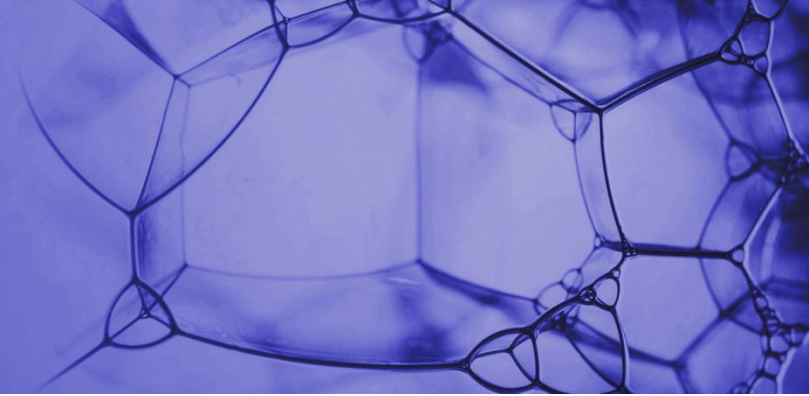


**LOSCHMIDT
LABORATORIES**



10. Molecular Biotechnology in Medicine

Outline

- ❑ definition of red biotechnology
- ❑ areas of red biotech applications
- ❑ molecular diagnostics
 - immunological diagnostic methods
 - nucleic acid diagnostic systems
- ❑ digital diagnostics
- ❑ personalized medicine

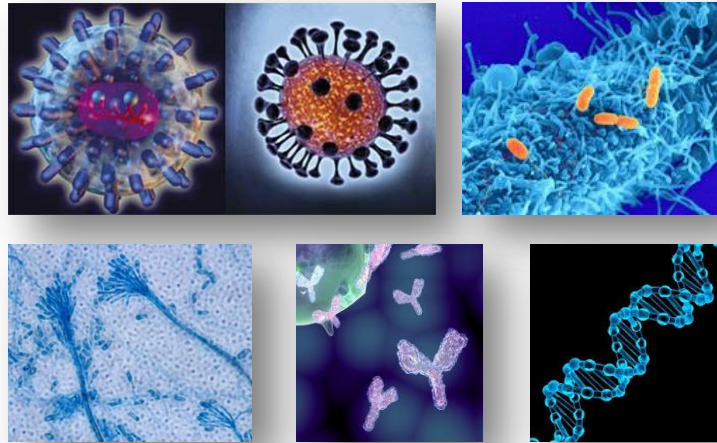
Red (medical) biotechnology

- ❑ biotechnology that deals specifically with **human health** care and methods of treatment
- ❑ aim at **prophylaxis**, accurate **diagnosis** and effective treatment
- ❑ **personalised medicine** - therapy tailored based on patient profile rather than the “one size fits all” approach
- ❑ promising areas of red biotech applications:
 - molecular diagnostics and genetic testing
 - vaccines, protein and nucleic acid therapeutics
 - tissue engineering and regenerative medicine
 - gene therapy and therapeutical cloning
 - drug delivery and nanomedicines

Clinical diagnostics

□ success of modern medicine depends on **specific detection** of

- viruses
- bacteria
- fungi
- proteins
- nucleic acids



□ medical laboratory methods contribute to **80% of diagnosis**

□ good detection method should have three characteristics

- **sensitivity** - ability to detect small amounts of target molecule
- **specificity** - positive result for the target molecule only
- **simplicity** - ability to run efficiently, inexpensively on a routine basis

❑ **classical methods**

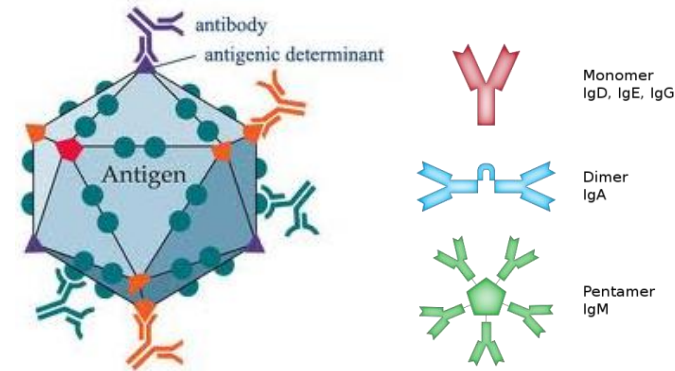
- cultivation, microscopic analysis, biochemical assays
- **POSITIVES:** simple, direct detection
- **NEGATIVES:** slow, laborious, low sensitivity, high skill level requirement, dangerous during cultivation infectious organisms

❑ **molecular diagnostics (past 20 years)**

- **immunological** and **nucleic acid diagnostic** systems
- **POSITIVES:** fast, simple, high sensitivity, automatable, safe
- **NEGATIVES:** not always specific, possible false positive or negative results

Immunological methods

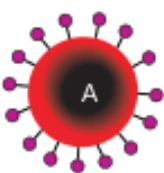
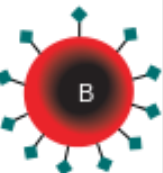
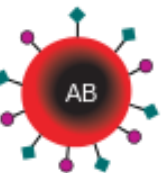







- ❑ sensitive, specific and simple
- ❑ based on **antigen-antibody interactions**
- ❑ protein >> sugar > nucleic acid
- ❑ wide range of applications in monitoring:
 - **hormones, vitamins, metabolites**, diagnostic **markers**
(e.g., insulin, testosterone, prostaglandins, corticoids)
 - **drugs**
(e.g., barbiturates, morphine, digoxin)
 - **infections**
(e.g., *Legionella*, HIV, hepatitis A, B)
 - **cancer**
(e.g., alpha-fetoprotein, carcino-embryonic antigen)



Immunological methods

□ agglutination

- **blood typing test**
- ABO blood-group antigens
(differences in the sugars on glyco-proteins)

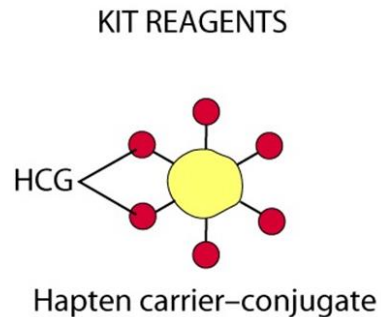
	Group A	Group B	Group AB	Group O
Red blood cell type				
Antibodies in Plasma	 Anti-B	 Anti-A	None	 Anti-A and Anti-B
Antigens in Red Blood Cell	 A antigen	 B antigen	 A and B antigens	None



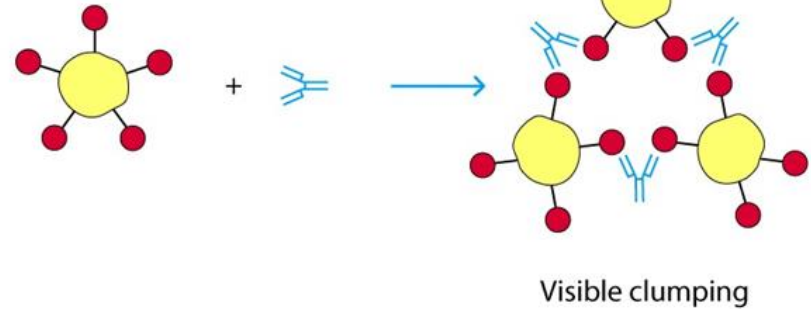
Immunological methods

□ agglutination

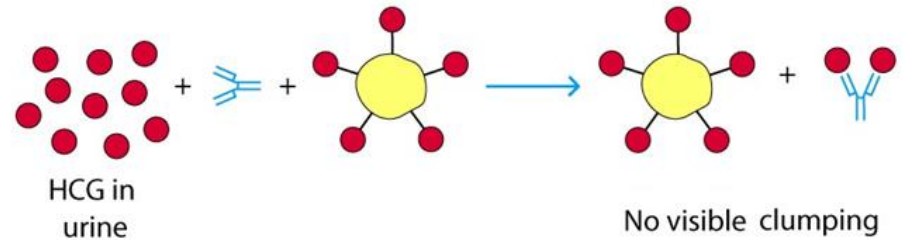
- pregnancy test
- inhibition in presence of human chorionic gonadotropin, hCG, glycoprotein hormone produced in pregnancy



⊖ reaction: not pregnant



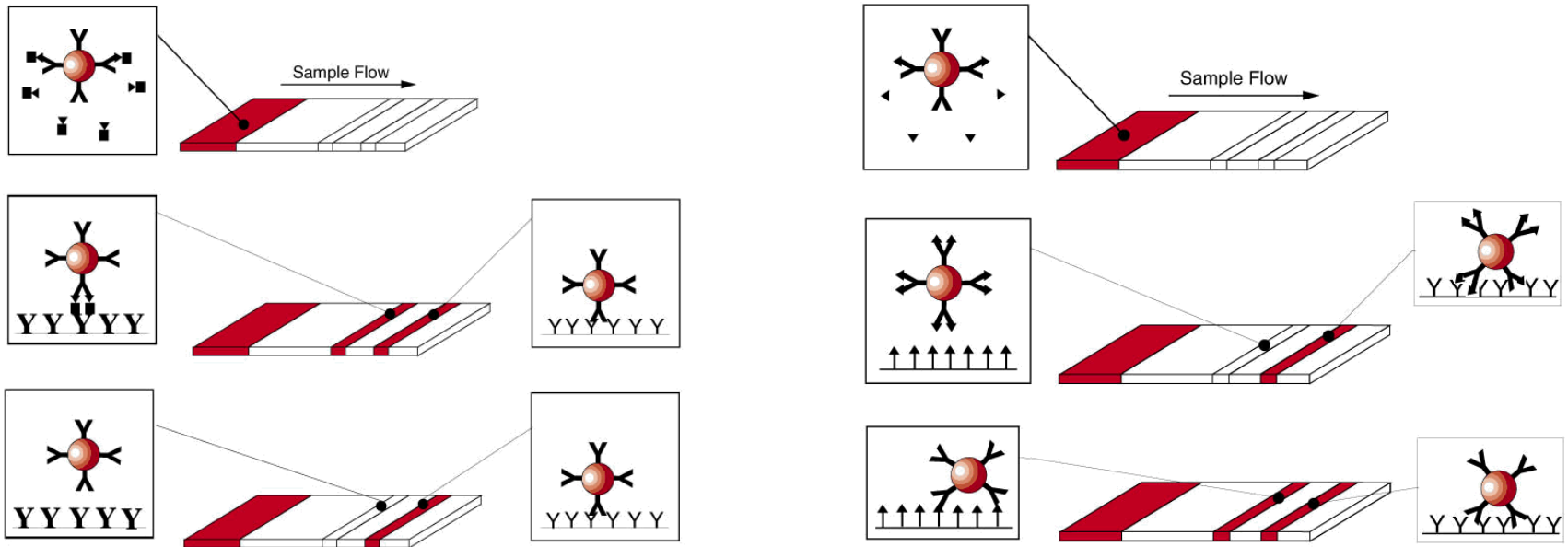
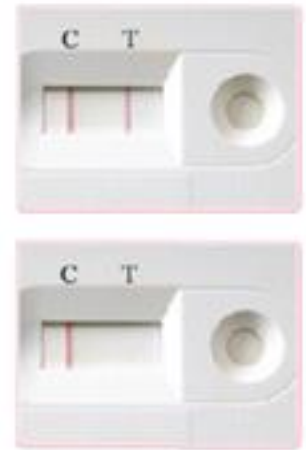
⊕ reaction: pregnant



Immunological methods

□ immuno-chromatographic assays

- simple devices to detect presence of analyte in sample
- no need for specialized equipment or sample treatment
- coloured particle - latex (blue) or nanosized gold (red)
- **sandwich** double antibody reaction scheme (e.g. HIV, hCG)
- **competitive** reaction scheme (small antigens)

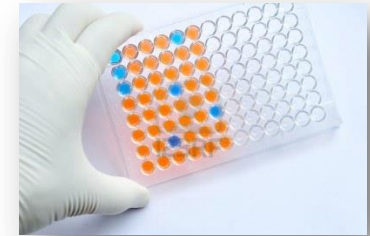
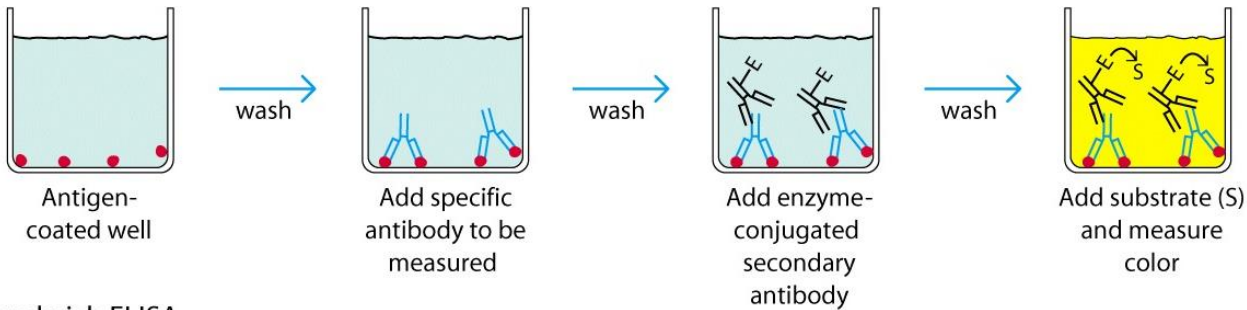


Immunological methods

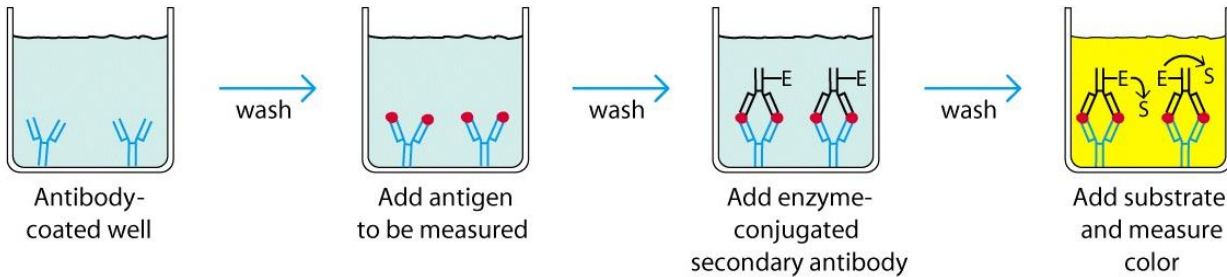
□ enzyme-linked immunosorbent assay (ELISA)

- enzyme based detection (e.g., HRP, β -galactosidase, phosphatase)
- fluorescence or colorimetric based detection

Indirect ELISA



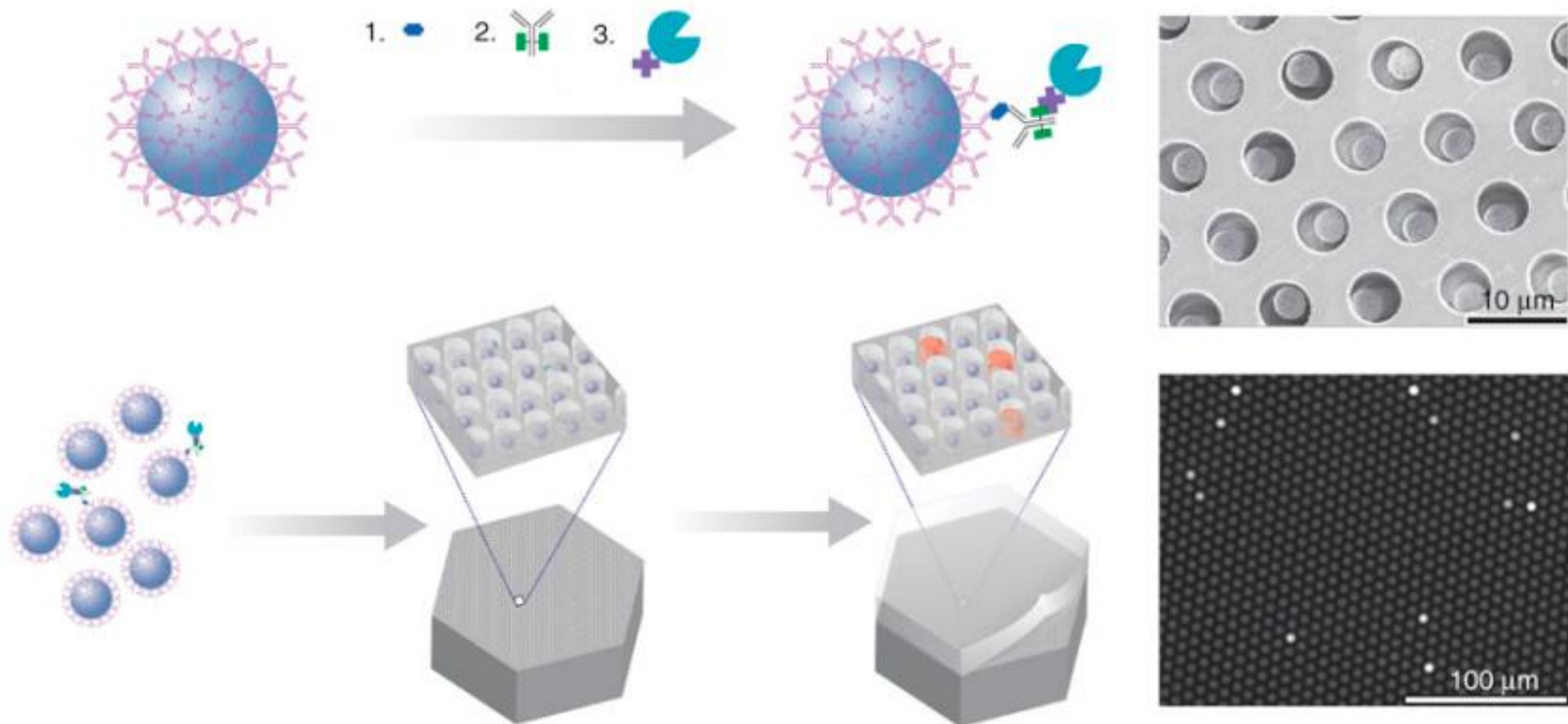
Sandwich ELISA



Immunological methods

□ digital immunoassay (single molecule ELISA)

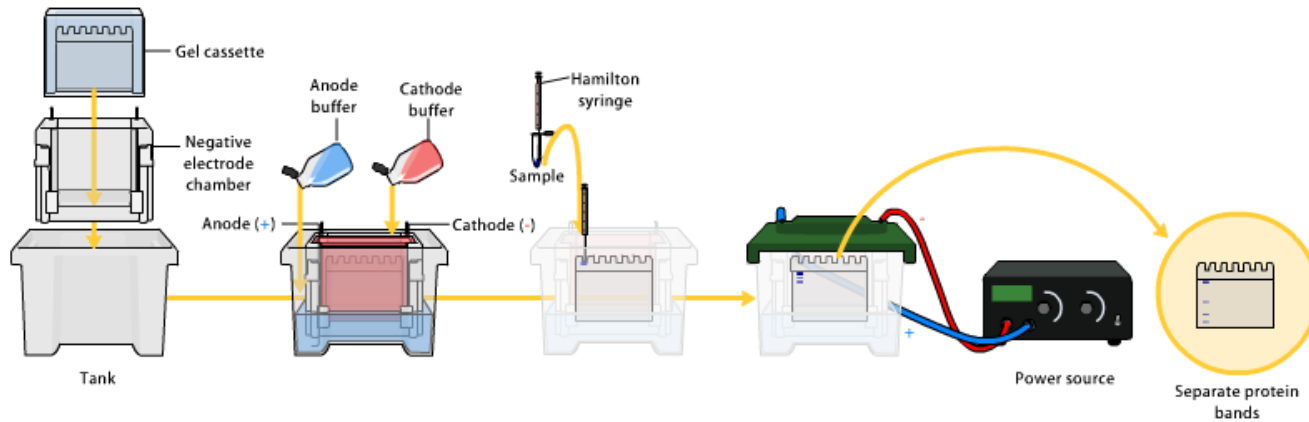
- detection volume decreased by a factor of 10^{10} (100mL to 10 fL)
- quantitative subfemtomolar range sensitivity



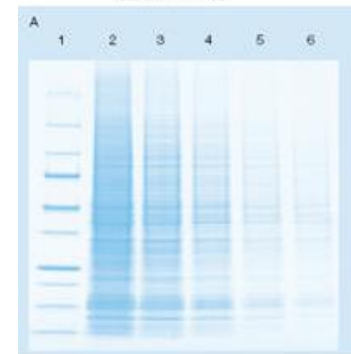
Immunological methods

□ western blotting

- **SDS-Page** - separates components according to molecular weight



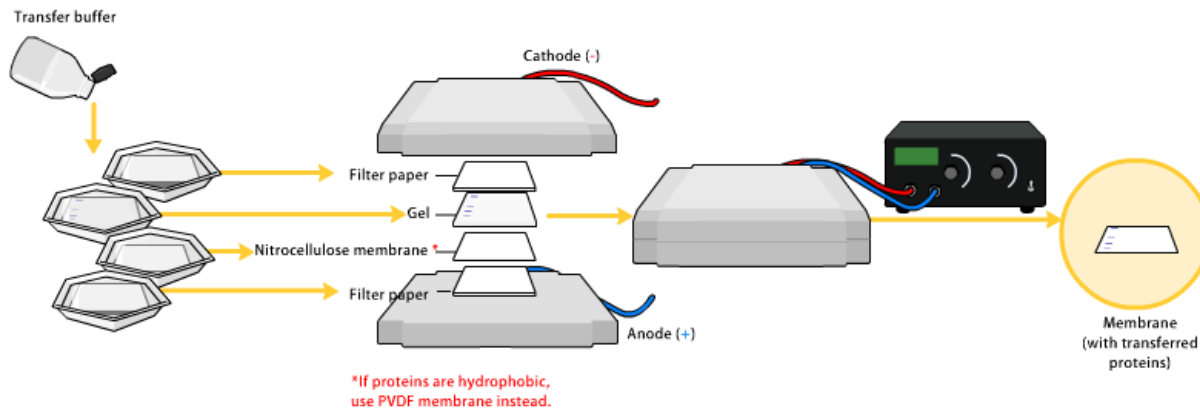
SDS-PAGE



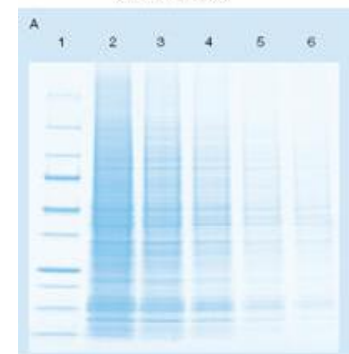
Immunological methods

□ western blotting

- **SDS-Page** - separates components according to molecular weight
- **Blot:** proteins in gel transferred to nitrocellulose or nylon



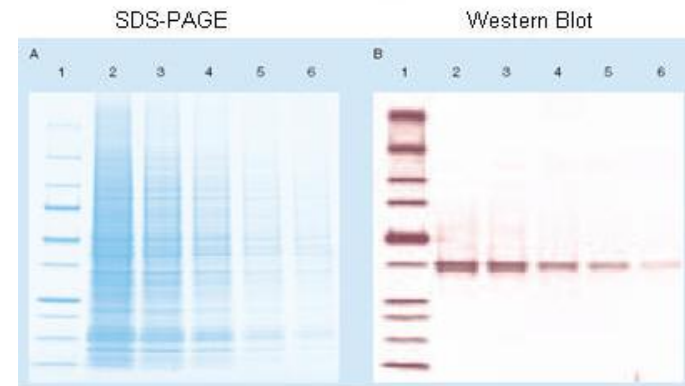
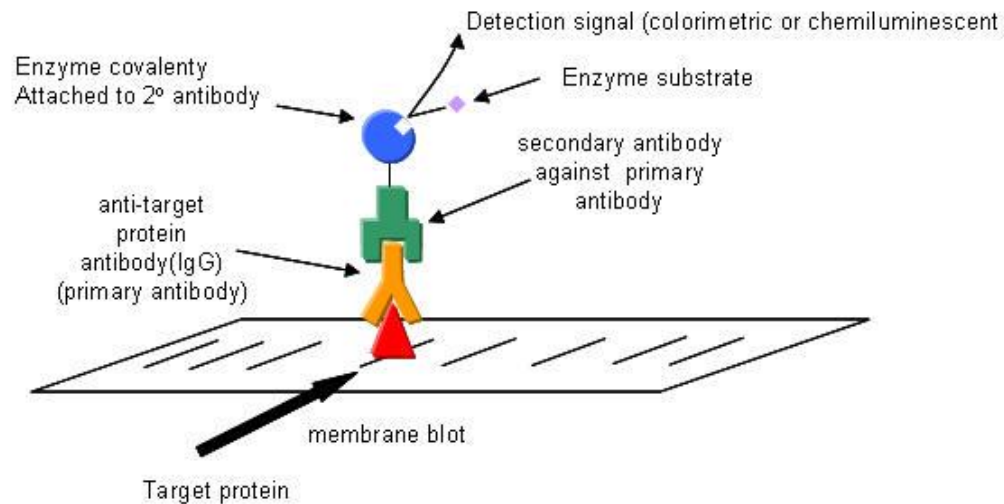
SDS-PAGE



Immunological methods

□ western blotting

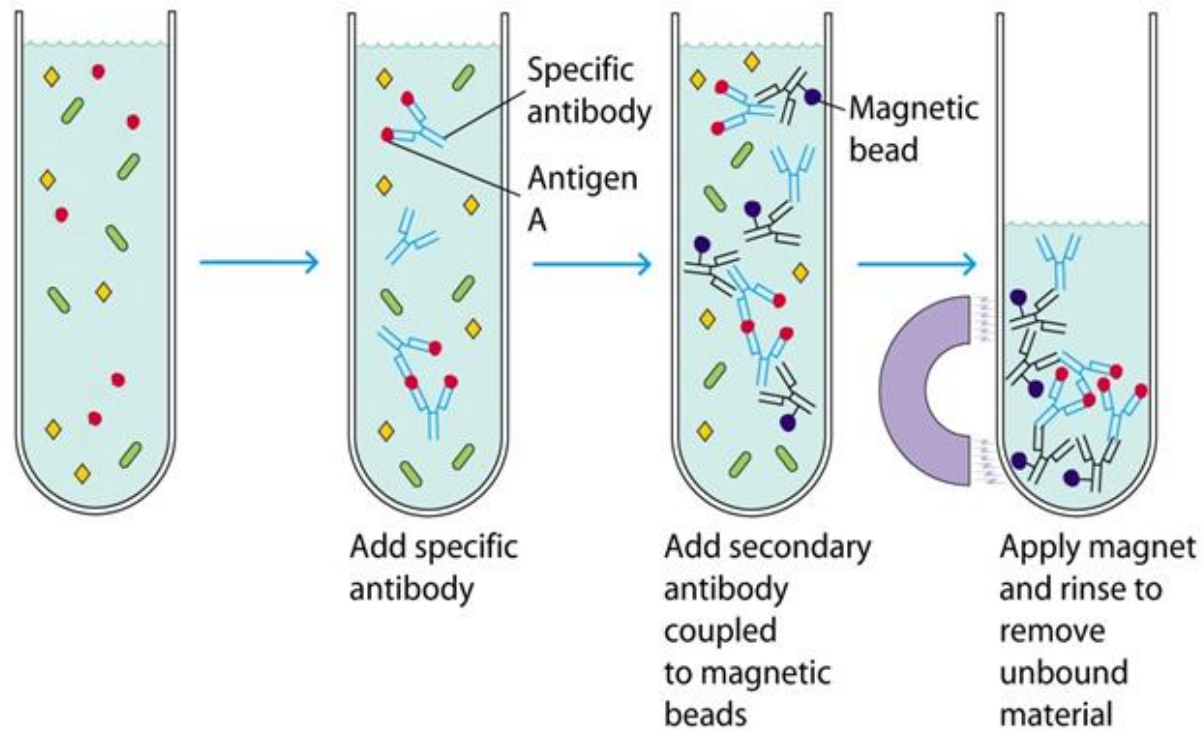
- **SDS-Page** - separates components according to molecular weight
- **Blot**: proteins in gel transferred to nitrocellulose or nylon
- **Immunoreaction**: after blocking (BSA) probed with primary and secondary antibody
- **Detection**: radioactivelabelling, colorimetry, florescence, (chemi)luminescence



Immunological methods

□ immunoprecipitation

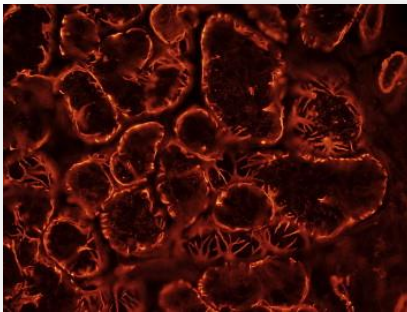
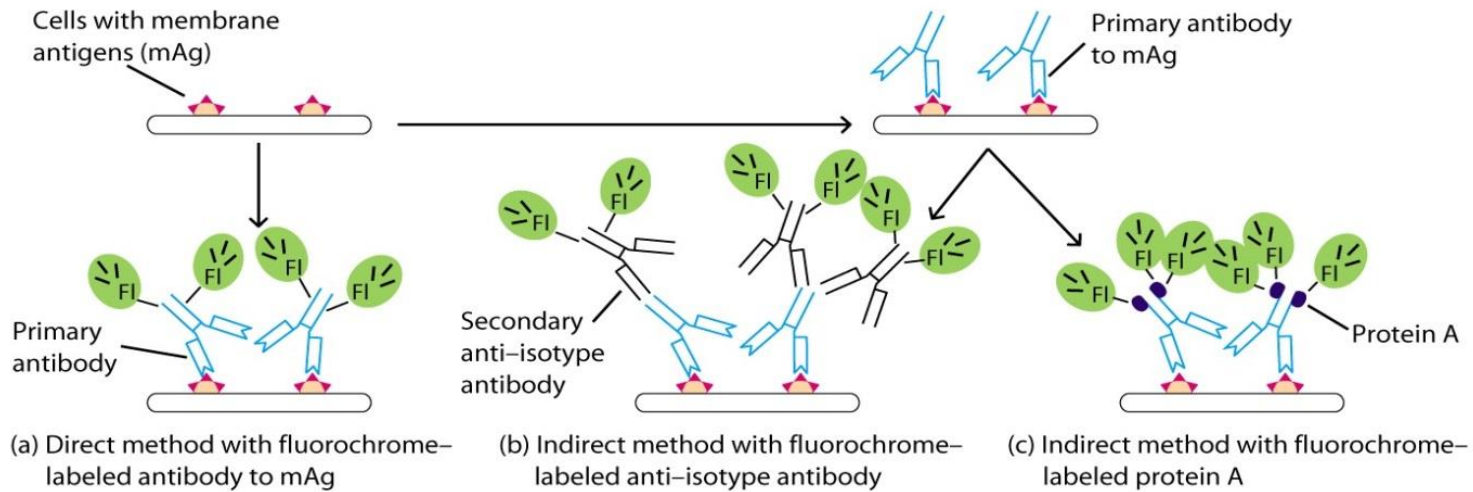
- collected by magnetic beads coupled to a secondary antibody



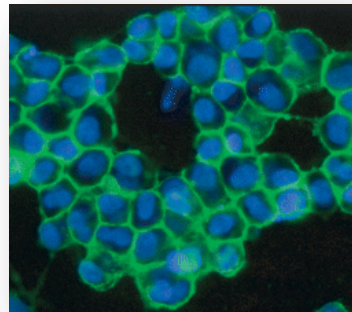
Immunological methods

□ immunofluorescence (microscopy methods)

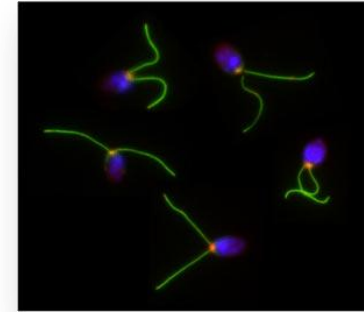
- fluorescence labelled antibody (e.g., fluorescein, rhodamine)



lacrimal gland myoepithel



virus infected cells



Chlamydomonas

Nucleic acid diagnostic systems

- ❑ most common object for testing is **DNA**, in some cases **RNA**
- ❑ areas of medical applications:
 - **prenatal diagnostics**: non-invasive detection of fetal diseases (e.g., Down syndrome, cystic fibrosis)
 - **genetic testing**: high throughput testing for genetic disorders (e.g., SNPs markers, insertions, deletions)
 - **infectious diseases**: pathogen identification and drug resistance (e.g. HIV, HBV, HCV)
 - **oncology**: early diagnosis of cancer (e.g., circulating tumor DNA, retinoblastoma gene)
 - **transplantation medicine**: non-invasive detection of organ rejection (e.g., urine testing for kidney rejection, human leukocyte antigen)
 - **pharmacogenomics**: influence of genetic variation on drug response
 - **DNA typing**: fingerprint of genotypic traits (paternity, crime suspects, ancestry)

Nucleic acid diagnostic systems

□ DNA hybridization

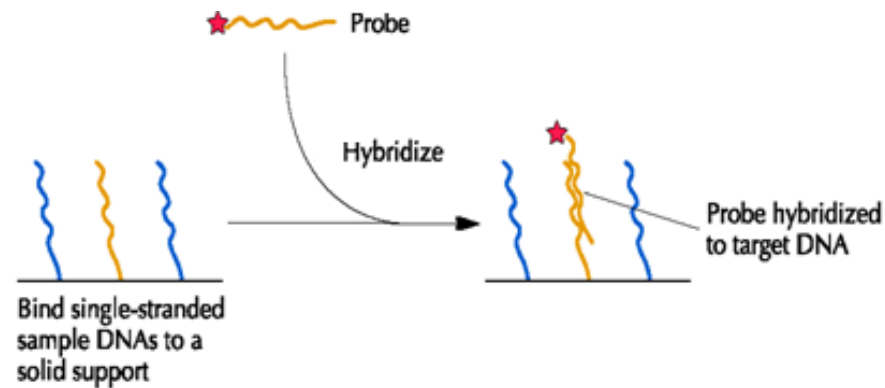
- **probe** which anneals to the target nucleic acid
- bacterial and viral **pathogens** contain specific gene(s)
- **genetic diseases** caused by mutation or absence of particular gene(s)

Nucleic acid diagnostic systems

□ DNA hybridization

▪ conventional method

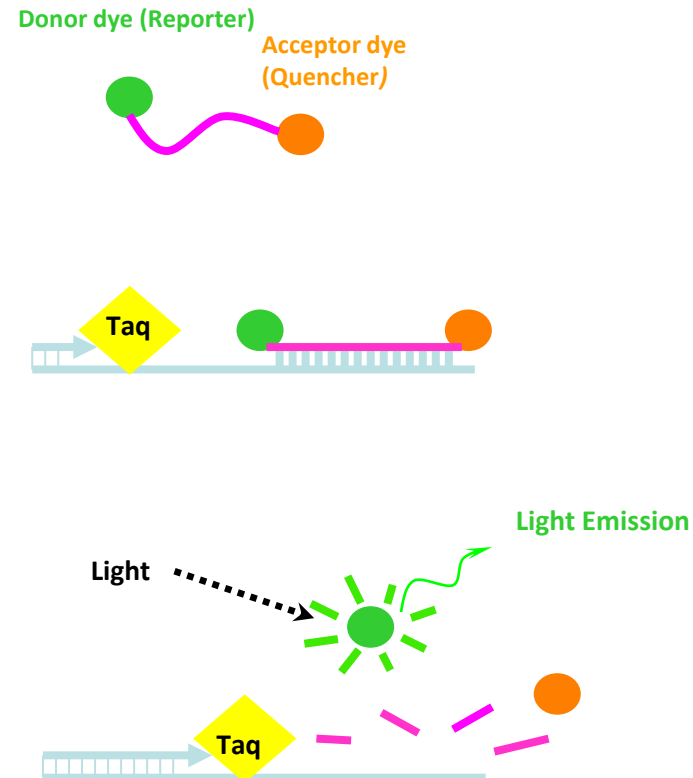
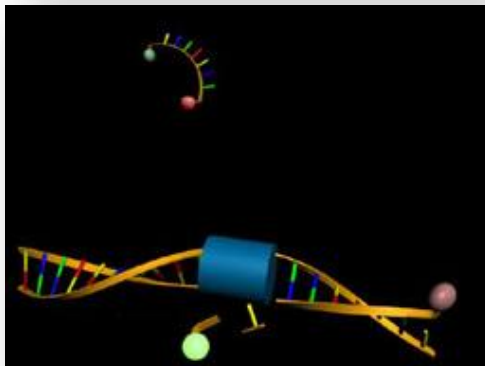
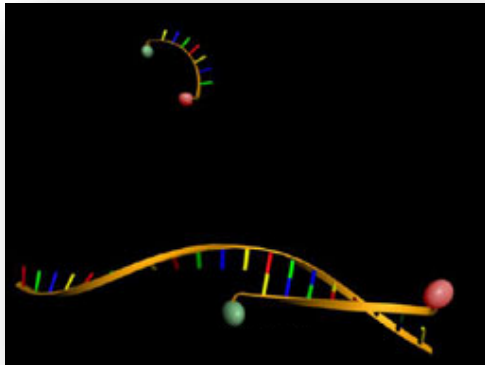
1. **attachement** of target DNA to solid matrix
2. **denaturation** of both probe and target
3. **annealing** probe to target DNA
4. washing and **detection**
(e.g. autoradiography, chemoluminiscence, fluorescence)



Nucleic acid diagnostic systems

□ DNA hybridization

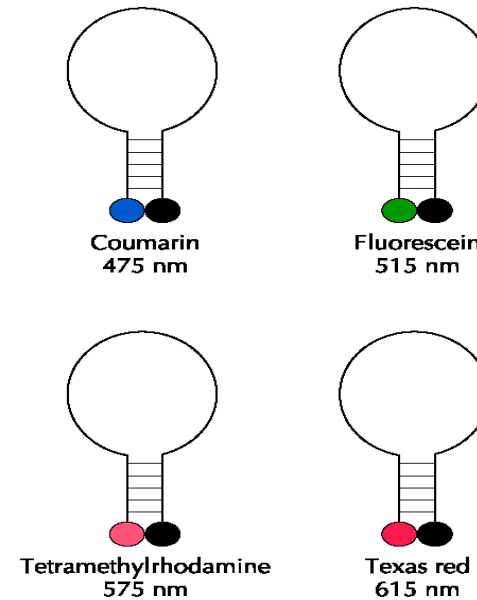
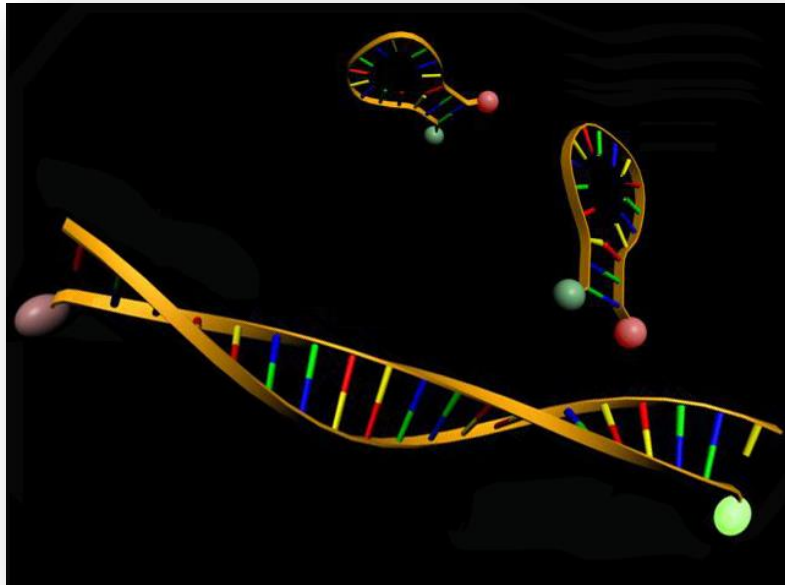
- conventional method
- TaqMan Probes - hydrolysis by Taq polymerase



Nucleic acid diagnostic systems

□ DNA hybridization

- conventional method
- **TaqMan Probes** - hydrolysis by Taq polymerase
- **molecular beacons** - hairpin DNA with internally quenched fluorophore



Nucleic acid diagnostic systems

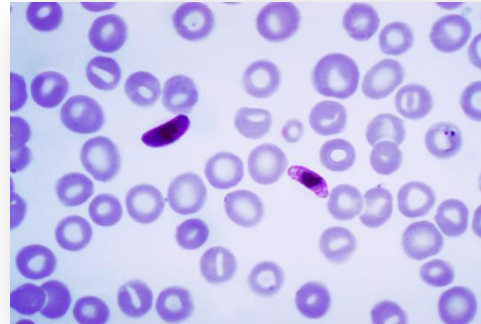
❑ DNA hybridization

❑ EXAMPLE: detection of parasite *Plasmodium falciparum*

- **microscopic** observations of blood smears is labour intensive
- **ELISA** does not differentiate between past and present infection
- **DNA** diagnostic system measure only current infection

Other examples: *Salmonella typhi* (food poisoning)

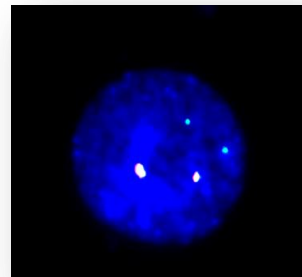
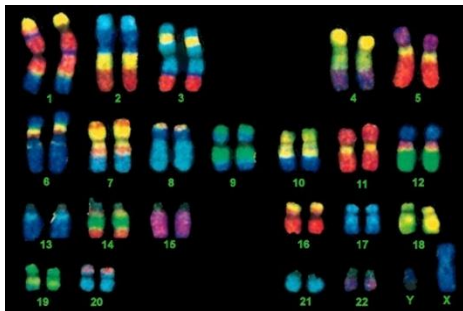
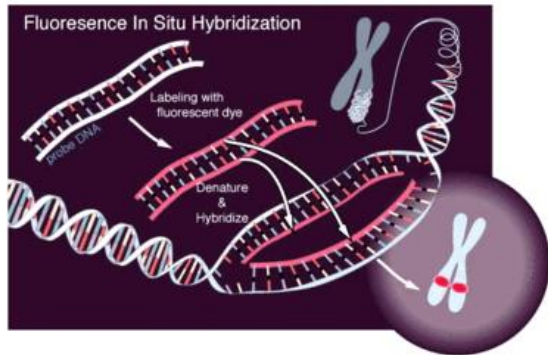
Escherichia coli (gastroenteritis)



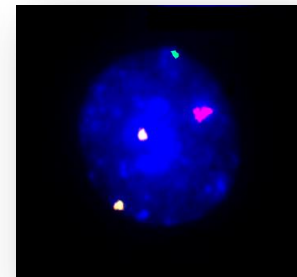
Nucleic acid diagnostic systems

□ DNA hybridization

- fluorescence in situ hybridisation (FISH)
 - new technique for **karyotyping**
 - **chromosome abnormalities** (segmental deletions and translocations)
 - **aneuploidy** (abnormal number of chromosomes)

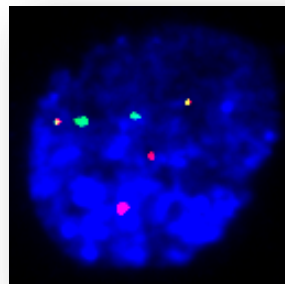


normal female

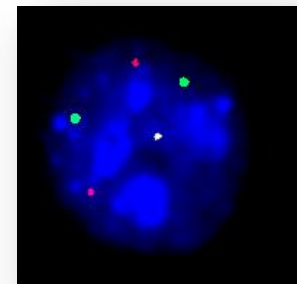


normal male

● X
● Y
● 16



normal 13, 14



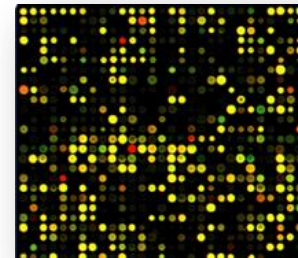
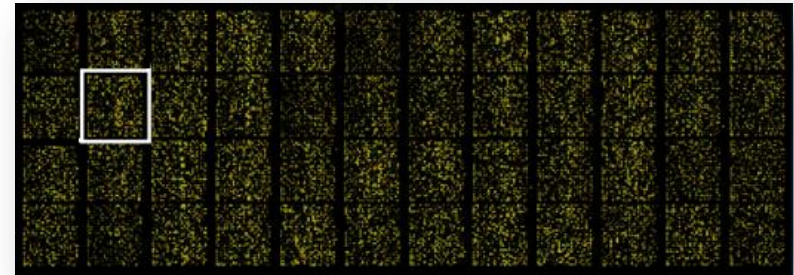
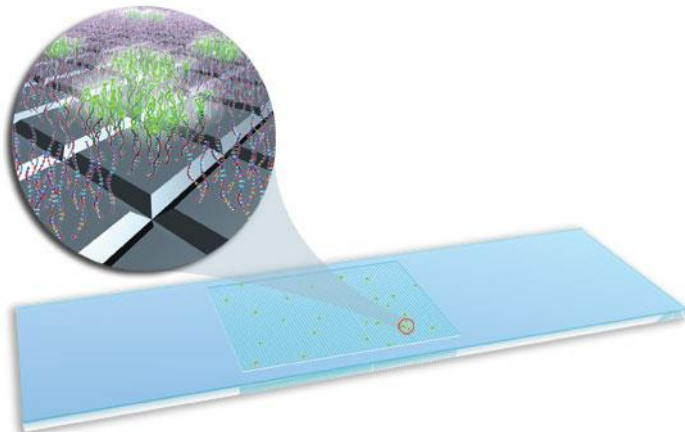
monosomy 14

● 13
● 14

Nucleic acid diagnostic systems

□ DNA hybridization

- **DNA microarray (DNA chip)**
 - **10^4 to 10^6 probes** (reporters)
 - **spot** - picomole (10^{-12} M) of oligo
 - **probe-target hybridization**
 - **labelling** by chemiluminescence, fluorophore or silver
 - **bioinformatics** data processing



Nucleic acid diagnostic systems

❑ polymerase chain reaction (PCR)

- amplify single or few copies of DNA to millions of copies
- the presence of the appropriate amplified size fragment (product) confirms the presence of the target
- specific primers are available for detection of bacteria (*E. coli*, *M. tuberculosis*), viruses (HIV), fungi
- early diagnosis of malignant diseases (leukemia)

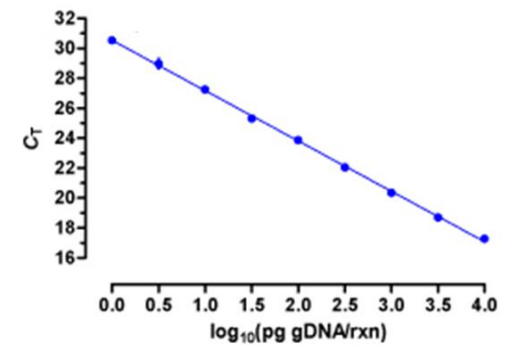
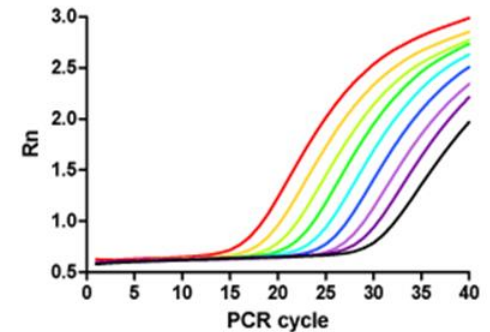
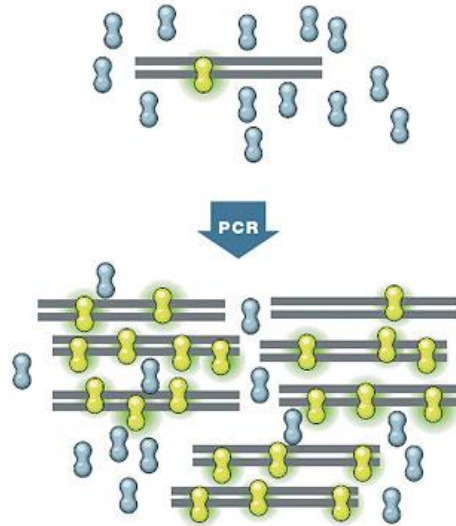
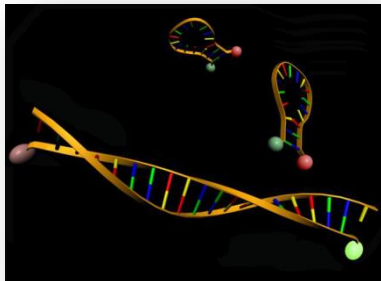
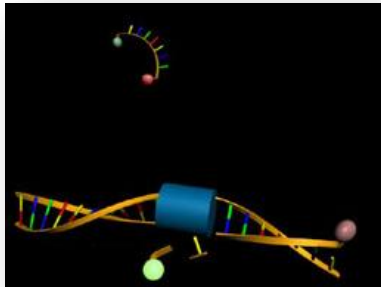


Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

▪ real-time PCR (qPCR)

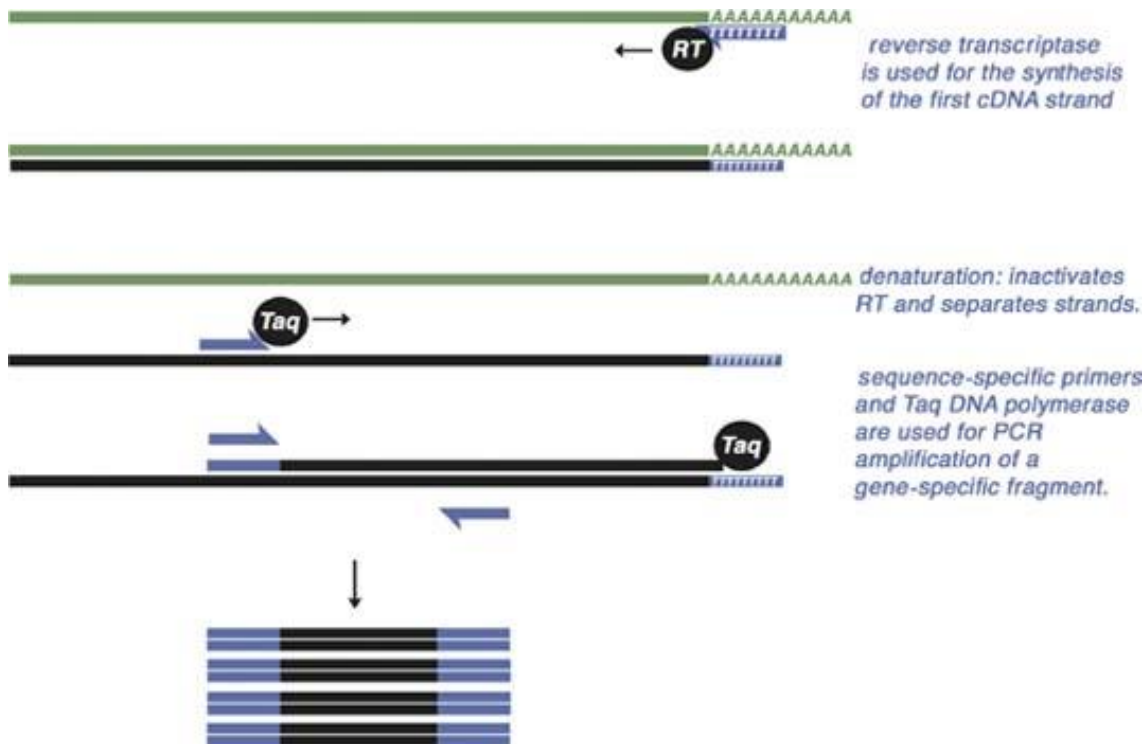
- non-specific fluorescent dyes that intercalate with dsDNA
- sequence-specific DNA probes, oligonucleotides labeled with fluorescent reporter



Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

- reverse transcription PCR (RT-PCR)
- real-time reverse-transcription PCR (qRT-PCR)



Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

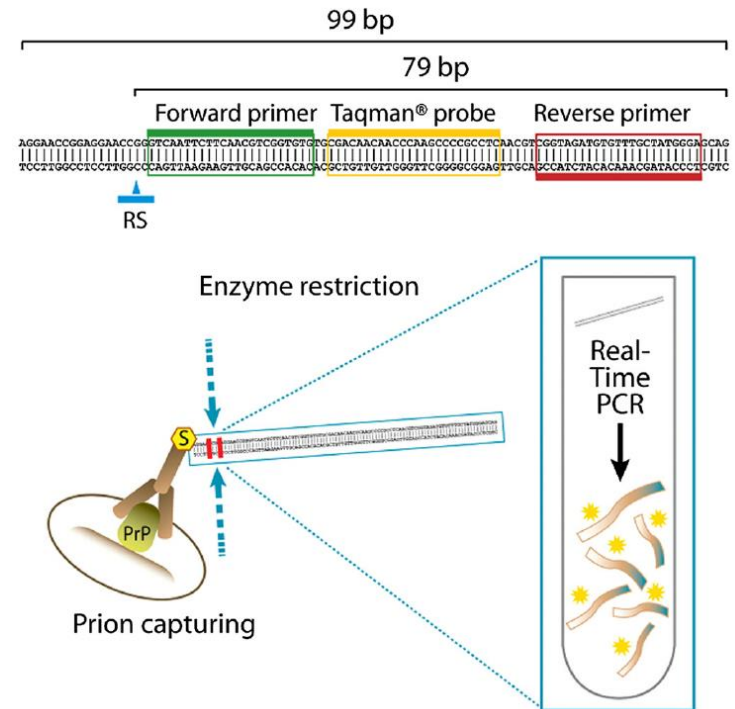
▪ immunoquantitative real-time PCR (iqPCR)

- combines **specificity** of antibodies and **sensitivity** of PCR
- overcome insufficient sensitivity of available immunological methods
- sensitive for **very low but still dangerous** levels of pathogens

▪ EXAMPLE: prion detection

detection limit 100 ng/L

10-fold lower than classical ELISA



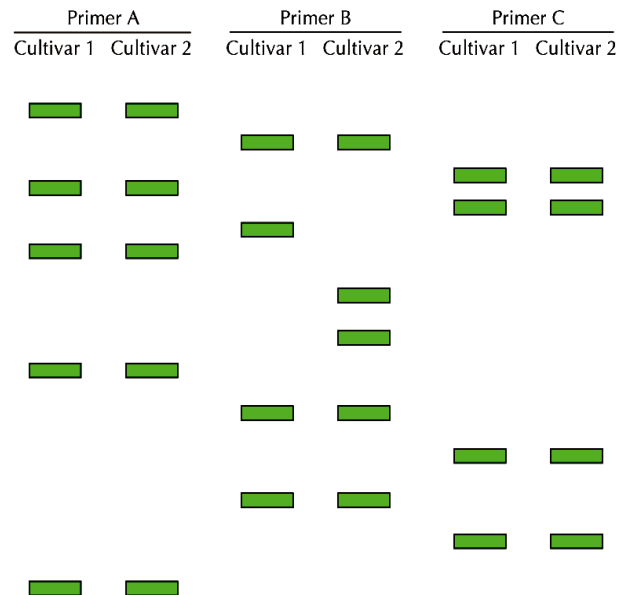
Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

▪ random amplified polymorphic DNA (RAPD)

- „random“ primers used to produce DNA fingerprint
- primers anneal in many places on template DNA and produce variety of sizes of amplified products

DNA fingerprinting



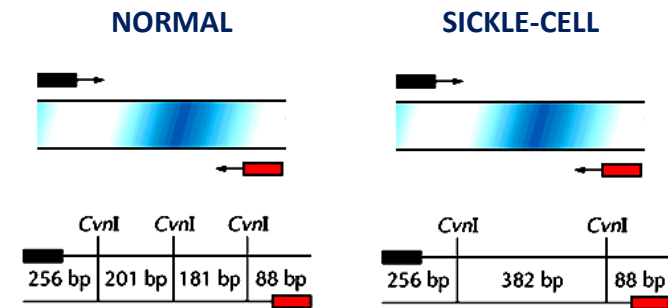
Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

- **restriction fragment length polymorphism (RFLP)**
 - many diseases caused by **single nucleotide change**
 - method dependent on **mutation within recognition site** of restriction enzyme

▪ EXAMPLE: **diagnostics of sickle cell anemia**

- anemia and damage to heart, lung, brain, joints and other organs
- single nucleotide change in **6th aa** of beta-chain of **hemoglobin (E6V)**
- normal DNA sequence **CCTGAGG (A)**
- mutant DNA sequence **CCTG**T**GG (S)**
- **homozygous state SS** red blood cells irregularly shaped



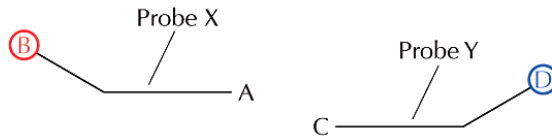
Size (bp)	Genotype		
	AA	AS	SS
382		■	■
256	■	■	■
201	■	■	
181	■	■	
88	■	■	■

Nucleic acid diagnostic systems

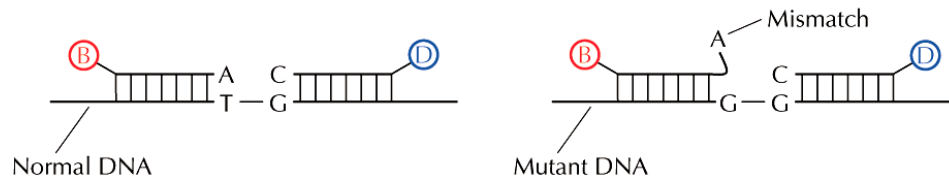
□ polymerase chain reaction (PCR)

▪ oligonucleotide ligation assay (PCR/OLA)

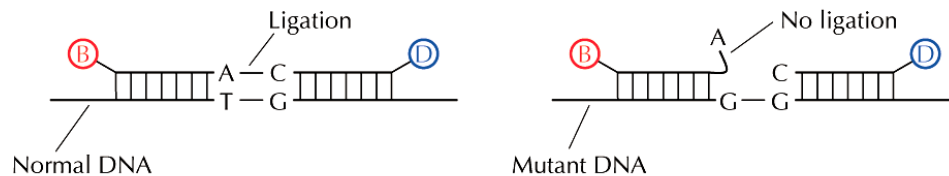
A Synthesize a pair of oligonucleotide probes



B Hybridize probes to PCR-amplified DNA



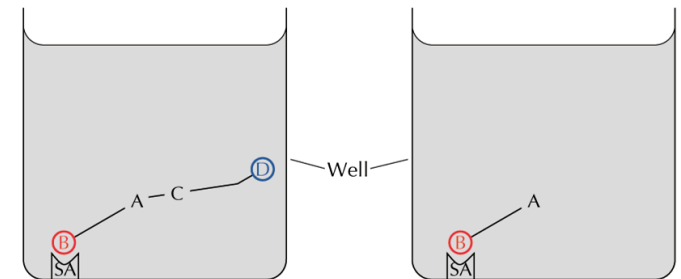
C Add ligase to hybridized DNA



D Bind probes to streptavidin; wash

Results with normal DNA

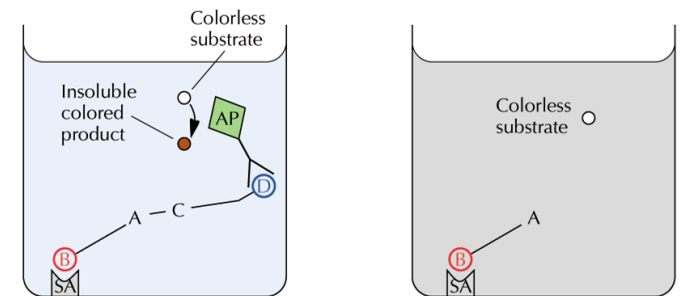
Results with mutant DNA



E Add antidigoxigenin antibody-alkaline phosphatase conjugate; wash; add substrate

Results with normal DNA

Results with mutant DNA

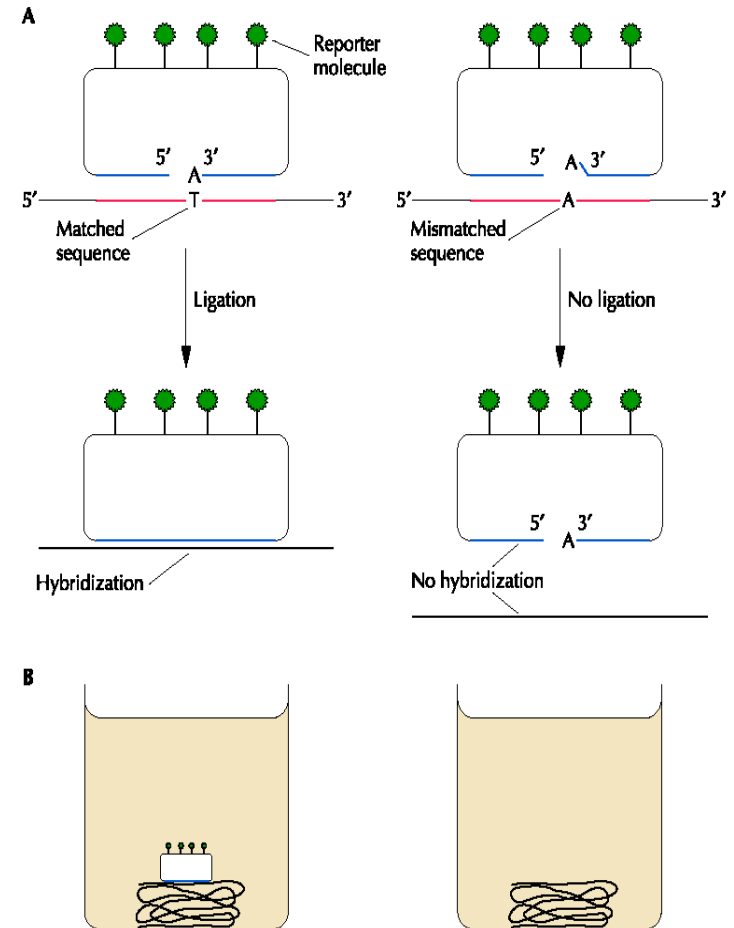


Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

▪ Padlock probe (PCR/PLP)

- target-complementary sequences at 5' and 3' ends
- ligate only if perfect match
- only ligated forms attach to target

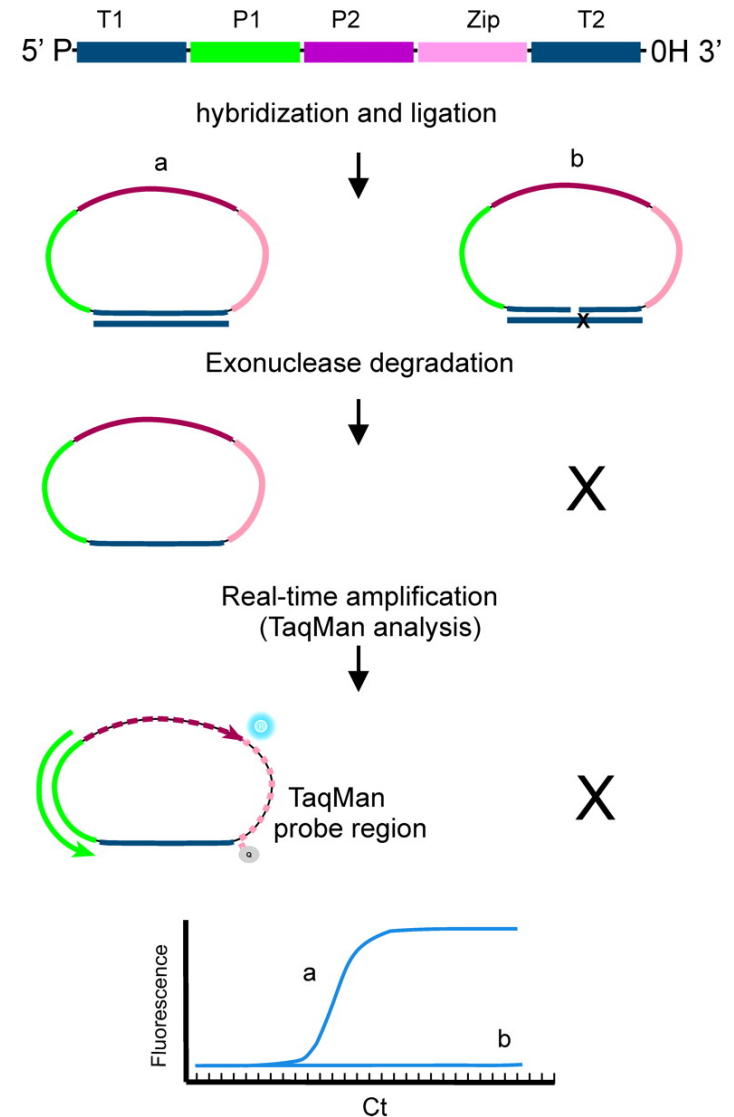


Nucleic acid diagnostic systems

□ polymerase chain reaction (PCR)

▪ Padlock probe (qPCR/PLP)

- target-complementary sequences at 5' and 3' ends (T1, T2)
- universal primer sites (P1, P2)
- reporter sequence (Zip)
- only ligated forms attach to target



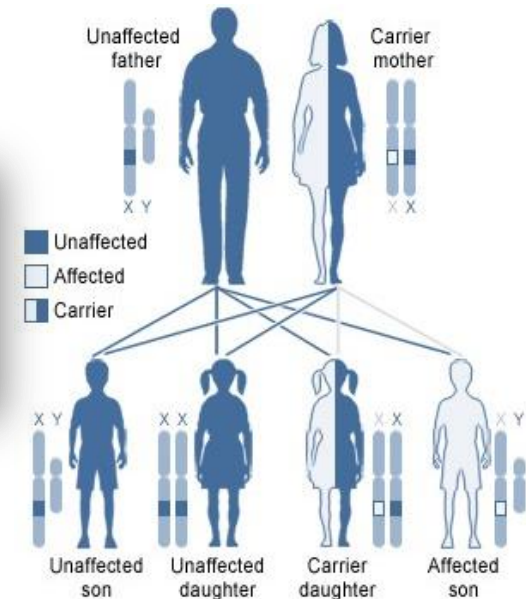
Nucleic acid diagnostic systems

□ DNA sequencing

- most direct method
- become cheap and fast, pushes other methods backward
- genes, genetic regions (i.e. gene clusters or operons), full chromosomes or entire genomes

□ EXAMPLE: Diagnostic for Duchenne muscular dystrophy (DMD)

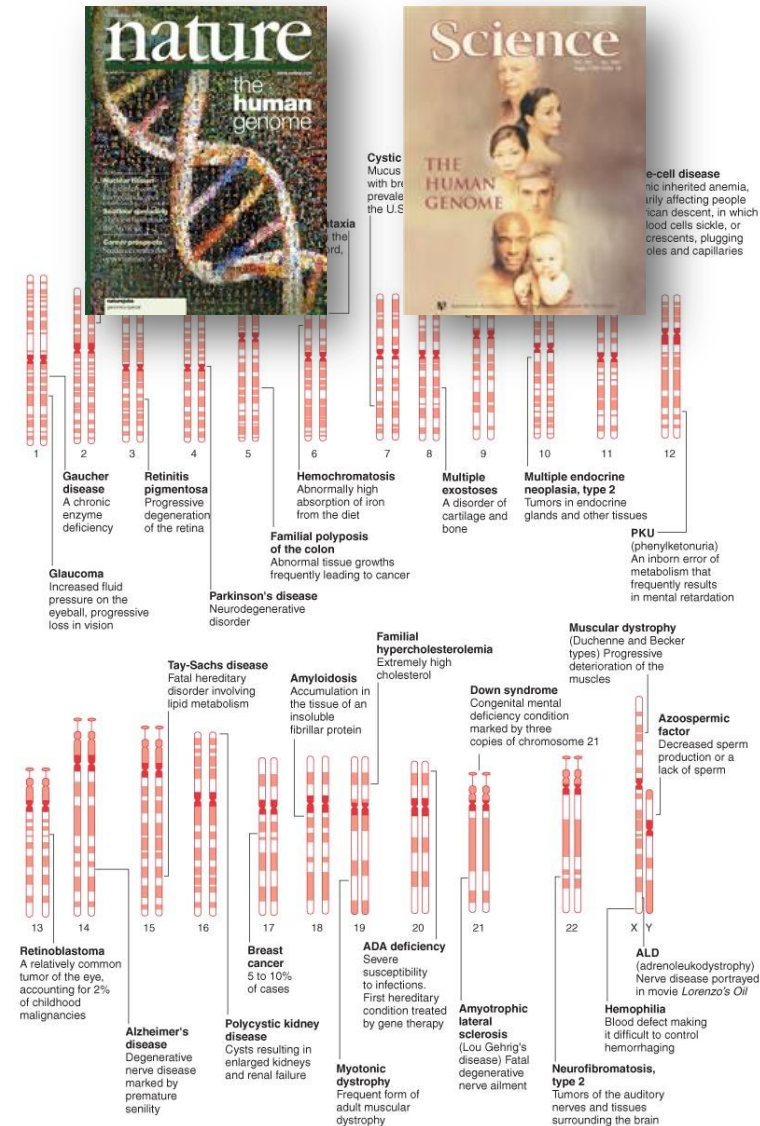
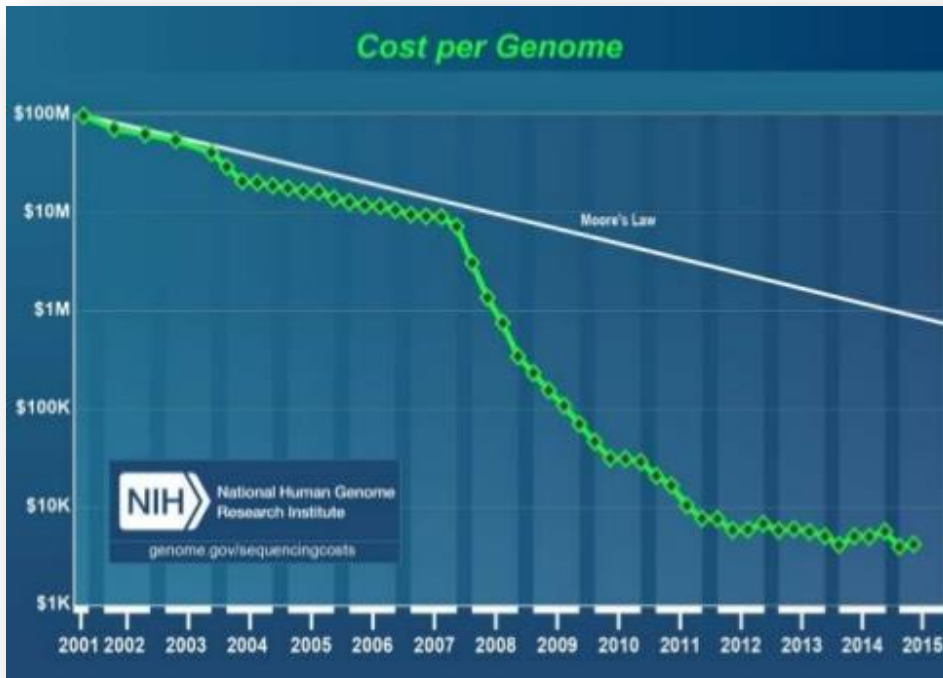
- mutated dystrophin ("implosion" of muscle cells)
- X-linked recessive, carrier mother
- dystrophin gene large (2,4 Mb)
- first mutation carrier often mosaic (blood may be not a mutation carrier)



Nucleic acid diagnostic systems

□ DNA sequencing

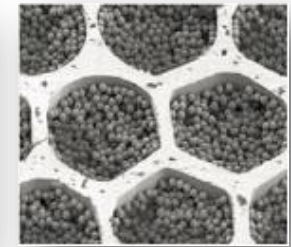
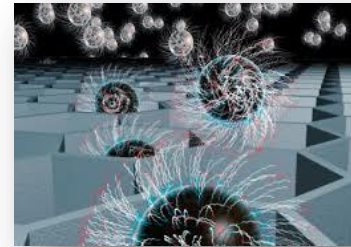
- 2003 Human Genom Project (13 years)
- 2008 James Watson genom (6 month)
- 2015 genome sequencing in (10 hours)



Digital diagnostics

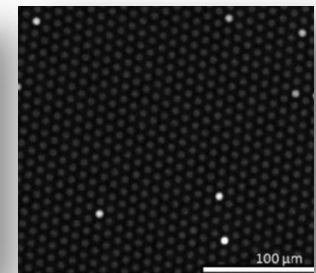
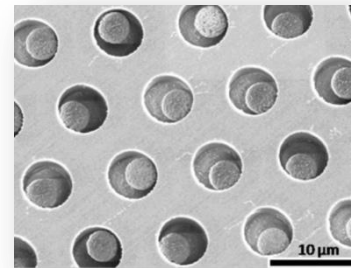
□ single molecule pyrosequencing

- droplets 1 picoliter (10^{-12} liters)
- 1 mil. reads/run, 1-10 USD/Mbase



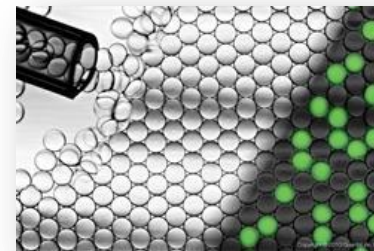
□ single molecule ELISA

- volume 10 femtoliter (10^{-15} liters)
- subfemtomolar range sensitivity



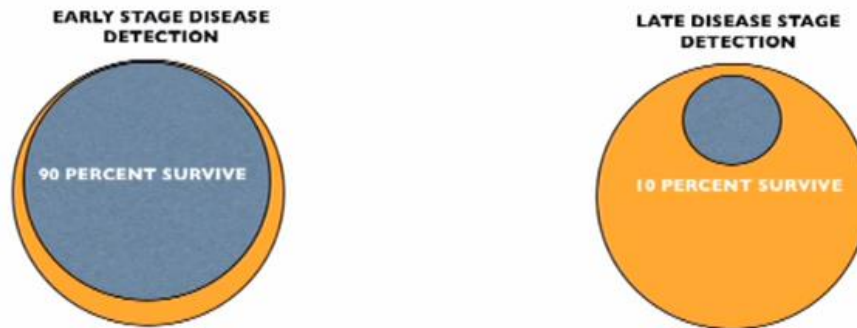
□ droplet digital PCR

- droplets 1 nanoliter (10^{-9} liters)
- 20 thousand reads/run



Personalized medicine

- ❑ medical practice and products **tailored to individual patient**
- ❑ **genetic, molecular or cellular diagnostics**
- ❑ genetic information has major role in personalized medicine (e.g. pharmacogenomics)
- ❑ miniaturization / simple handheld devices
- ❑ medical **diagnostics** from hospital/clinics to **office/home**



STAGE **ZERO** MEDICINE

Personalized medicine

☐ pharmacogenomics

- designing the most effective drug therapy based on **specific genetic profile of patient**
- different drug effects - genetic **polymorphisms**

Individuals respond differently to the anti-leukemia drug 6-mercaptopurine.

Most people metabolize the drug quickly. Doses need to be high enough to treat leukemia and prevent relapses.



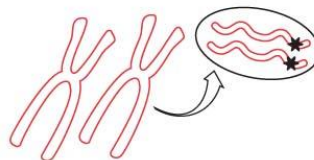
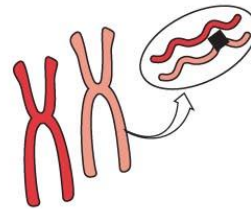
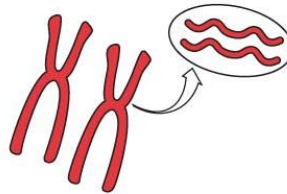
Others metabolize the drug slowly and need lower doses to avoid toxic side effects of the drug.



A small portion of people metabolize the drug so poorly that its effects can be fatal.



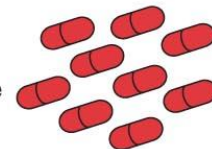
The diversity in responses is due to variations (mutations, ■ or ★) in the gene for an enzyme called TPMT, or thiopurine methyltransferase.



After a simple blood test, individuals can be given doses of medication that are tailored to their genetic profile.



Normal dose

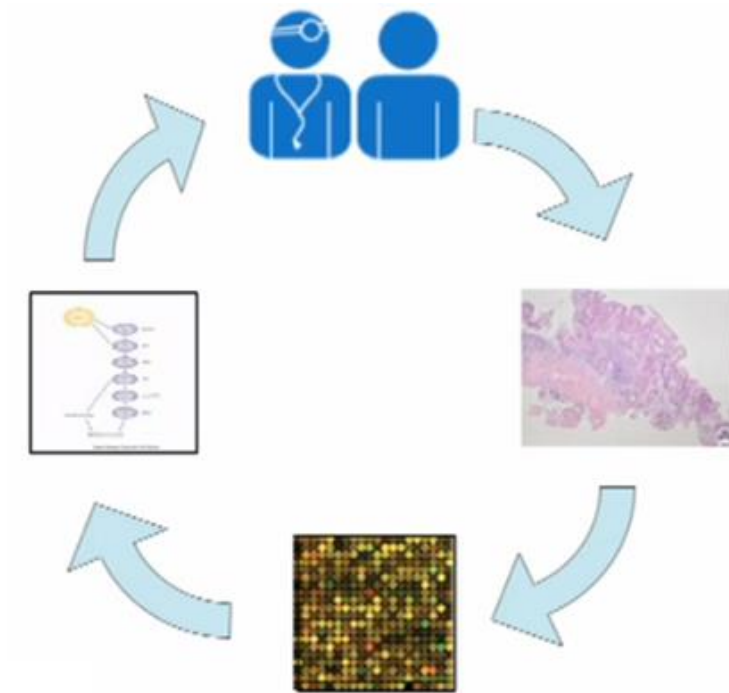


Dose for an extra slow metabolizer (TPMT deficient)



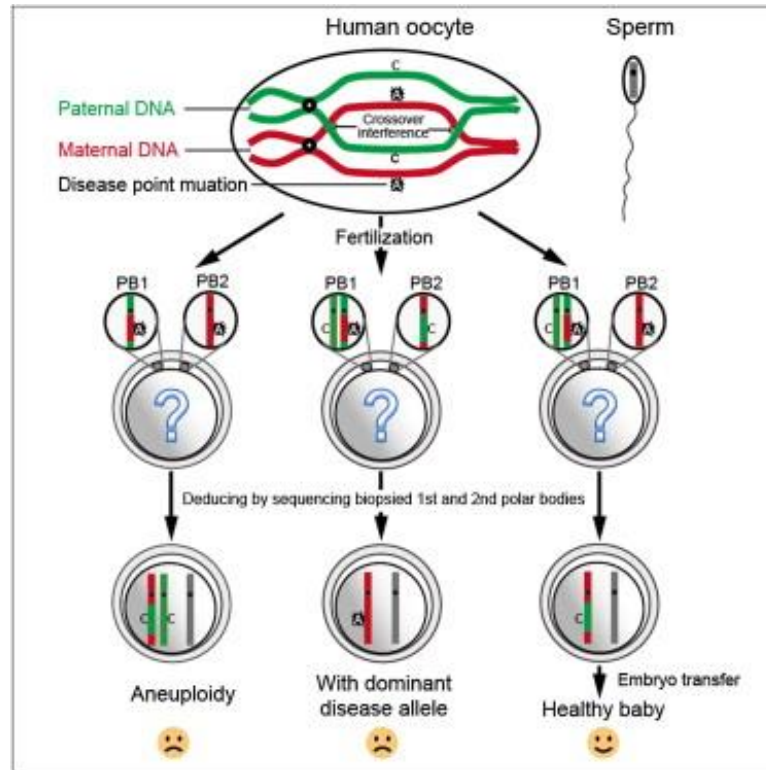
Personalized medicine

- ❑ **pharmacogenomics**
- ❑ **personalized oncology**
 - analyse tumor
 - design specific treatment



Personalized medicine

- ❑ pharmacogenomics
- ❑ personalized oncology
- ❑ pre-implantation genetic diagnosis (PGD)
 - 7000 genetic diseases 4000 known (Mendelian heretige)



Personalized medicine

- ❑ **pharmacogenomics**
- ❑ **personalized oncology**
- ❑ **pre-implantation genetic diagnosis (PGD)**
- ❑ **gene editing (gene therapy)**

Personalized medicine

- pharmacogenomics
- personalized oncology
- pre-implantation genetic diagnosis (PGD)
- gene editing (gene therapy)
- WHAT NEXT?