Introduction to Diagnostics and Biosensors

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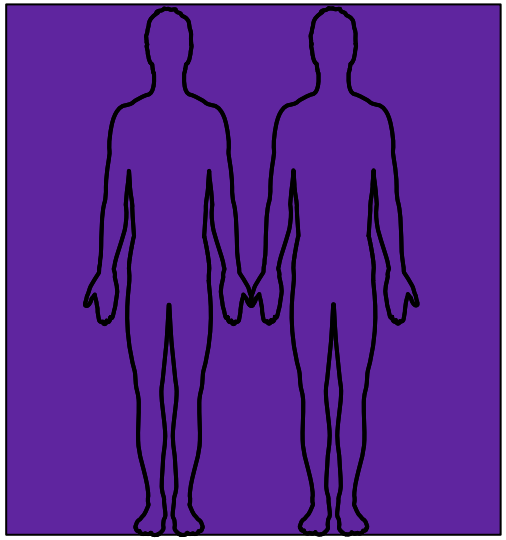
INSPIRING INNOVATION INNOVANTE PAR TRADITION

Diagnostics

- **Part I: Why do we need to detect disease**
  - **What are the major causes of death worldwide & current diagnostics**
    - **Infectious disease**
    - **Heart disease**
    - **Cancer**
- **Part II: Newer technologies for disease detection**
  - **Biosensors for diagnostics**
  - **Biofunctionalisation**
  - **Label free technologies**

Reasons for interfacing with biology



Health

?

The Environment

Fundamentals

Treatment

Why Detect/Study disease?

Disease detection is important for :

- Health of individual and population
- Food safety
- Water safety
- Environmental Pollution (algae, fungi etc.)
- Drug development
- Basic Research

toxicology

Leading Causes of Mortality and Burden of Disease (2004)

Mortality

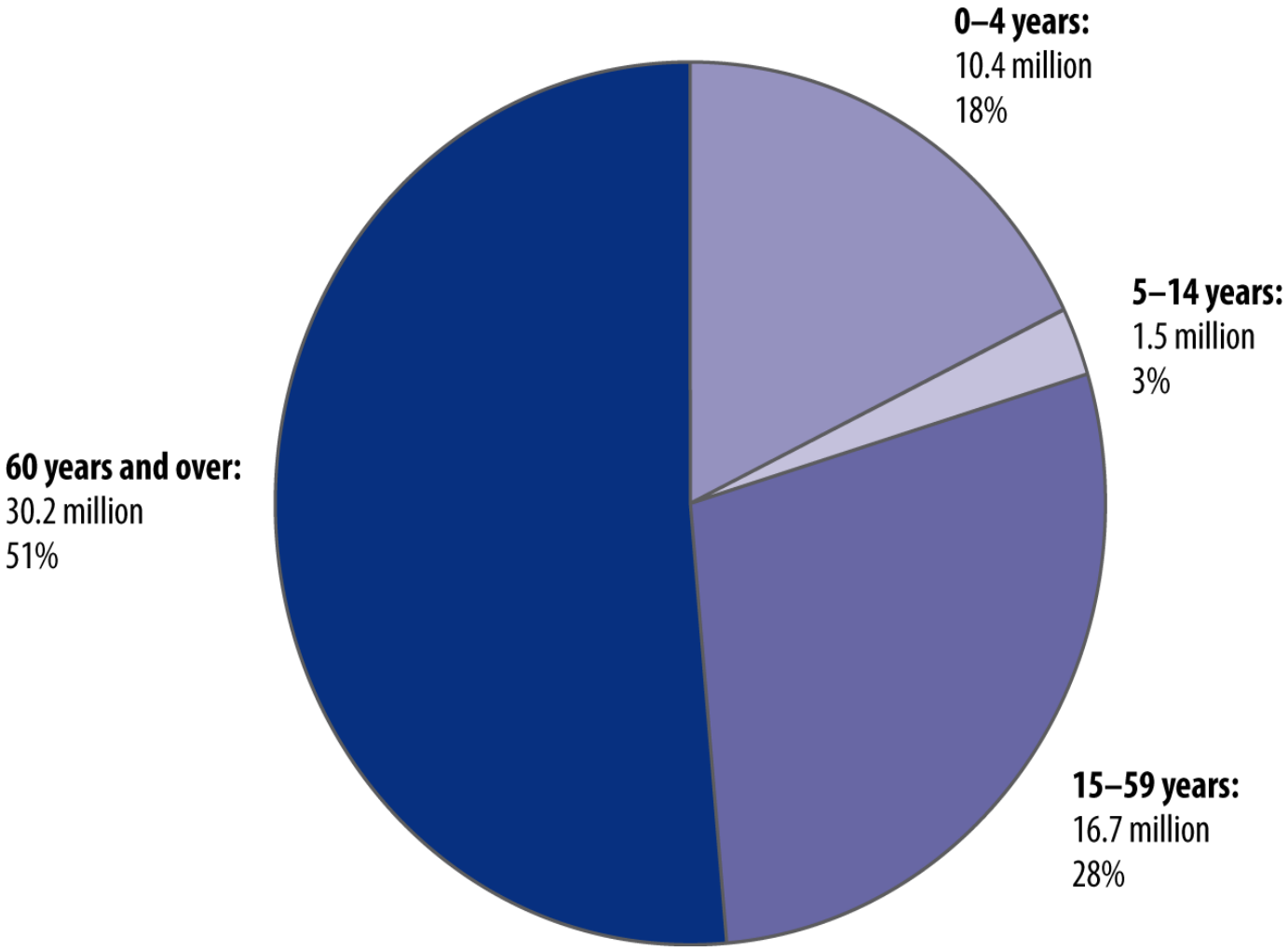
		%
1.	Ischaemic heart disease	12.2
2.	Cerebrovascular disease	9.7
3.	Lower respiratory infections	7.1
4.	COPD	5.1
5.	Diarrhoeal diseases	3.7
6.	HIV/AIDS	3.5
7.	Tuberculosis	2.5
8.	Trachea, bronchus, lung cancers	2.3
9.	Road traffic accidents	2.2
10.	Prematurity, low birth weight	2.0

DALYs

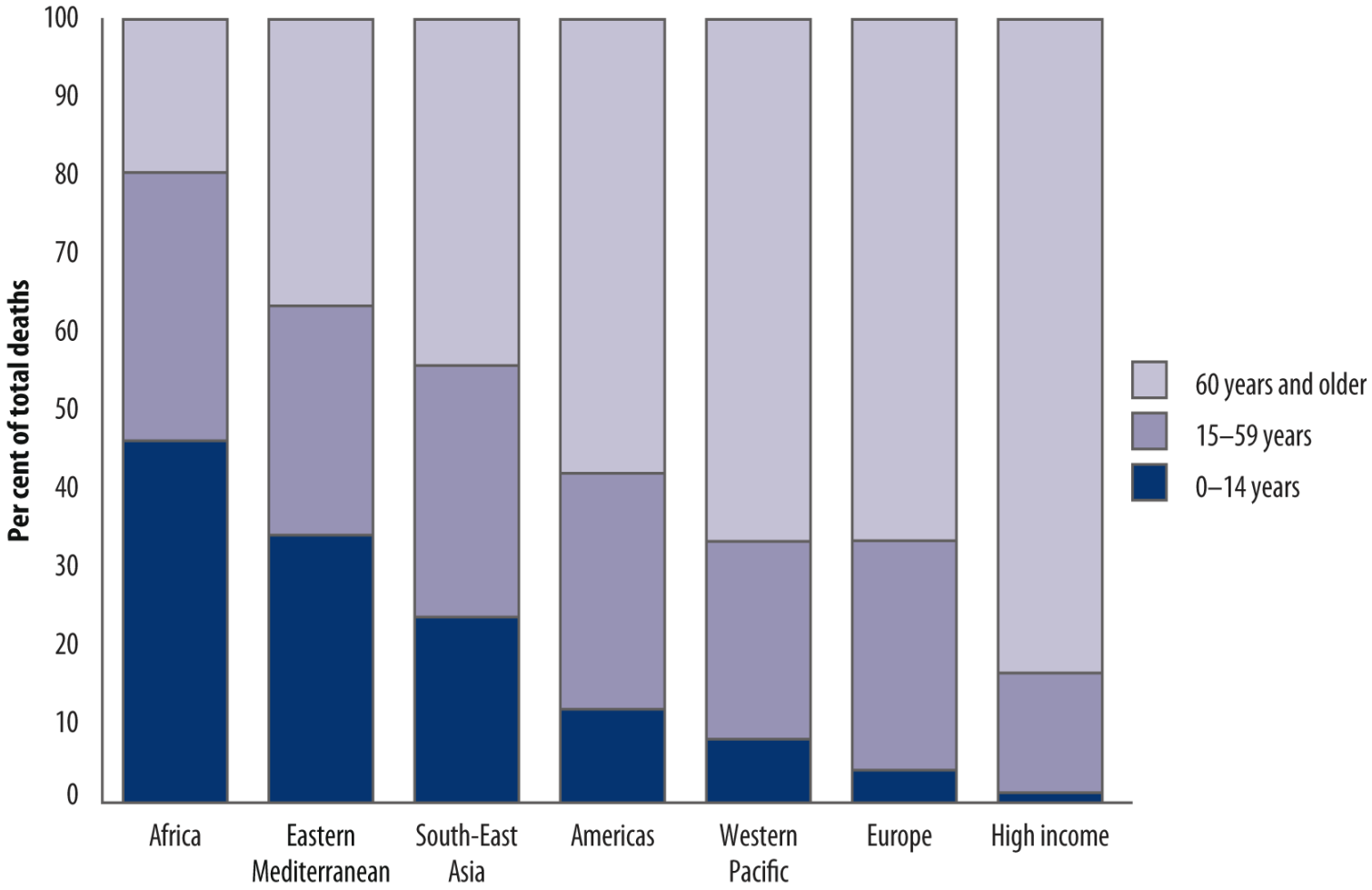
		%
1.	Lower respiratory infections	6.2
2.	Diarrhoeal diseases	4.8
3.	Depression	4.3
4.	Ischaemic heart disease	4.1
5.	HIV/AIDS	3.8
6.	Cerebrovascular disease	3.1
7.	Prematurity, low birth weight	2.9
8.	Birth asphyxia, birth trauma	2.7
9.	Road traffic accidents	2.7
10.	Neonatal infections and other	2.7

One DALY can be thought of as one lost year of "healthy" life. The sum of DALYs across the population, or the burden of disease, can be thought of as a measurement of the gap between current health status and an ideal health situation where the entire population lives to an advanced age, free of disease and disability.

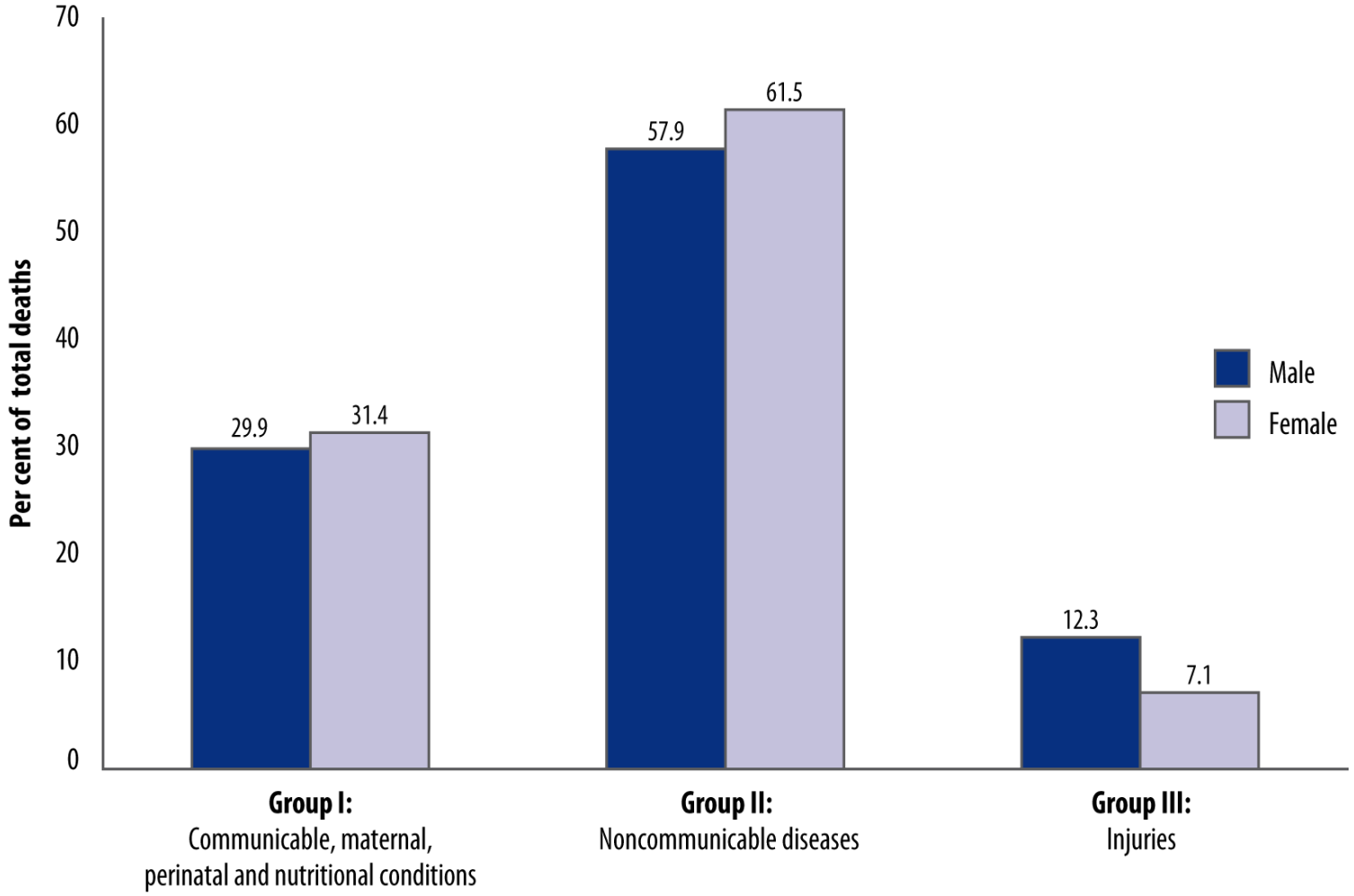
Distribution of age at death and numbers of deaths, world, 2004



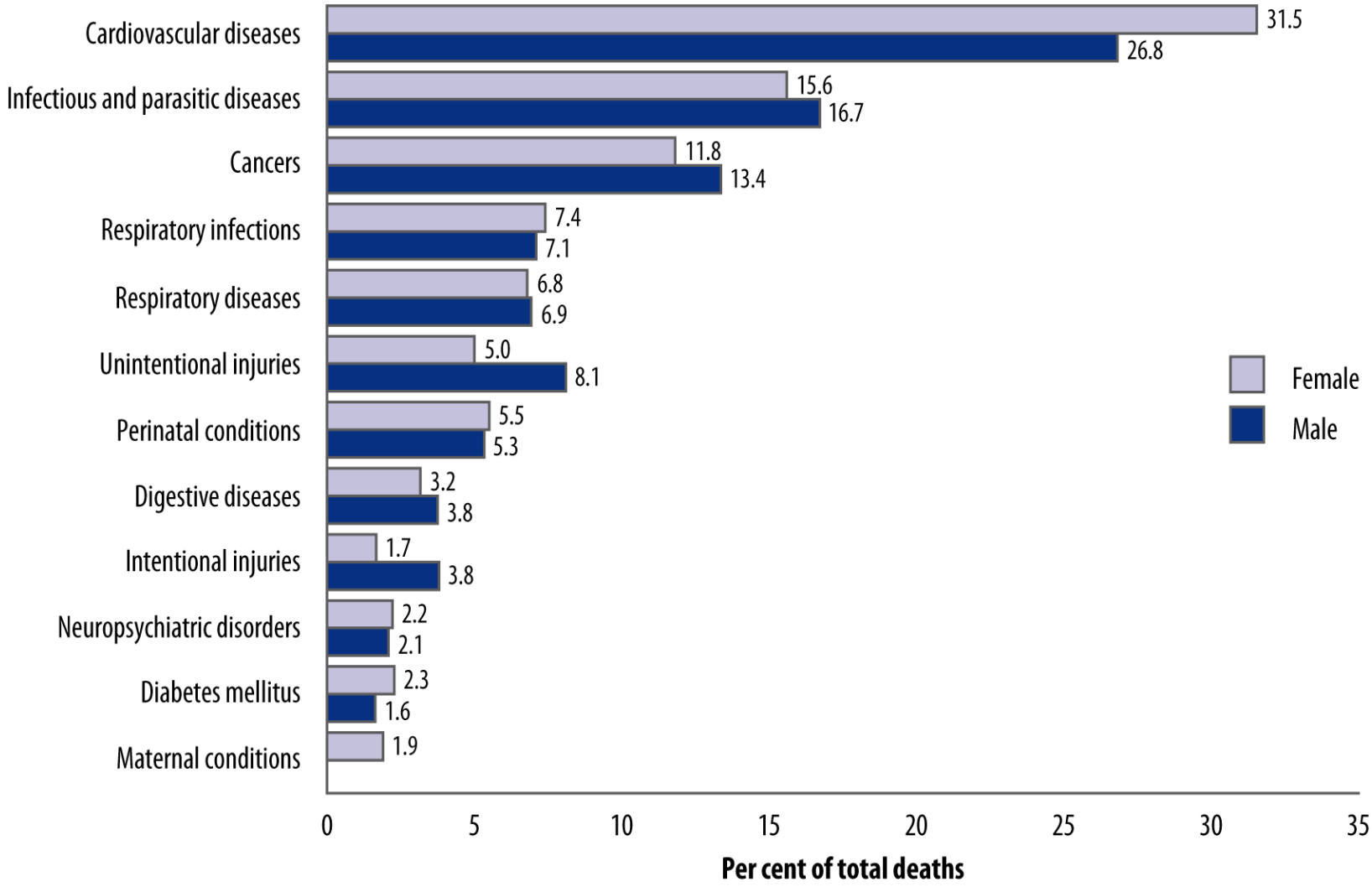
Per cent distribution of age at death by region, 2004



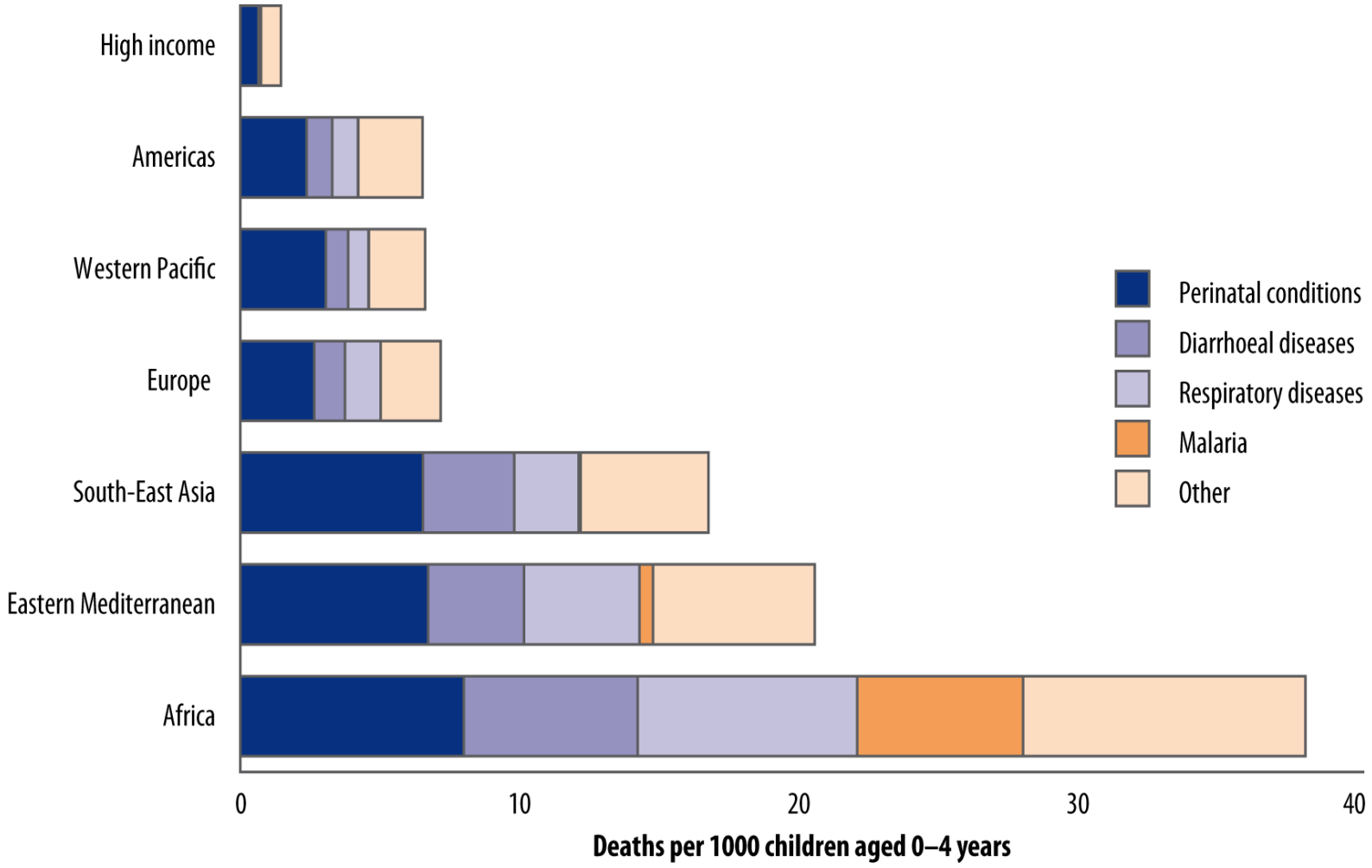
Distribution of deaths in the world by sex, 2004



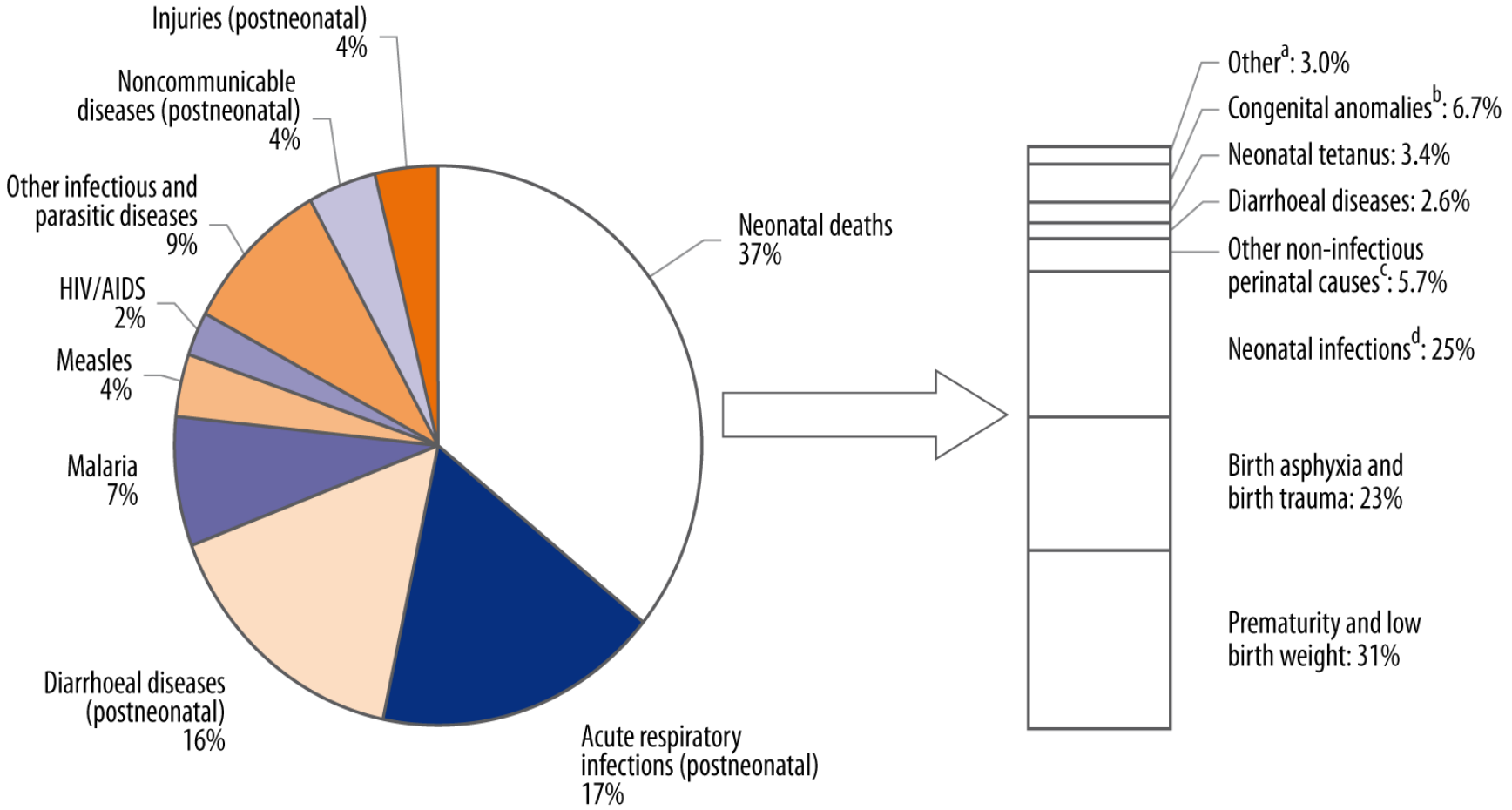
Distribution of deaths by leading cause groups (2004)



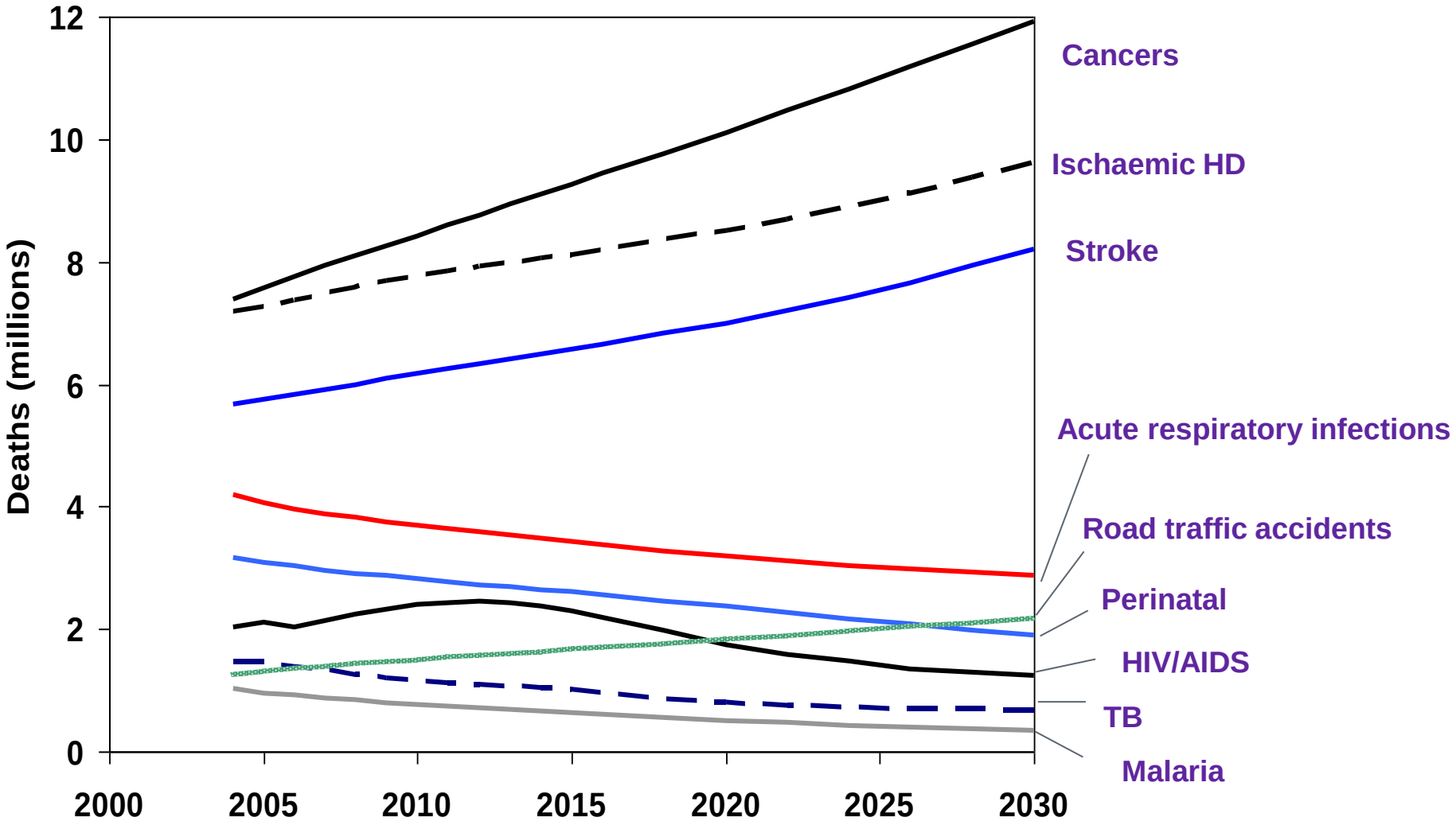
Child mortality rates by cause and region, 2004



Distribution of causes of death among children under 4, 2004



Global projections for selected causes 2004 to 2030



Updated from Mathers and Loncar, PLoS Medicine, 2006

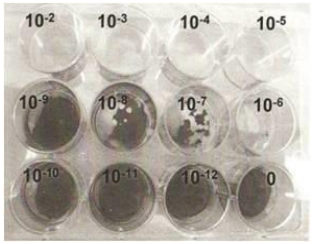
Ten leading causes of burden of disease: 2004 and 2030

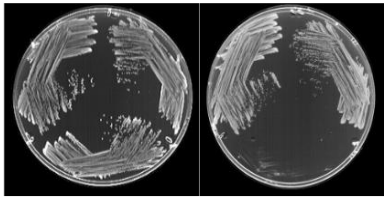
2004 Disease or injury	As % of total DALYs	Rank	Rank	As % of total DALYs	2030 Disease or injury
Lower respiratory infections	6.2	1	1	6.2	Unipolar depressive disorders
Diarrhoeal diseases	4.8	2	2	5.5	Ischaemic heart disease
Unipolar depressive disorders	4.3	3	3	4.9	Road traffic accidents
Ischaemic heart disease	4.1	4	4	4.3	Cerebrovascular disease
HIV/AIDS	3.8	5	5	3.8	COPD
Cerebrovascular disease	3.1	6	6	3.2	Lower respiratory infections
Prematurity and low birth weight	2.9	7	7	2.9	Hearing loss, adult onset
Birth asphyxia and birth trauma	2.7	8	8	2.7	Refractive errors
Road traffic accidents	2.7	9	9	2.5	HIV/AIDS
Neonatal infections and other[a]	2.7	10	10	2.3	Diabetes mellitus
COPD	2.0	13	11	1.9	Neonatal infections and other[a]
Refractive errors	1.8	14	12	1.9	Prematurity and low birth weight
Hearing loss, adult onset	1.8	15	15	1.9	Birth asphyxia and birth trauma
Diabetes mellitus	1.3	19	18	1.6	Diarrhoeal diseases

How do we detect disease?

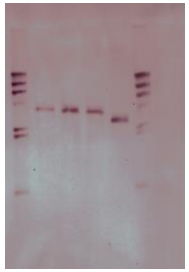
Traditional methods for detecting disease

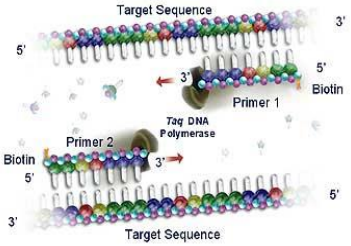
- Culture



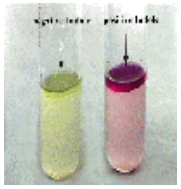


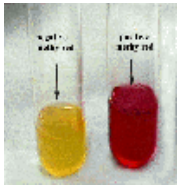
- PCR



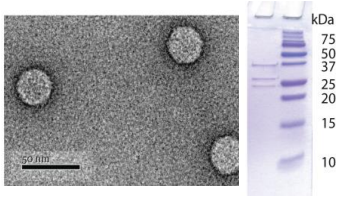


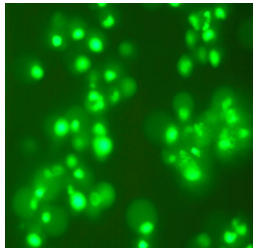
- Biochemical tests



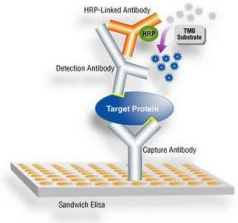


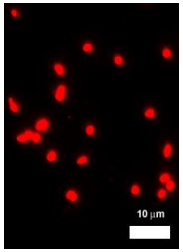
- Microscopy



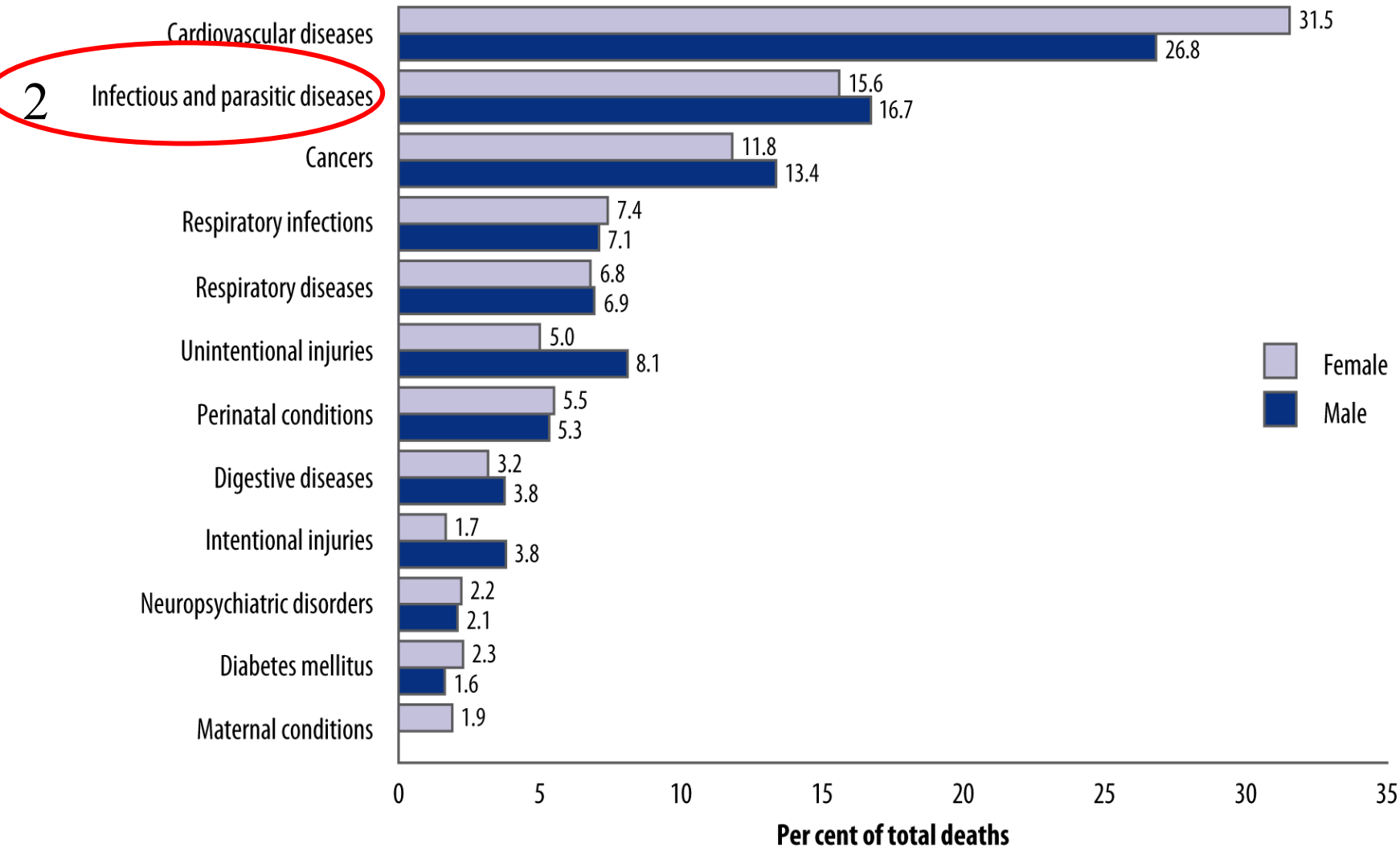


- Immunoassays



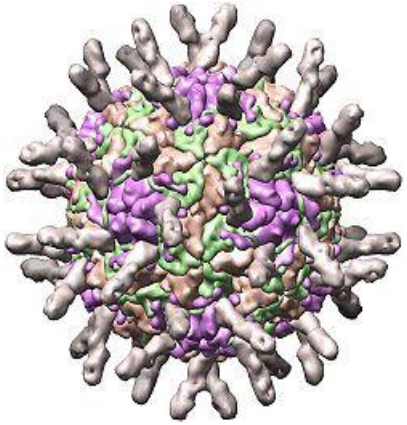


Distribution of deaths by leading cause groups (2004)



Detection of infectious disease

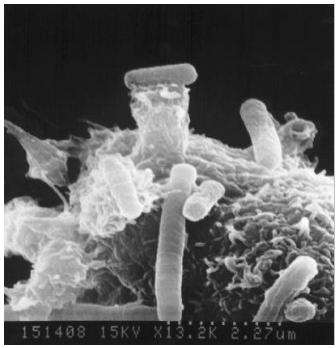
Human Rhinovirus

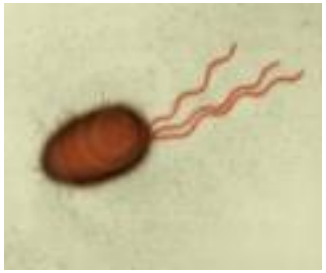


an **infectious** biological agent that causes disease to its host

Viruses (Flu)
Fungi (Candida)
Bacteria (Tuberculosis)
Protozoa (Cryptosporidium)
Parasites (Malaria)
Prions (Mad cow disease)

Enteric bacteria





Severe diarrhea!!!!

Worldwide mortality due to infectious diseases WHO

Rank	Cause of death	Deaths 2002	Percentage of all deaths	Deaths 1993	1993 Rank
N/A	All infectious diseases	14.7 million	25.9%	16.4 million	32.2%
1	Lower respiratory infections	3.9 million	6.9%	4.1 million	1
2	HIV/AIDS	2.8 million	4.9%	0.7 million	7
3	Diarrheal diseases	1.8 million	3.2%	3.0 million	2
4	Tuberculosis (TB)	1.6 million	2.7%	2.7 million	3
5	Malaria	1.3 million	2.2%	2.0 million	4
6	Measles	0.6 million	1.1%	1.1 million	5
7	Pertussis	0.29 million	0.5%	0.36 million	7
8	Tetanus	0.21 million	0.4%	0.15 million	12
9	Meningitis	0.17 million	0.3%	0.25 million	8
10	Syphilis	0.16 million	0.3%	0.19 million	11
11	Hepatitis B	0.10 million	0.2%	0.93 million	6
12-17	Tropical diseases	0.13 million	0.2%	0.53 million	9, 10, 16-18

Note: Other causes of death include maternal and perinatal conditions (5.2%), nutritional deficiencies (0.9%), noncommunicable conditions (58.8%), and injuries (9.1%).

Identification of Microorganisms

- **How to identify unknown specimens ??????**
- **Labs can grow, isolate and identify most routinely encountered bacteria within 48 hrs of sampling.**
- **The methods microbiologists use fall into three categories:**
  - ✓ **Phenotypic**- morphology (micro and macroscopic)
  - ✓ **Immunological**- serological analysis
  - ✓ **Genotypic**- genetic techniques

Phenotypic Methods

- **'Old fashioned' methods via biochemical, serological and morphological are still used to identify many microorganisms.**
- **Phenotypic Methods**
- **Microscopic Morphology include a combination of cell shape, size, Gram stain, acid fast reaction, special structures e.g. Endospores, granule and capsule can be used to give an initial presumptive identification.**

Phenotypic Methods

- **Macroscopic morphology are traits that can be accessed with the naked eye e.g. appearance of colony including texture, shape, pigment, speed of growth and growth pattern in broth.**

- **Physiology/Biochemical characteristic are traditional mainstay of bacterial identification**

- **These include enzymes (Catalase, Oxidase, Decarboxylase), fermentation of sugars, capacity to digest or metabolize complex polymers and sensitivity to drugs can be used in identification.**

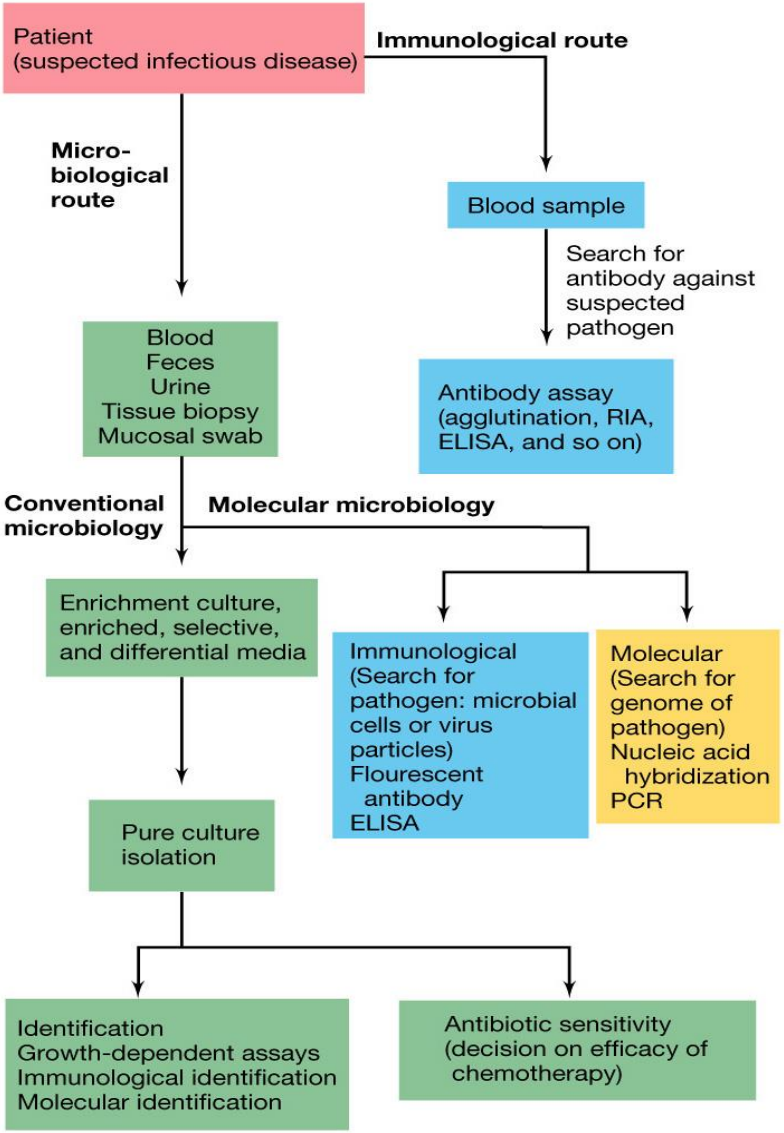
Immunological Methods

- **Immunological methods involve the interaction of a microbial antigen with an antibody (produced by the host immune system).**
- **Testing for microbial antigen or the production of antibodies is often easier than test for the microbe itself.**
- **Lab kits based on this technique is available for the identification of many microorganisms.**

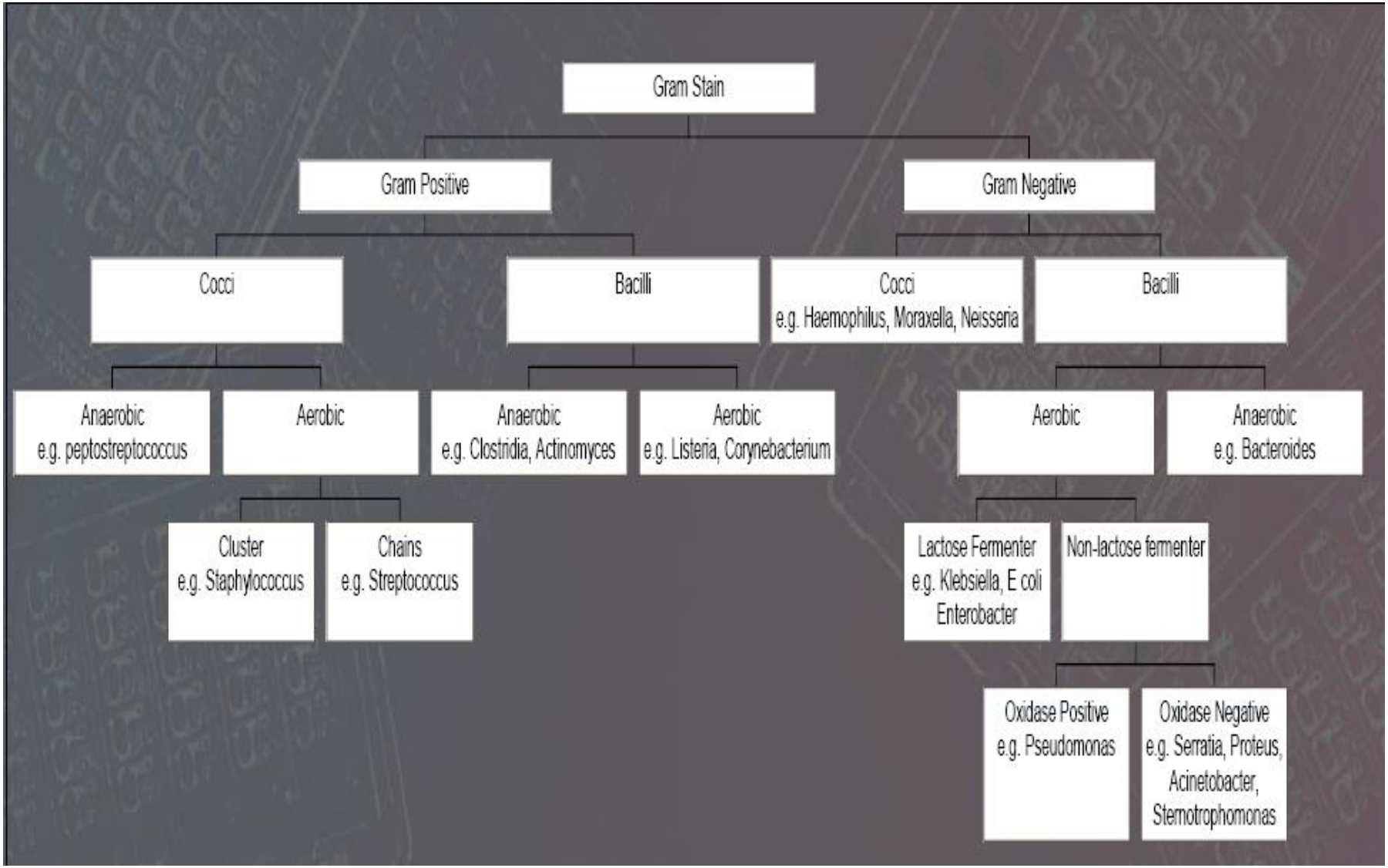
Genotypic Methods

- **Genotypic methods involve examining the genetic material of the organisms and has revolutionized bacterial identification and classification.**
- **Genotypic methods include PCR, (RT-PCR, RAPD-PCR),use of nucleic acid probes, RFLP and plasmid fingerprinting.**
- **Increasingly genotypic techniques are becoming the sole means of identifying many microorganisms because of its speed and accuracy.**

Microbe Identification Scheme



Bacterial Classification



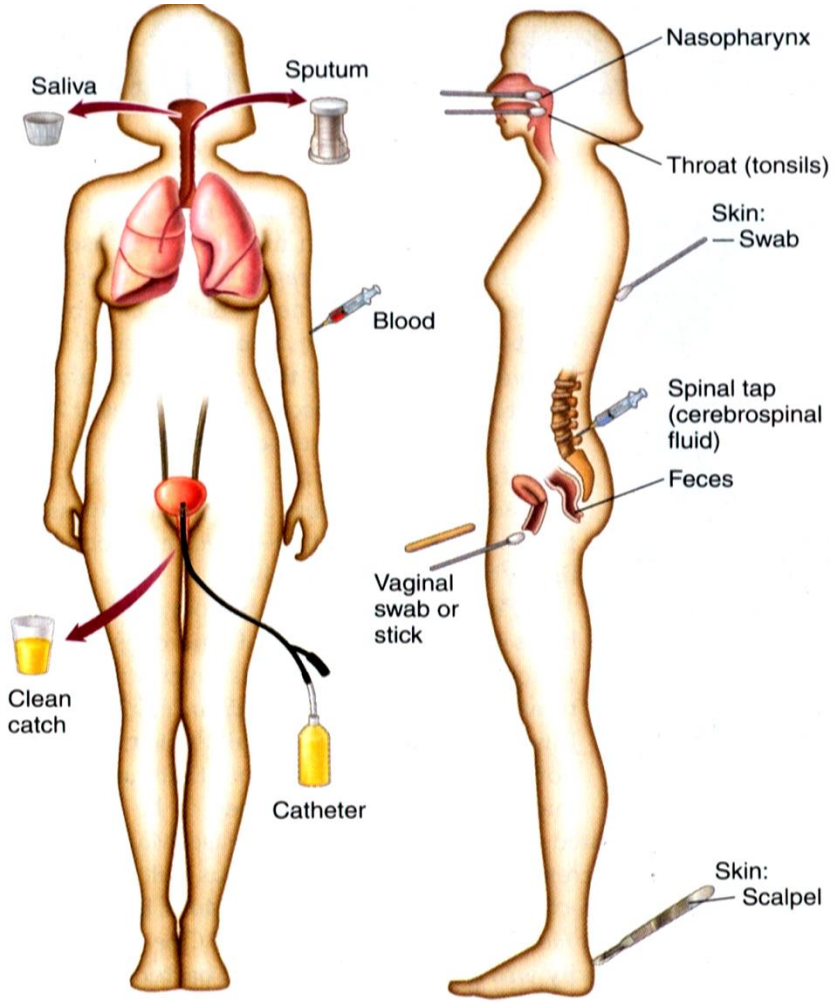
Microbe Identification

- **The successful identification of microbe depends on:**
  - Using the proper **aseptic techniques**.
  - **Correctly** obtaining the specimen.
  - Correctly **handling the specimen**
  - **Quickly transporting** the specimen to the lab.
  - Once the specimen reaches the lab it is **cultured and identified**
  - Use care and tact to **avoid patient harm**

The specimen is the beginning. All diagnostic information from the laboratory depends upon the knowledge by which specimens are chosen and the care with which they are collected and transported.

—Cynthia A. Needham

Specimen Collection



Microbe Identification

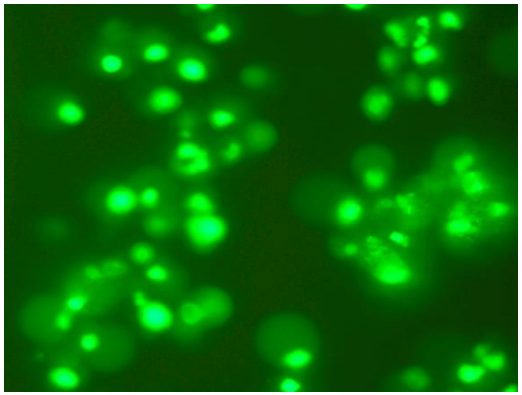
- **Identification measures include:**
  - **Microscopy (staining)**
  - **growth on enrichment, selective, differential or characteristic media**
  - **specimen biochemical test (rapid test methods)**
  - **immunological techniques**
  - **molecular (genotypic) methods**
- **After the microbe is identified for clinical samples it is used in susceptibility tests to find which method of control is most effective.**

Staining Reactions

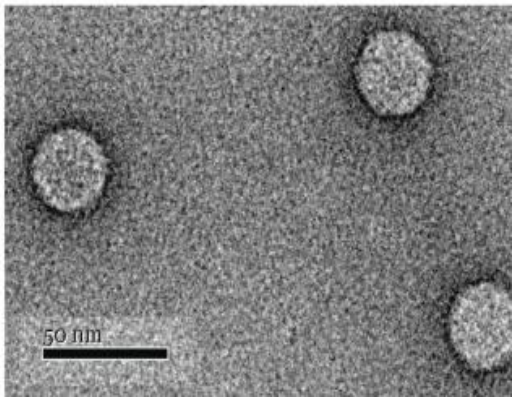
The presence of certain structures and staining reactions aids in their identification and classification

1) **To render microscopic and semitransparent objects visible**
2) **To reveal their shape and size**
3) **To produce specific physical or chemical reactions**
4) **To produce specific physical or chemical reactions.**

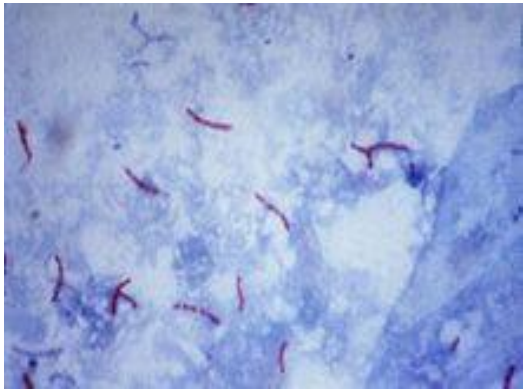
Microscopy/Histology



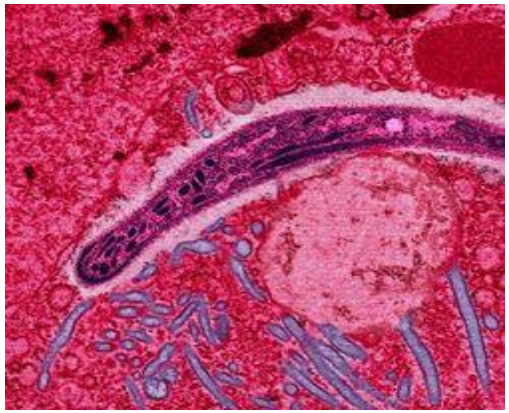
RNA stained HRV infected into HeLa cells



TEM of purified HRV



M. tuberculosis bacteria using acid-fast Ziehl-Neelsen stain; Magnified 1000 X.



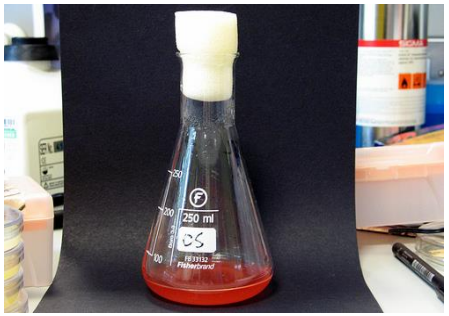
False-colored EM of malaraial sporozoite migrating throughcytoplasm of midgut epithelia.

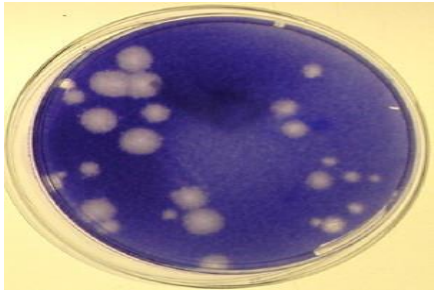
Staining Reactions

- Simple staining bring out the morphology the best
- Differential and special stains are necessary to bring out characteristics like flagella, capsules, spores and metachromatic granules.
- Gram stain divides bacteria into Gram positive and Gram negative
- Ziehl-Neelsen stain divides them into acid fast and non acid fast
- Fluorescent dyes bring out special characteristics and fluorescent antibody technique enables to identify them.

Culture of microorganisms

- A method of multiplying microorganisms in controlled lab conditions
- Bacteria: in liquid or solid culture
- Use different types of media/agar depending on the bacterium
- Viruses: cultivate in animal cells





Cultural Characteristics

- **Provides additional information for the identification of the bacterium. The characters revealed in different types of media are noted**

- **While studying colonies on solid media following characteristics are observed :**

  Size, Shape, Margins, Surface, Their elevations, Edge, colour, structure, consistency.

- **In fluid medium following characteristics are observed :**

  Degree of growth – Absence, scanty, moderate, abundant etc.

  presence of turbidity and its nature

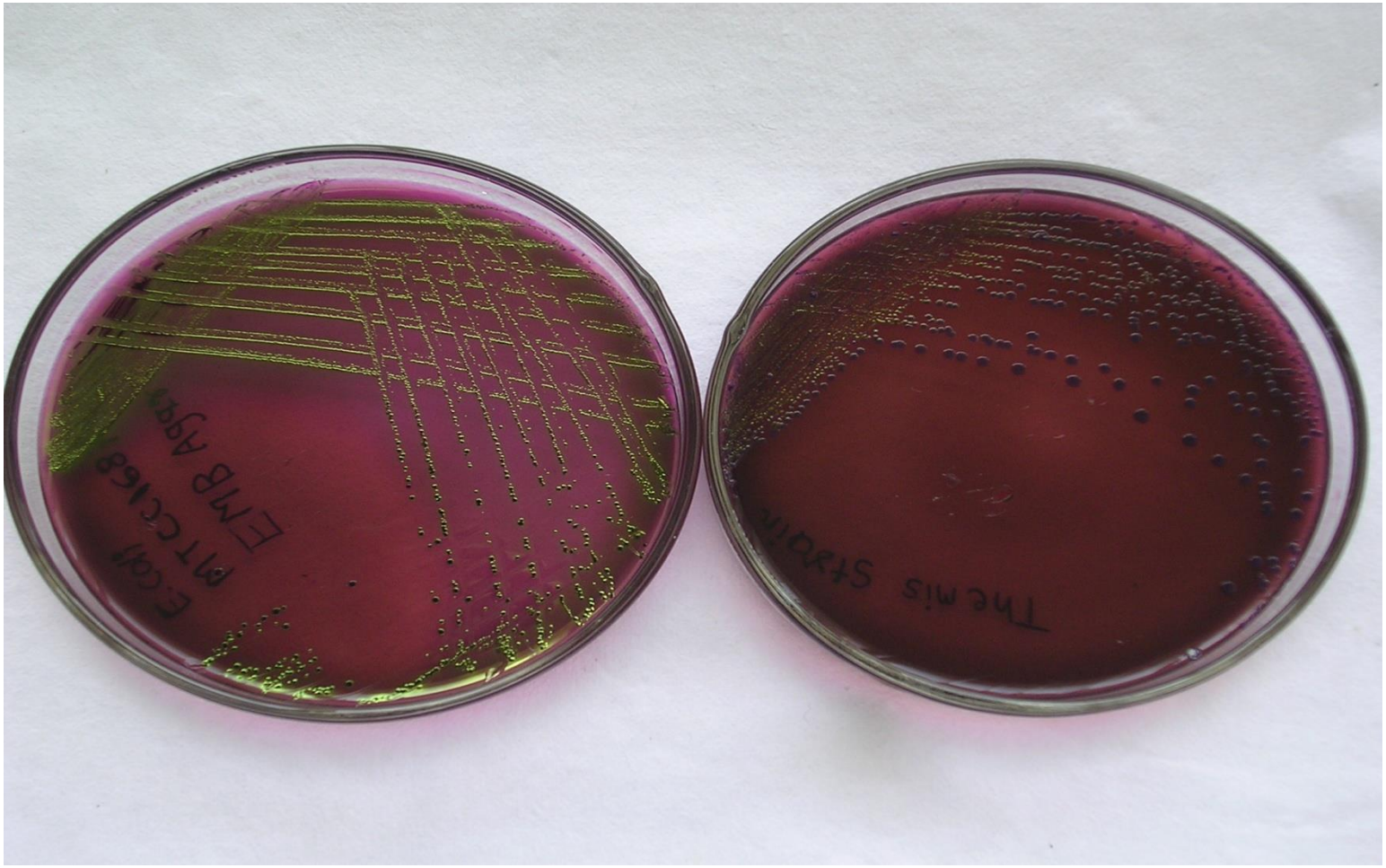
  presence of deposit and its character

  Nature of surface growth

  Ease of disintegration and odour

MacConkey's Agar

Plates showing differentiating characteristics



Resistance

The resistance of the organism is tested against number of parameters which helps differentiation and identification of the organisms

- **Heat**
- **Low concentration of disinfectants**
- **Antibiotics**
- **Chemotherapeutic agents**
- **Bacteriocins etc.**

Metabolism

- **To classify and differentiate species following aspects are studied**

- Requirements of oxygen

- The need for CO_2

- Capacity to form pigments

- power of haemolysis

Biochemical properties

- Tests for Metabolism of Carbohydrates and related compounds
- Tests for Metabolism of Proteins and Amino acids
- Test for metabolism of Lipids
- Tests for Enzymes
- Combined Tests

Biochemical properties

Tests for Metabolism of Carbohydrates and related compounds

- **Tests to distinguish b/w aerobic and anaerobic breakdown of carbohydrates**

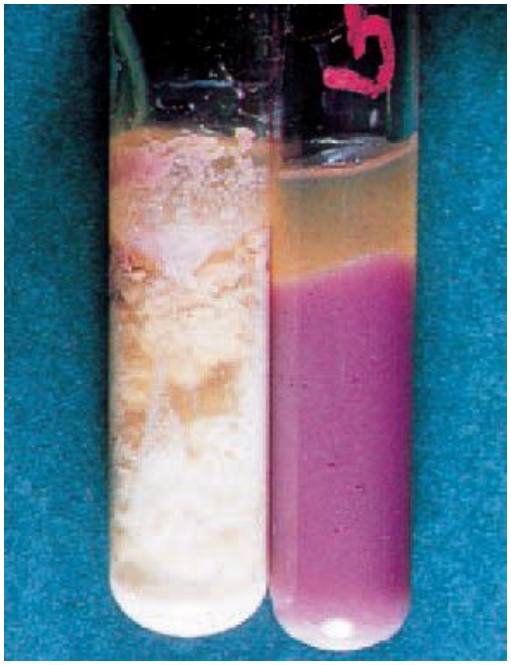
  - O/F test depends upon the use of a semi-solid tubed medium containing the carbohydrate (usually glucose) along with the pH indicator

- **Tests to show the range of carbohydrates and related compounds that can be attacked**

  - A large variety of carbohydrate compounds are used and they are often regarded as 'sugars'

Sugar fermentation – Acid production

Litmus milk – Acid or alkali production, clot formation, peptonisation or saponification. Disruption of clot due to gas production.

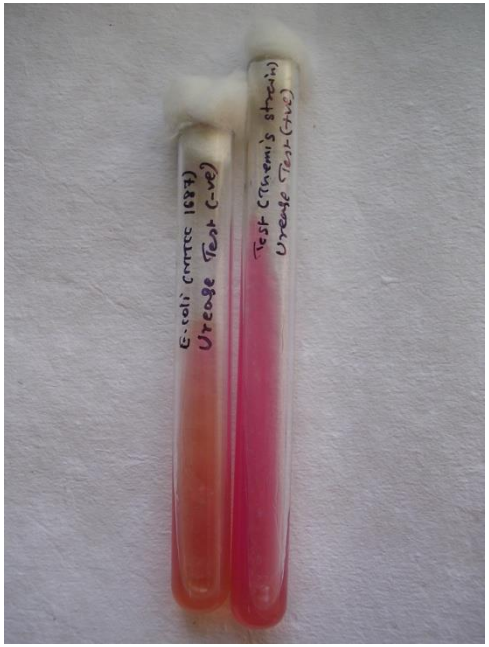


Stormy Fermentation of Litmus Milk.
The tube on the left shows fermentation; the tube on the right is negative for stormy fermentation.
Used for the identification of *Clostridium* species.

Sugar fermentation: Acid and gas production

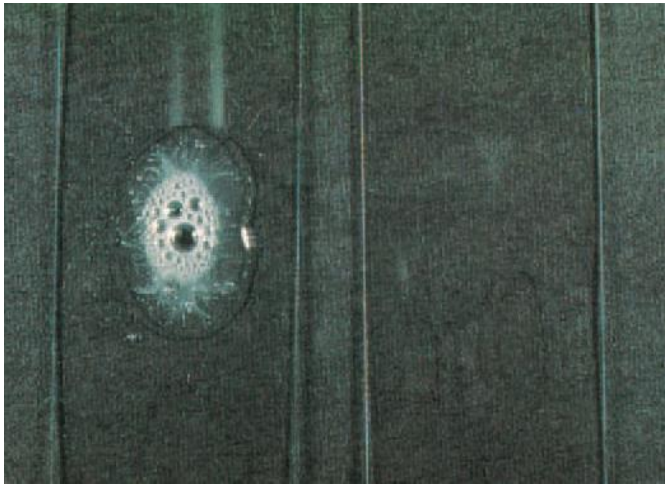
Biochemical properties

Urease



coagulase test

(a) (b)

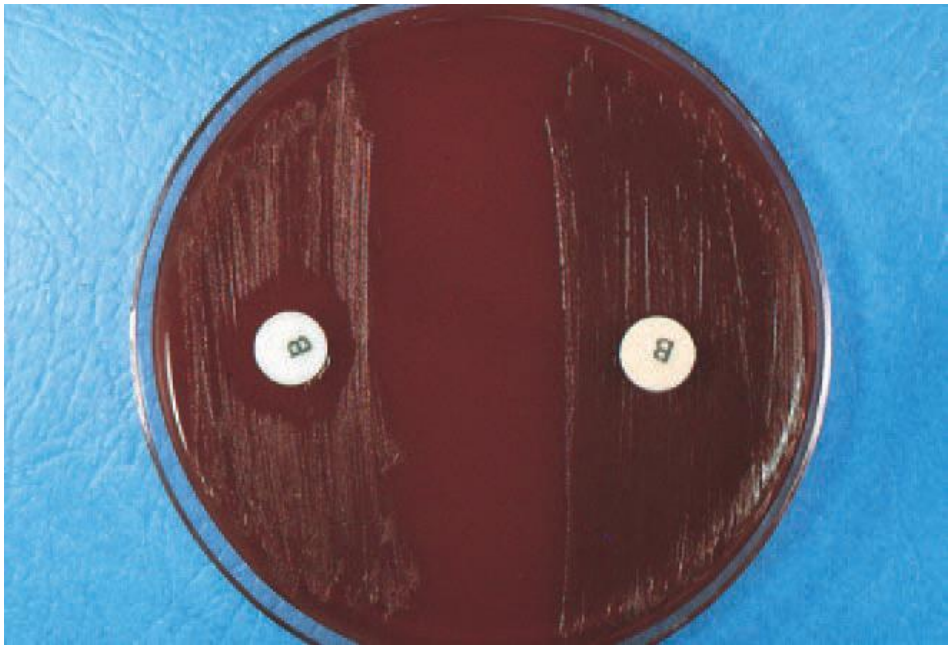


Tube and slide Catalase Test.

Differentiation of two organisms

Biochemical properties

- Miscellaneous tests
- Antibiotic tolerance (resistance) test, dye tolerance and other chemical inhibition tests
- KCN test – Ability to grow in a medium containing KCN. (Should be handled carefully)
- Detection of motility
  - Slide test (Hanging drop technique)
  - Tube test (Semisolid Agar)

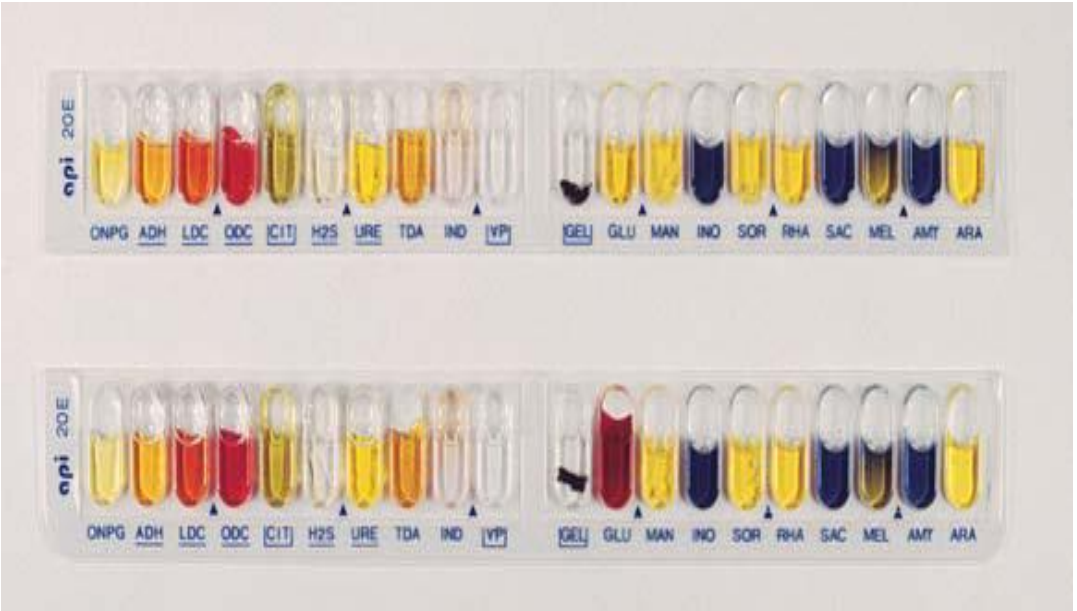


API Strips - Rapid Tests

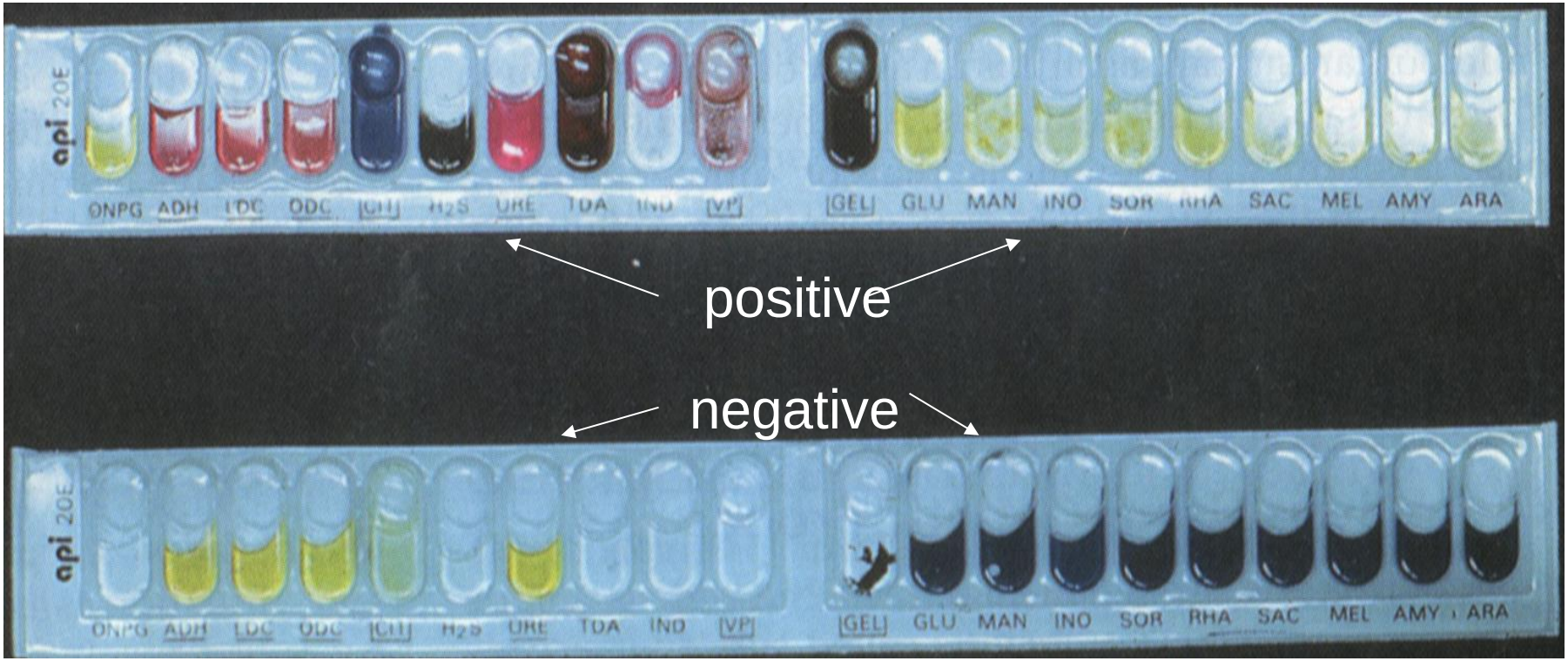
Commercial miniaturized biochemical test panels - Cover a significant number of clinically-important groups of bacteria, as well as food- and water-associated microorganisms.

The earliest, is the Analytical Profile Index (API) panel.

Identification of Enterobacteriaceae using API 20E, a standardized microplate method. Positive and negative reactions are shown by color reactions.



Rapid Tests



ONPG (β galactosidase); ADH (arginine dihydrolase); LDC (lysine decarboxylase); ODC (ornithine decarboxylase); CIT (citrate utilization); H_2S (hydrogen disulphide production); URE (urease); TDA (tryptophan deaminase); IND (indole production); VP (Voges Proskauer test for acetoin); GEL (gelatin liquefaction); the fermentation of glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC); Melibiose (MEL), amygdalin (AMY), and arabinose (ARA); and OXI (oxidase).

Immunologic Techniques

- **The culturing of certain viruses, bacteria, fungi, and parasites from clinical specimens may not be possible because the methodology remains undeveloped (*Treponema pallidum; Hepatitis A, B, C;* and *Epstein-Barr virus*), is unsafe (rickettsias), or is impractical for all but a few clinical microbiology laboratories (*Mycobacteria*).**
- **Cultures also may be negative because of prior antimicrobial therapy. Under these circumstances, detection of antibodies or antigens may be quite valuable diagnostically**
- **Immunologic systems for the detection and identification of pathogens from clinical specimens are easy to use, give relatively rapid reaction endpoints, and are sensitive and specific**

Antigens

- **Antigens have 2 characteristics**
  - Immunogenicity – ability to provoke immune response
  - Reactivity – ability of antigen to react specifically with antibodies it provoked
  - Entire microbes may act as antigen
  - Typically, just certain small parts of large antigen molecule triggers response (epitope or antigenic determinant)

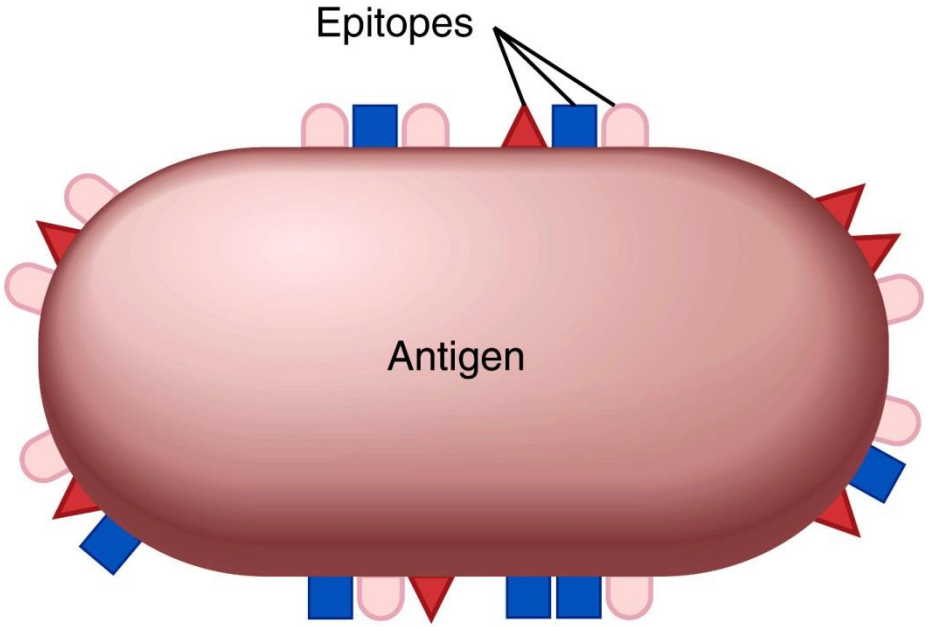


Figure 22.12 Tortora - PAP 12/e

Antibodies (Ab)

- Can combine specifically with epitope of the antigen that triggered its production
- Belong to group of glycoproteins called globulins
  - Ab are immunoglobulins (Igs)
- 4 polypeptide chains – 2 heavy (H) chains, 2 light (L) chains
- Hinge region – antibody can be T shape or Y shape
- Variable (V) region at tips of each H and L chain
  - 2 antigen-binding sites - bivalent
- Constant (C) region – remainder of H and L chain
  - Same in each 5 classes – determines type of reaction

Antibody actions

- Neutralizing antigen
- Immobilizing bacteria
- Agglutinating and precipitating antigen
- Enhancing phagocytosis
- Activating complement
  - Defensive system of over 30 proteins
  - Destroy microbes by causing phagocytosis, cytolysis, and inflammation
  - Acts in a cascade – one reaction triggers another
  - 3 different pathways ass activate C3
  - C3 then begins cascade that brings about phagocytosis, cytolysis, and inflammation

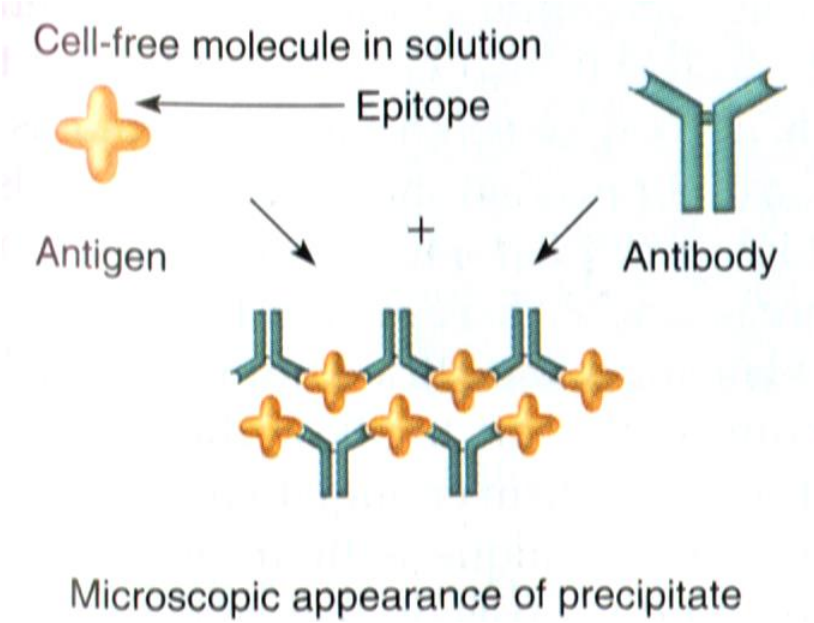
serology

- **The branch of medical immunology concerned with antigen-antibody reactions in vitro is serology [serum and -ology]. The usefulness of serological test is dependent on its sensitivity and specificity.**

  - **False Negatives/Positives**
  - High sensitivity prevents false negatives.
  - False negatives occurs when there is no reaction when the Ag or Ab is present.
  - High specificity prevents false positives.
  - False positives occurs when there is cross reaction with another molecule.

Precipitation Reactions

- **Precipitation is the interaction of a soluble Ag with an soluble Ab to form an insoluble complex.**
- **The complex formed is an aggregate of Ag and Ab.**



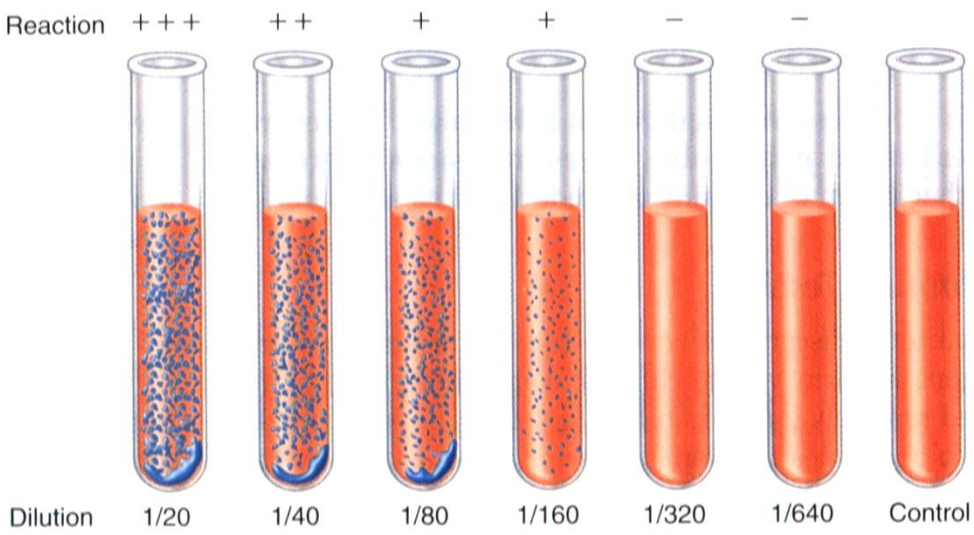
- Precipitation reactions occur maximally only when the **optimal proportions** of Ag and Ab are present.
- Precipitation can also be done in agar referred to as **immunodiffusion**.

Agglutination Reactions

- **Agglutination is the visible clumping of an Ag when mixed with a specific Ab.**
- **Widely used because they are simple to perform, highly specific, inexpensive and rapid.**
- **Standardized tests are available for the determination of blood groups and identification of pathogens and their products.**

Direct agglutination occurs when a soluble Ab results in clumping by interaction with an Ag which **is part of a surface of a cell.** E.g. Blood typing and detection of *mycoplasma pneumonia.*

Indirect (passive) agglutination. Ab/Ag is **adsorbed or chemically coupled** to the cell, latex beads or charcoal particles which serves as an inert carrier.



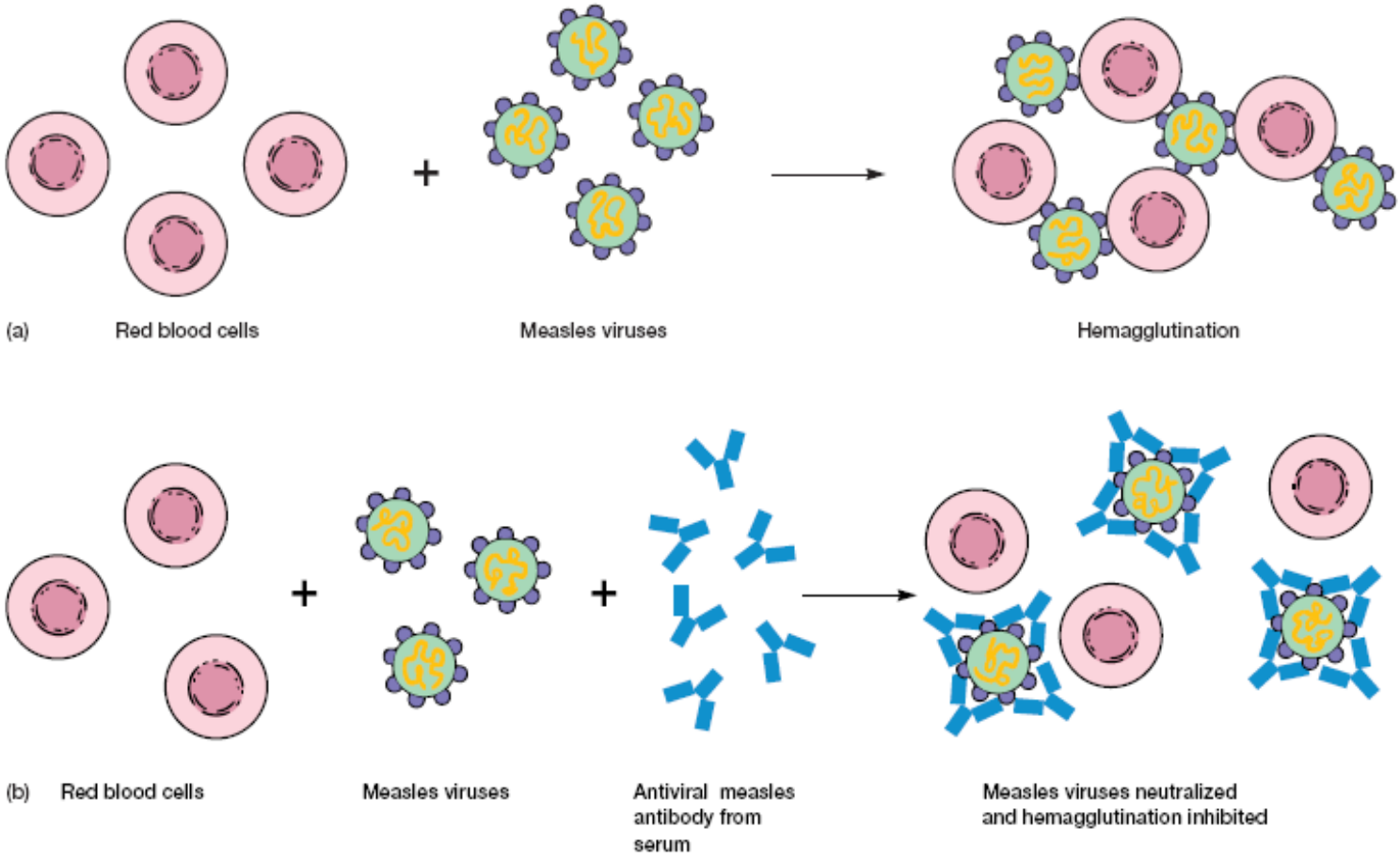
The latex beads can be used to detect for surface Ag.

Viral hemagglutination

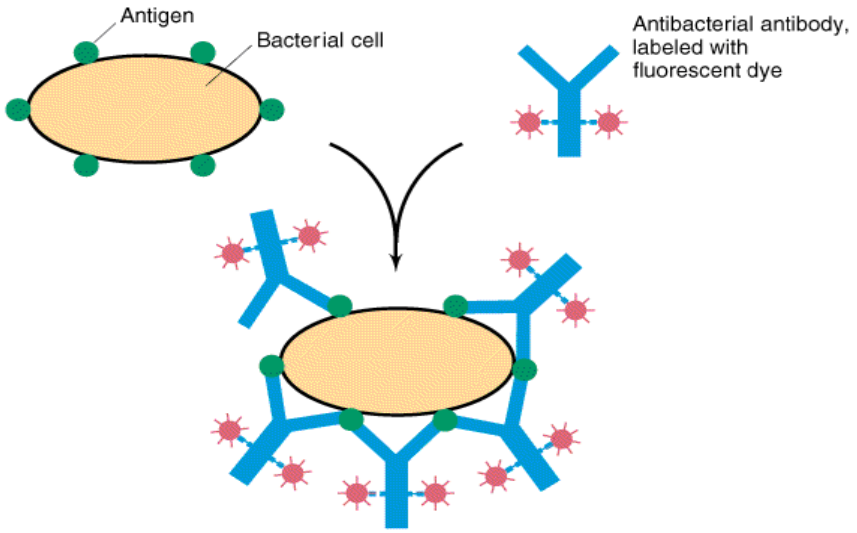
Viral Hemagglutination.

(a) Certain viruses can bind to red blood cells causing hemagglutination.

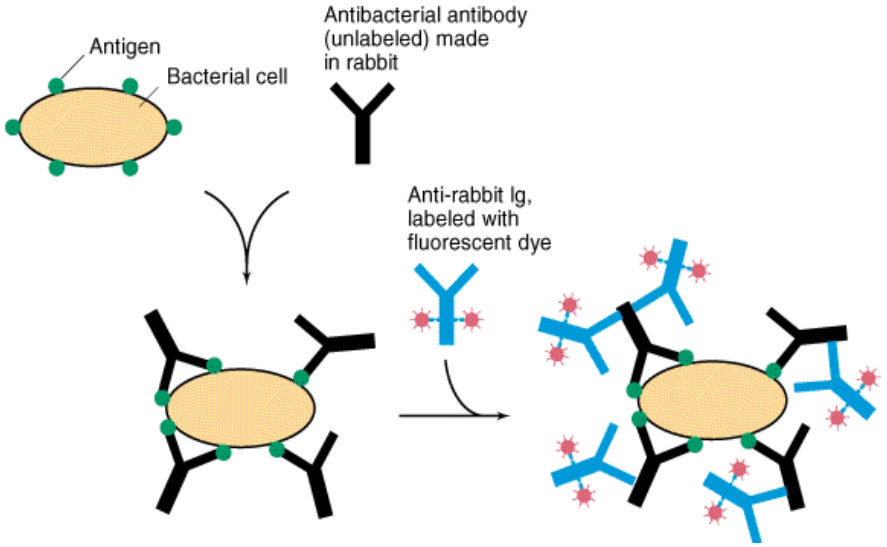
(b) If serum containing specific antibodies to the virus is mixed with the red blood cells, the antibodies will neutralize the virus and inhibit hemagglutination (a positive test).



Using fluorescent antibodies



Direct method



Indirect method

- Abs can be chemically modified with Fluorescent dyes such as Rhodamine B, Fluorescent Red, Fluorescein Isothiocynate and Fluoresces Yellow Or Green.
- Cells with bound fluorescent Ab emit a bright red, orange, yellow or green light depending on the dye used.
- There are two distinct fluorescent Ab procedure: direct and indirect.

Fluorescent antibodies

- **In the direct method the fluorescent Ab is directed to surface Ag of the organism.**

- **In the indirect method a non-fluorescent Ab reacts with the organism's Ag and a fluorescent Ab reacts with the non-fluorescent Ag-Ab complex.**

Fluorescent Ab can be used to detect microorganisms **directly in tissue**, long before a primary isolation technique yield the suspected pathogen.

Fluorescent Ab have been used for the detection of *Bacillus anthracis* and HIV virus.

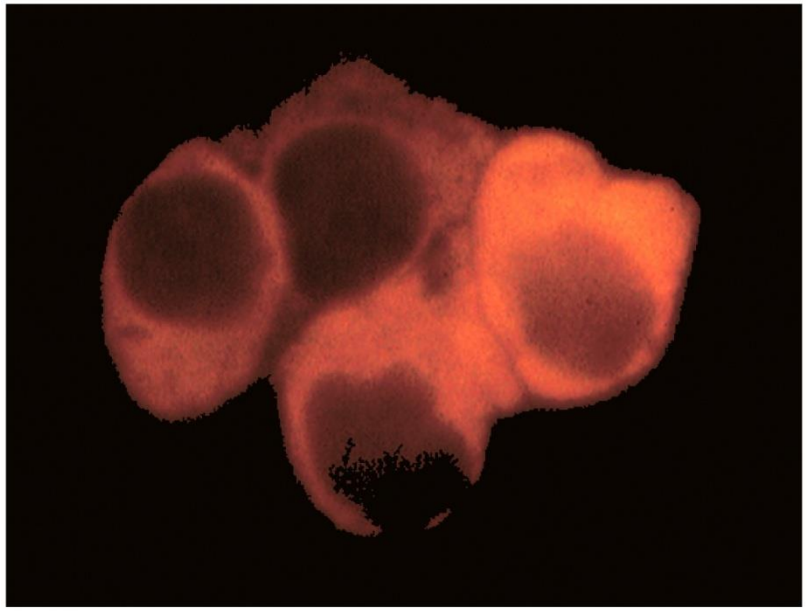
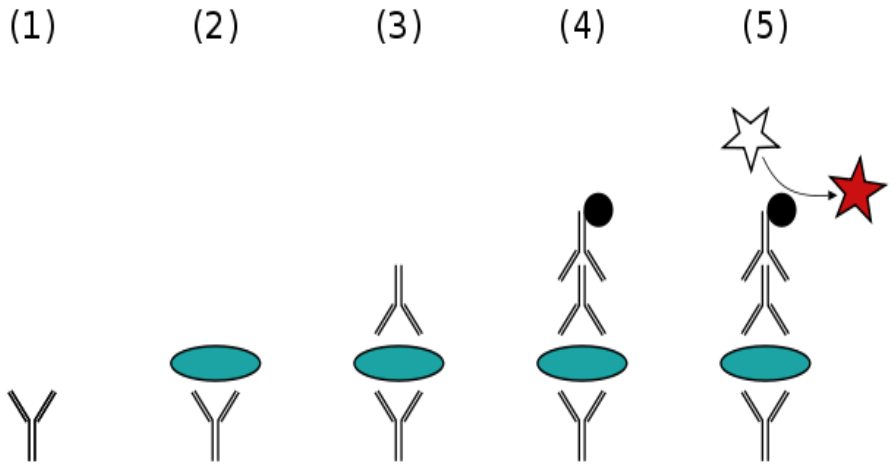


Figure 6-14d
Kuby IMMUNOLOGY, Sixth Edition

Immunoassays



A sandwich ELISA. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form

Usually with blood sample:

- Measures Abs against pathogen
- Requires use of standard curve
- Usually done in a 96-well plate
- Needs fluorescence plate reader
- About 2-3 hours minimum

Reagents

- Primary antibody
- Secondary antibody with label
- Substrate for secondary antibody
- Wash buffers

ELISA - Enzyme-linked immunosorbent assay

- The enzyme-linked immunosorbent assay (ELISA) has become one of the most widely used serological tests for antibody or antigen detection. This test involves the linking of various “label” enzymes to either antigens or antibodies.
- Enzymes used in ELISA include Alkaline Phosphate, Peroxidase and ß Galactosidase.
- During indirect ELISA the Ag is trapped between two Ab molecules (sandwich ELISA)

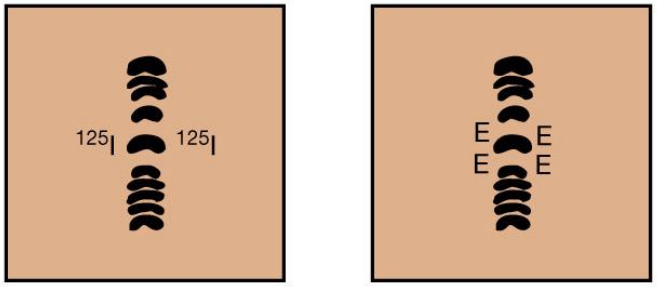
Immuno/Western Blot

- **Immunoblot is used as a confirmatory test for HIV**
- **The ELISA test for HIV often yields false positive and the immunoblot test is used to confirm a positive ELISA results**
- **To perform the HIV immunoblot purified HIV is treated with SDS to solubilize the proteins and inactivate the virus**
- **The proteins (at least 7) are resolved by polyacrylamide gel electrophoresis and the proteins are blotted unto a membrane and incubated with the test serum.**

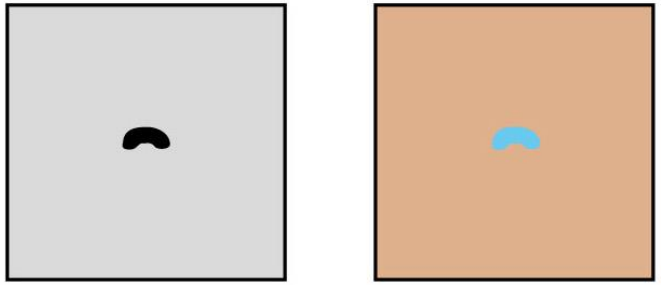
HIV Immunoblot Test

- **The test is considered positive if bands occur at, 2 locations e.g. gp160 and gp 120 or p24 and gp 41-45.**

4. Treat nitrocellulose paper containing blotted proteins with antibodies; each antibody recognizes and binds to a specific protein, labeling the protein for detection



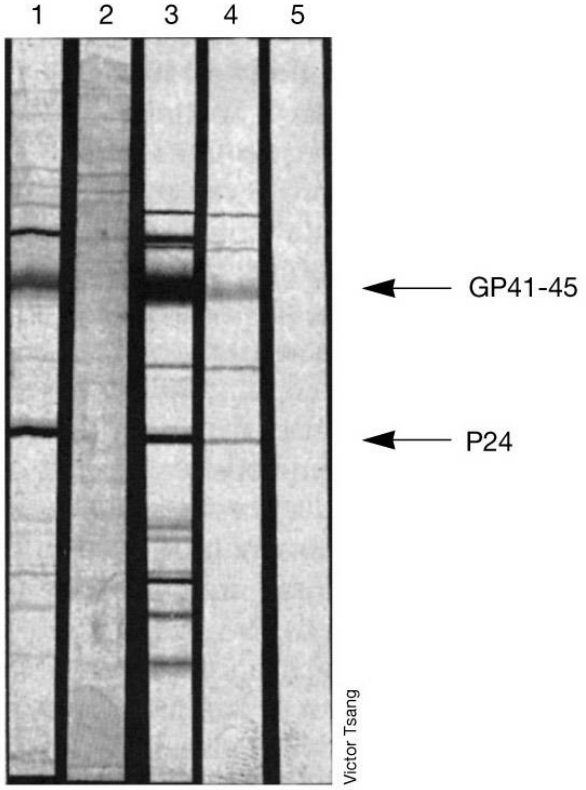
5. Add marker to bind to antigen–antibody complexes, either (left) radioactive *Staphylococcus* protein A–^{125}I, or (right) antibody containing conjugated enzyme



X-ray film

Nitrocellulose with enzyme-produced colored spot

(a)



(b)

Immunoassays

TABLE 6-3 Sensitivity of various immunoassays

Assay	Sensitivity* (μg antibody/ml)
Precipitation reaction in fluids	20–200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10–50
Ouchterlony double immunodiffusion	20–200
Immunoelectrophoresis	20–200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006–0.06
Agglutination inhibition	0.006–0.06
Radioimmunoassay (RIA)	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	~0.0001–0.01
ELISA using chemiluminescence	~0.00001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.006–0.06

*The sensitivity depends on the affinity of the antibody used for the assay as well as the epitope density and distribution on the antigen.
†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Updated and adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology,* 5th ed., American Society for Microbiology, Washington, DC.

Table 6-3
Kuby IMMUNOLOGY, Sixth Edition

Problems With Traditional Methods

- Cultivation-based methods insensitive for detecting some organisms.
- Cultivation-based methods limited to pathogens with known growth requirements.
- Poor discrimination between microbes with common behavioral features.
- Failure to detect infections caused by uncultivated (e.g., novel) organisms, or organisms that fail to elicit a detectable host immune response.
- Visual appearance of microorganisms is nonspecific.

Problems With Traditional Methods

Examples of Failures With Traditional Approaches

- Detection and speciation of slow-growing organisms takes weeks
- (e.g., *M. tuberculosis*).
- A number of visible microorganisms cannot be cultivated (e.g., *Whipple bacillus*).
- Diseases presumed to be infectious remain ill-defined with no detected microorganism (e.g., abrupt fever after tick bite).

Genotypic methods

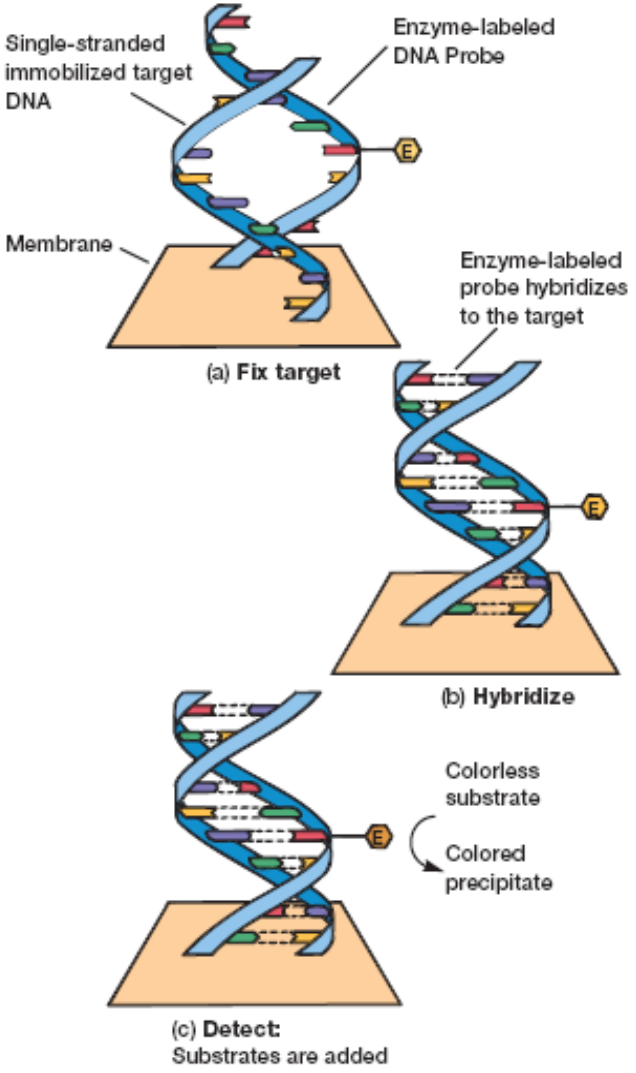
- **The initiation of new molecular technologies in genomics and proteomics is shifting traditional techniques for bacterial classification, identification, and characterization in the 21st century toward methods based on the elucidation of specific gene sequences or molecular components of a cell.**
- **Genotypic methods of microbe identification include the use of :**
  - ✓ Nucleic acid probes
  - ✓ PCR (RT-PCR, RAPD-PCR)
  - ✓ Nucleic acid sequence analysis
  - ✓ 16s rRNA analysis
  - ✓ RFLP
  - ✓ Plasmid fingerprinting.

Nucleic acid probes

- **Nucleic acid hybridization is one of the most powerful tools available for microbe identification.**
- **Hybridization detects for a specific DNA sequence associated with an organism.**
- **The process uses a nucleic acid probe which is specific for that particular organism.**
- **The target DNA (from the organism) is attached to a solid matrix such as a nylon or nitrocellulose membrane.**

Nucleic Acid Probes

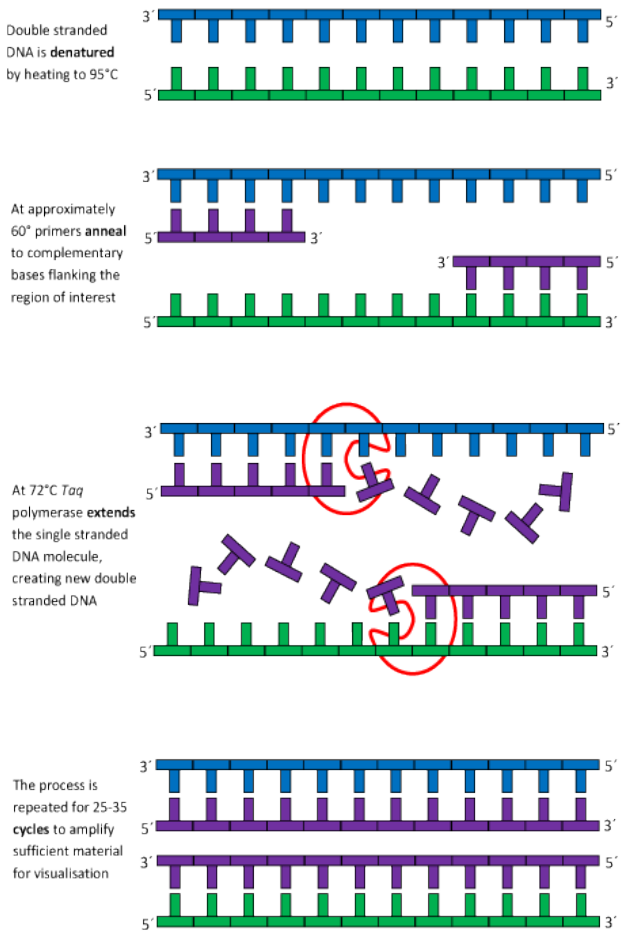
- A single stranded probe is added and if there is sequence complementality between the target and the probe a positive hybridization signal will be detected.
- Hybridization is detected by a reporter molecule (radioactive, fluorescent, chemiluminescent) which is attached to the probe.
- Nucleic acid probes have been marketed for the identification of many pathogens such as *N. gonorrhoeae*.

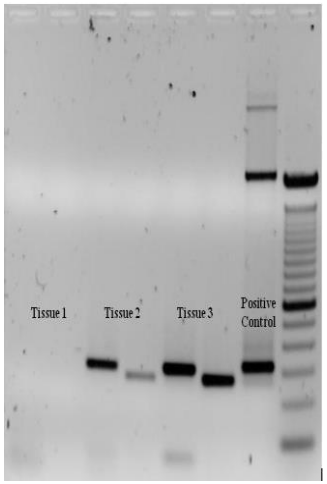


Advantages of Nucleic Acid Probes

- Nucleic acid probes has many advantages over immunological methods.
- Nucleic acid are more stable at high temperature, pH, and in the presence of organic solvents and other chemicals.
- This means that the specimen can be treated very harshly to destroy interfering materials.
- Nucleic acid probes can be used to identify microorganisms which are no longer alive.
- Furthermore nucleic acid probes are more specific than antibodies.

Polymerase Chain Reaction (PCR)





Agarose gel showing amplified fragments

Usually with blood sample:

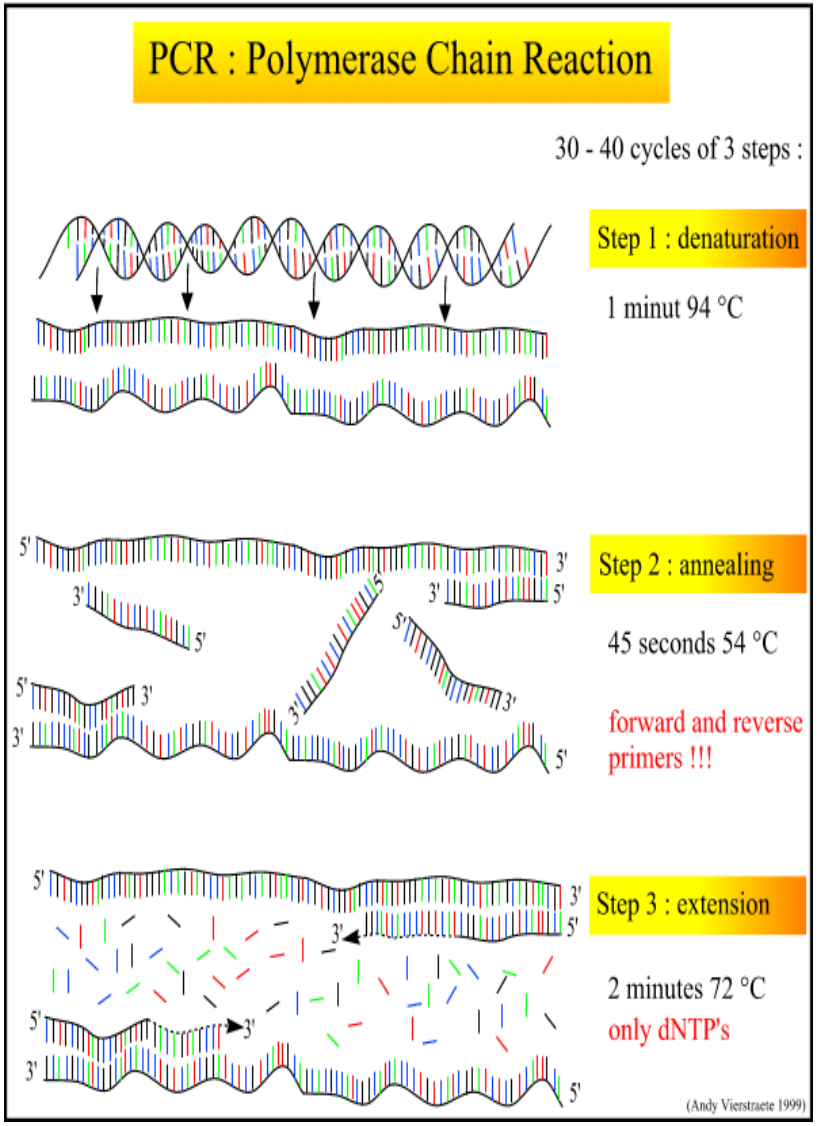
- Measures presence of pathogen
- Amplifies from live/dead
- Can be multiplexed
- Is very sensitive
- About 2-3 hours minimum

Reagents:

- dNTP
- ATP/Mg
- Taq polymerase
- primers

Polymerase Chain Reaction (PCR)

- PCR is widely used for the identification of microorganisms.
- Sequence specific primers are used in PCR for the amplification of DNA or RNA of specific pathogens.
- PCR allows for the detection even if only a few cells are present and can also be used on viable nonculturables.
- The presence of the appropriate amplified PCR product confirms the presence of the organisms.



Limitations of End-Point PCR

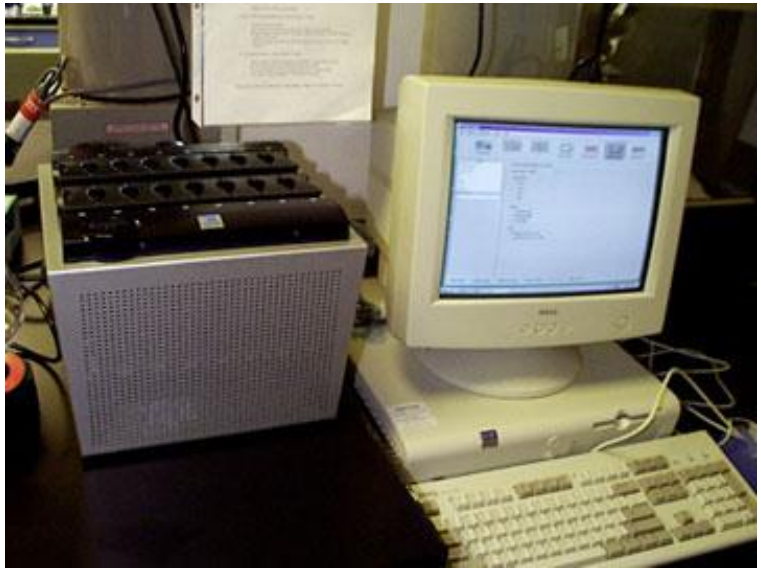
Agarose gel results are obtained from the end point of the reaction. Endpoint detection is very time consuming. Results may not be obtained for days. Results are based on size discrimination, which may not be very precise. The end point is variable from sample to sample. Gels may not be able to resolve these variability in yield, real-time PCR is sensitive enough to detect these changes. Agarose Gel resolution is very poor, about 10 fold. Real-Time PCR can detect as little as a two-fold change!

Some of the problems with **End-Point Detection:**

- Poor Precision
- Low sensitivity
- Short dynamic range < 2 logs
- Low resolution
- Non - Automated
- Size-based discrimination only
- Results are not expressed as numbers
- Ethidium bromide for staining is not very quantitative
- Post PCR processing

Real Time PCR

- Currently many PCR tests employ real time PCR.
- This involves the use of fluorescent primers.
- The PCR machine monitors the incorporation of the primers and display an amplification plot which can be viewed continuously thru the PCR cycle.
- Real time PCR yields immediate results.



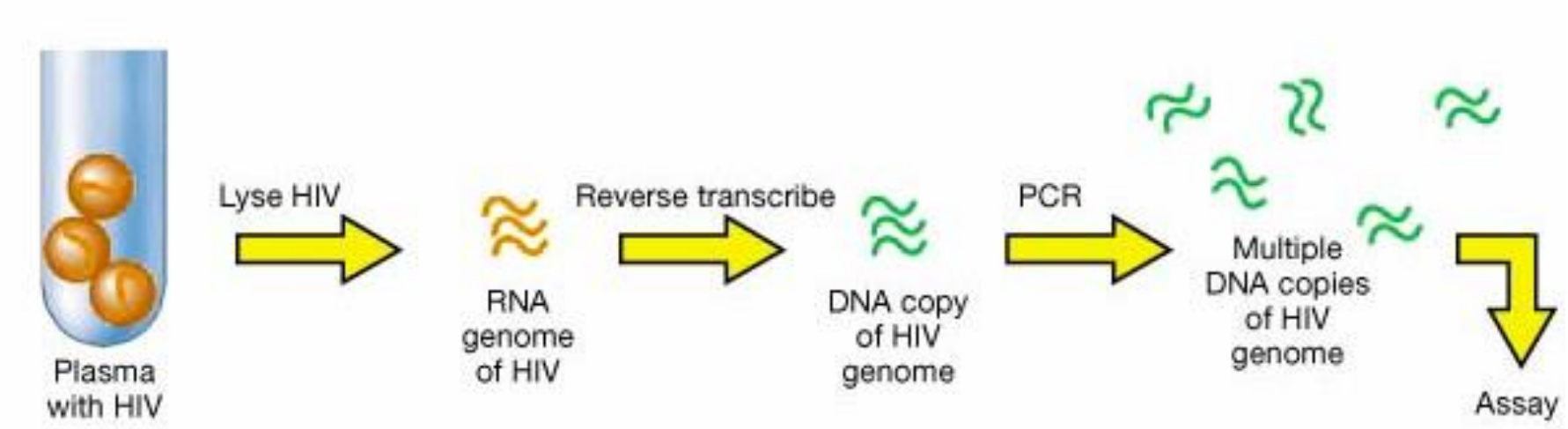
Real-time PCR

- Rapid detection and identification of several bacterial strains
- Promising tool for distinguishing specific sequences from a complex mixture of DNA and therefore is useful for determining the presence and quantity of pathogen-specific or other unique sequences within a sample
- Facilitates a rapid detection of low amounts of bacterial DNA accelerating therapeutic decisions and enabling an earlier adequate antibiotic treatment

RT-PCR (reverse trancriptase PCR

- **RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available.**
- **Compared to the two other commonly used techniques for quantifying mRNA levels, Northern blot analysis and RNase protection assay, RT-PCR can be used to quantify mRNA levels from much smaller samples.**
- **This technique is sensitive enough to enable quantitation of RNA from a single cell.**

RT-PCR


Plasma with HIV
Lyse HIV
RNA genome of HIV
Reverse transcribe
DNA copy of HIV genome
PCR
Multiple DNA copies of HIV genome
Assay

DNA sequencing (16s rDNA)

- **The rRNA is the most conserved (least variable) gene in all cells. Portions of the rDNA sequence from distantly-related organisms are remarkably similiar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure.**
- **For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16s rDNA sequence can show evolutionary relatedness among microorganisms.**

This work was pioneered by Carl Woese, who proposed the **three Domain system of classification - Archaea, Bacteria,** and **Eucarya** - based on such sequence information.

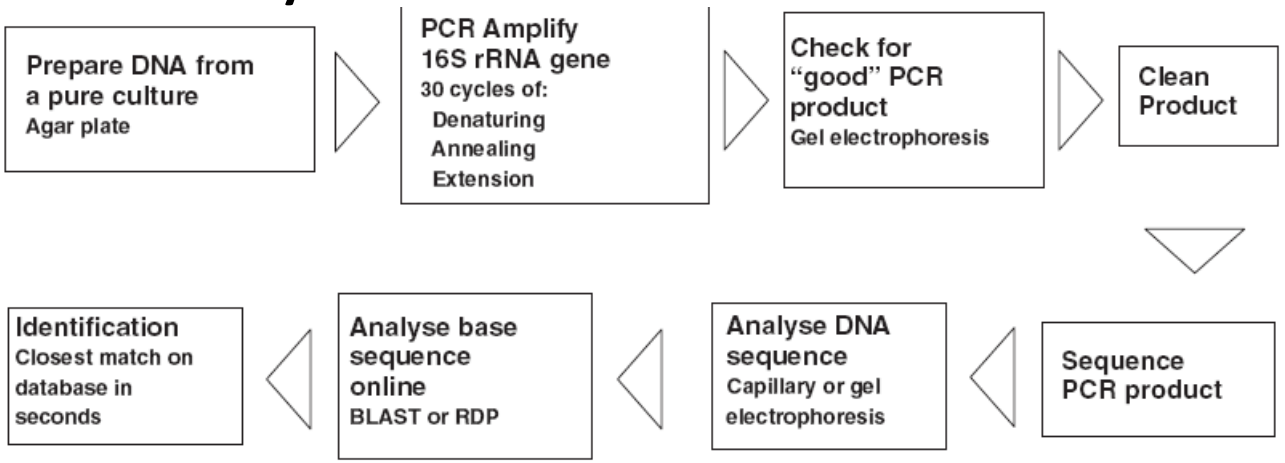
DNA or RNA extraction
In vitro amplification
e.g., PCR-based assays to detect specific DNA target
Sequence determination
i.e., analyze the PCR product
Computer-aides sequence analysis
e.g., BLAST search using the NCBI GenBank database[a]
[a] Basic Local Alignment Search Tool (BLAST) is a computational method for sequence comparison alignment which is available for public use

DNA Sequencing

- **Computer analysis of 16SrRNA sequence has revealed the presence of signature sequences, short oligonucleotides unique to certain groups of organisms and useful in their identification.**

- **rRNA sequence can be used to fine tune identity at the species level e.g differentiating between *Mycobacterium* and *Legionella*.**

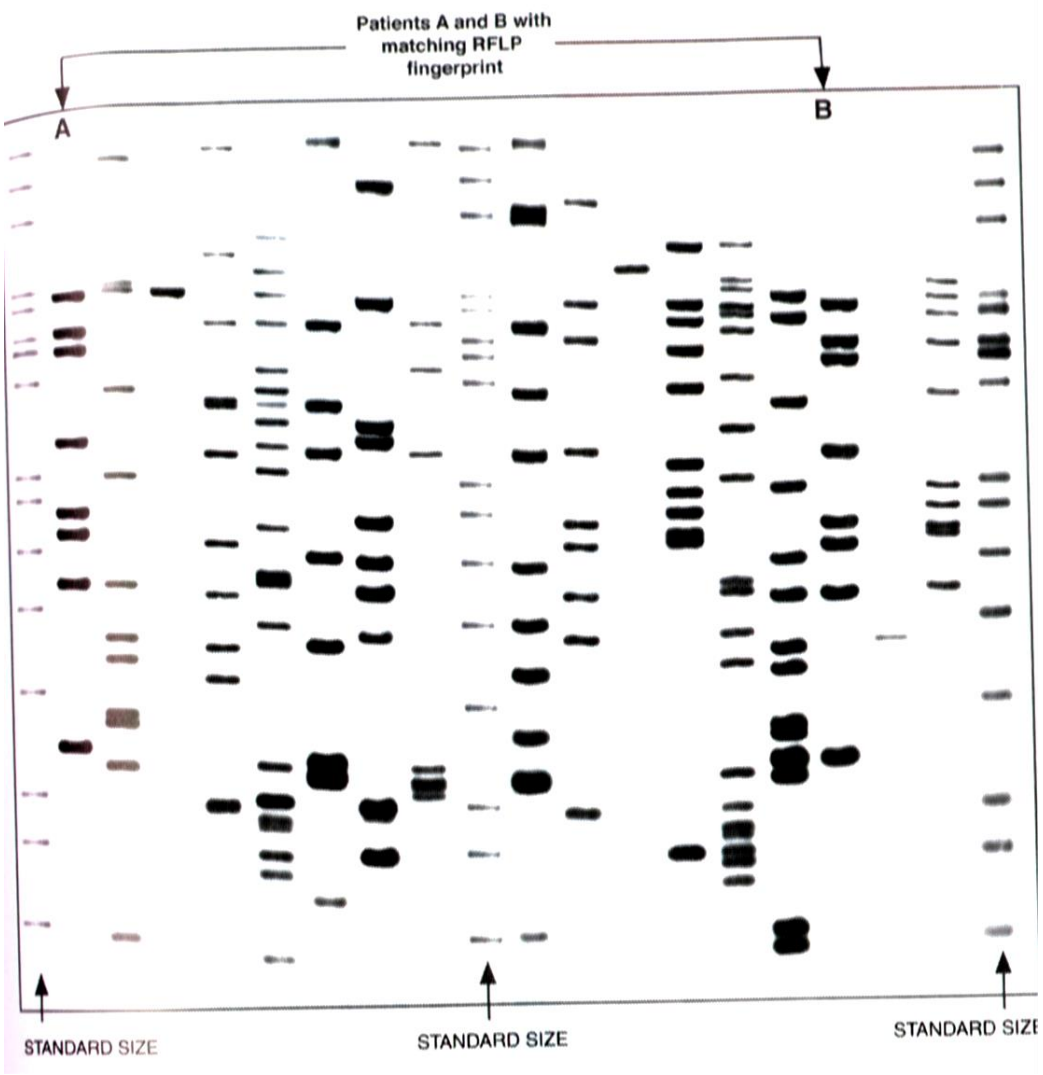
- **16srRNA sequence can also be used to identify microorganisms from a microbial community.**



Restriction Fragment Length Polymorphism

- **RFLP involves digestion of the genomic DNA of the organism with restriction enzymes.**
- **The restricted fragments are separated by agarose gel electrophoresis.**
- **The DNA fragments are transferred to a membrane and probed with probes specific for the desired organisms.**
- **A DNA profile emerges which can be used for microbe identification.**

Restriction Fragment Length Polymorphism



RFLP of *M. tuberculosis* from 17 patients

Plasmid fingerprinting

- **Plasmid fingerprinting identifies microbial species or similar strains as related strains often contain the same number of plasmids with the same molecular weight.**

- **Plasmid of many strains and species of *E. coli, Salmonella, Camylobacter* and *Psseudomonas* has demonstrated that this methods is more accurate than phenotypic methods such as biotyping, antibiotic resistance patterns , phage typing and serotyping.**

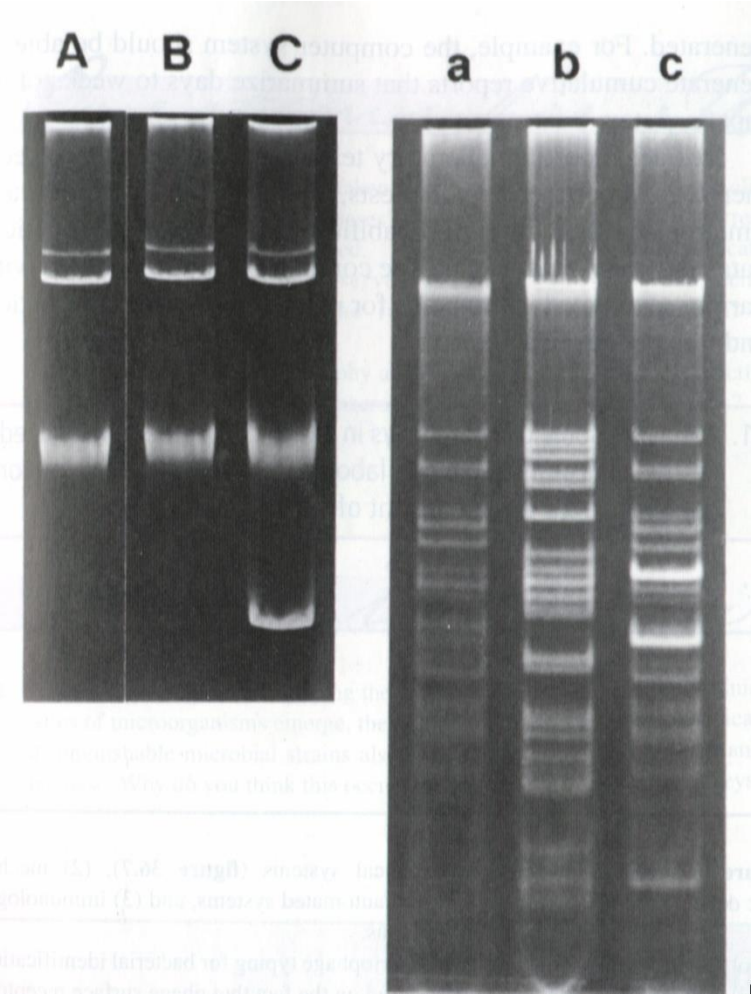
Plasmid fingerprinting

- **The procedure involves:**

The bacterial strains are grown, the cells lysed and harvested.

The plasmids are separated by agarose gel electrophoresis

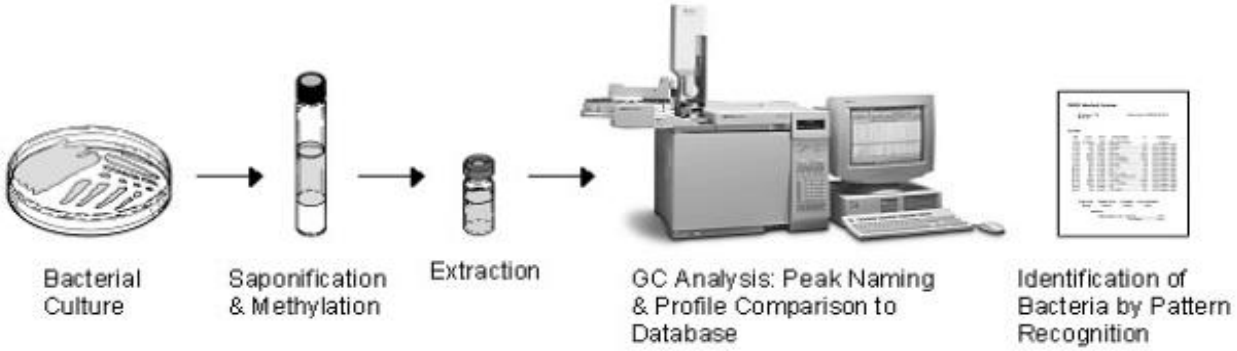
The gels are stained with EtBr and the plasmids located and compared.

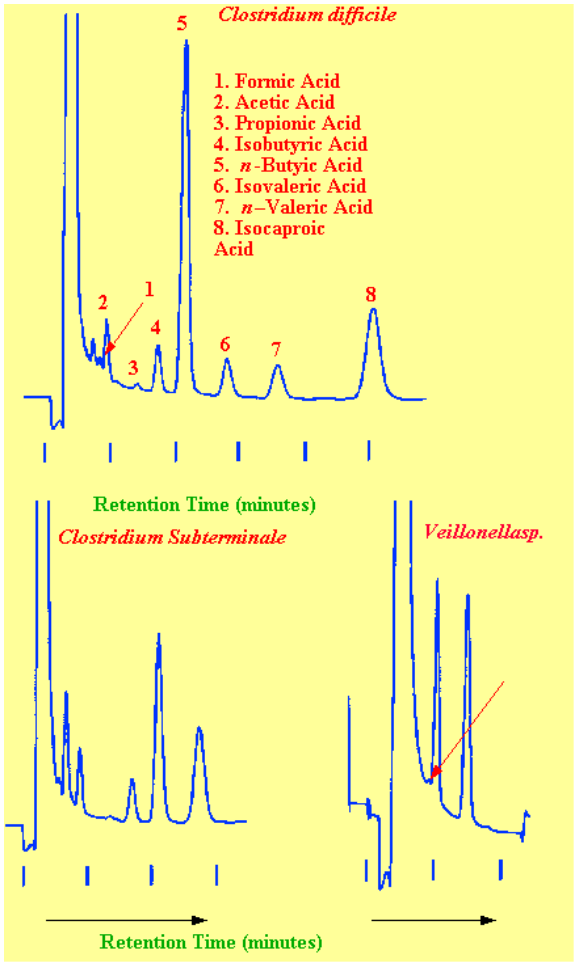


Gas-liquid chromatography

In (GLC), specific microbial metabolites, cellular fatty acids, and products from the pyrolysis (a chemical change caused by heat) of whole bacterial cells are analyzed and identified.

These compounds are easily removed from growth media by extraction with an organic solvent such as ether. The ether extract is then injected into the GLC system. Both volatile and nonvolatile acids can be identified. Based on the pattern of fatty acid production, common bacteria isolated from clinical specimens can be identified.





Volatile Fatty Acid Profiles from Different Bacteria.

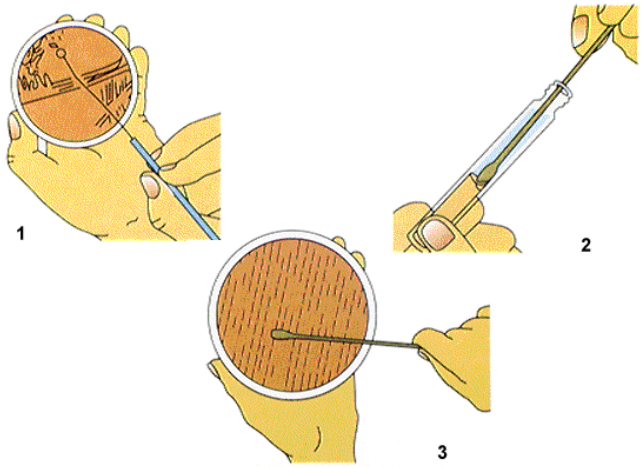
Bacteriophage Typing

- Bacteriophage typing is based on the specificity of phage surface receptor for the cell surface receptor.
- Only those phages that can attach to the surface receptors can cause lysis

The procedure involves:

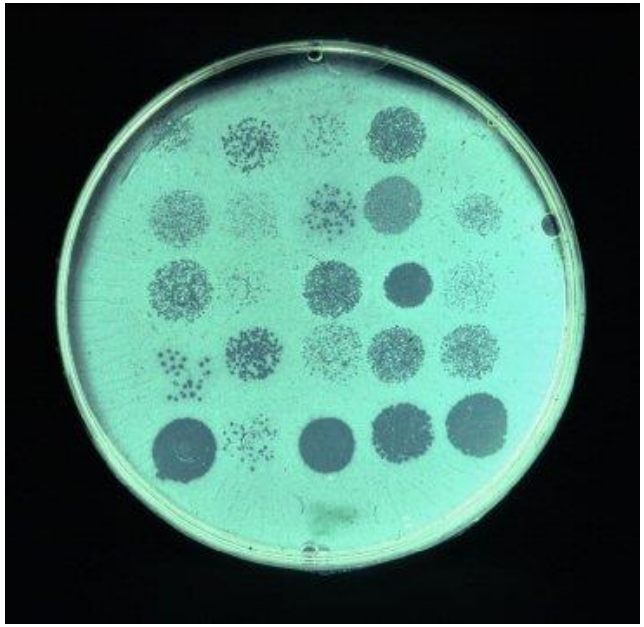
- A plate is heavily inoculated so that there are no uninoculated areas
- The plate is marked off in squares (15-20 mm) and each square inoculated with a drop of suspension for different phages.

Heavily Inoculated Plate

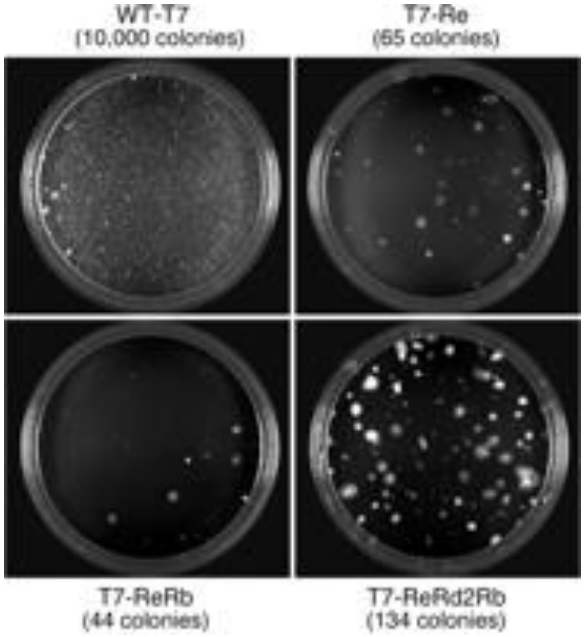


Bacteriophage Typing

- The plate is incubated for 24 hrs then observed for plaques.
- The phage type is reported as a specific genus and species followed by the types that can infect the bacterium.
- E.g. 10/16/24 means that the bacteria is sensitive to phages 10, 16 and 24.
- Phage tying remain a tool for research and reference labs.



A bacterial lawn inoculated with a range of bacteriophage



Unculturable Organisms

- **Environmental researchers estimate that < 1% of microorganisms are culturable and therefore it is not possible to use phenotypic methods of identification.**

- **These microorganisms are called viable nonculturable (VNC).**

Flow Cytometry

- **Classical techniques are not successful in identification of those microorganisms that cannot be cultured.**
- **Flow cytometry allows single or multiple microorganisms detection an easy, reliable and fast way.**
- **In flow cytometry microorganisms are identified on the basis of the cytometry parameters or by means of certain dyes called fluorochromes that can be used independently or bound to specific antibodies.**

Flow Cytometry

- The cytometer forces a suspension of cells through a laser beam and measures the light they scatter or the fluorescence the cell emits as they pass through the beam.
- The cytometer also can measure the cell's shape, size and the content of the DNA or RNA.

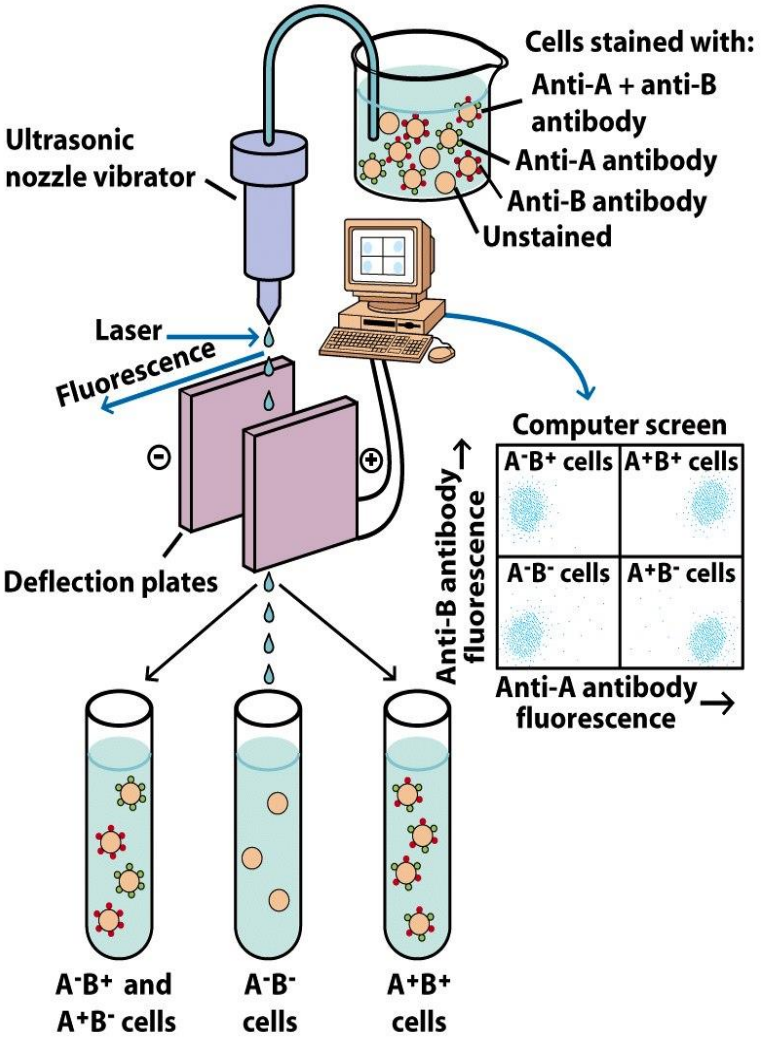
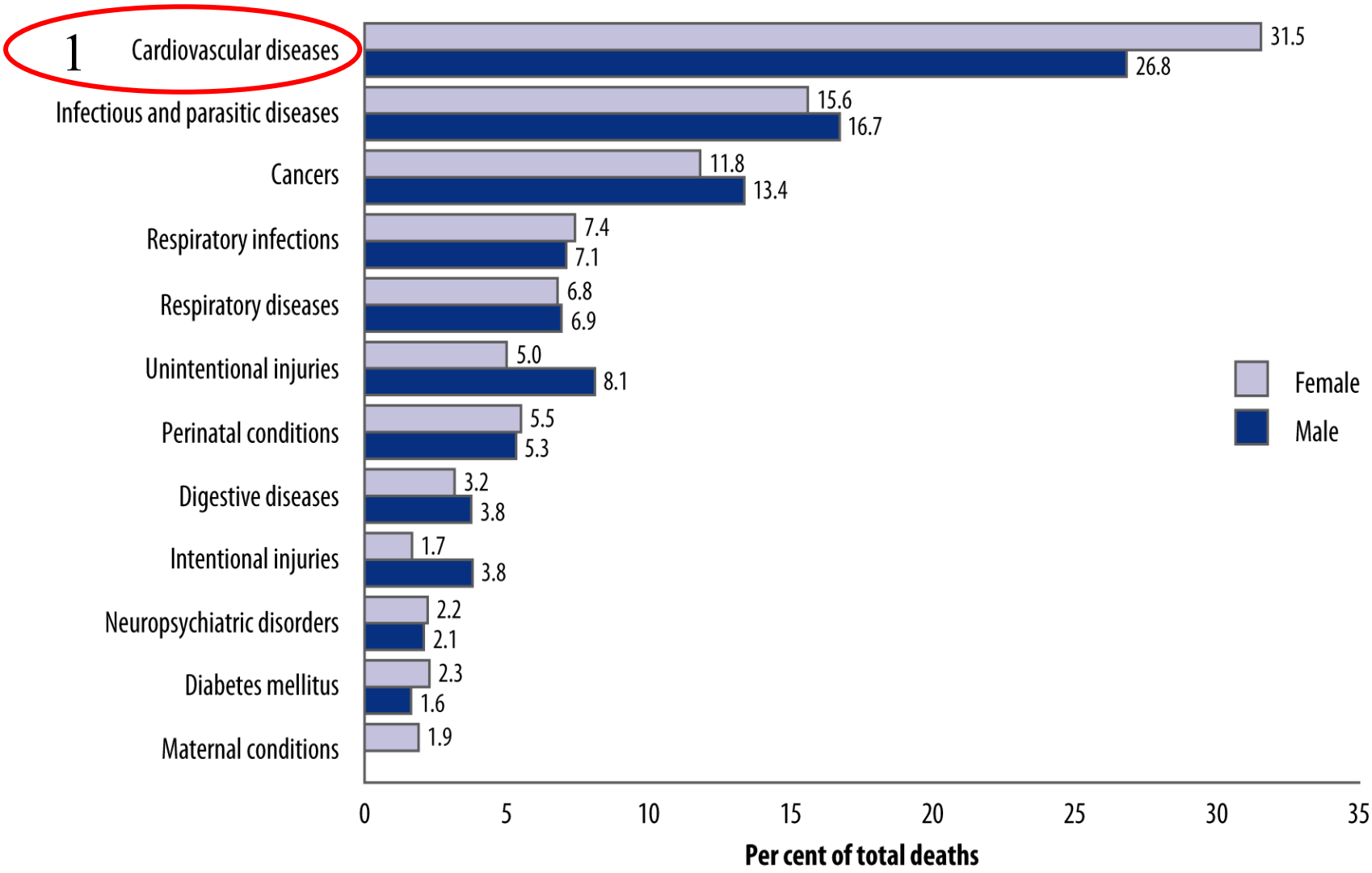


Figure 6-15
Kuby IMMUNOLOGY, Sixth Edition

Distribution of deaths by leading cause groups (2004)



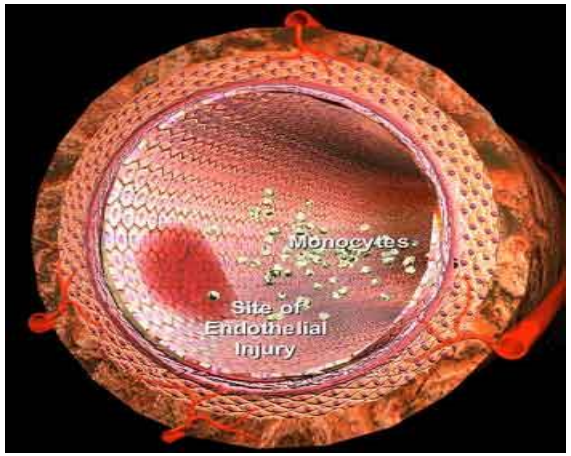
Detection of heart disease

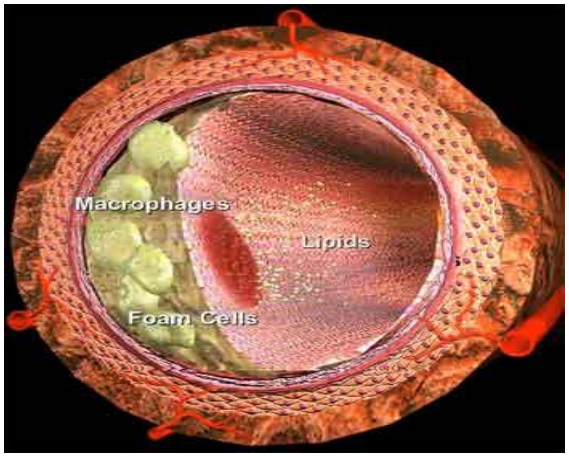
- **Coronary Heart Disease (CHD)**

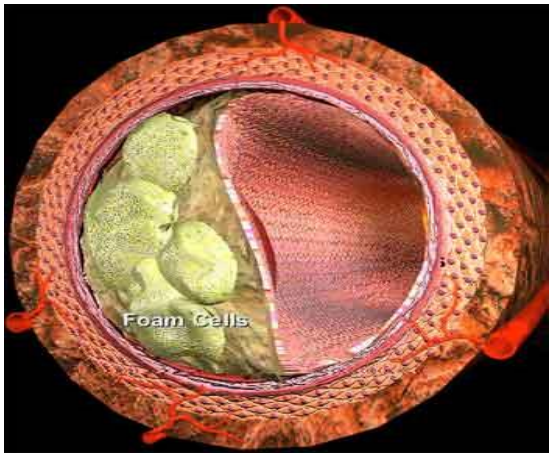
  The major underlying cause is *atherosclerosis*. *Atherosclerosis* is a slow, progressive disease which begins in childhood and takes decades to advance

Plaque (the build-up of lipid/cholesterol) in the artery wall forms as a response to *injury* to the endothelium in the artery wall

Plaque formation

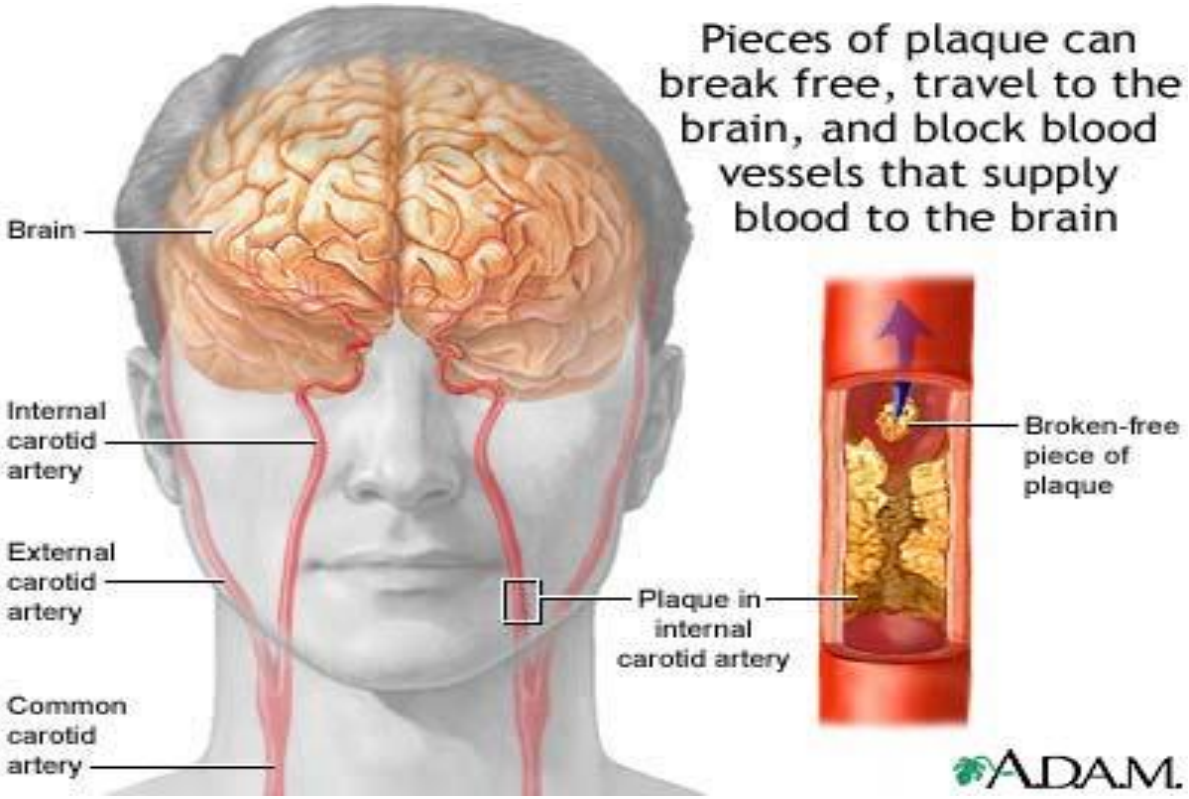




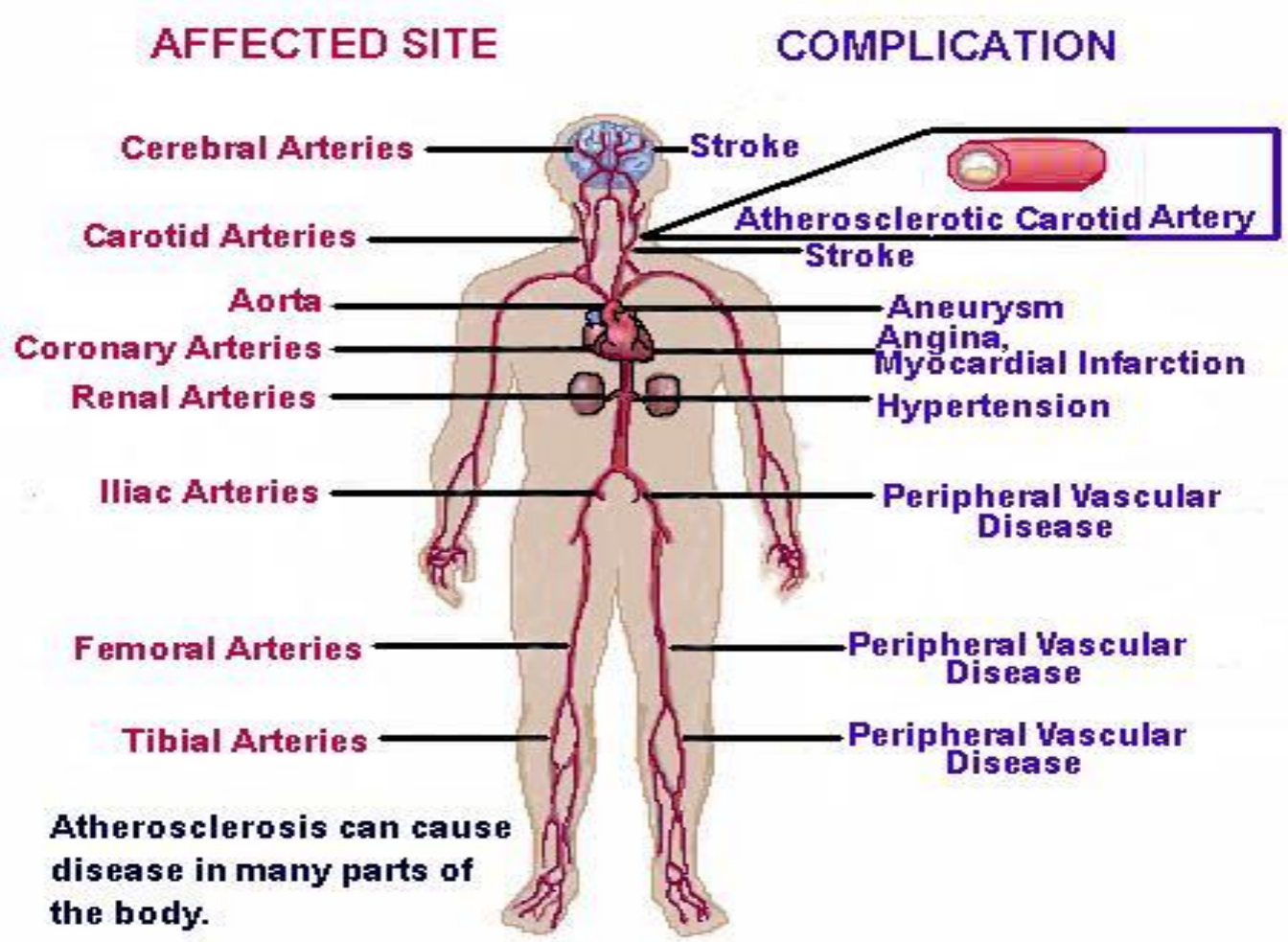


https://www.youtube.com/watch?v=NZ14XjOQoFY

Plaque formation



Plaque formation

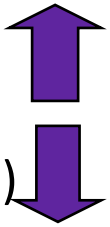


Risk Factors for coronary heart disease

- **Hypertension**
  - Appears to weaken the artery wall at points of high pressure leading to injury and invasion of cholesterol.
- **Cigarette Smoking**
  - #1 cause of preventable death in US
  - 1 in 5 CHD deaths attributable to smoking
- **Diabetes**
  - 50% of deaths related to DM is due to CHD
- **Inactivity**
  - Sedentary person has 2x risk for developing CHD as a person who is active.
- **Obesity**

Risk Factors for coronary heart disease

Abnormal Blood Lipids



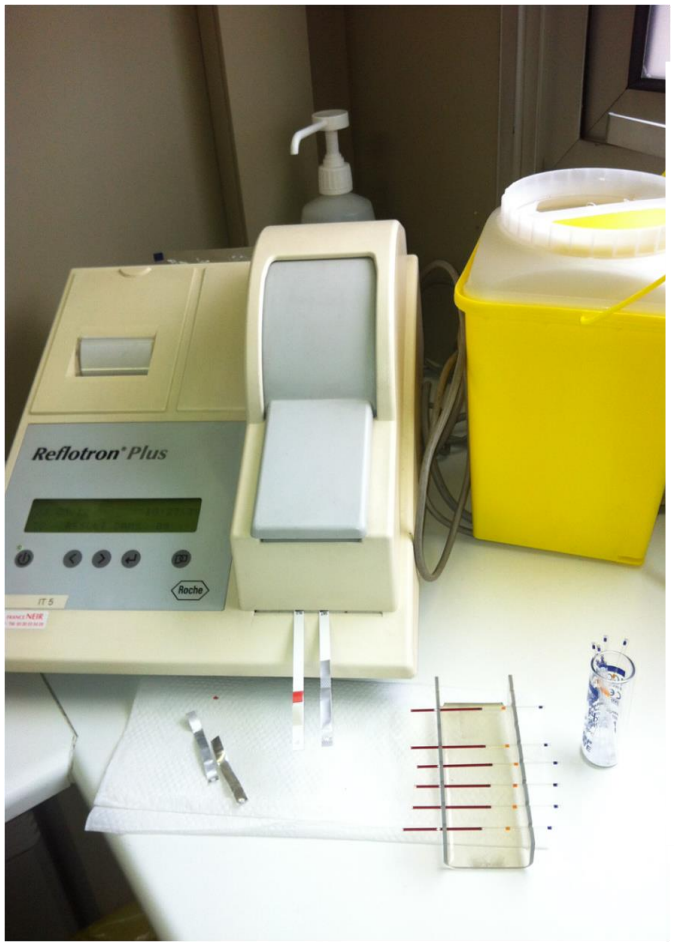
- LDL Cholesterol (low density lipoprotein)
- HDL Cholesterol (high density lipoprotein)

- Some LDL-C can be oxidized and takes up by endothelial cells and macrophages in the arterial wall, which leads to the first stages of atherosclerosis
- HDL-C is thought to be involved in the transport of excess cholesterol from membranes to the liver for removal from the body
- HDL-C is increased following exercise, loss of weight, and moderate consumption of ETOH.
- HDL-C is lowered:
  - Obesity, inactivity, cigarette smoking, some oral contraceptives and steroids, hypertriglyceridemia and some genetic factors.

Risk Factors for coronary heart disease

Blood Lipid	Classification
Cholesterol:	
<200 mg/dl	Desirable
200-239 mg/dl	Borderline high-risk
>240 mg/dl	High-risk
HDL Cholesterol	
<40 mg/dl	Low
>60 mgl/dl	High, negates one risk factor
LDL Cholesterol	
<100 mg/dl	Desirable
100 – 129 mg/dl	Above optimal
130-159 mg/dL	Borderline High
>160 mg/dl	High Risk

Metabolite testing



Heure prise alimentaire : 7H30

Nature : BOL DE CEREALES + LAIT + THE AU LAIT NON SUCRE + CAFE SANS SUCRE (9H)

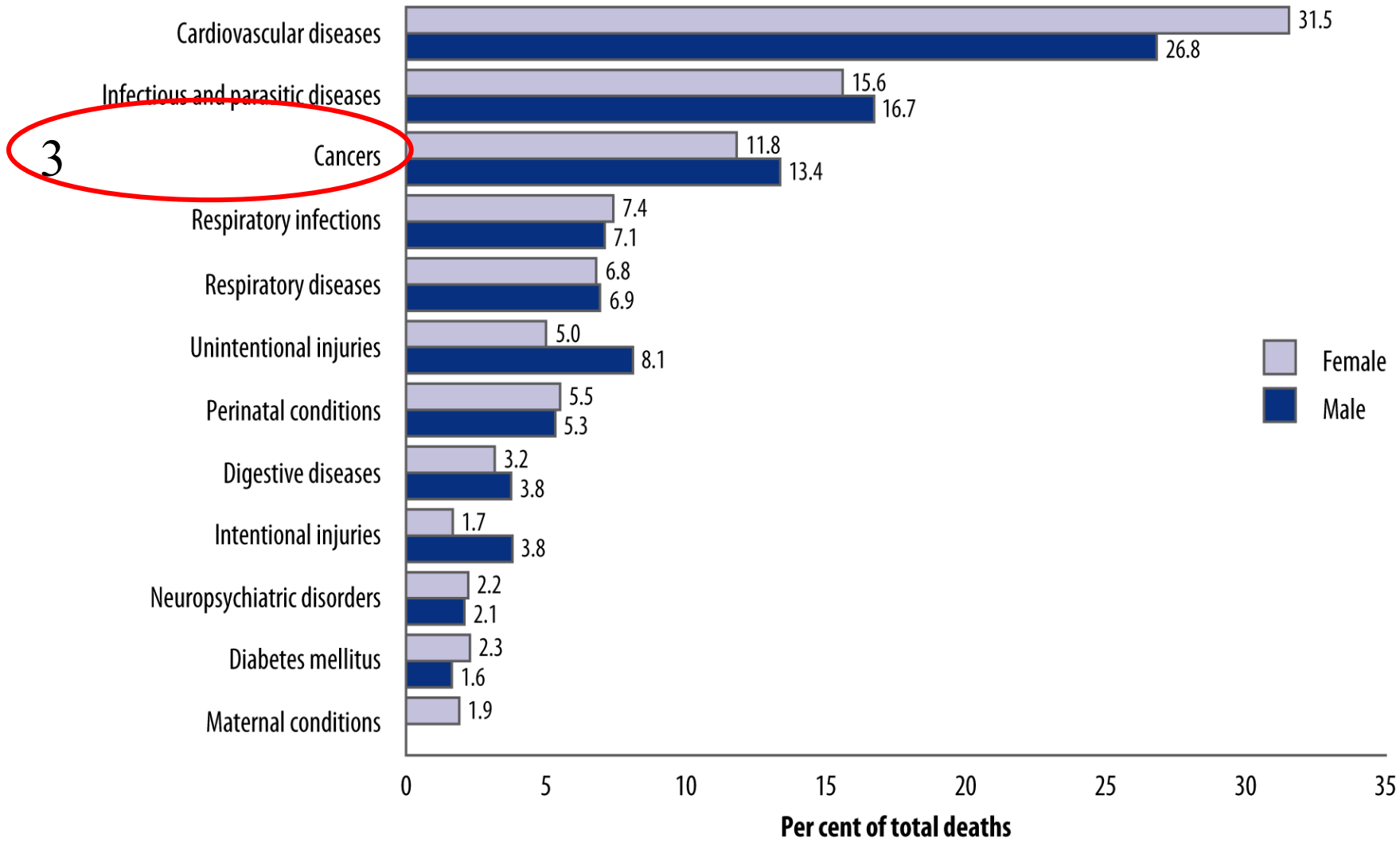
X	GLUCOSE	76 à 110 mg/dl	
X	CHOLESTEROL	Moins de 200 mg/dl (selon l'âge)	
	CHOLESTEROL HDL		
X	TRIGLYCÉRIDES	Moins de 200 mg/dl (selon l'âge)	
	CRÉATININE	H 0,5–1,10 mg/dl	F 0,5 à 0,9 mg/dl
	ACIDE URIQUE	H 7 mg/dl	F 5,7 mg/dl
	URÉE	10 à 50 mg/dl	
	BILIRUBINE	0,5 à 12 mg/dl	
X	GAMMA GT	H 11 à 40 UI/l	F 7 à 32 UI/l
X	S.G.O.T.	H 40 UI/l	F 33 UI/l
X	S.G.P.T.	H 41 UI/l	F 32 UI/l
	HÉMOGLOBINE	5 à 20 mg/l	

```
23.03.12          10:29:09
16
TG     91.3       mg/dl

23.03.12          10:31:52
17
CHOL   196        mg/dl

23.03.12          10:34:16
18
GPT    7.51 U/l     25°C

23.03.12          10:36:23
19
GLU    95.9       mg/dl

23.03.12          10:38:23
20
GGT    4.56 U/l     25°C

23.03.12          10:40:39
21
GOT    13.3 U/l     25°C
```

Other markers of CHD

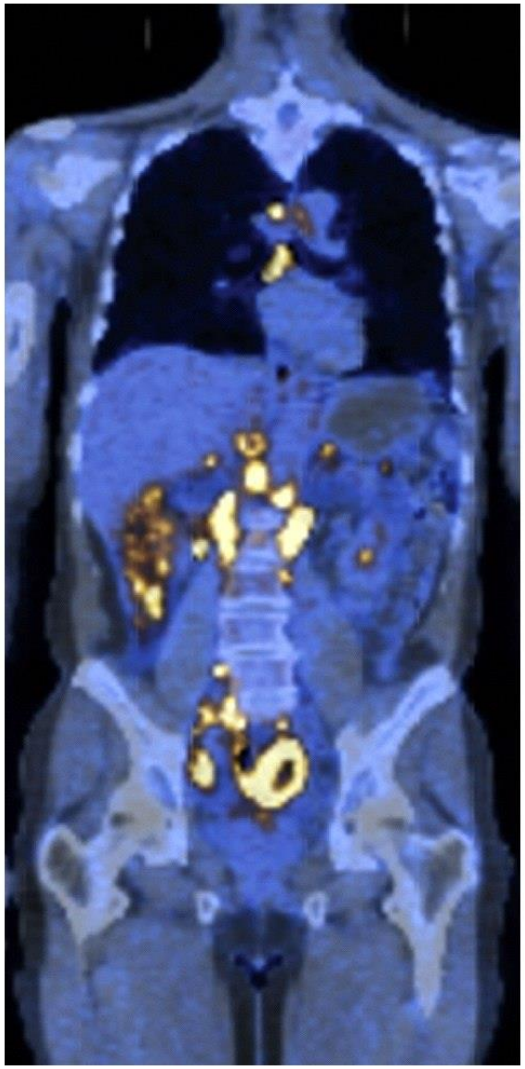
Recent research suggests that patients with elevated basal levels of CRP are at an increased risk of diabetes, hypertension and cardiovascular disease

Distribution of deaths by leading cause groups (2004)



3

Cancer: Where do we stand?



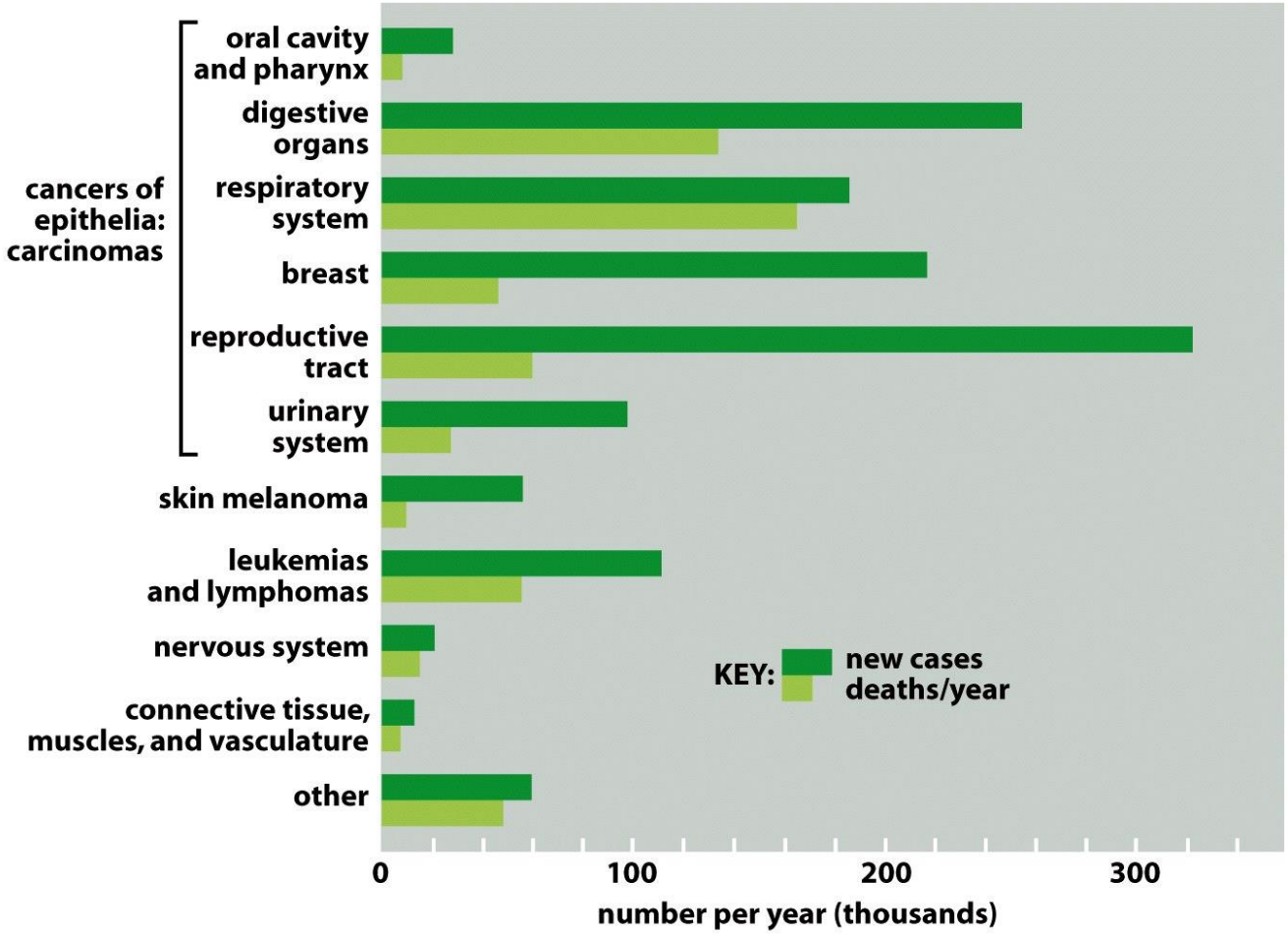


Figure 20-1 *Molecular Biology of the Cell* (© Garland Science 2008)

Early detection is key!

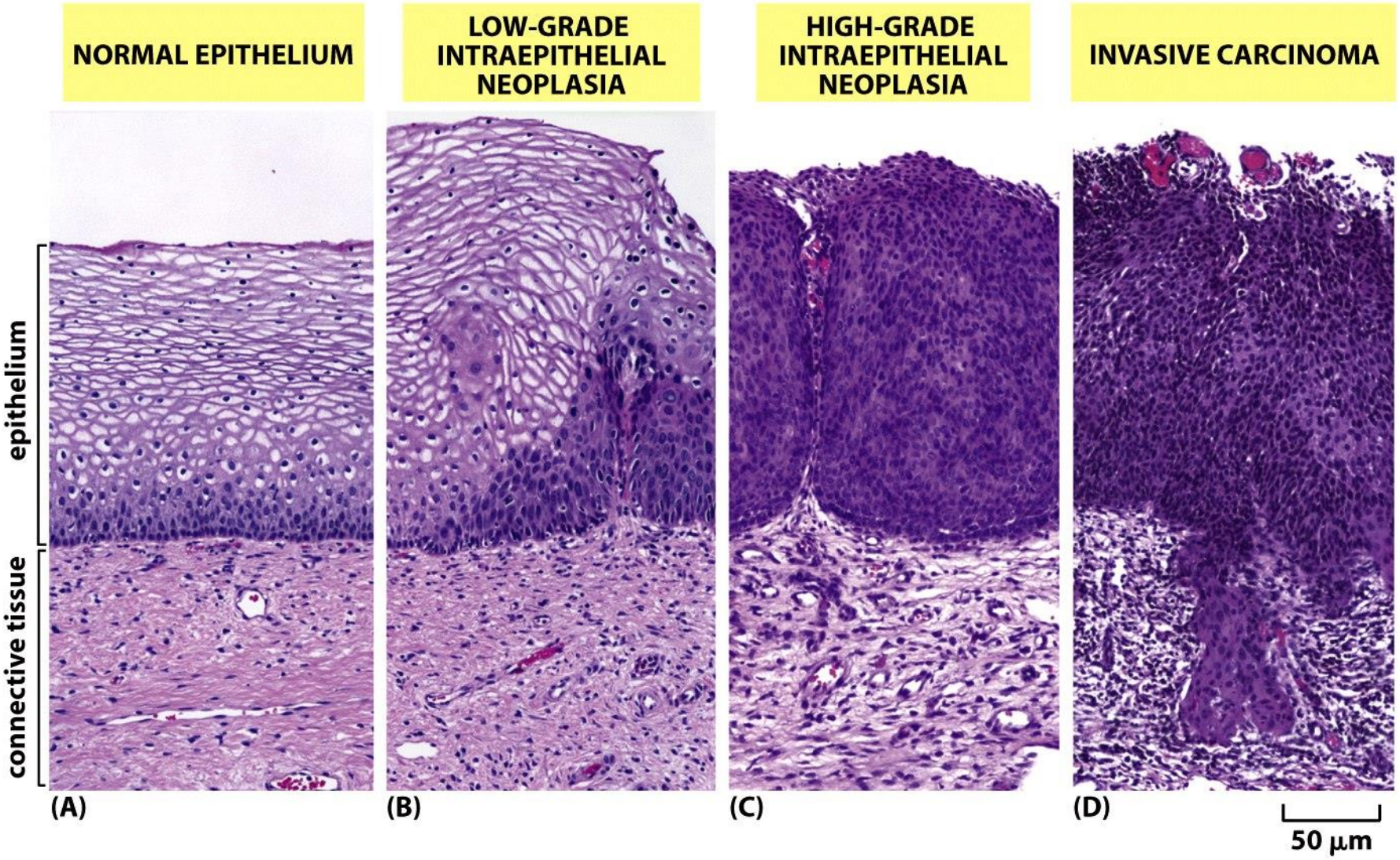


Figure 20-9 *Molecular Biology of the Cell* (© Garland Science 2008)

Cancer

Early detection is key: pap smear

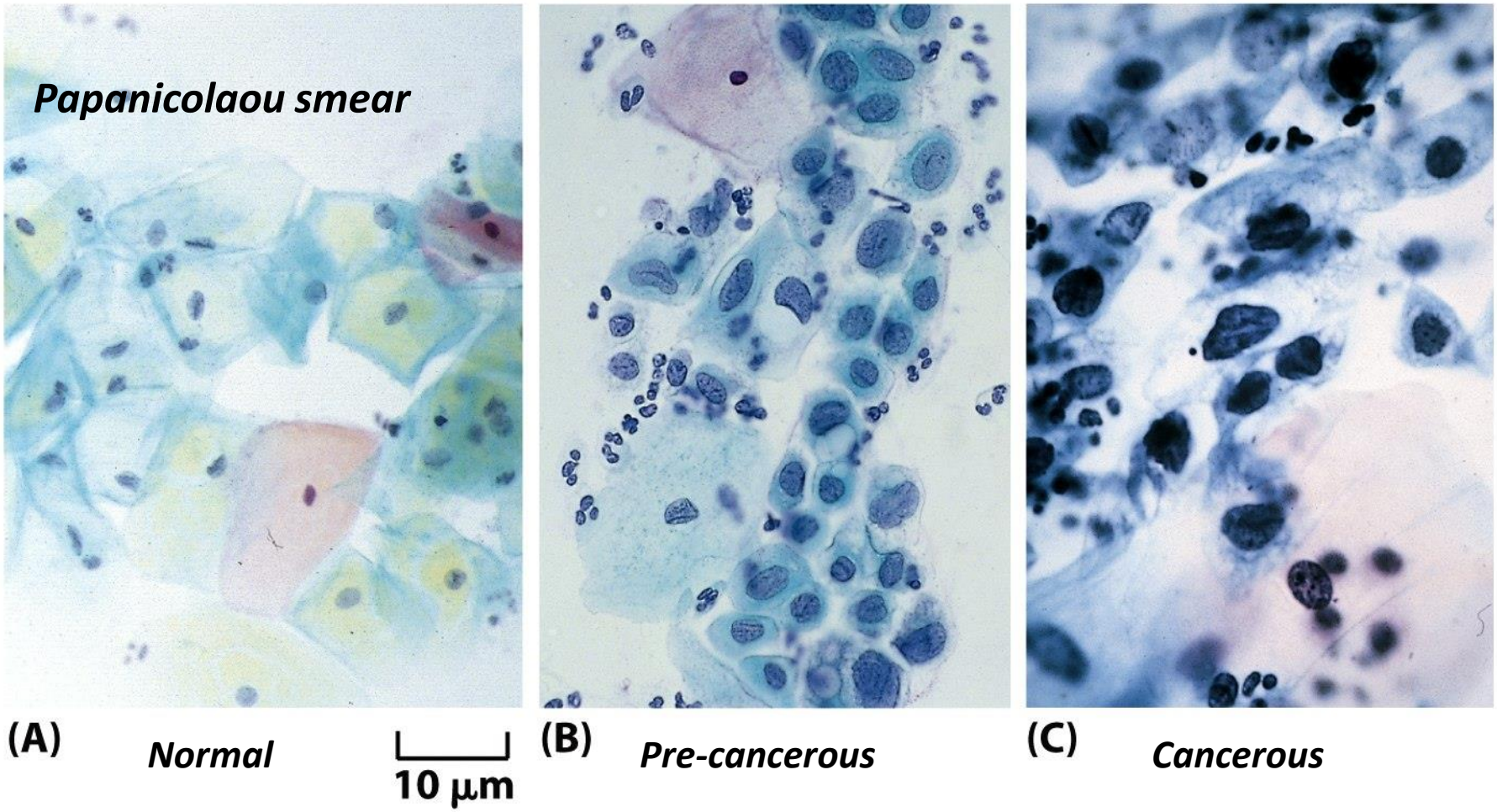
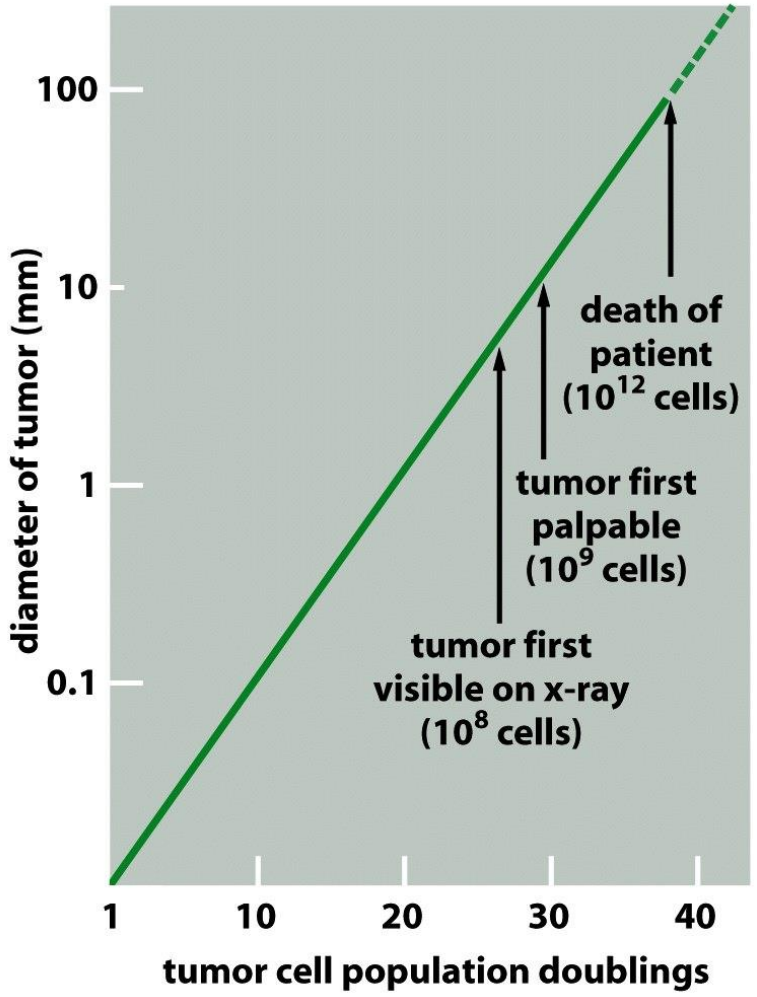


Figure 20-10 *Molecula* 2008)

MINES Saint-Étienne

Cancer

Cancer statistics



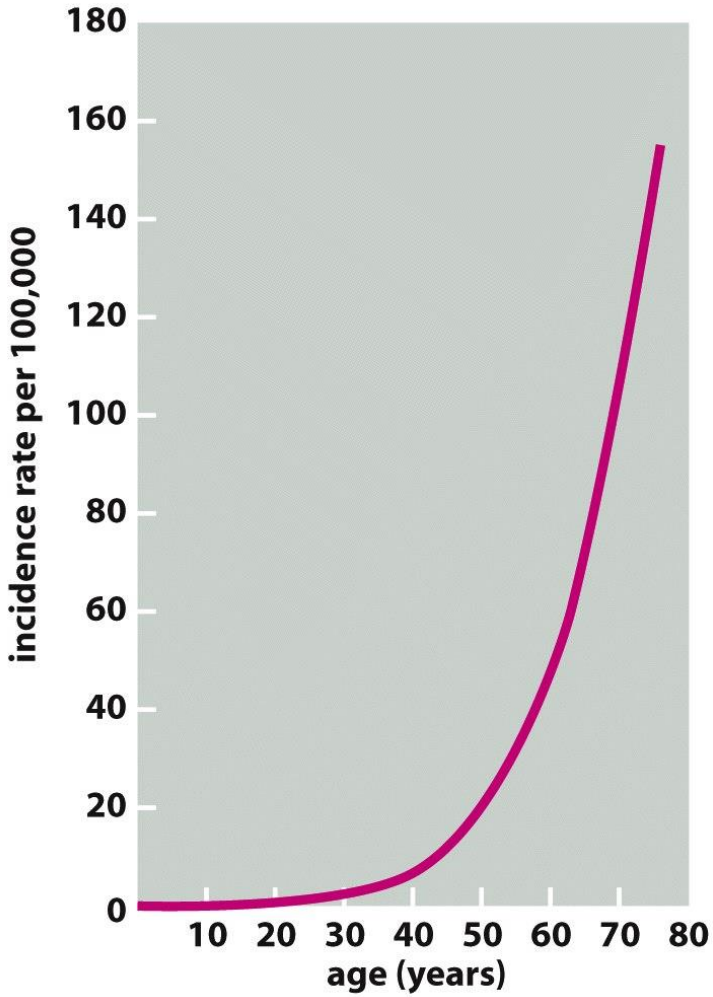


Figure 20-4 *Molecular Biology of the Cell* (© Garland Science 2008)

Tumor markers

What are they?

- Are substances usually proteins, that are produced by the body in response to cancer growth or by the cancer tissue itself and certain benign (noncancerous) conditions
- Detected in higher than normal amounts in the blood, urine, or body tissues
- Some tumor markers are specific for one type of cancer, while others are seen in several cancer types
- Measurements can be useful – when used along with x-rays, or other tests in the detection and diagnosis of some types of cancer

Cancer

Metastasis

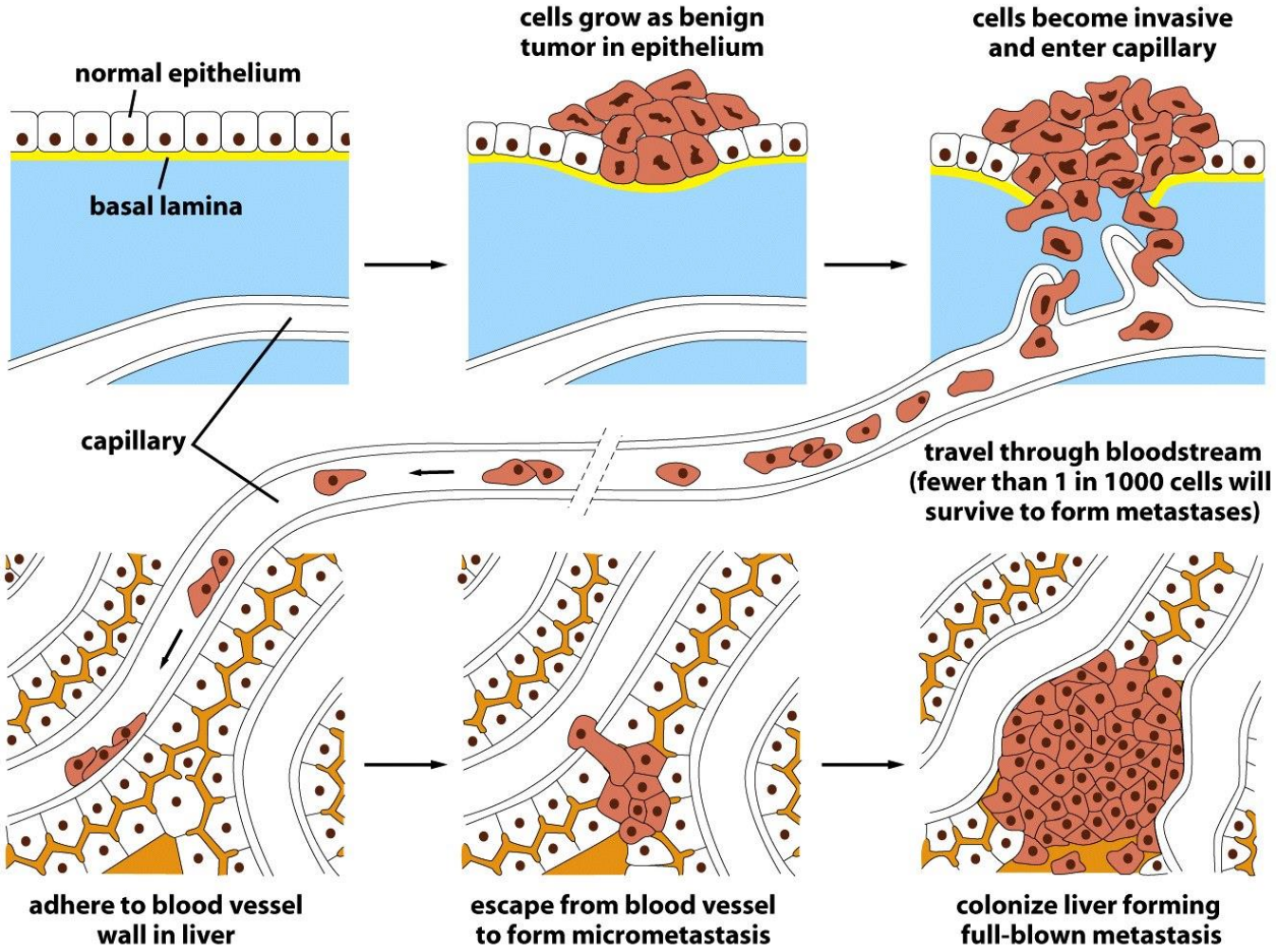


Figure 20-17 *Molecular Biology of the Cell* (© Garland Science 2008)

Cancer

Metastasis

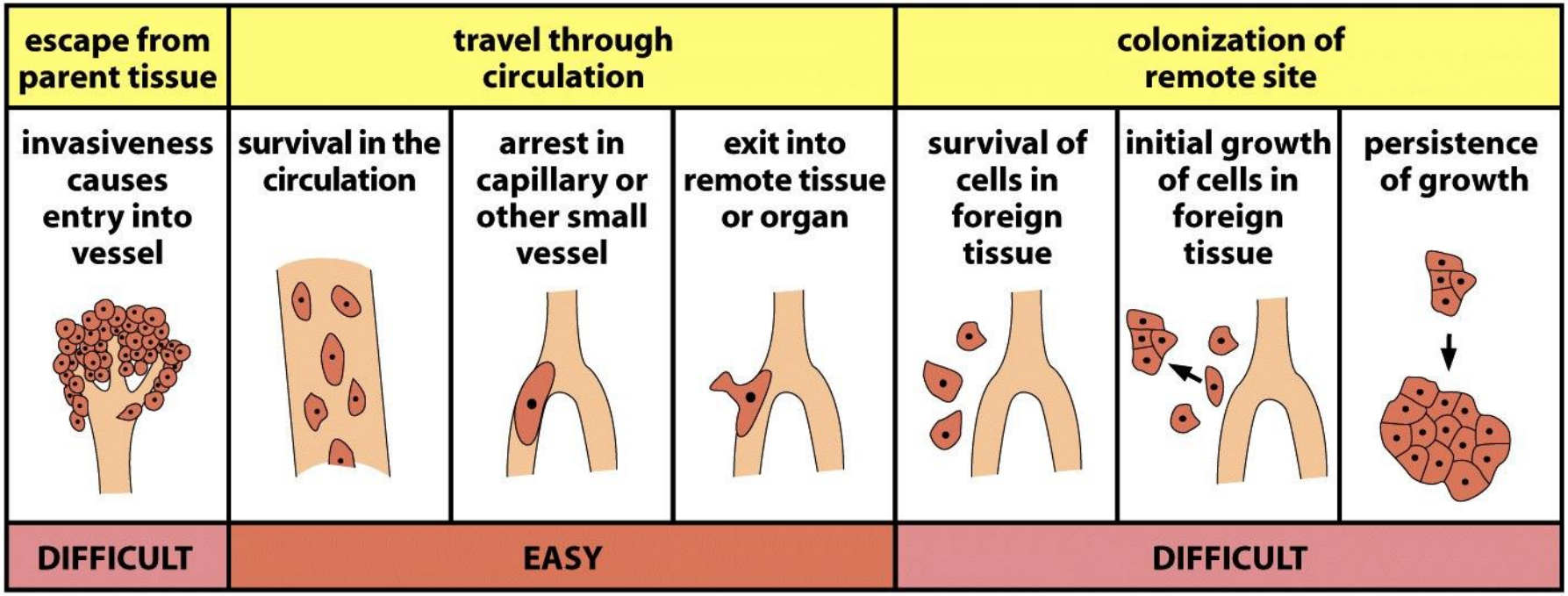


Figure 20-44 *Molecular Biology of the Cell* (© Garland Science 2008)

Colon adenocarcinoma metastasis in lung

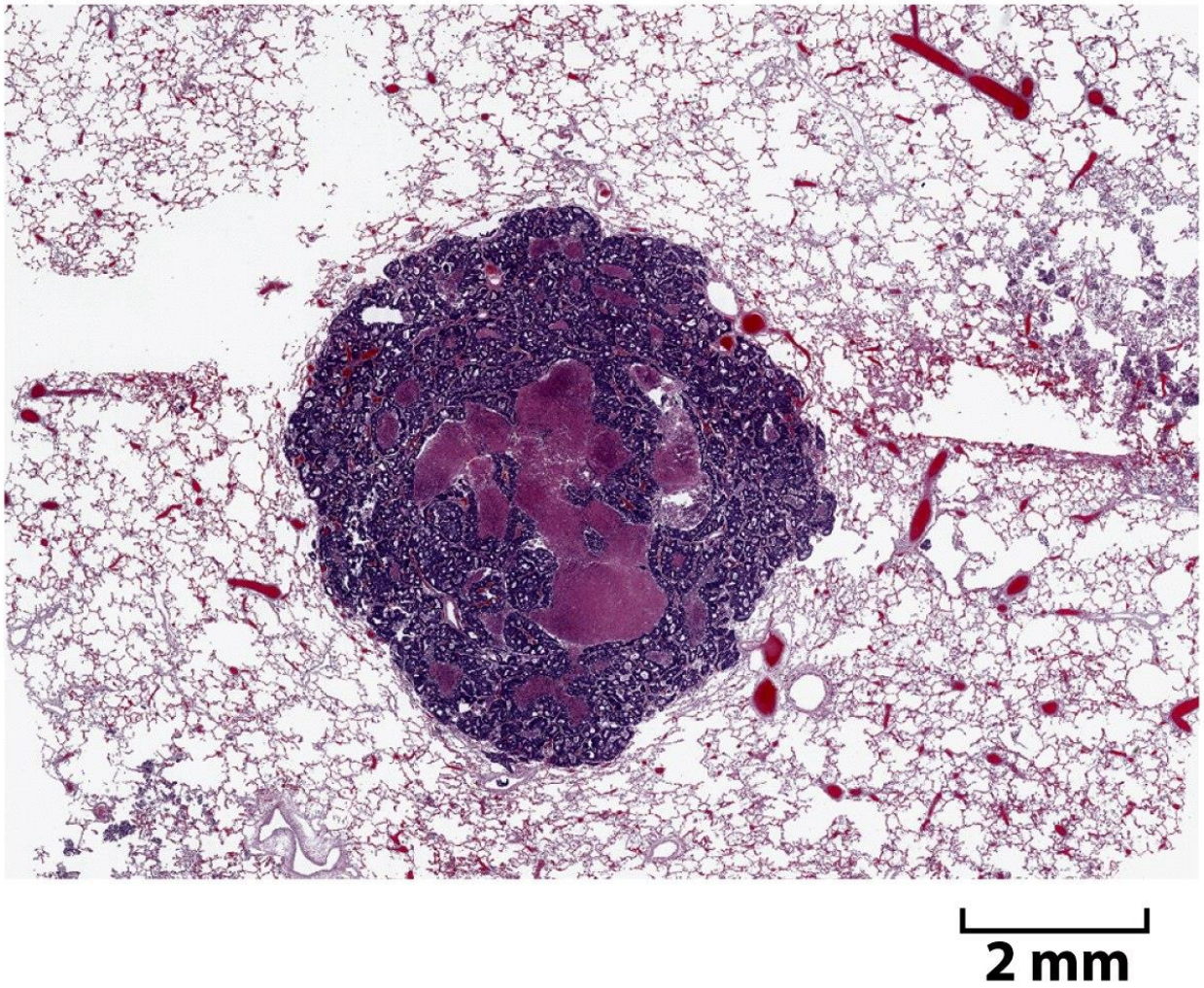


Figure 20-18 *Molecular Biology of the Cell* (© Garland Science 2008)

Tumor markers

- **Measurements of tumor marker levels alone are not sufficient to diagnose cancer for the following reasons:**
  - Tumor marker levels can be elevated in people with benign conditions
  - Tumor marker levels are not elevated in every person with cancer – especially in early stages of the disease
  - Many tumor markers are not specific to a particular type of cancer

Tumor markers

Characteristics required of the “ideal” tumor marker

- The following are desirable
  - 100% accuracy in differentiating between healthy individuals and tumor patients
  - Ability to detect all tumor patients, if possible at a very early stage
  - Organ specificity, so that information is provided on the localization of the tumor
  - Correlation between the concentration of the marker freely circulating in serum and the individual tumor stages
  - Ability to indicate all changes in tumor patients receiving treatment
  - Prognostic value of the tumor marker concentration

Tumor markers

Clinical Uses of Tumor Markers

- Early detection of the tumor
- Differentiating benign from malignant conditions
- Evaluating the extent of the disease
- Monitoring the response of the tumor to therapy
- Predicting the recurrence of the tumor

Tumor markers

- **CARCINO-EMBRYONIC ANTIGEN (CEA)**
  - A complex glycoprotein with a MW of approximately 180,000 daltons
  - First discovered in patients with adenocarcinoma of the colon in 1965
  - Metabolized primarily by the liver with a circulating half-life ranging from 1 to 8 days
  - Hepatic diseases, including extrahepatic biliary obstruction, intrahepatic cholestasis and hepatocellular disease, may impede clearance rates and increase serum concentrations

Tumor markers

- **CARCINO-EMBRYONIC ANTIGEN (CEA)**
  - Normally, it is present in the fetal intestine, pancreas and liver during the first 2 trimesters of gestation
  - Normal colonic mucosa and pleural and lactating mammary tissue bind to anti-CEA antiserum; however, the quantity of CEA or CEA-like molecules expressed in these tissues is much less than that observed in malignant tumors
  - Normal range is from 0 to 2.5 to 3.0 ng/ml as determined by radioimmunoassay

Tumor markers

- **CARCINO-EMBRYONIC ANTIGEN (CEA)**

  Benign conditions that cause elevated CEA

  - Cigarette smoking
  - Emphysema
  - Gastric ulcer
  - Pancreatitis
  - Diverticulitis
  - BPH
  - Bronchitis
  - Gastritis
  - Hepatic disease
  - Polyps of colon & rectum
  - Crohn’s disease
  - Renal disease

Tumor markers

CARCINO-EMBRYONIC ANTIGEN (CEA)

- Malignant conditions causing elevation of CEA in addition to adenocarcinoma of colon & rectum --- Ca of the pancreas, lung, breast, stomach, thyroid gland and female reproductive tract
- Of these non-colonic CA, levels of CEA are most commonly elevated in CA of the pancreas (65-90%) and lung (52-77%)
- The magnitude of elevation of levels of CEA correlates with stage of disease to a lesser extent

Tumor markers

- **Alpha-FETOPROTEIN (AFP)**
  - An oncofetal protein that was first discovered in 1963 in the serum of mice with hepatoma
  - Normal fetal protein synthesized by the liver, yolk sac, and GIT that shares sequence homology with albumin
  - A major component of fetal plasma, reaching a peak concentration of 3mg/ml at 12 weeks of gestation -- following birth, it clears rapidly from the circulation, having a half-life of 3.5 days
  - Concentration in adult serum <20ng/ml

Tumor markers

- **Alpha-FETOPROTEIN (AFP)**
  - Benign conditions causing elevation of AFP
    - 2nd and 3rd trimesters of pregnancy
    - Cirrhosis
    - Acute and chronic hepatitis
    - Hepatic necrosis

Tumor markers

Alpha-FETOPROTEIN (AFP)

- Malignant conditions causing elevation of AFP aside from hepatoma
  - Teratocarcinoma of the testis and embryonal Ca (70%)
  - Carcinoma of the pancreas (23%)
  - Carcinoma of the stomach (18%)
  - Carcinoma of the lung (7%)
  - Carcinoma of the colon (5%)

  *** In patients with hepatoma, the incidence of elevation of levels of AFP correlates with tumor burden

Tumor markers

- **CA 19-9**
  - A monoclonal antibody generated against a colon carcinoma cell line to detect a monosialoganglioside found in patients with gastrointestinal adenocarcinoma
  - Elevated in gastric cancer (21-42%), colon cancer (20-40%), pancreatic cancer (71-93%)

Tumor markers

PROSTATE-SPECIFIC ANTIGEN (PSA)

- Found in normal prostatic epithelium and secretions but not in other tissues
- It is a glycoprotein whose function may be to lyse the seminal clot
- Highly sensitive for the presence of prostatic cancer
- Elevation correlated with stage and tumor volume
- Predictive of recurrence and response to treatment
- Has prognostic value in patients with very high values prior to surgery are likely to relapse

Tumor markers

- **PROSTATE-SPECIFIC ANTIGEN (PSA)**
  - Present in low concentrations in the blood of adult males
  - It is produced by both normal and abnormal prostate cells
  - Benign elevations – prostatitis and BPH

Common tumour markers currently in use

Tumor Markers	Cancers	What else?	Sample
AFP (Alpha-fetoprotein)	Liver, germ cell cancers of ovaries or testes	Also elevated during pregnancy	blood
CA 15-3	Breast and others including lung and ovaries	Also elevated in benign breast conditions;	blood
CA 19-9	Pancreatic, sometimes colorectal and bile ducts	Also elevated in pancreatitis and inflammatory bowel disease	blood
CA 125	Ovarian	Also elevated with endometriosis, some other diseases and benign conditions; not recommended as a general screen	blood

Criteria for diagnostics

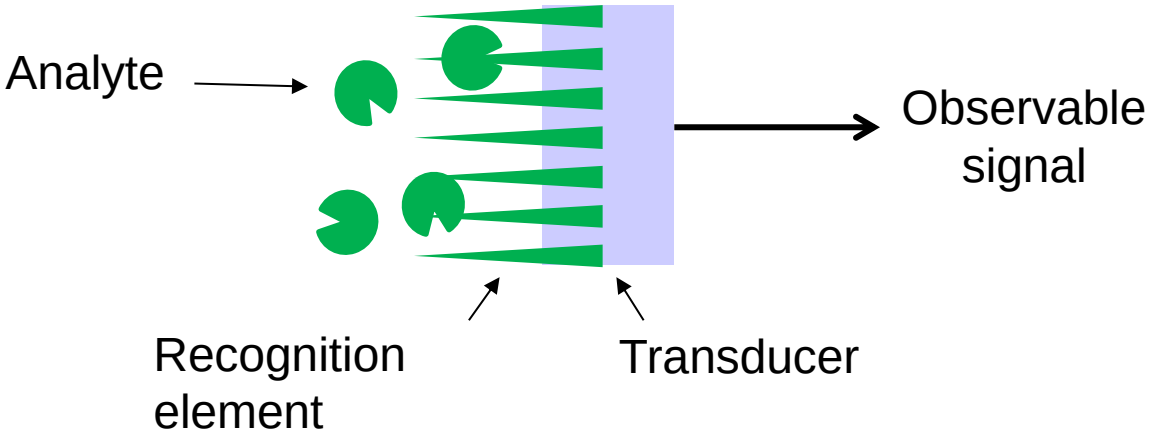
There are three golden Ss for diagnostics:

- ❑Speed
- ❑Sensitivity
- ❑Specificity

An important fourth parameter is COST

- Multiplexing becoming essential
- Miniaturisation

What is a biosensor?



Recognition element: interacts selectively with a biological or chemical analyte.

Transducer: translates this specific interaction into an observable signal.

Biosensor classification

Biosensors can be classified according to three schemes:

***(a) the receptor type,* e.g., an immunosensor**

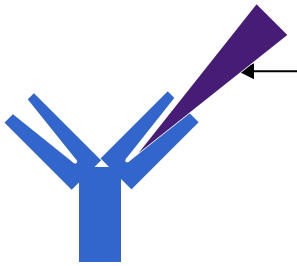
***(b) the physics of the transduction process*, e.g., an amperometric sensor**

***(c) the application*, e.g., a medical biosensor**

Transducer types

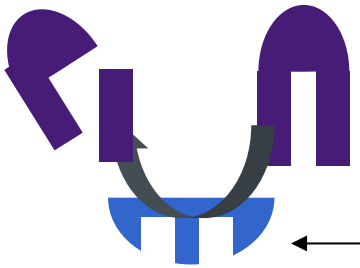
- **Electrical (amperometric, potentiometric)**
- **Mechanical (piezoelectric, acoustic microbalances)**
- **Optical (e.g. the eye, absorbance readers, SPR….**

Biorecognition elements



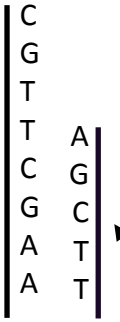
antigen
(analyte)

antibody
(biorecognition element)



biomolecule
(analyte)

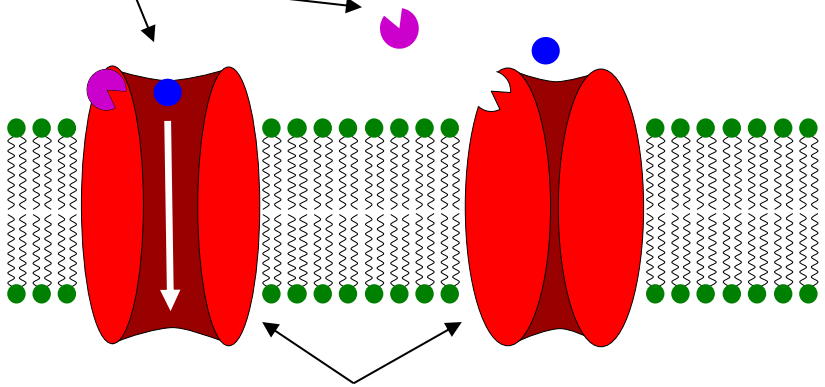
enzyme
(biorecognition element)



DNA
(analyte)

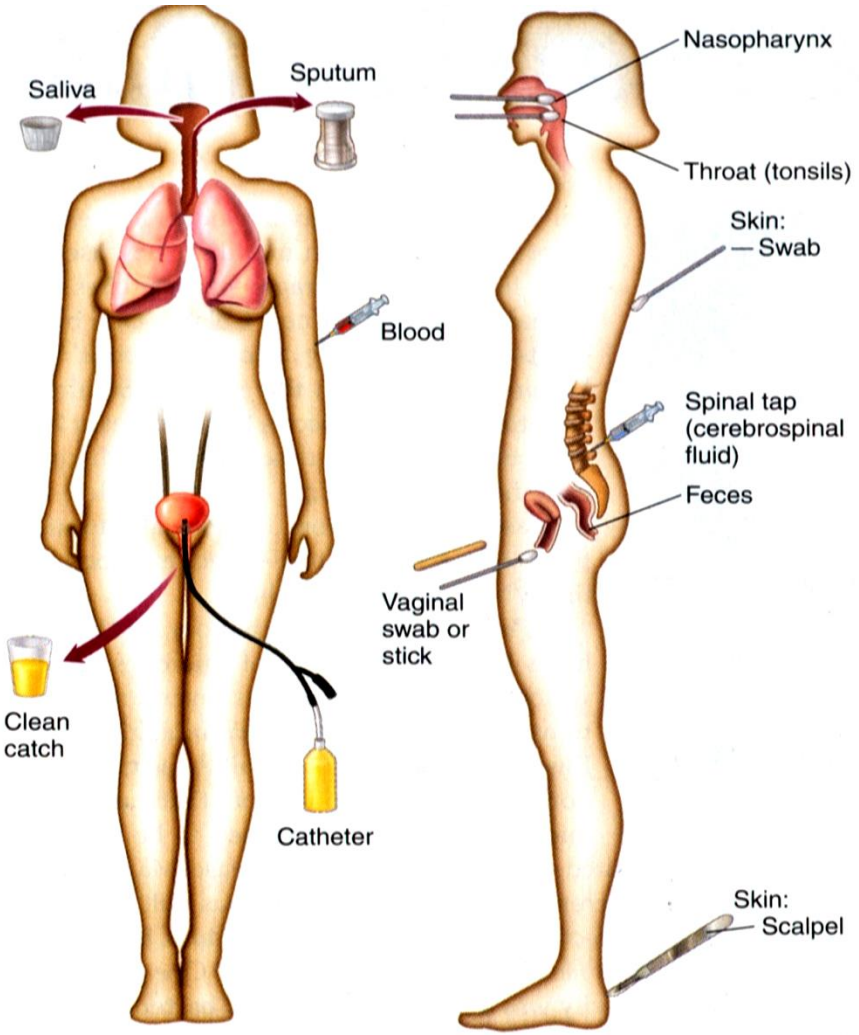
oligonucleotide
(biorecognition element)

biomolecule
(analyte)



gated ion channel
(biorecognition element)

Sample volume



Invasiveness determines sample volume

Transducer types

Classify these sensor types

- Fluorescence
- DNA Microarray
- Surface plasmon resonance (SPR)
- Impedance spectroscopy
- Scanning probe microscopy (SPM) (AFM, STM)
- Quartz crystal microbalance (QCM)
- Surface Enhanced Raman Spectroscopy(SERS)
- Electrochemical

Electrical Biosensors

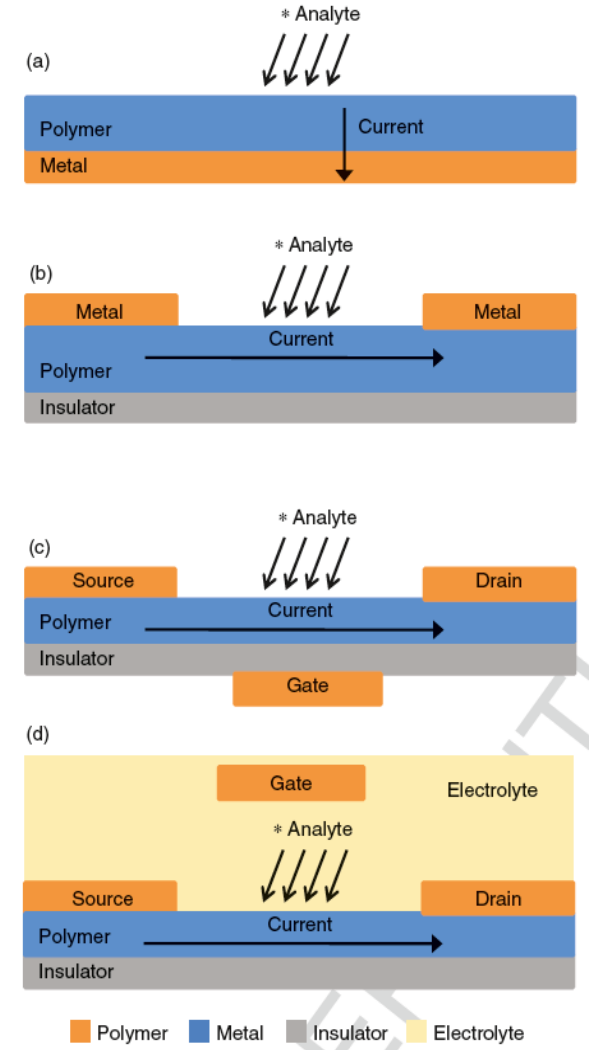


Figure 2 Different configurations for electrical sensors: (a) electrochemical sensor; (b) chemiresistor; (c) organic field-effect transistor; and (d) organic electrochemical transistor

Electrochemical

Chemiresistor

OFET

OECT

Optical biosensors

- The eye!
- Fluorescence spectroscopy
- Absorbance spectroscopy
- Surface plasmon resonance

Mechanical biosensors

- Piezoelectric…

Why is Label-free Important?

- Labels in biological assays are chromophores/fluorophores or other tags added to allow detection of a desired molecule with a given platform
- Often required for specificity and sensitivity BUT:
  - Labelling of probes adds cost, time and steps
  - Labelling can generate artefacts
  - Limits user to kit from manufacturer, or available products

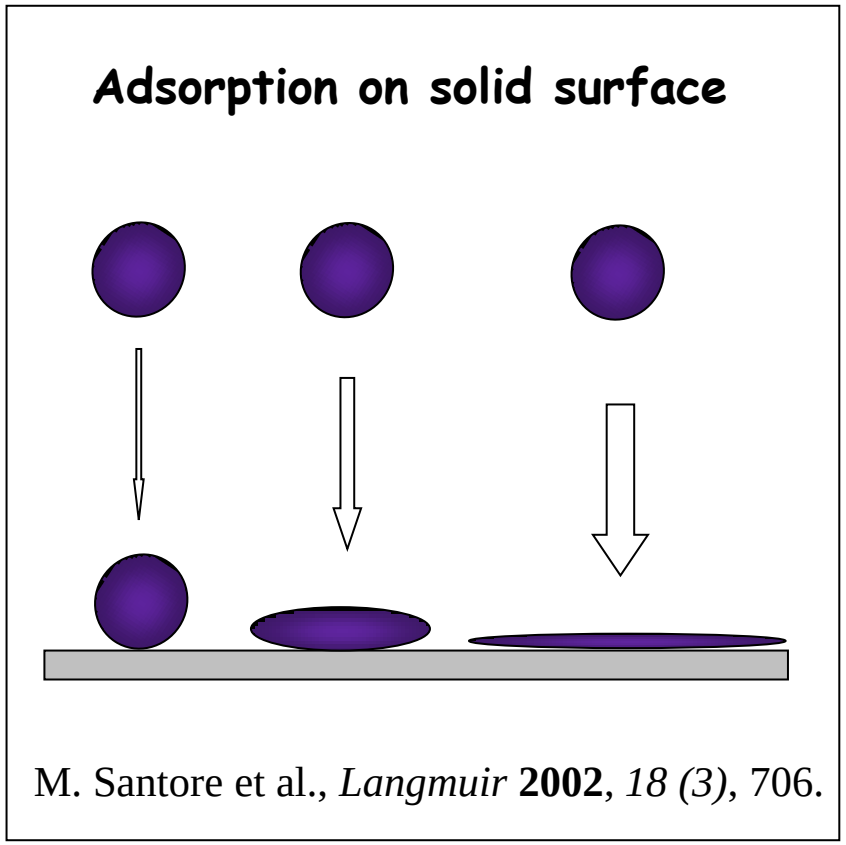
Some Examples of Label-free Pathogen Detection

1. Surface Plasmon Resonance (Biacore –GE healthcare)
2. Refractive Waveguide Grating Optical Sensor (Epic – Corning LifeSciences)
3. The Organic Electrochemical Transistor

Biofunctionalisation

1. Adsorption of the biological receptor directly to the biosensor's surface
2. Physical entrapment near the biosensor's surface (e.g., in a polymer layer)
3. Covalent coupling of the biological receptor directly to the biosensor's surface
4. Covalent coupling to a polymer layer on the biosensor's surface
5. Use of a chemical/biochemical "capture system"

Direct Adsorption on biosensor surface



M. Santore et al., *Langmuir* **2002**, *18 (3)*, 706.

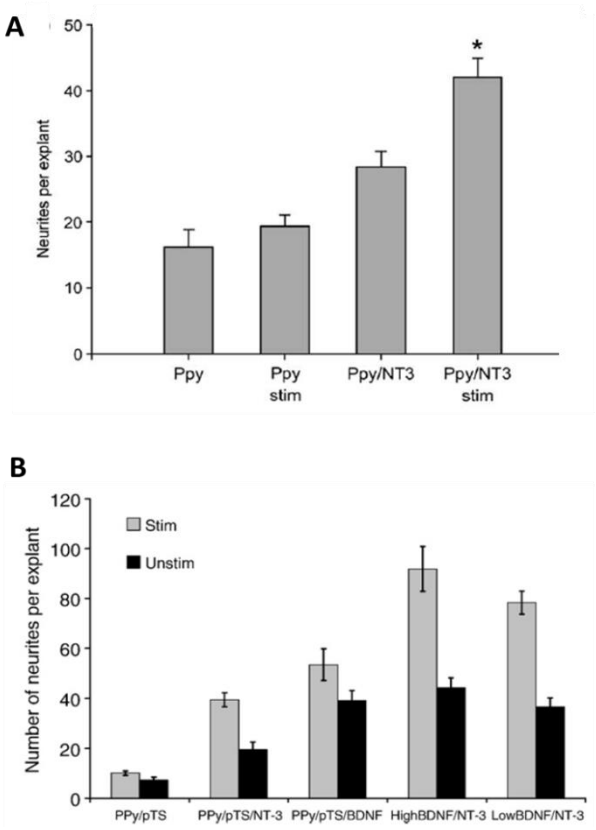
Denaturation of proteins by adsorption on solid surfaces

- strong attraction by van der Waals or hydrophobic interaction

⇒ Loss of biological function

Physical Entrapment

Electropolymerisation



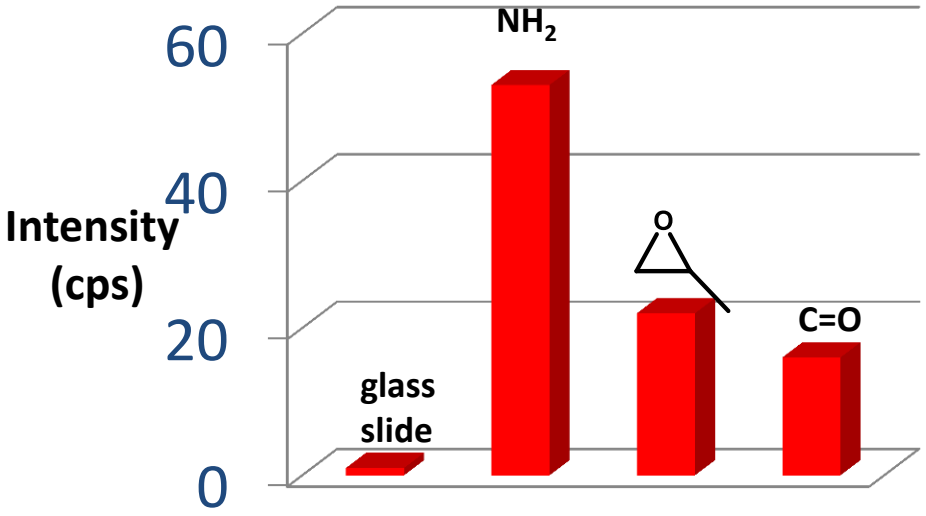
Richardson *et al, Biomaterials* (2007)

Thompson *et al*, *J Control Release* (2010)

Covalent modification of surface

1. Problem = immobilization of bioactive molecules for bio sensing
2. Solution = covalent binding of the proteins

Immobilised model protein with Cy5 dye – recorded fluorescence intensity



Antibody Immobilization on Silica Surface

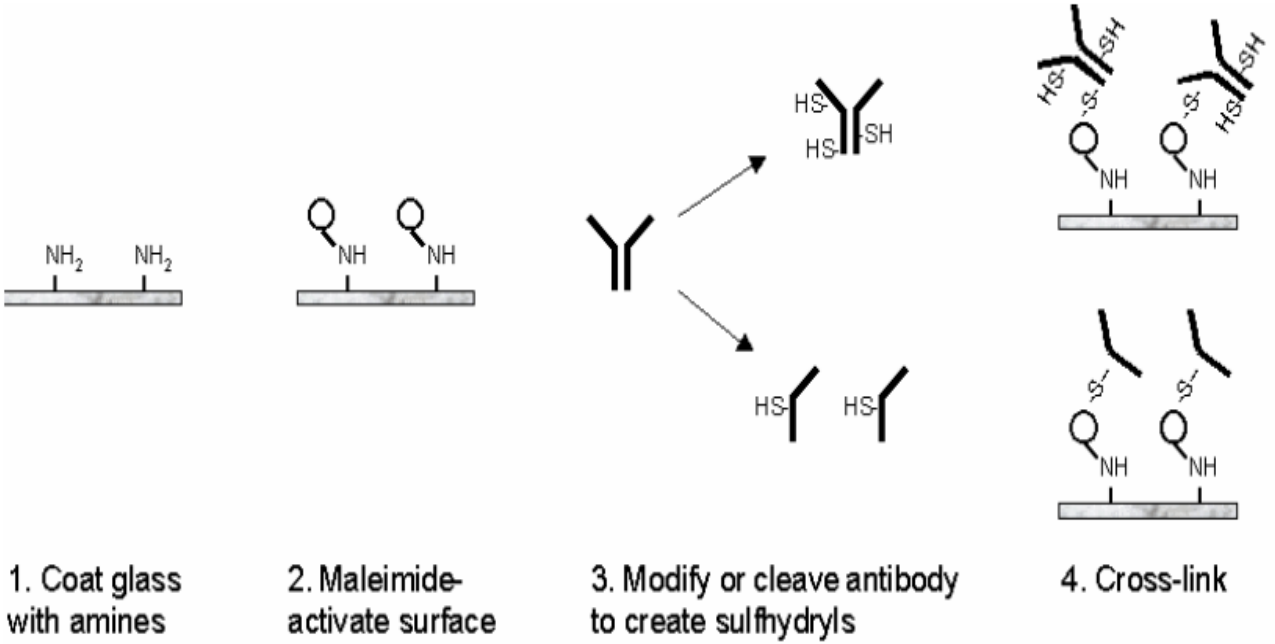
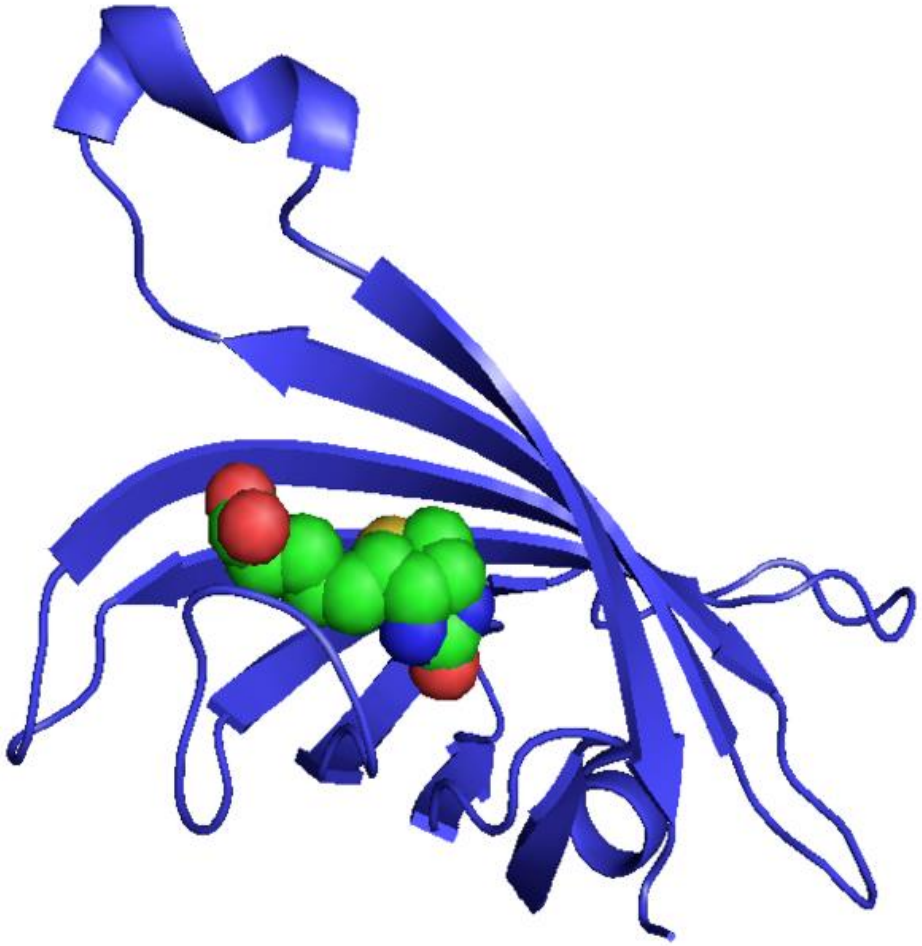


Figure 1. Basic steps involved in attaching an antibody onto a glass surface. See text and protocol for details. Functional groups and molecules are not drawn to scale.

Basic steps involved in attaching an antibody onto a glass surface (Pierce)

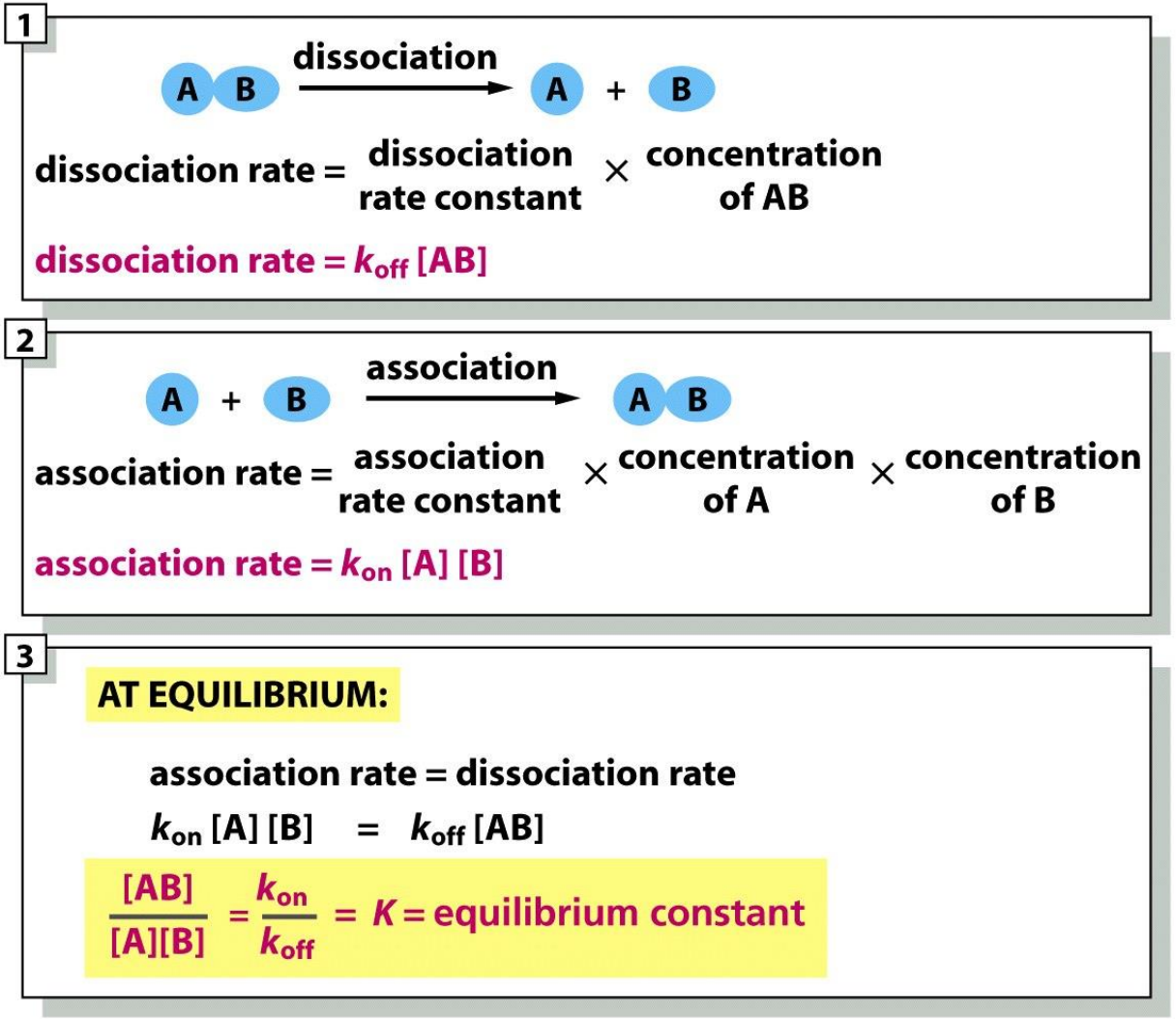
Use of a biochemical capture system

Streptavidin-biotin



- K_d of $\approx 10^{-14}$ mol/L
- One of the strongest non-covalent interactions known in nature.
- Streptavidin-biotin complex is highly resistant to organic solvents, denaturants proteolytic enzymes, and extremes of temperature and pH.

Equilibrium dissociation constant

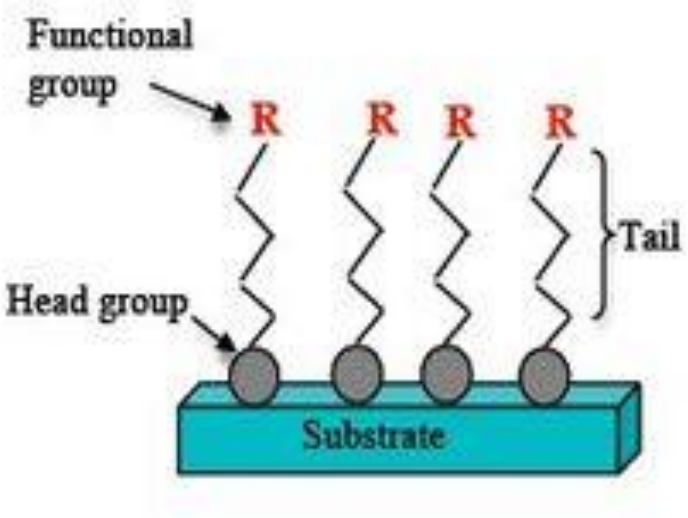


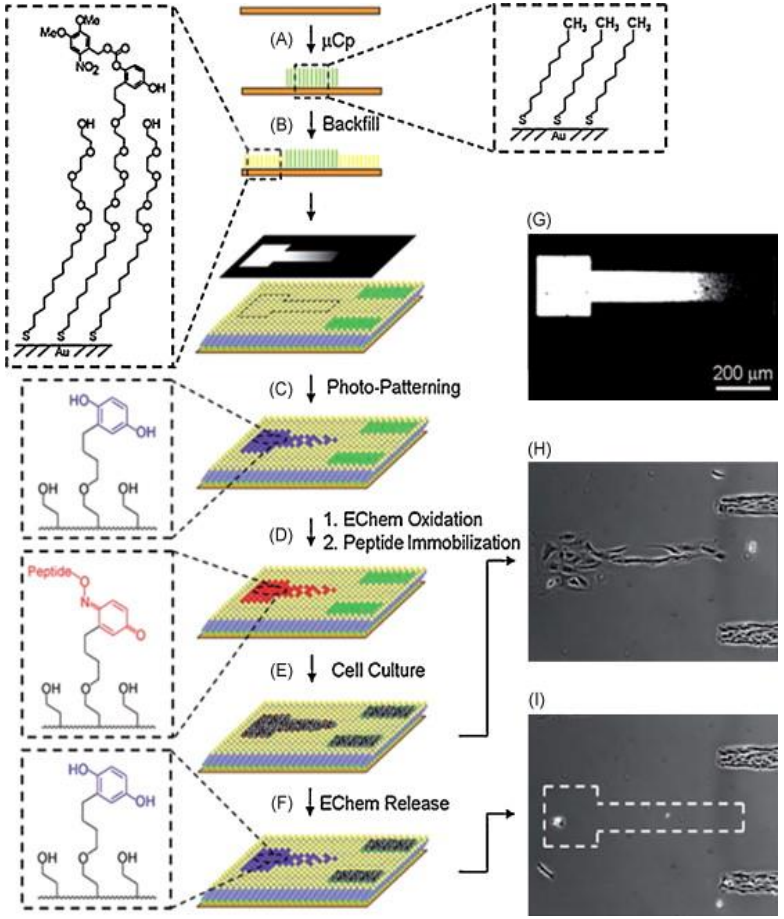
$$K_D = [A][B]/[AB]$$

Figure 3-43a *Molecular Biology of the Cell* (© Garland Science 2008)

Covalent coupling to a polymer layer on the sensor surface

Surface assembled monolayers





- Micro-contact printing
- Electrodeposition
- Vapor phase deposition

Biofunctionalisation considerations

- Surface chemistry is not the same as solution chemistry
- Denaturation of protein/biomolecule on surface
- Availability of binding region
- Orientation of biomolecule on surface
- Repulsion of ligands due to surface charge/chemistry

Protein stability

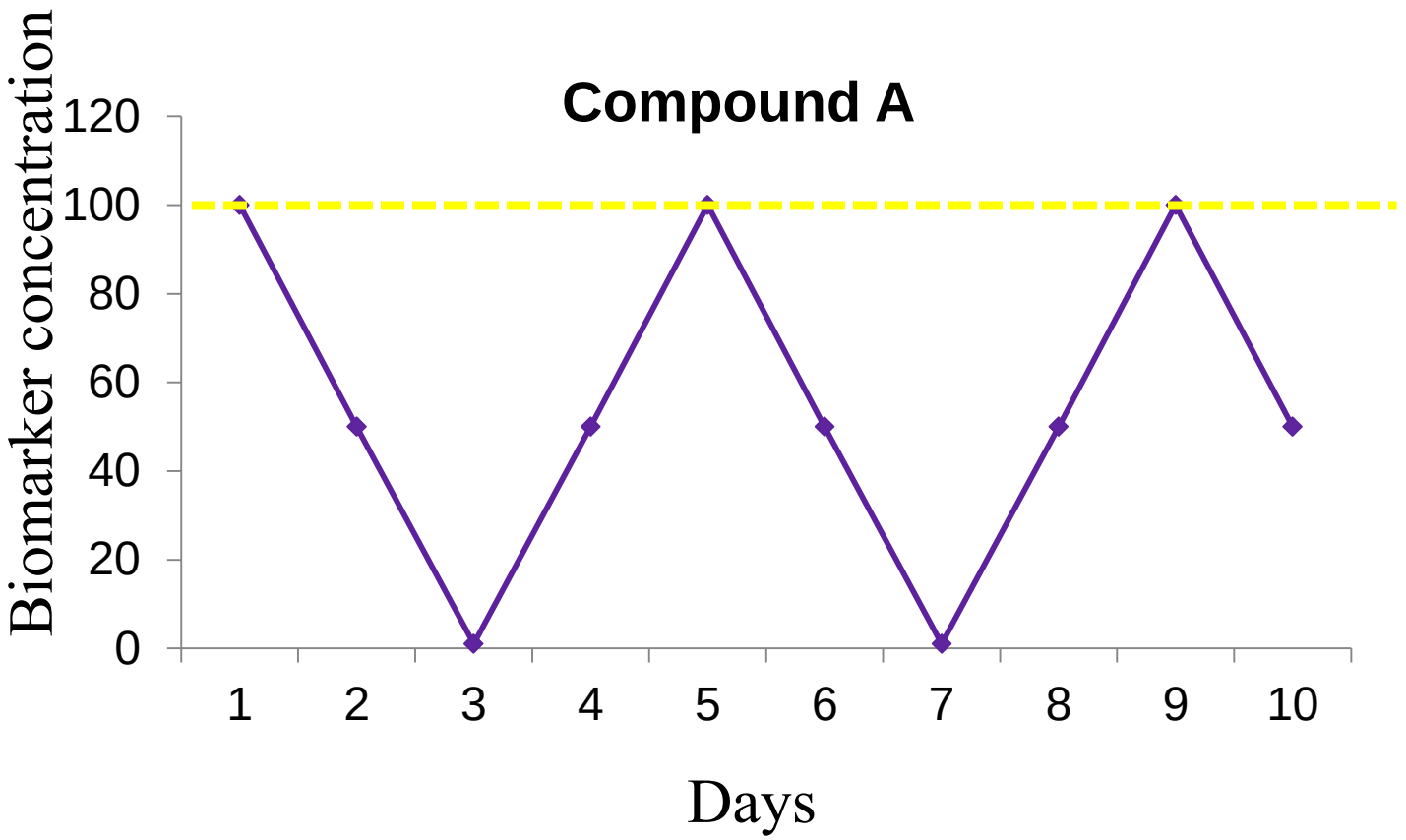
- **Electrostatic forces**
  - Interactions of positive and negative charges
- **Ionic interactions (ion pair/salt bridge)**
- **Dipole-dipole interactions e.g Van der Waals**
  - Very weak, but very numerous
- **Hydrogen bonding**
  - 12-30 kJ/mol
- **Hydrophobic forces**
  - Hydrophobic go in, hydrophillic out
- **Disulfide bonds**
  - Between 2 SH groups (251kJ/mol)

Protein denaturation

- **Low conformation stability => highly susceptible to denaturation**
  - Changes in temperature (Tm less than 1OO°C) but small changes in folding result in almost total loss of 3D structure
  - pH changes: Ionisation of side groups change – changes H-bonding, charge distrib.
  - Detergents: change hydrophobic interactions
  - Organic substances: interfere with hydrophobic interactions (have their own interaction with water)
  - Salts: can stabilise (raise Tm) Ils can be good
  - Chaotropic agents: urea, Guanidinium – increase solubility of nonpolar substances in water – disrupt hydrobphobic interactions (expel water)

Protein Engineering

Why do continuous, label-free monitoring?



- ***Label-free monitoring avoids use of fluorophores/chromophores which may introduce artefacts***
- ***Also possibility to go below opical diffraction limit of 250 nm***