LOSCHMIDT LABORATORIES

10. Molecular Biotechnology in Medicine I.

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**
- **personalized 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

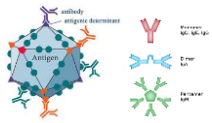
- medical laboratory methods contribute to **80% of diagnosis**
- good detection method should have three characteristics
  - **sensitivity** - ability to detect small amounts of target molecule
  - **specificity** - positive result for the target molecule only
  - **simplicity** - ability to run efficiently, inexpensively on a routine basis

Clinical diagnostics

- **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

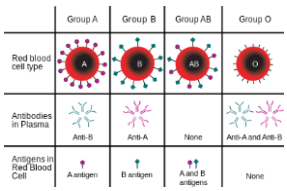
Immunological methods

- 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)



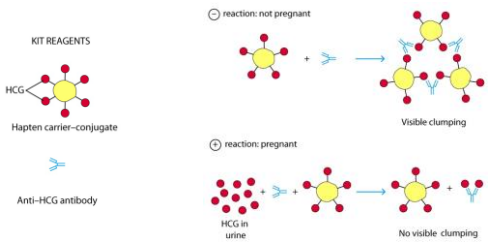
Immunological methods

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



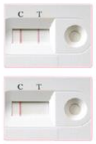
Immunological methods

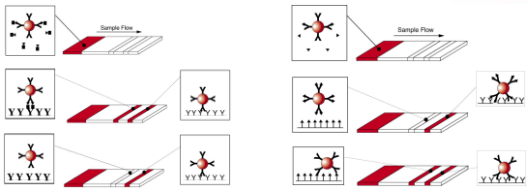
- **agglutination**
  - **pregnancy test**
  - inhibition in presence of human chorionic gonadotropin, hCG, glycoprotein hormone produced in pregnancy



Immunological methods

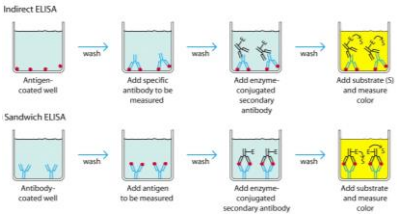
- **immuno-chromatographic assays**
  - simple devices to detect presence of analyte in sample
  - no need for specialized equipment or sample treatment
  - colored particle - latex (blue) or nanosized gold (red)
  - **sandwich** double antibody reaction scheme (e.g. HIV, hCG)
  - **competitive** reaction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





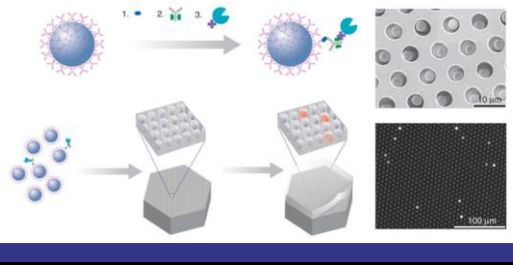
Immunological methods

- **enzyme-linked immunosorbent assay (ELISA)**
  - enzyme based detection (e.g., HRP, β-galactosidase, phosphatase)
  - florescence or colorimetric based detection



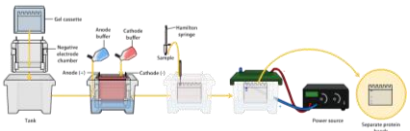
Immunological methods

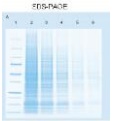
- **digital immunoassay** (single molecule ELISA)
  - detection volume decreased by a factor of 10^{10} (100µL to 10 fL)
  - fluorescence detection of fL-array
  - quantitative subfemtomolar range sensitivity



Immunological methods

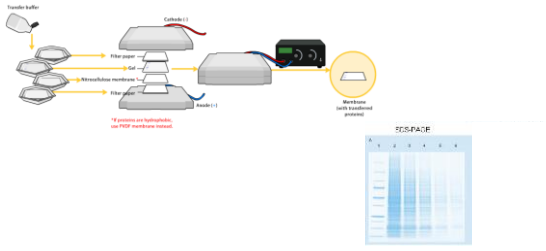
- **western blotting**
  - **SDS-Page** - separates components according to molecular weight





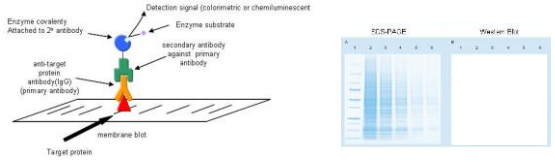
Immunological methods

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



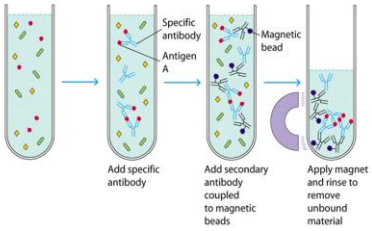
Immunological methods

- **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



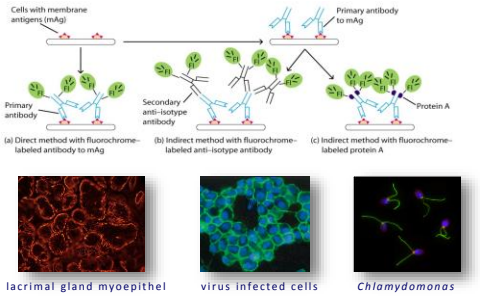
Immunological methods

- **immunoprecipitation**
  - collected by magnetic beads coupled to a secondary antibody



Immunological methods

- **immunofluorescence** (microscopy methods)
  - fluorescence labelled antibody (e.g., fluorescein, rhodamine)



Nucleic acid diagnostic systems

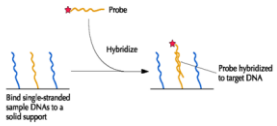
- most common object for testing is **DNA**, in some cases **RNA**
- areas of medical applications:
  - **prenatal diagnostics**: non-invasive detection of fetal diseases (e.g., Down syndrome, cystic fibrosis)
  - **genetic testing**: high throughput testing for genetic disorders (e.g., SNPs markers, 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)

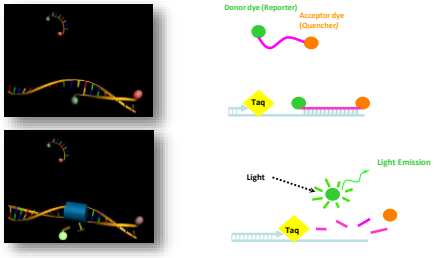
Nucleic acid diagnostic systems

- **DNA hybridization**
  - **conventional method**
    1. **attachment** 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)



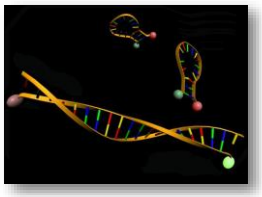
Nucleic acid diagnostic systems

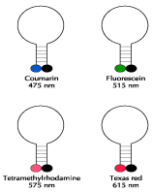
- **DNA hybridization**
  - **conventional method**
  - **TaqMan Probes** - hydrolysis by Taq polymerase



Nucleic acid diagnostic systems

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

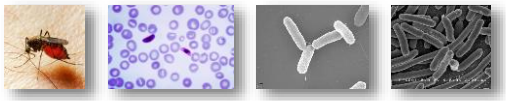




Nucleic acid diagnostic systems

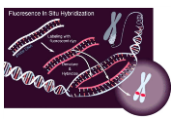
- **DNA hybridization**

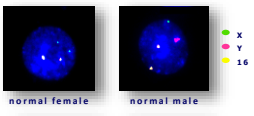
- EXAMPLE: detection of parasite *Plasmodium falciparum*
  - **microscopic** observations of blood smears is labor intensive
  - **ELISA** does not differentiate between past and present infection
  - **DNA** diagnostic system measure only current infection
- Other examples: *Salmonella typhi* (food poisoning)
  Escherichia coli (gastroenteritis)

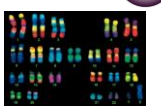


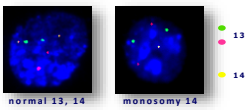
Nucleic acid diagnostic systems

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



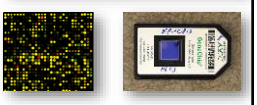


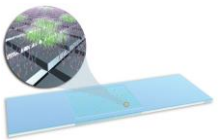




Nucleic acid diagnostic systems

- **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





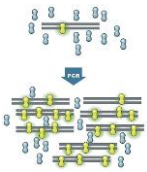
Nucleic acid diagnostic systems

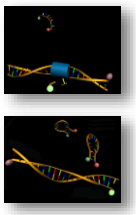
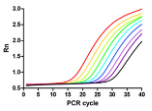
- **polymerase chain reaction (PCR)**
  - amplify single or few copies of DNA to millions of copies
  - the presence of the appropriate amplified size fragment (product) confirms the presence of the target
  - specific primers are available for detection of bacteria (*E. coli*, *M. tuberculosis*), viruses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

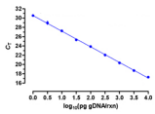


Nucleic acid diagnostic systems

- **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

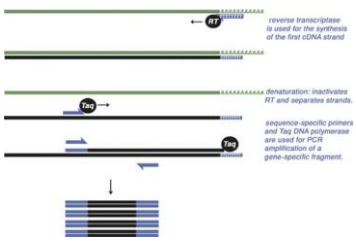




Nucleic acid diagnostic systems

- **polymerase chain reaction (PCR)**
  - **reverse transcription PCR (RT-PCR)**
  - **real-time reverse-transcription PCR (qRT-PCR)**

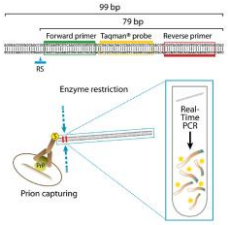


Nucleic acid diagnostic systems

- **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**

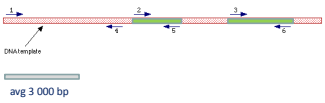
    detection limit 100 ng/L

    10-fold lower than classical ELISA



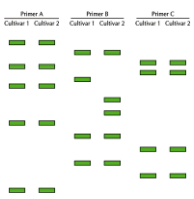
Nucleic acid diagnostic systems

- **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



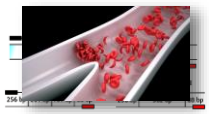
Which region(s) will be amplified?

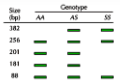
DNA fingerprinting



Nucleic acid diagnostic systems

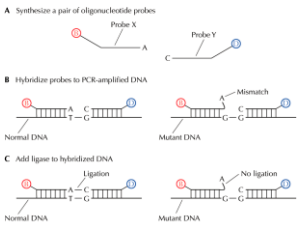
- **polymerase chain reaction (PCR)**
  - **restriction fragment length polymorphism (RFLP)**
    - many diseases caused by **single nucleotide change**
    - method dependent on **mutation within recognition site** of restriction enzyme
  - EXAMPLE: **diagnostics of sickle cell anemia**
    - anemia 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 CCT**GAG**G (A)
    - mutant DNA sequence CCT**GTG**G (S)
    - **homozygous state SS** red blood cells irregularly shaped

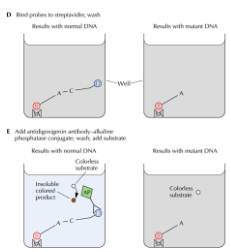




Nucleic acid diagnostic systems

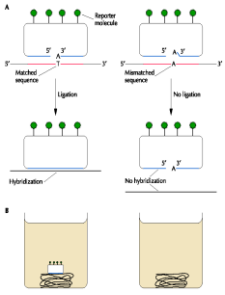
- **polymerase chain reaction (PCR)**
  - **oligonucleotide ligation assay (PCR/OLA)**





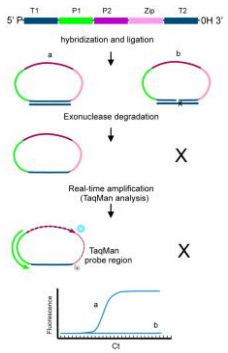
Nucleic acid diagnostic systems

- **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

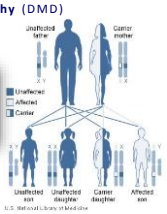
- **polymerase chain reaction (PCR)**
  - **Padlock probe (qPCR/PLP)**
    - target-complementary sequences at 5′ and 3′ ends (T1, T2)
    - universal primer sites (P1, P2)
    - reporter sequence (Zip)



Szemes et al. 2005. Nucl. Acids Res. 33: e70

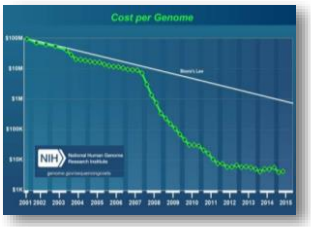
Nucleic acid diagnostic systems

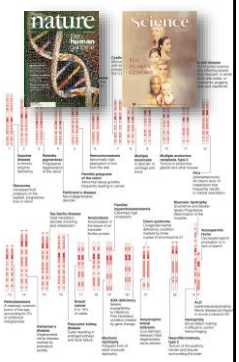
- **DNA sequencing**
  - most direct method
  - become cheap and fast, pushes other methods backward
  - genes, genetic regions (i.e. gene clusters or operons), full chromosomes or entire genomes
- EXAMPLE: **Diagnostic for Duchenne muscular dystrophy** (DMD)
  - mutated dystrophin ("implosion" of muscle 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

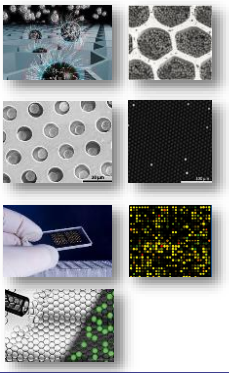
- **DNA sequencing**
  - 2003 Human Genome Project (**13 years**)
  - 2008 James Watson genome (**6 month**)
  - 2016 genome sequencing (**10 hours**)





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
- **DNA microarray (DNA chip)**
  - up to 10^6 probes (reporters)
  - picomole (10^{-12} M) of oligo per spot
- **droplet digital PCR**
  - droplets 1 nanoliter (10^{-9} liters)
  - 20 thousand reads/run

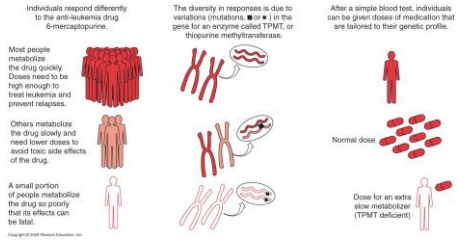


Personalized medicine

- medical practice and products **tailored to individual patient**
- **effective** 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**

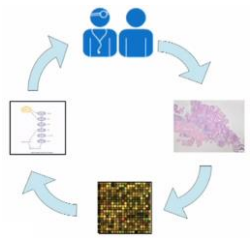
Personalized medicine

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



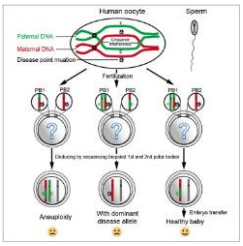
Personalized medicine

- pharmacogenomics
- **personalized oncology**
  - analyze tumor / patient **profile**
  - **design** specific treatment



Personalized medicine

- pharmacogenomics
- personalized oncology
- **pre-implantation genetic diagnosis (PGD)**
  - 7000 genetic deseases 4000 known (Mendelianian heretige)



Personalized medicine

- pharmacogenomics
- personalized oncology
- pre-implantation genetic diagnosis (PGD)
- **gene editing**
  - DNA is inserted, replaced, or removed from a genome
  - artificially engineered nucleases
  - **gene therapy** replaces defective gene at natural location