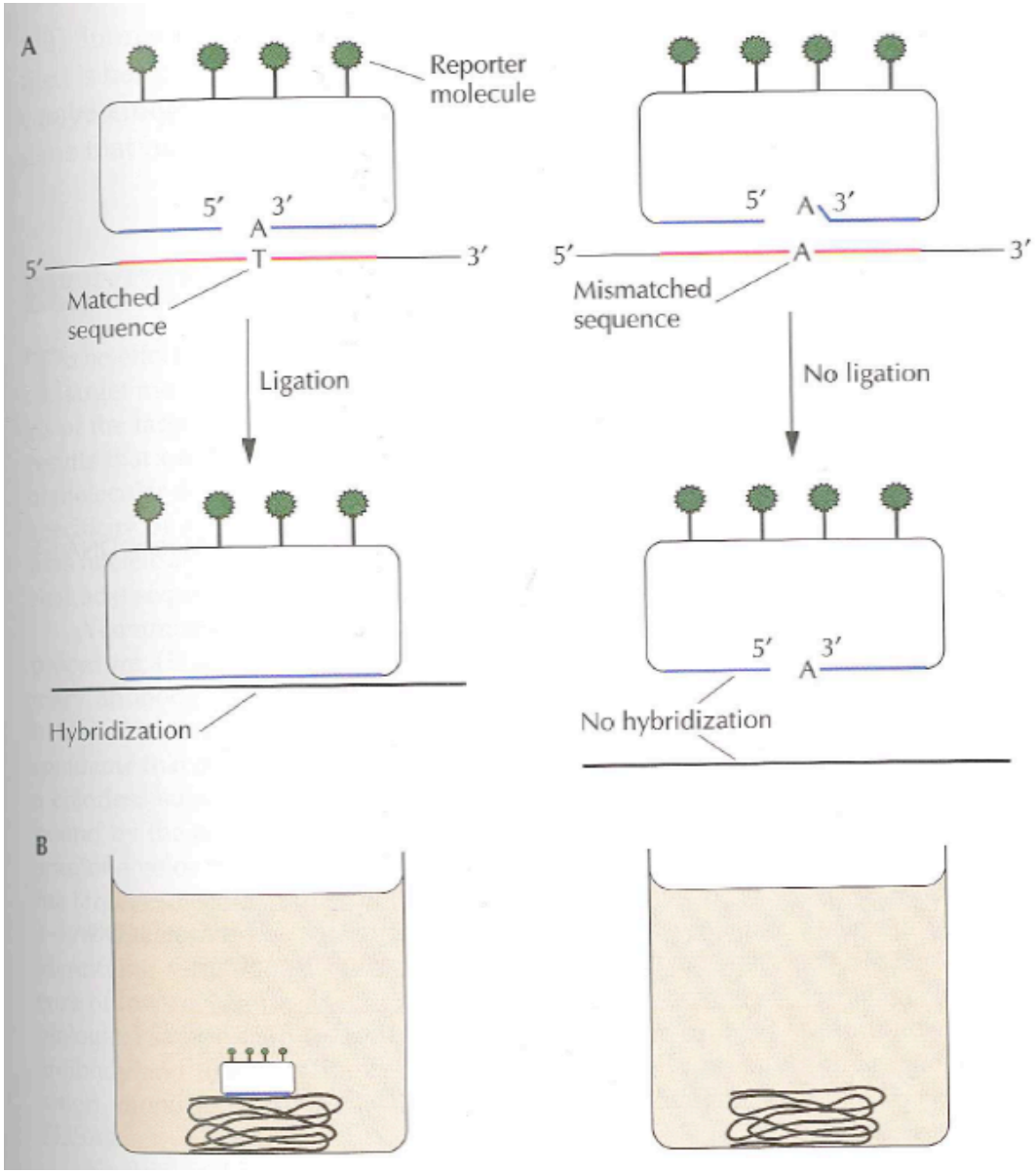
Molecular Diagnosis of Genetic Disease

A combined PCR oligonucleotide ligation assay (PCR/OLA)



B = biotin
D = digoxigenin

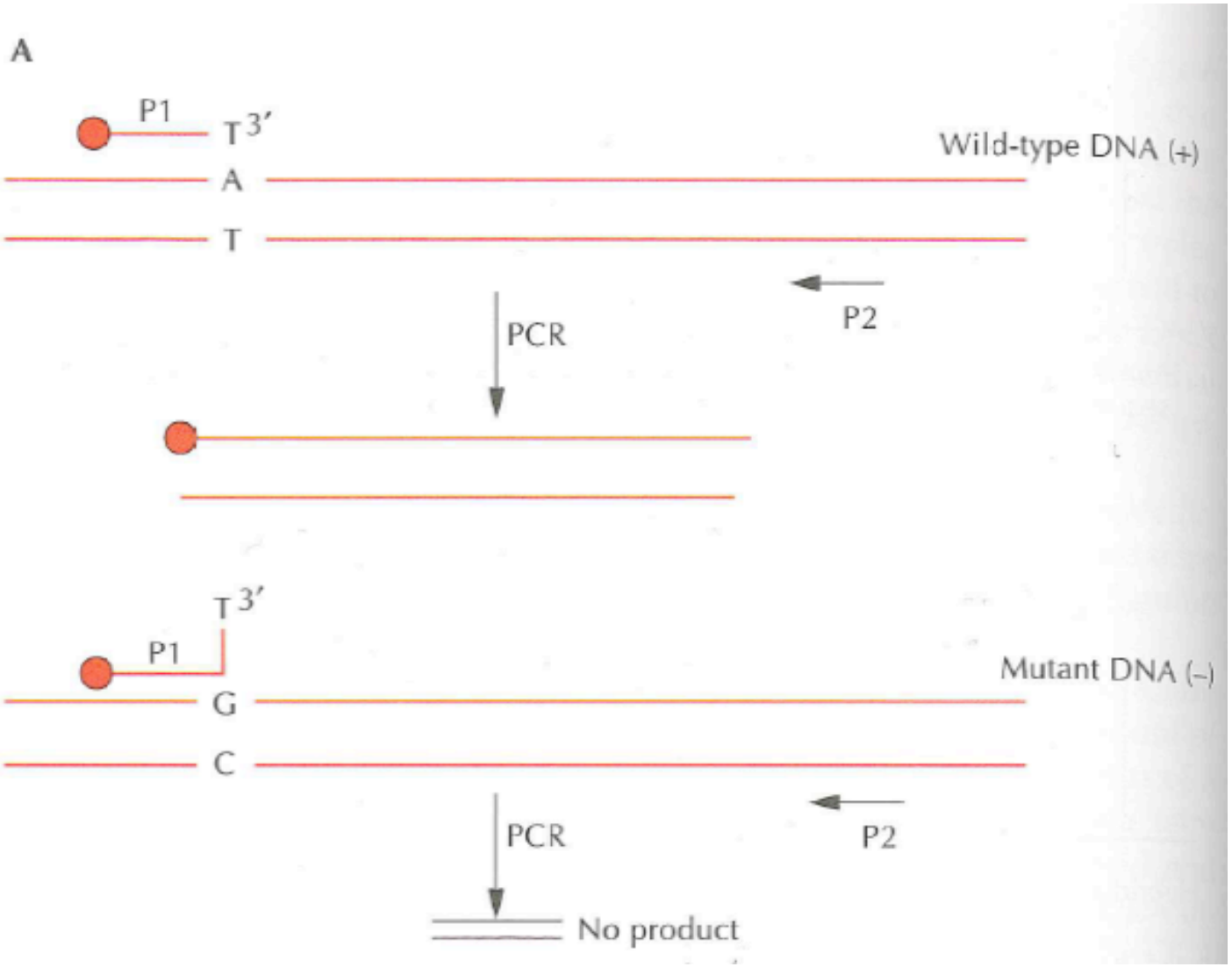
Padlock Probes



The probes typically have 15 to 20 nucleotides at the 5’ and 3’ ends complementary to the target DNA, and a central region of ~50 nucleotides

Detection of Point Mutations by PCR

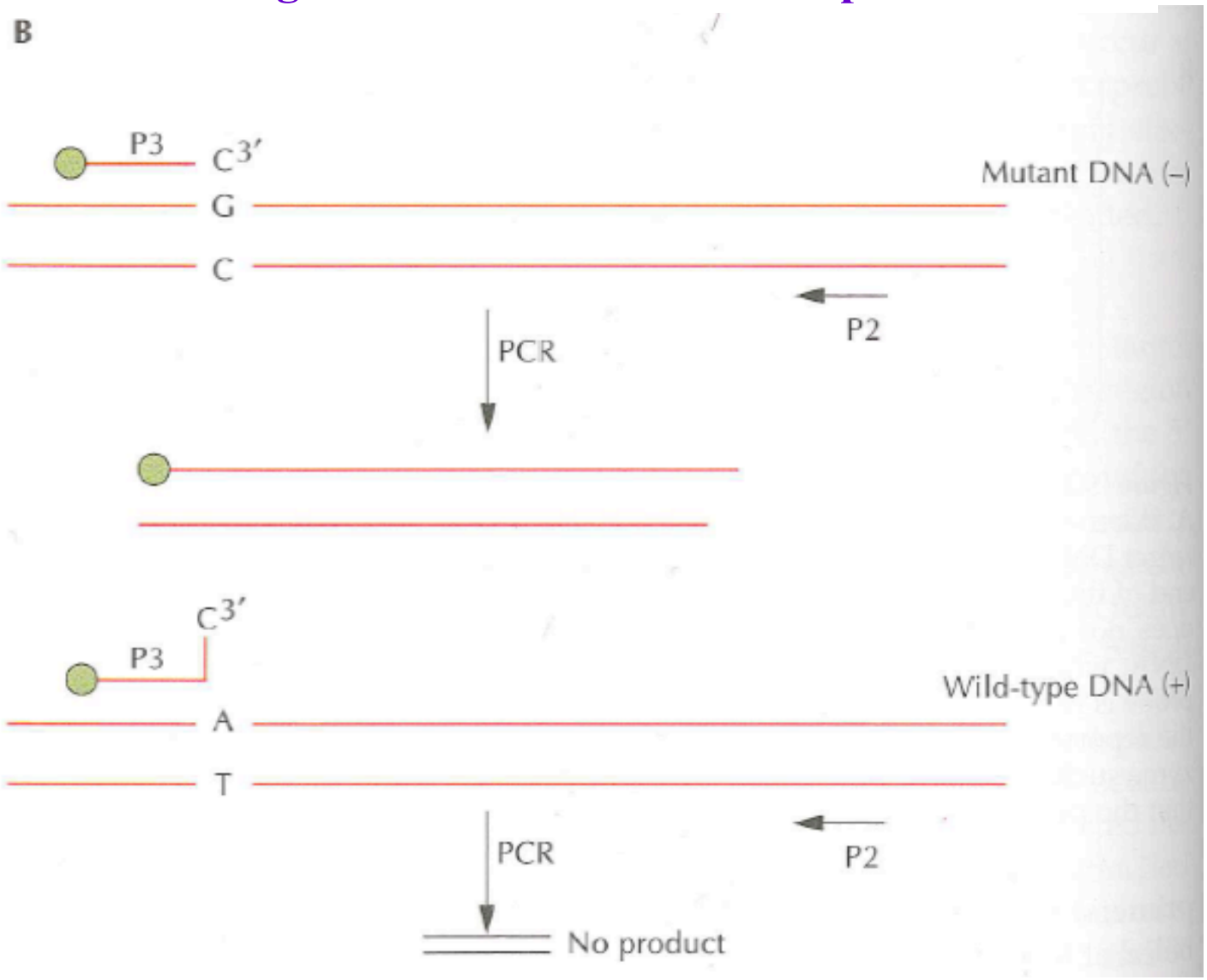
Using fluorescent-labelled PCR primers



Primer 1 is labeled with rhodamine (red) and only amplifies the wild-type gene

Detection of Point Mutations by PCR

Using fluorescent-labelled PCR primers

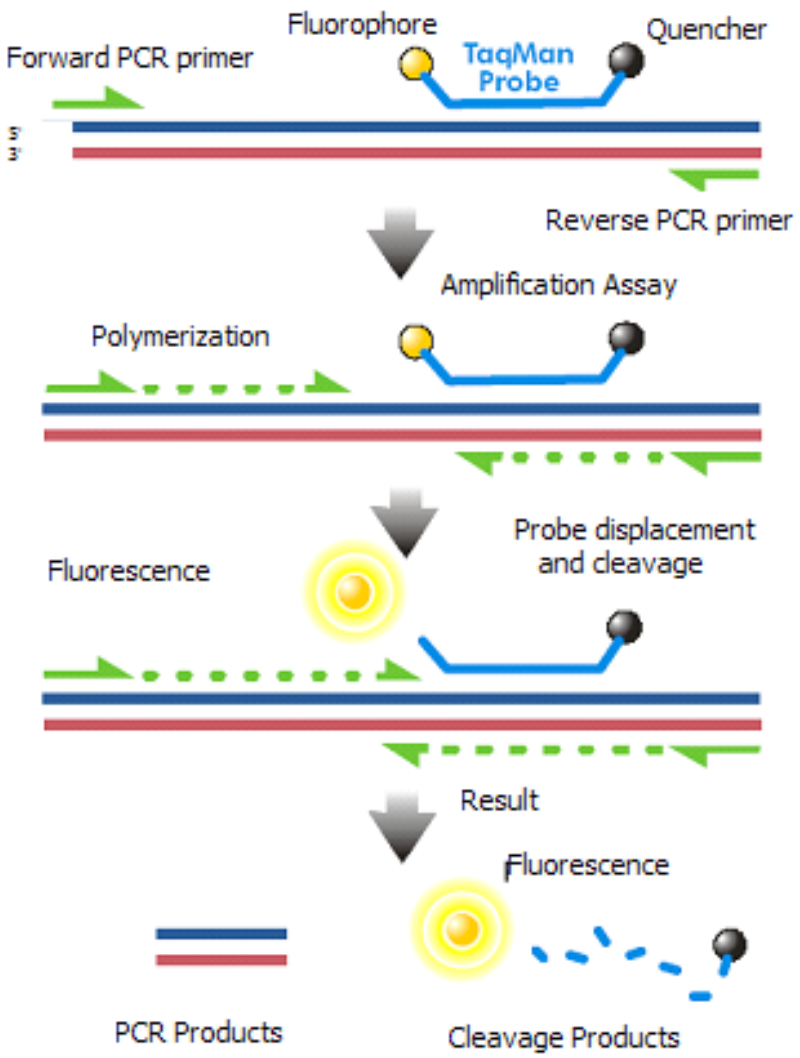


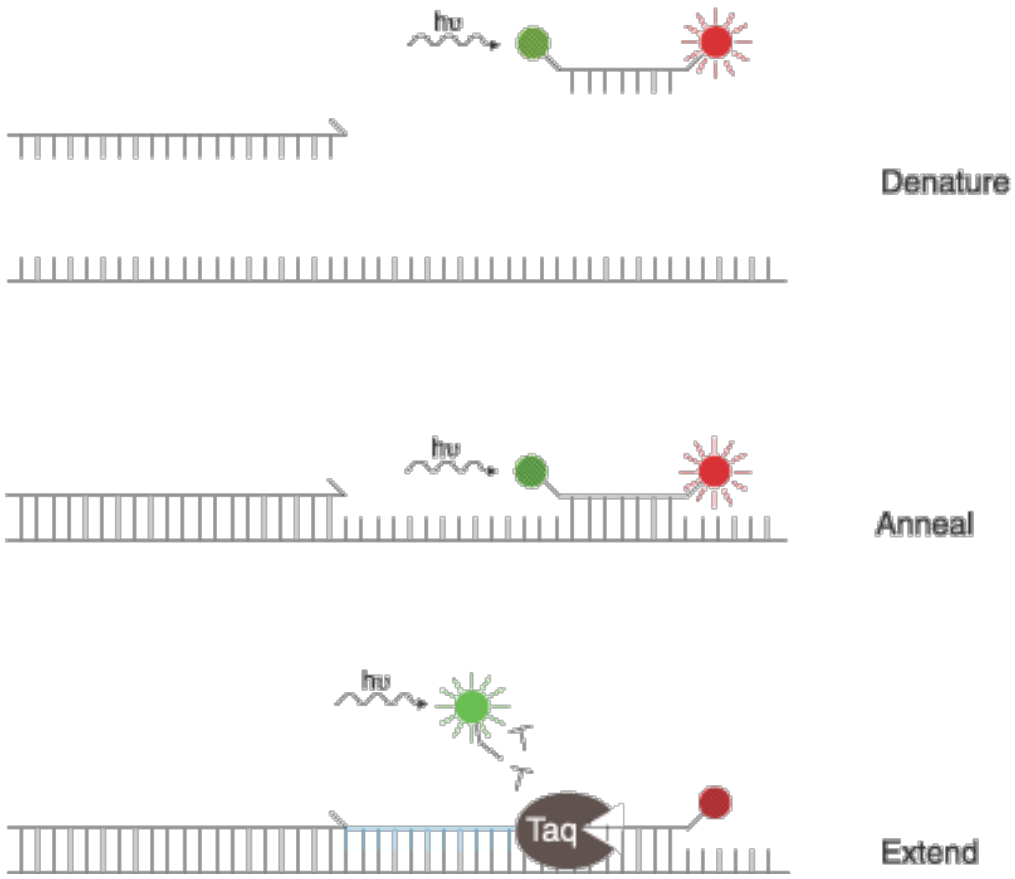
Primer 3 is labeled with fluorescein (green) and only amplifies the mutant gene

Hence genotype 1/1 = red; 1/2 = yellow; 2/2 = green

TaqMan

Quantitative fluorogenic PCR reactions

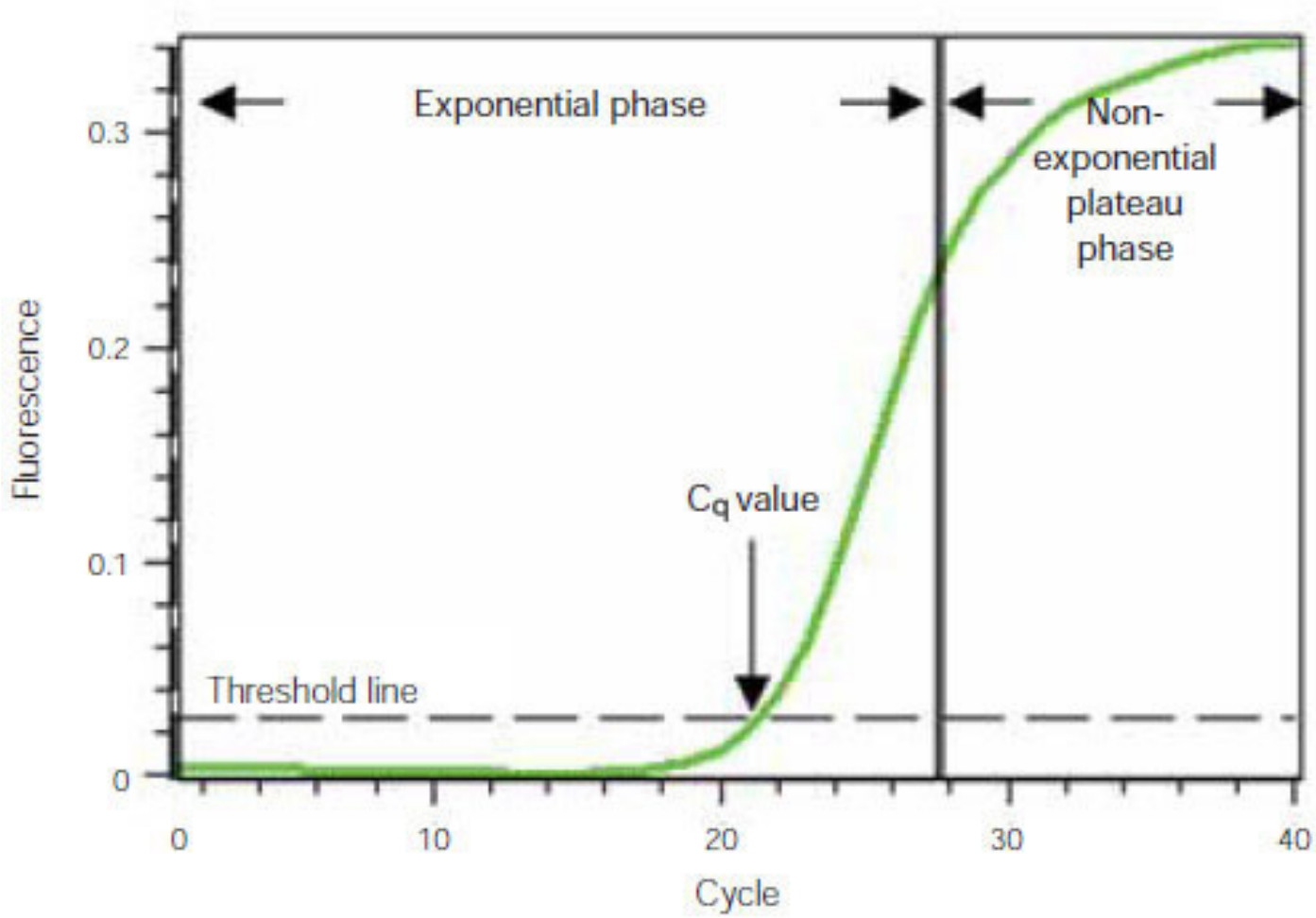




This is the most widely used system to detect specific genes and gene mutations today

TaqMan

Quantitative fluorogenic PCR reactions



The concentration of template DNA in a sample can be determined by measuring the fluorescence in real time as the PCR reaction proceeds.

The cycle at which the fluorescence exceeds a threshold value can be used to calculate the concentration of the template DNA