Molecular diagnostics. L3
Lecturer: Zhussupova A.I.

Review

The Role of the Pathologist in the Next-Generation Era of Tumor Molecular Characterization

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Abstract: Current pathology practice is being shaped by the increasing complexity of modern medicine, in particular of precision oncology, and major technological advances. In the "next-generation technologies era", the pathologist has become the person responsible for the integration and interpretation of morphologic and molecular information and for the delivery of critical answers to diagnostic, prognostic and predictive queries, acquiring a prominent position in the molecular tumor boards.

This "molecular revolution" is providing pathologists with the unique opportunity to gain a novel pivotal role in the therapeutic decision-making process and to be the main actors of the translation of biomarkers discovery into clinical application. In order to take the lead in the genomic transition, pathologists must be equipped with the ability to interpret molecular data and exploit molecular technologies and also willing to expand their horizons to other scientific disciplines, such as bioinformatics and artificial intelligence. However, while embracing the future, molecular pathologists must not underestimate the value of traditional histomorphology in order to provide a comprehensive morpho-molecular diagnosis. Moreover, the adoption of cutting-edge technologies is not without challenges. Many preanalytical and analytical issues should be addressed in order to efficiently integrate molecular profiling in the pathology workflow.

https://doi.org/10.3390/diagnostics11020339

Characteristics of a Detection System

- A good detection system should have 3 qualities:
  - ➢ Sensitivity
  - ➢ Specificity
  - ➢ Simplicity

- **Sensitivity** means that the test must be able to **detect very small amounts of target** even in the presence of other molecules.

- **Specificity**: the test yields a **positive result for the target molecule only.**

- **Simplicity**: the test must be able to **run efficiently and inexpensively** on a routine basis.

MassARRAY Diagnostics Are Being Developed For Multiple Disease Areas

Genetic Testing

- High throughput testing for genetic disorders including single nucleotide polymorphisms (SNPs) markers, insertions, deletions
- Examples: Factor II, Factor V, CFTR

Prenatal Diagnostics

- Non-invasive detection of fetal diseases
- Examples: Down syndrome, cystic fibrosis

Oncology

- Early diagnosis of cancer
- Example: circulating tumor DNA

Transplantation Medicine

- Non-invasive, early detection of organ rejection
- Example: urine testing for kidney rejection

Infectious Disease

- Pathogen identification and early detection
- Examples: identification of multi drug resistant mycobacteria, early detection of drug-resistant viral strains, e.g., HIV, HBV, HCV

- Progress is being made in all these areas

- Each of these areas are commercially attractive

- In some cases, the MassARRAY platform is uniquely qualified for specific tests

- More tests will be added to the platform as these tests are rolled out

Arrayed Primer Extension Reaction for Genotyping on Oligonucleotide Microarray
-> for Identification of allele specific mutations

Method based on 2 steps:

1. targeting of DNA hybridization to the complementary oligoprimers

2. single base extension of these primers with appropriate dyelabeled ddNTPs that match the nucleotide on polymorphic site by DNA polymerase or Reverse transcriptase
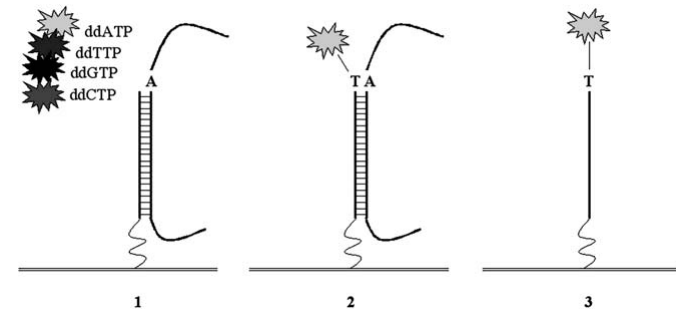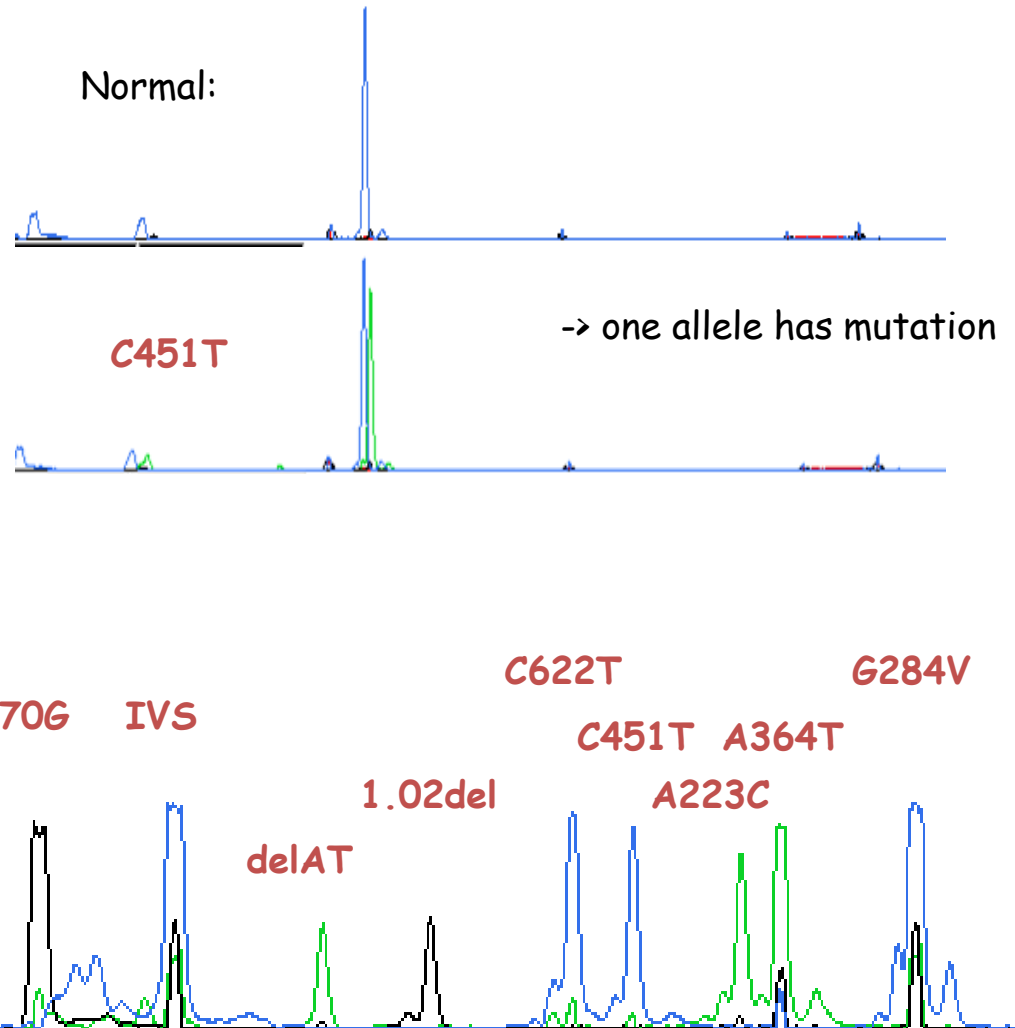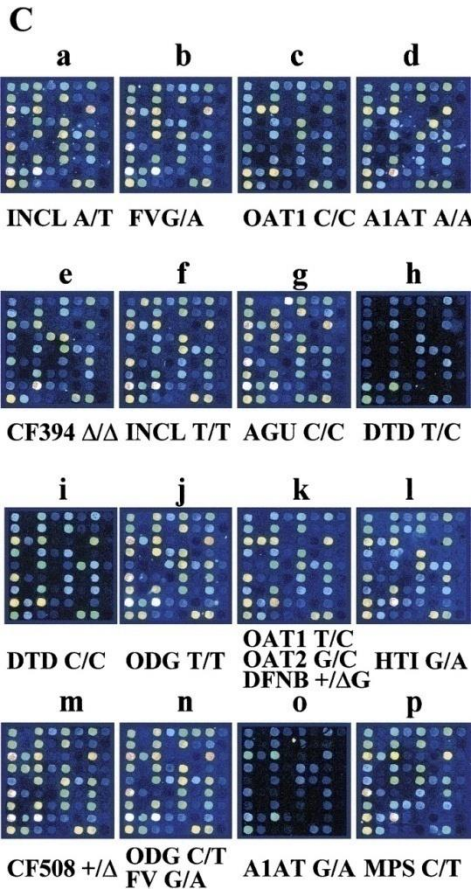
Primer design:
-> each base is identified by 2 unique 25-mer oligos (one for each strand) with their 3'-end one base upstream of the base to be identified

-> detects allele-specific mutations
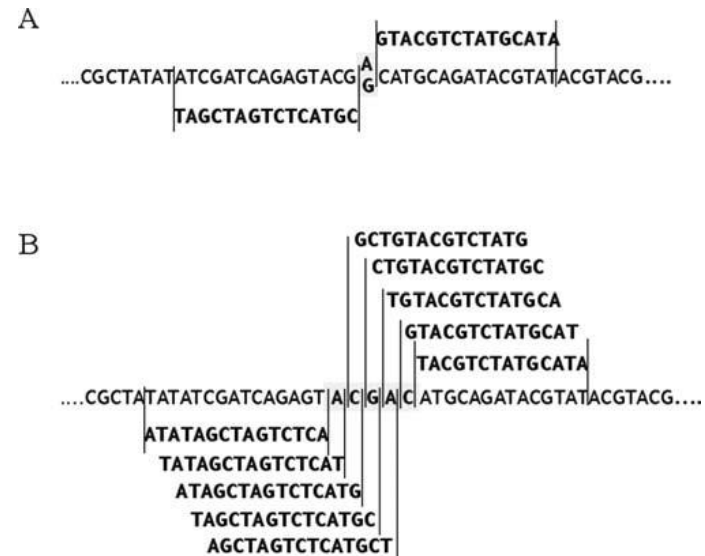
Arrayed Primer Extension Reaction for Genotyping on Oligonucleotide Microarray -> for Identification of NCL Mutations
(Neuronal Ceroid Lipofuscinoses)

Normal:

C451T

-> one allele has mutation

C70G   IVS   1.02del   delAT   C622T   C451T   A223C   A364T   G284V

Immunological Diagnostics Methods

Applications of Immunoassays

- Analysis of hormones, vitamins, metabolites, diagnostic markers
  - Eg. ACTH, FSH, T3, T4, Glucagon, Insulin, Testosterone, vitamin B12, prostaglandins, glucocorticoids,
- Therapeutic drug monitoring:
  - Barbiturates, morphine, digoxin
- Diagnostic procedures for detecting infection
  - HIV, Hepatitis A, B, etc…

Based on Antigen-Antibody Interactions

- a bimolecular association
  involving various non-covalent interactions
- Is similar to an enzyme-substrate interactions,
  but not lead to an irreversible chemical alteration
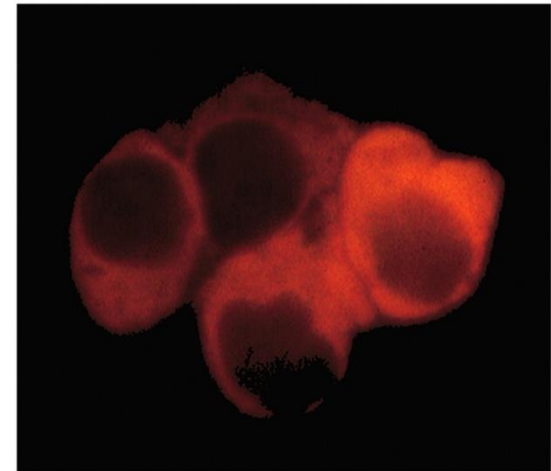
Immunological Diagnostics Methods

1. Strength of Antigen-Antibody Interactions

2. Cross-Reactivity

3. Agglutination Reactions

4. Radioimmunoassay

5. Enzyme-Linked ImmunoSorbent Assay (ELISA)

6. Western Blotting

7. Immunoprecipitation

8. Immunofluorescence

9. Flow Cytometry and Fluorescence

10. Alternatives to Antigen-Antibody Reactions

11. Immunoelectron Microscopy

Immunological Diagnostics Methods

Strength of Antigen-Antibody Interactions

Antibody affinity

– is a quantitative measure of binding strength
- combined strength of the noncovalent interactions between a binding site on an Ab & monovalent Ag

Antibody avidity (describes the binding intensity of multiple bond interactions)
-True strength of the Ab-Ag interaction within biological systems
- The interaction at one site will increase the possibility of reaction at a second site
- High avidity can compensate for low affinity
  (IgM may have low affinity but it has high avidity due to its 10 weak binding sites contrary to the two strong binding sites of IgG.)

IgM (pentamer)
μ chain

IgG
γ chain
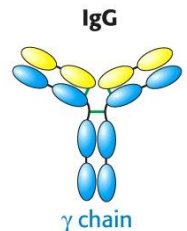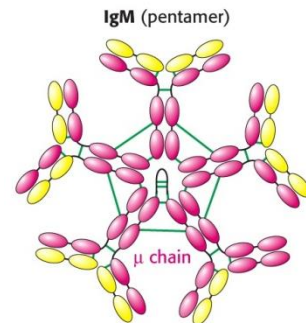
Forward & reverse rate constants (k1 & k-1)
Association & dissociation constants (Ka & Kd) for 3 ligand-Ab interaction

Antibody	Ligand	k_1	k_{-1}	K_a	K_d
Anti-DNP	ϵ-DNP-L-lysine	8×10^7	1	1×10^8	1×10^{-8}
Anti-fluorescein	Fluorescein	4×10^8	5×10^{-3}	1×10^{11}	1×10^{-11}
Anti-bovine serum albumin (BSA)	Dansyl-BSA	3×10^5	2×10^{-3}	1.7×10^8	5.9×10^{-9}

SOURCE: Adapted from H. N. Eisen, 1990, *Immunology*, 3rd ed., Harper & Row Publishers.

– High affinity complexes have high Ka values
- Very stable complexes have very low values of Kd

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	0.0006−0.006
Enzyme-linked immunosorbent assay (ELISA)	<0.0001−0.01
ELISA using chemiluminescence	<0.0001−0.01[†]
Immunofluorescence	1.0
Flow cytometry	0.06−0.006

[*]The sensitivity depends upon the affinity of the antibody as well as the epitope density and distribution.

[†]Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology,* 5th ed., American Society for Microbiology, Washington, D.C.

Immunological Diagnostics Methods

Cross-reactivity

- Antibody elicited by one Ag can cross-react with unrelated Ag.
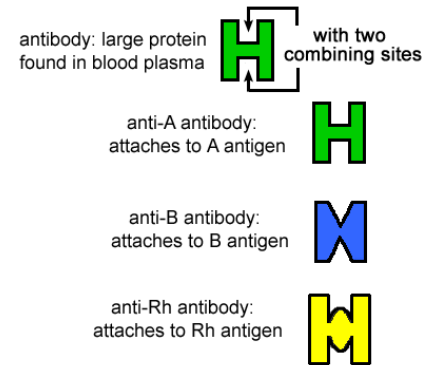- occurs if two different Ags share identical or very similar epitope

(i) Cowpox antigens in vaccinia virus (also used for vaccination) are
   cross-reactive to smallpox antigens in variola virus (share
   similar or identical epitope)

(ii) Streptococcus pyogenes infection --->>> heart & Kidney damage
   following the infection (cell wall proteins called M antigens vs
   Myocardial & skeletal muscle proteins).

(iii) Original antigenic sin.
   - The existence of long-lived lymphocytes & crossreactivity
   - Vaccination with one strain of flu elicited Ab responses to
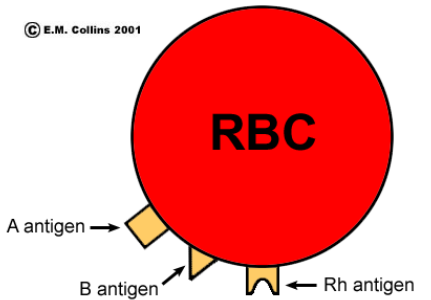   another flu strain.

Smallpox

Immunological Diagnostics Methods
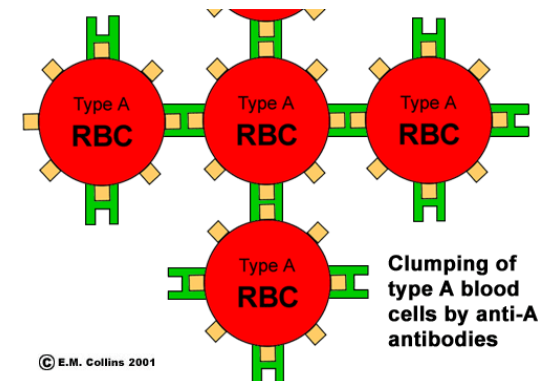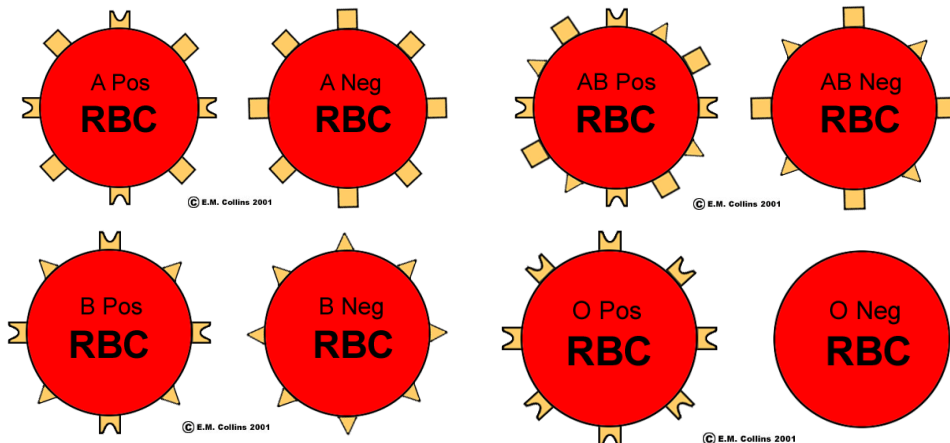
Cross-reactivity + agglutination

antibody: large protein found in blood plasma — with two combining sites

anti-A antibody: attaches to A antigen

anti-B antibody: attaches to B antigen

anti-Rh antibody: attaches to Rh antigen

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ABO blood types		
Blood type	**Antigens on RBCs**	**Serum antibodies**
A	A	Anti-B
B	B	Anti-A
AB	A and B	Neither
O	Neither	Anti-A and anti-B

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RBC

A antigen →
B antigen →
← Rh antigen

- The antibodies are induced by exposure to cross-reacting microbial antigens present on common intestine bacteria.
- ABO blood-group antigens have differences in the sugars on glyco-proteins in RBC (Red blood cells).
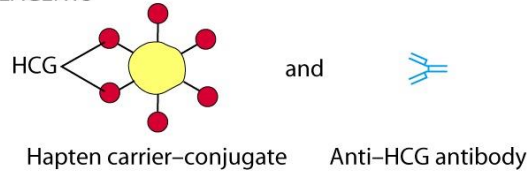- Providing the basis for blood typing test in blood transfusion

A Pos RBC   A Neg RBC   AB Pos RBC   AB Neg RBC
© E.M. Collins 2001   © E.M. Collins 2001

B Pos RBC   B Neg RBC   O Pos RBC   O Neg RBC
© E.M. Collins 2001   © E.M. Collins 2001

Type A RBC   Type A RBC   Type A RBC
Type A RBC

Clumping of type A blood cells by anti-A antibodies

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Immunological Diagnostics Methods

Agglutination

Home pregnancy kit

KIT REAGENTS

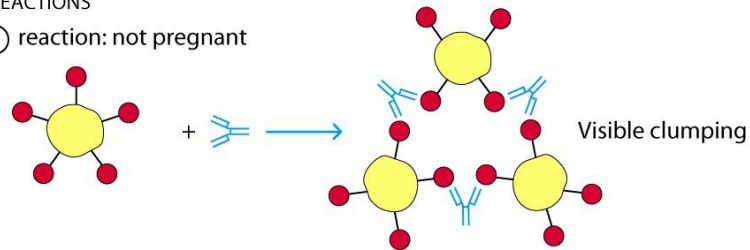HCG — Hapten carrier–conjugate   and   Anti–HCG antibody

TEST PROCEDURE

Urine + Anti–HCG →(Incubate)→ + HCG carrier conjugate → Observe for visible clumping
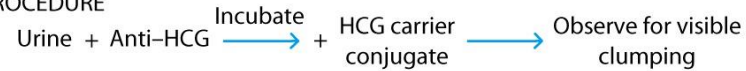
POSSIBLE REACTIONS

(−) reaction: not pregnant

+ → Visible clumping

(+) reaction: pregnant

HCG in urine + + → No visible clumping +
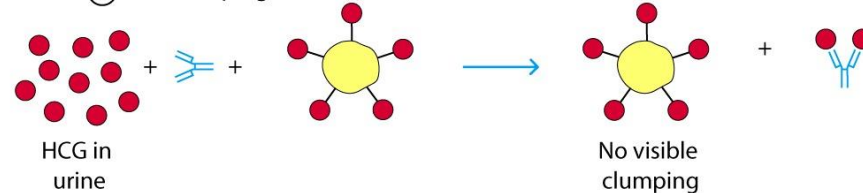
- Based on <u>hapten inhibition</u> (<u>agglutination inhibition</u>) to determine the presence or absence of <u>h</u>uman <u>c</u>horionic <u>g</u>onadotropin (HCG; a glycoprotein hormone produced in pregnancy) >>> The kits currently on the market use <u>ELISA-based assays</u>.
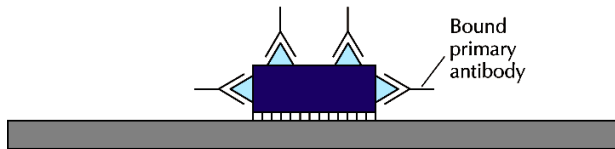- Also used to determine the use of illegal drugs, & immunity (Ab) to virus (rubella).

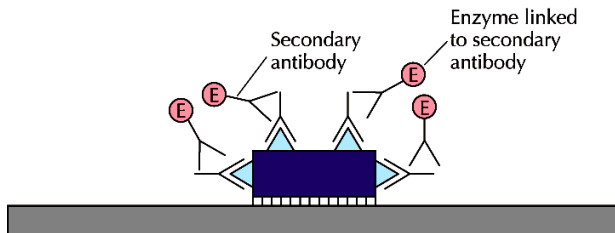# Immunological Diagnostics Methods
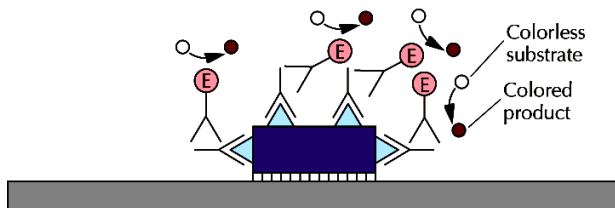
ELISA

A Bind sample to support

Antigenic site
Target molecule
Support
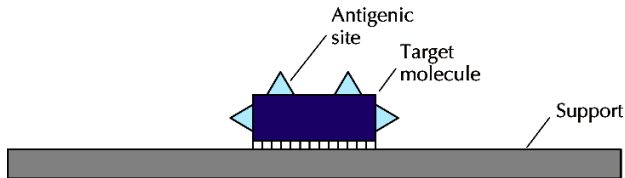
B Add primary antibody; wash

Bound primary antibody

C Add secondary antibody–enzyme conjugate; wash

Secondary antibody
Enzyme linked to secondary antibody

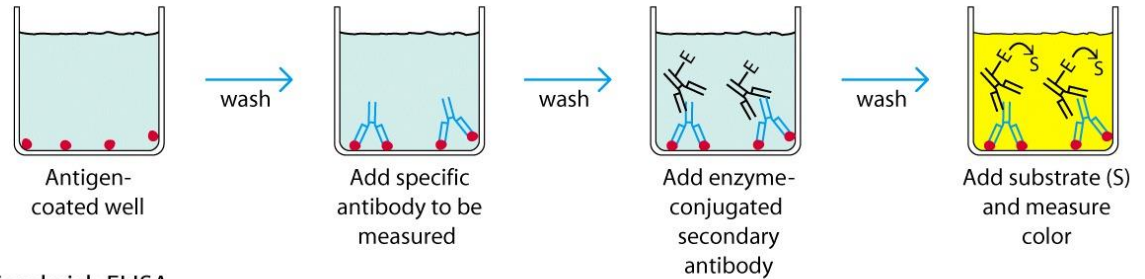**D** Add substrate

Colorless substrate
Colored product

- Addition of a **specific antibody** (primary antibody) which will bind to the test molecule if it is present.

- **Washing** to remove unbound molecules.

- Addition of **secondary antibody** which will bind to the primary antibody.

- The secondary antibody usually has attached to it an **enzyme** e.g., **alkaline phosphatase**.

- **Wash** to remove unbound antibody.

- Addition of a **colourless substrate** which will react with the secondary antibody to give a **colour reaction** which indicates a positive result.
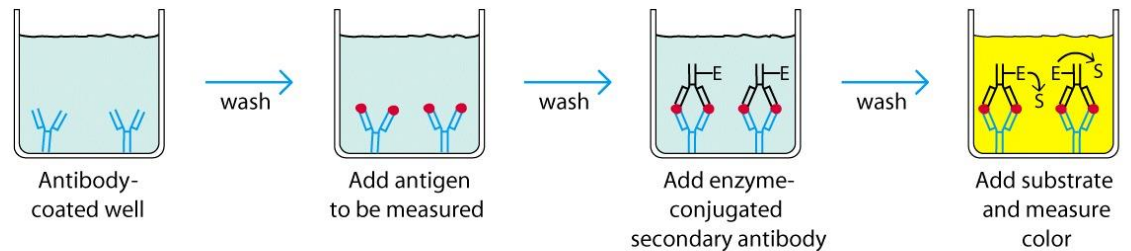
-> can be used for quasi High-throughput!!!
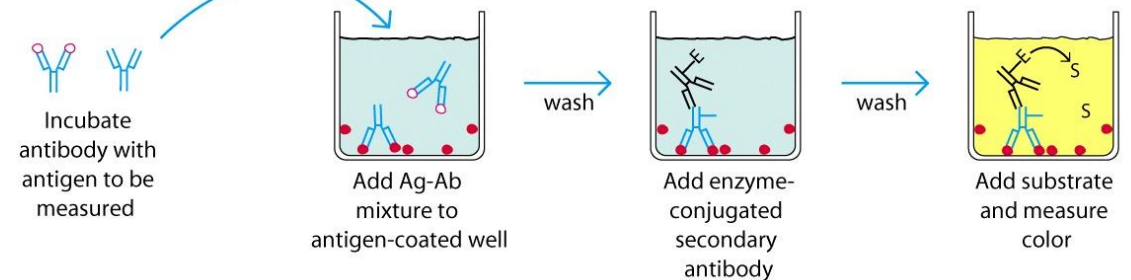
Immunological Diagnostics Methods

ELISA -Variants
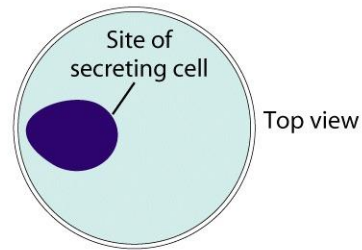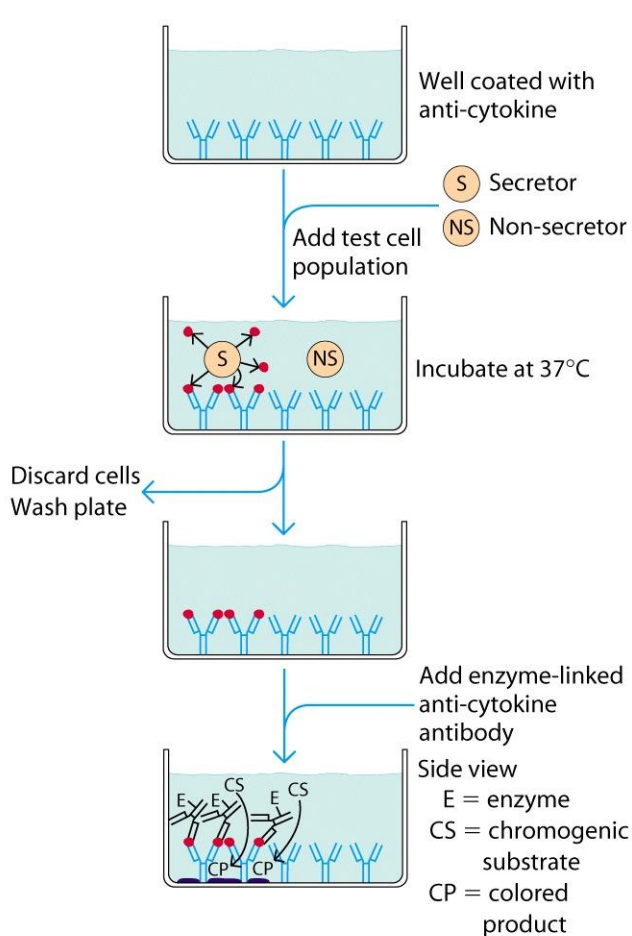
Detection based on enzyme catalyzed reactions:

1. alkaline Ⓟ
2. horseradish peroxidase
3. β-galactosidase

Detection based on fluorescent labeled secondary antibody

(a) Indirect ELISA

Antigen-coated well → wash → Add specific antibody to be measured → wash → Add enzyme-conjugated secondary antibody → wash → Add substrate (S) and measure color

(b) Sandwich ELISA

Antibody-coated well → wash → Add antigen to be measured → wash → Add enzyme-conjugated secondary antibody → wash → Add substrate and measure color

(c) Competitive ELISA

Incubate antibody with antigen to be measured → Add Ag-Ab mixture to antigen-coated well → wash → Add enzyme-conjugated secondary antibody → wash → Add substrate and measure color

Immunological Diagnostics Methods
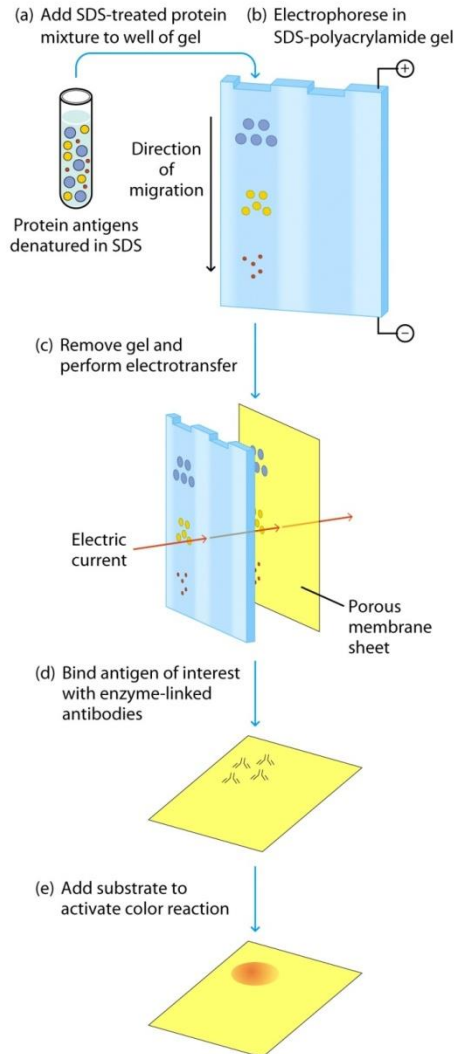
ELISA -Variants

The ELISPOT assay -> to determine quantitatively

the # of cells in a population that are producing specific Ab or cytokine.

-> precipitates & forms a spot only on the areas of the well where cytokine-secreting cells had been deposited.

Immunological Diagnostics Methods

Western blot

(a) Add SDS-treated protein mixture to well of gel

(b) Electrophorese in SDS-polyacrylamide gel

Direction of migration

Protein antigens denatured in SDS

(c) Remove gel and perform electrotransfer

Electric current

Porous membrane sheet

(d) Bind antigen of interest with enzyme-linked antibodies

(e) Add substrate to activate color reaction

SDS-Page: separates the components according to their molecular weight.

Blot: the proteins in the gel are transferred to the sheet of nitrocellulose or nylon by the passage of an electric current.
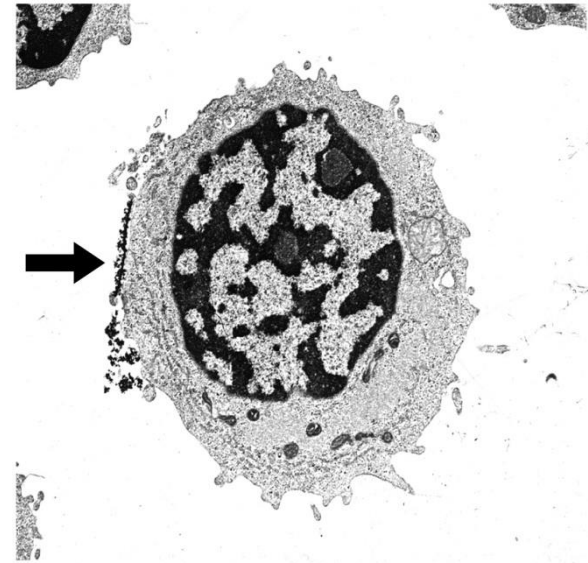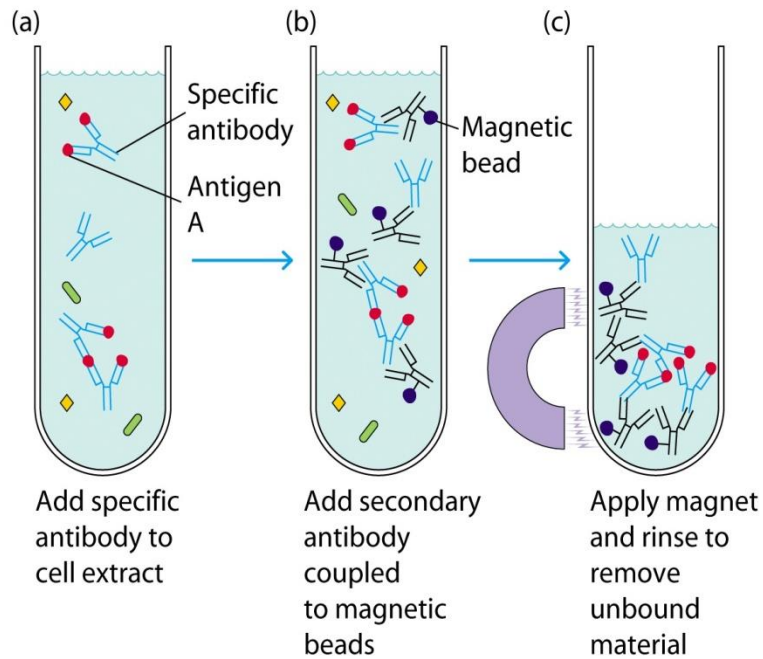
Immunoreaction: probed with Ab & then radiolabeled or enzyme-linked 2nd Ab.

Detection: a position is visualized by means of an ELISA reaction.
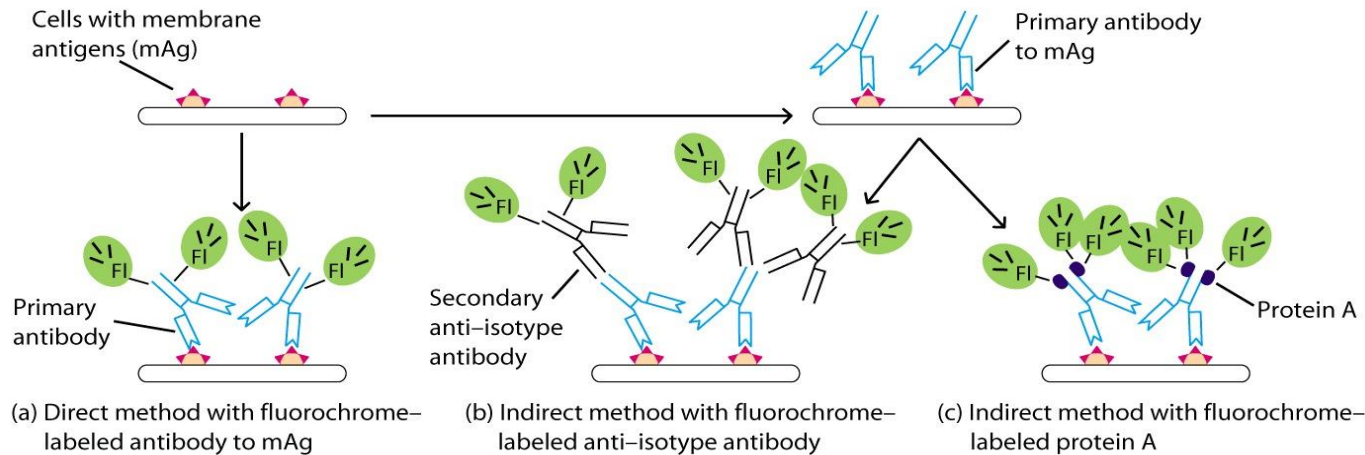
Immunological Diagnostics Methods

Immunoprecipitation

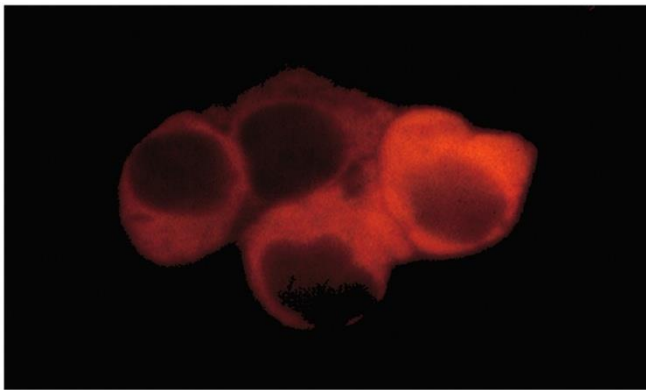Immuno-precipitates can be collected using magnetic beads coupled to a secondary antibody.

(a) Add specific antibody to cell extract — Specific antibody, Antigen A
(b) Add secondary antibody coupled to magnetic beads — Magnetic bead
(c) Apply magnet and rinse to remove unbound material

EM showing a cell with magnetic beads attached to its surface via antibodies.

Immunological Diagnostics Methods

Immunofluorescence

Cells with membrane antigens (mAg)

Primary antibody to mAg

Primary antibody

Secondary anti–isotype antibody

Protein A

(a) Direct method with fluorochrome–labeled antibody to mAg

(b) Indirect method with fluorochrome–labeled anti–isotype antibody

(c) Indirect method with fluorochrome–labeled protein A

Protein A has the ability to bind to IgG

Fluorochromes
- Fluorescein (490→517nm)
- Rhodamine (515→546nm)
- Phycoerythrin

mIgM-producing B cells indirectly stained with rhodamine-conjurated secondary Ab under a <u>fluorescence microscope</u>.
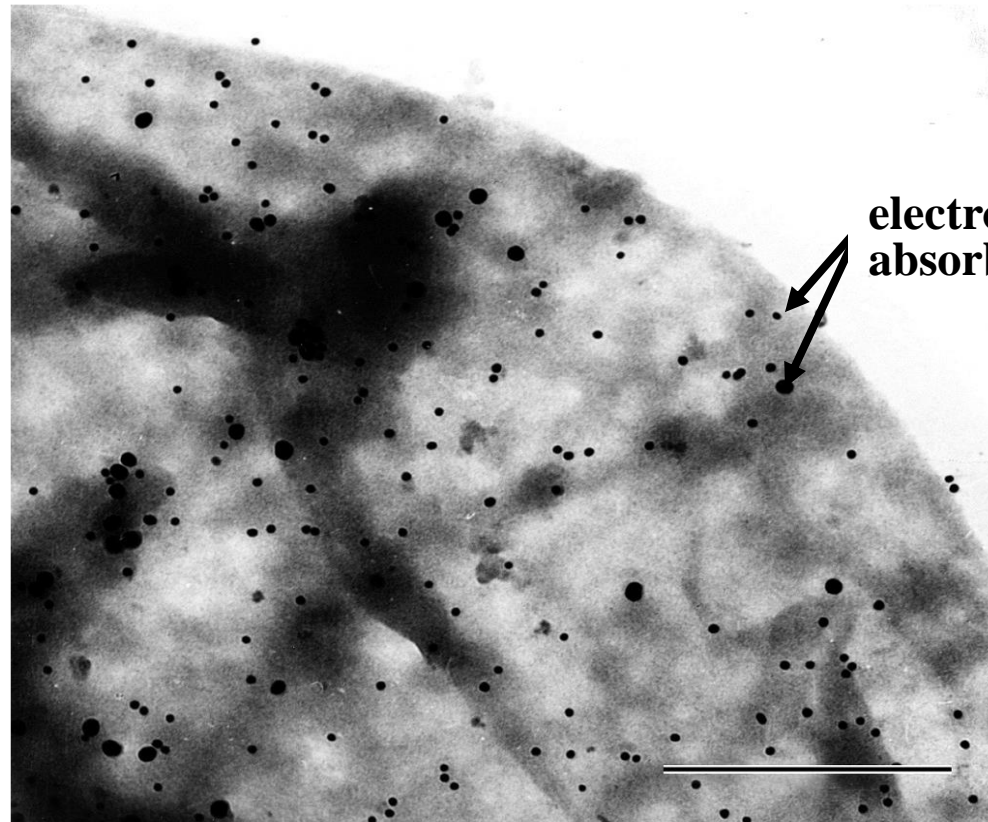
Immunological Diagnostics Methods

Immuno Electron Microscopy

An immunoelectronmicrograph of the surface of a B-cell lymphoma was stained with two antibodies (Ab against class II MHC labeled with 30nm gold particles, & another Ab against class I MHC w/ 15nm gold particles. (The density of class I exceeds that of class II)

- Electron-dense label (ferritin or colloidal gold) is conjugated to the Fc portion.

electron-dense labels absorb electrons.

Alternatives to Ag-Ab Reactions

Instead of Ag-Ab-Ab*:

Ag-IgG-A/G*:
① Protein A (from staphylococcus) & protein G (from streptococcus)
  - bind to rhe (human rheumatoid factor) Fc region (fragment crystallizable
    region – constant) of lgG molecules ($k_a \sim 10^8$)
  - used to detect lgG molecules in the Ag-Ab complexes
  - used to isolate lgG molecules in the affinity columns

Ag-Ab-biotin-(a)vidin*
② Avidin (from egg whites) & streptavidin (from streptomyces avidinii)
  conjugated with an enzyme, fluorochrome, radioactive label)
  - bind to biotin (a vitamin) with higher affinity ($k_a \sim 10^{15}$)
  - Ab can be labeled with ($k_a \sim 10^{18}$)

DNA Diagnostic Systems

Problematics & Solutions

Ask the right question:

- Does THIS patient have ANY mutation in ANY gene that would explain his disease?
  -> NOT POSSIBLE TO SAY

- Does THIS patient have ANY mutation in THIS gene that might cause his disease?
  -> NEED LOTS OF EFFORTS TO ANSWER

- Does THIS patient have a 3-bp deletion of Phe codon in CFTR gene?
  -> THAT IS A RIGHT QUESTION !!!

The choice of material to test:

- DNA most common; tested by PCR
  Sometimes tested by Southern blotting
- RNA RT-PCR allow to test genes directly, without breaking them into exons.
- Allow to detect alternative spliced isoforms.

DNA Diagnostic Systems

Problematics & Solutions

How to obtain DNA specimen:

- Blood sample (most common for adult testing);
- Mouthwashes or buccal scrapes (non-invasive);
- *Chorionic villus* biopsy samples (fetal DNA);
- Hair, semen (criminology)
- One or two cells removed from 8-cell embryo (*in vitro* fertilisation)
- Archived pathological specimens (typing dead peoples, tumor samples in paraffin blocks);
- Paper cards with blood drops on them

Methods of mutation scanning (when we do not know where is our mutation)

- Sequencing -- most direct method;
- Detecting mismatches or heteroduplex DNA molecules;
- PCR based Single-strand conformational polymorphism (SSCP) analysis;
- Protein truncation test (PTT);
- Detecting of deletions;
- Detection of methylation

DNA Diagnostic Systems

DNA Diagnostic Systems include:

- DNA Hybridization
- DNA Sequencing
- PCR
- Restriction endonuclease analysis
- RAPD (random amplified polymorphic DNA)
- DNA fingerprinting

DNA Diagnostic Systems

Hybridization methods

- Bacterial and viral pathogens may be pathogenic because of the presence of **specific genes** or sets of genes.
- Genetic diseases often are due to **mutations** or **absence** of particular gene or genes.
- These genes (DNA) can be used as diagnostic tools.

Example: Detection of Malaria

- Malaria is caused by the parasite *Plasmodium falciparum*.
- The parasite infects and destroys **red blood** cells.
- Symptoms include fever, rashes and damage to brain, kidney and other organs.
- Current testing involves **microscopic observations** of blood smears, which is labour intensive.
- A DNA diagnostic system would only measure **current infection**
- Find a **probe** that just hybrisized with *Plasmodium falciparum* DNA and not with human DNA
- The probe is able to detect 10 pg of purified DNA or 1 ng of DNA in blood smear.

- Other DNA probes were developed for the following diseases:
- *Salmonella typhi* (food poisoning)
- *E. coli* (gastroenteritis)
- *Trypanosoma cruzi* (chagas' disease)

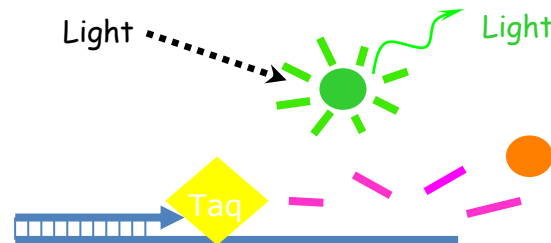# DNA Diagnostic Systems

Hybridization

TaqMan® Probes

Donor dye
(Reporter)

Acceptor dye
(Quencher)

Unbound probe free in solution, Donor in close poximity -> signal quenched

Taq

Only if probe binds specifically to DNA reaction occurs

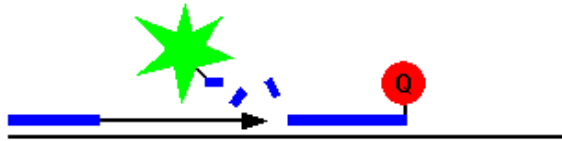Light

Light Emission

Taq

Taq extends and hydrolyzes probe, donor dye free to emit fluorescence --> accumulation of signal

-> Signal proportional to used probe
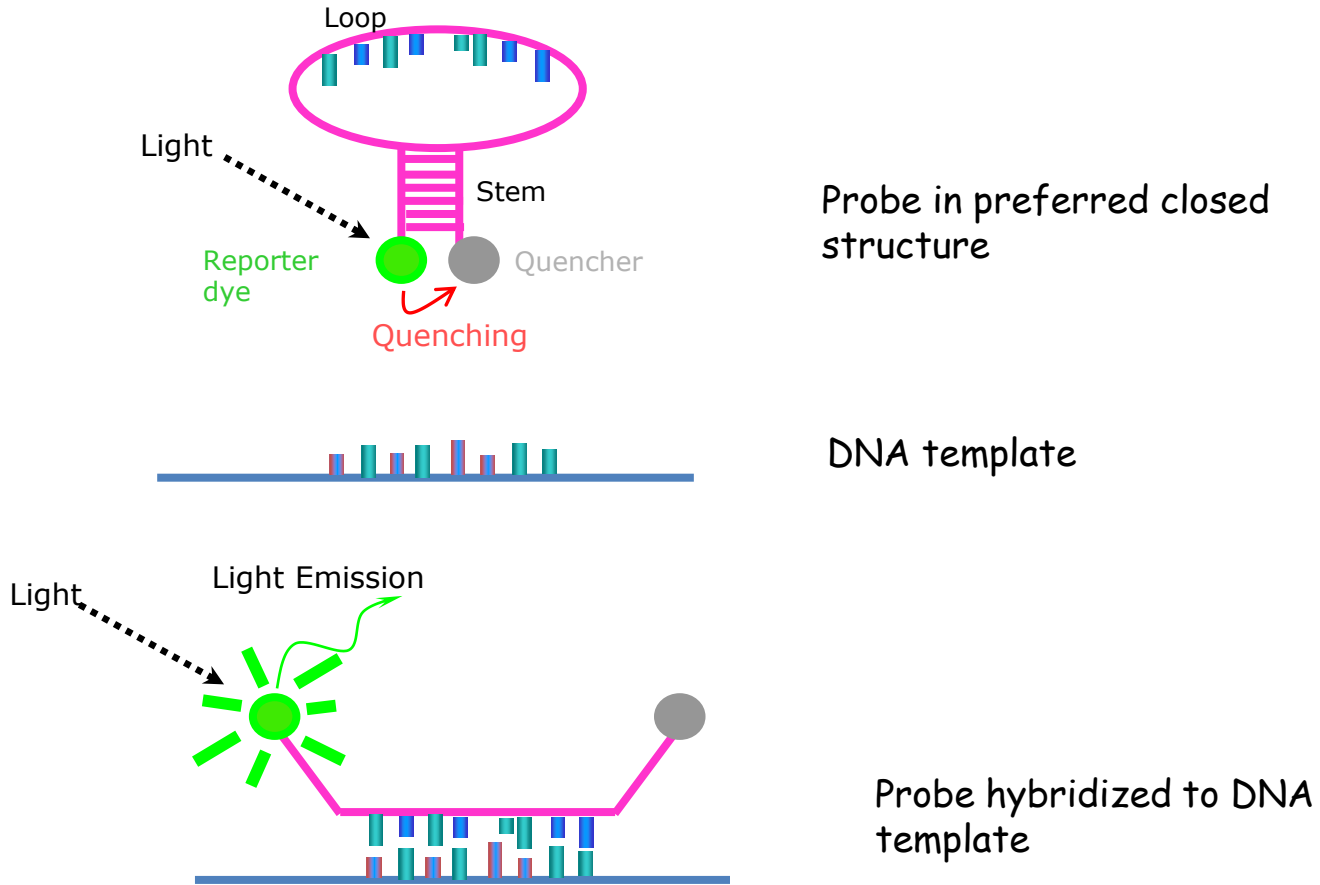
DNA Diagnostic Systems

Hybridization

TaqMan® Probe design

- 20-30 bp in length, Tm 10°C higher than primer.
- 35-65% G/C; more Cs than G's. Can try as high as 80% or as low as 20% if the region is particularly GC or AT rich.
- Avoid runs of 3+ of the same nucleotide, especially G's.
- 5' base ≠ G.
- When the probe and primer anneal to the target, the 5' end of probe should be 3 nucleotides from the 3' end of the primer on same strand (max of 10-12).
- Test that primer and probe are not complementary to each other. (delta G free energy at 25C should be greater than -2)

DNA Diagnostic Systems

Hybridization

Molecular Beacons

Loop

Light

Stem

Reporter dye

Quencher

Quenching

Probe in preferred closed structure

DNA template

Light

Light Emission

Probe hybridized to DNA template

DNA Diagnostic Systems

Hybridization
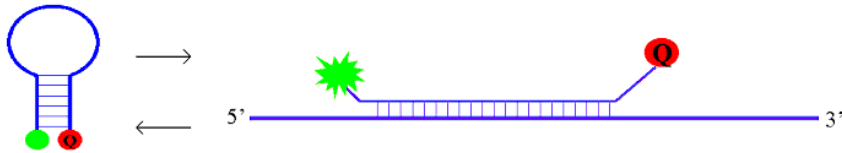
Molecular Beacon design

Stem (bp)	Approx. Tm
5	55-60°C
6	60-65°C
7	65-70°C

- Tm of probe region should be 7-10°C above target annealing temp.
- To the chosen sequence add a stem
- 5-7 bp in length, with similar Tm
- as the probe region.
- Check that there is no complementarity between primers and probe.
- Tm of probe alone and probe + complement should be verified experimentally

Beacon + Target

Beacon + 1bp mismatched target

Beacon alone
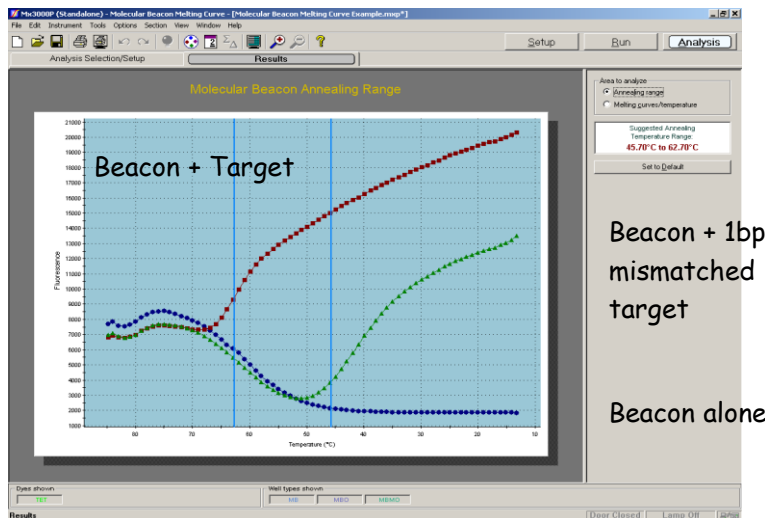
- Properly designed Molecular Beacons can effectively discriminate between targets with a single bp mismatch.

DNA Diagnostic Systems

Hybridization

FISH diagnosis -> used for Preimplantation Genetic Diagnosis (PGD):

- Analyse chromosomes

- Sexing for X-linked disease

- Chromosome abnormalities

- Age related aneuploidy (abnormal number of chromosomes)
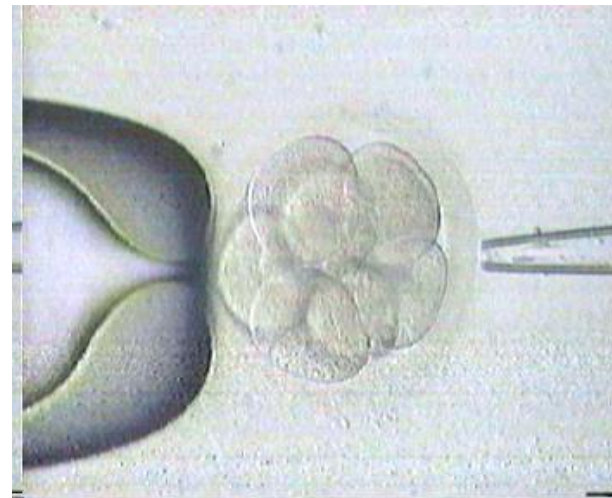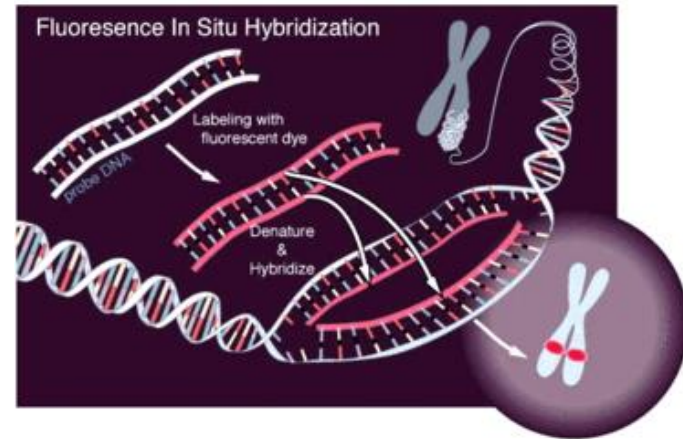
Cleavage Stage Biopsy

DNA Diagnostic Systems

Chromosomes in human embryos

- NORMAL
  - All cells uniformly diploid
- ABNORMAL
  - All cells uniformly abnormal eg trisomy 21
- MOSAIC
  - Two or more cell lines present
    - often diploid with aneuploid or tetraploid cells
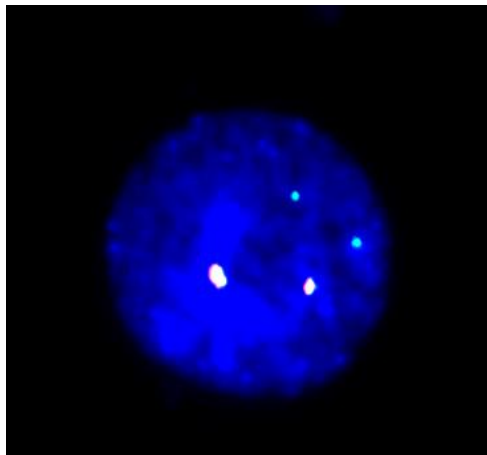- CHAOTIC
  - Different chromosome pattern in every cell
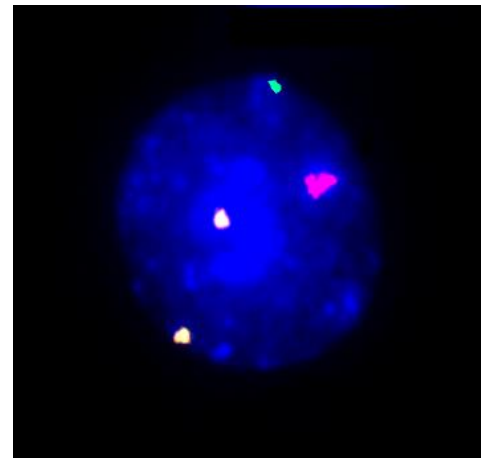
DNA Diagnostic Systems

Hybridization

FISH diagnosis -> used for Preimplantation Genetic Diagnosis (PGD):

Sexing Embryos for PDG: FISH analysis of interphase nuclei

🟢 Chromosome X　　🔴 Chromosome Y　　🟡 Chromosome 16

Normal Female

Normal Male

DNA Diagnostic Systems

Hybridization

FISH diagnosis -> used for Preimplantation Genetic Diagnosis (PGD):

Chromosome Abnormalities

- Translocations (rearrangement of parts between nonhomologous chromosomes)
  - Robertsonian
    - Occurres in chromosome 13,14,15,21,22
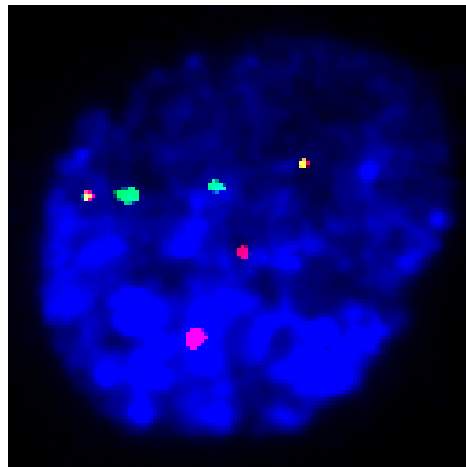 - Reciprocal
- Insertions
- Inversions
- Ring Chromosomes

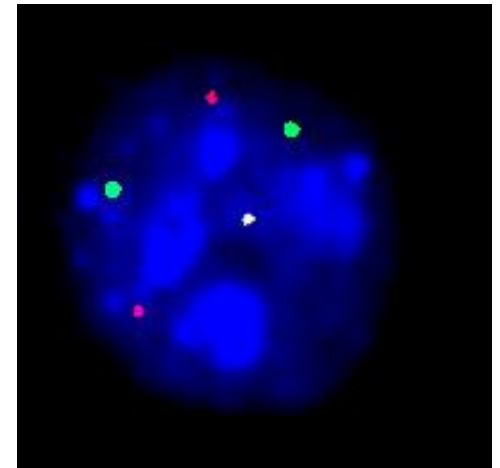PGD of Chromosome Abnormalities:
Robertsonian Translocation

Chromosome 13   Chromosome 14

Normal for Chromosomes 13 & 14   Monosomy 14

Monosomy 14 -> presence of only one chromosome (instead of the typical two in humans) 14 from a pair, Fetuses usually are not viable.

DNA Diagnostic Systems

Hybridization

FISH diagnosis -> used for Preimplantation Genetic Diagnosis (PGD):

Aneuploidy Screening

- incorrect number of chromosomes
- Older women likely to produce abnormal oocytes
- Leads to chromosomally abnormal embryos
  - increase in miscarriage
  - lower pregnancy rate
- Chromosomes commonly involved
  - 13, 16, 18, 21, X and Y
- Used for older women with
  - recurrent IVF (*in vitro* fertilization) failure
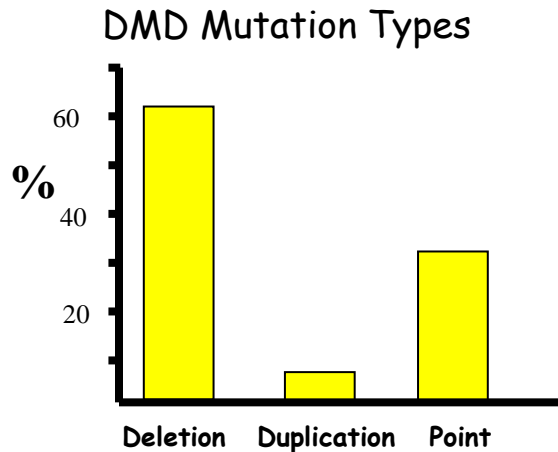 - recurrent miscarriage

DNA Diagnostic Systems

Sequencing → (cost – DKK 50,00 per run)

As sequencing becomes more and more cheap,
it pushes other methods backward.

For sequencing of genomic DNA,
every exon is amplified separately
(Typical sequencing run – 500bp; typical exon size – 145 bp)

Example: Diagnostic for Duchenne Muscular Dystrophy (DMD)

DMD Mutation Types

- X-linked and affect mainly males an estimated 1 in 3500 boys worldwide
- DMD encodes a large structural protein: dystrophin
- strengthen muscle cells by anchoring elements of the internal cytoskeleton to the surface membrane
- Mutated dystrophin leads to "implosion" of muscle cells

Duchenne muscular dystrophy

Dongsheng Duan, Nathalie Goemans, Shin'ichi Takeda, Eugenio Mercuri & Annemieke Aartsma-Rus ✉

https://www.nature.com/articles/s41572-021-00248-3

Abstract

Duchenne muscular dystrophy is a severe, progressive, muscle-wasting disease that leads to difficulties with movement and, eventually, to the need for assisted ventilation and premature death. The disease is caused by mutations in *DMD* (encoding dystrophin) that abolish the production of dystrophin in muscle. Muscles without dystrophin are more sensitive to damage, resulting in progressive loss of muscle tissue and function, in addition to cardiomyopathy. Recent studies have greatly deepened our understanding of the primary and secondary pathogenetic mechanisms. Guidelines for the multidisciplinary care for Duchenne muscular dystrophy that address obtaining a genetic diagnosis and managing the various aspects of the disease have been established. In addition, a number of therapies that aim to restore the missing dystrophin protein or address secondary pathology have received regulatory approval and many others are in clinical development.

DNA Diagnostic Systems

Sequencing

Minisequencing by primer extension

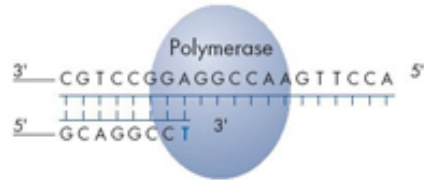**DNA polymerase + one of the four labeled dNTPs**
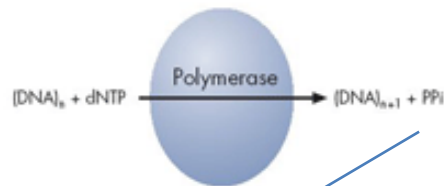**= sequencing of one nucleotide**
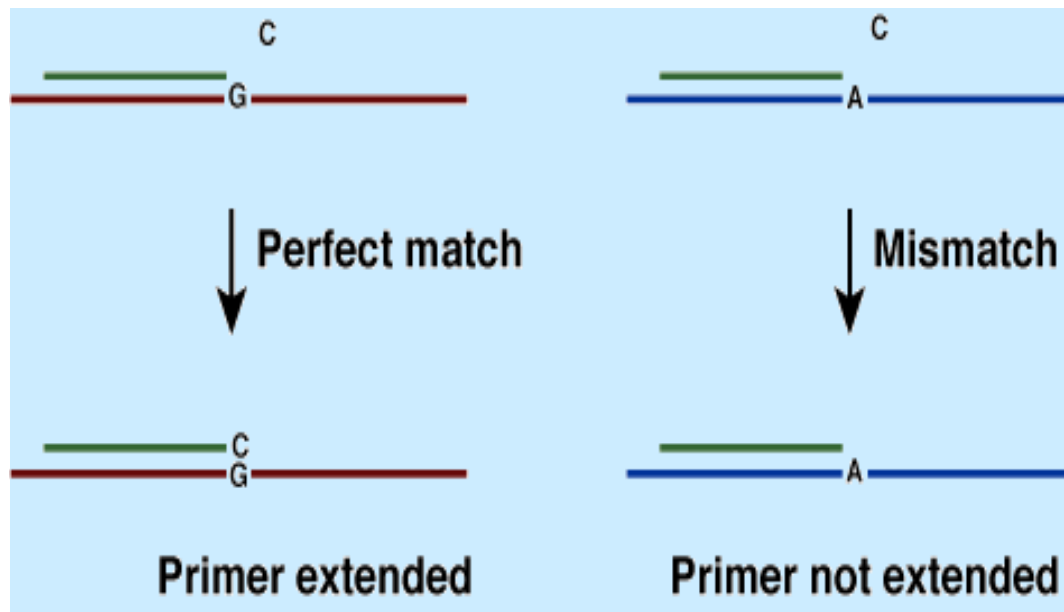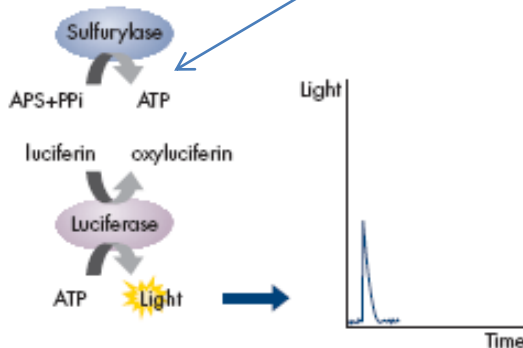

-> HPLC analysis

Sequencing

Pyrosequencing

Step 1
A sequencing primer is hybridized to a single-stranded PCR amplicon that serves as a template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin.

Step 2
The first of the four deoxribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

Step 3
ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.

Nucleotide incorporation generates light seen as a peak in the Pyrogram trace

DNA Diagnostic Systems
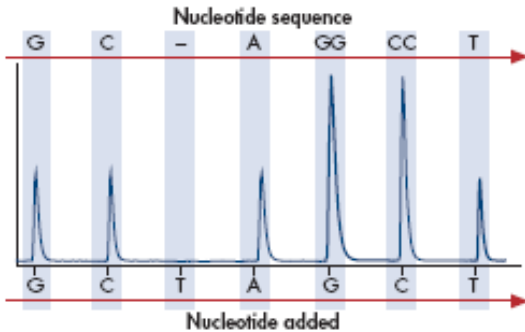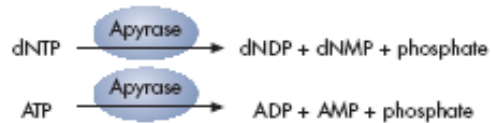
Sequencing

Pyrosequencing

Step 4
Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

Step 5
Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

Sequencing

Problems arising in mutation scanning:

Example: Duchenne muscular dystrophy

Problems:
1. Gene is large, **2,4 Mb, 79 exons**
Hard to find point mutation

2. High Frequency
of new mutations
(30% of cases);

3. First mutation carrier
is often a mosaic
(blood may be
not a mutation carrier)

Diagnosis of Duchenne Muscular Dystrophy in a Presymptomatic Infant Using Next-Generation Sequencing and Chromosomal Microarray Analysis: A Case Report

Eun-Woo Park [1], Ye-Jee Shim [2], Jung-Sook Ha [3], Jin-Hong Shin [4], Soyoung Lee [1], Jang-Hyuk Cho [1]

Affiliations + expand

PMID: 34064562   PMCID: PMC8151037   DOI: 10.3390/children8050377

Free PMC article

Abstract

Duchenne muscular dystrophy is a progressive and lethal X-linked recessive neuromuscular disease caused by mutations in the dystrophin gene. It has a high rate of diagnostic delay; early diagnosis and treatment are often not possible due to delayed recognition of muscle weakness and lack of effective treatments. Current treatments based on genetic therapy can improve clinical results, but treatment must begin as early as possible before significant muscle damage. Therefore, early diagnosis and rehabilitation of Duchenne muscular dystrophy are needed before symptom aggravation. Creatine kinase is a diagnostic marker of neuromuscular disorders. Herein, the authors report a case of an infant patient with Duchenne muscular dystrophy with a highly elevated creatine kinase level but no obvious symptoms of muscle weakness. The patient was diagnosed with Duchenne muscular dystrophy via next-generation sequencing and chromosomal microarray analysis to identify possible inherited metabolic and neuromuscular diseases related to profound hyperCKemia. The patient is enrolled in a rehabilitation program and awaits the approval of the genetic treatment in Korea. This is the first report of an infantile presymptomatic Duchenne muscular dystrophy diagnosis using next-generation sequencing and chromosomal microarray analysis.

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DNA Diagnostic Systems

PCR based methods

-> The presence of the appropriate **amplified size fragment** confirms the presence of the target.
-> Specific primers are now available for the detection of many pathogens including bacteria (*E. coli, M. tuberculosis*), viruses (HIV) and fungi.

Example: Using PCR to Detect for HIV

- **RT-PCR** (reverse transcriptase PCR).
- HIV has a ssRNA genome.

Other examples:
-> Using PCR to Detect DMD deletions (60% of mutations are deletions)
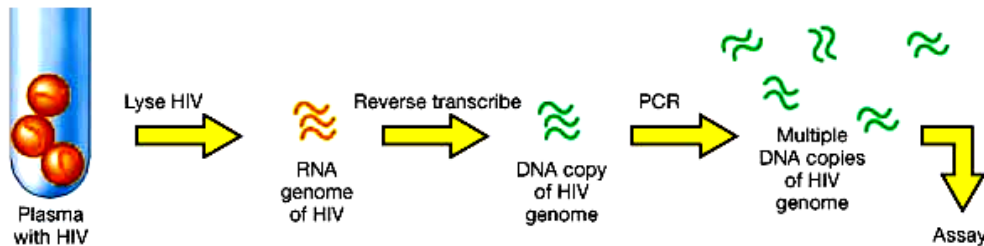
- Specific primers are used to amplify a 156 bp portion of the HIV *gag gene*.
- Using standards the amount of PCR product can be used to determine the **viral load**.
- PCR can also be used as a prognostic tool to determine viral load.
- This method can also be used to determine the effectiveness antiviral therapy.

DNA Diagnostic Systems

PCR based methods

DNA Fingerprinting

- **RFLP** = **Restriction Fragment Length Polymorphism**
- Regular fingerprinting analyses **phenotypic** traits.
- DNA fingerprinting analyses **genotypic** traits.
- DNA fingerprinting (DNA typing) is used to characterize biological samples e.g.

 -> In legal proceedings to identify suspects and clear others.

 -> Paternity testing

Restriction fragment length polymorphism (RFLP):

- Very simple; dependent on mutation within recognition site of restriction enzyme
- Former used with southern blot experiments
- Even as many restriction enzymes are known,
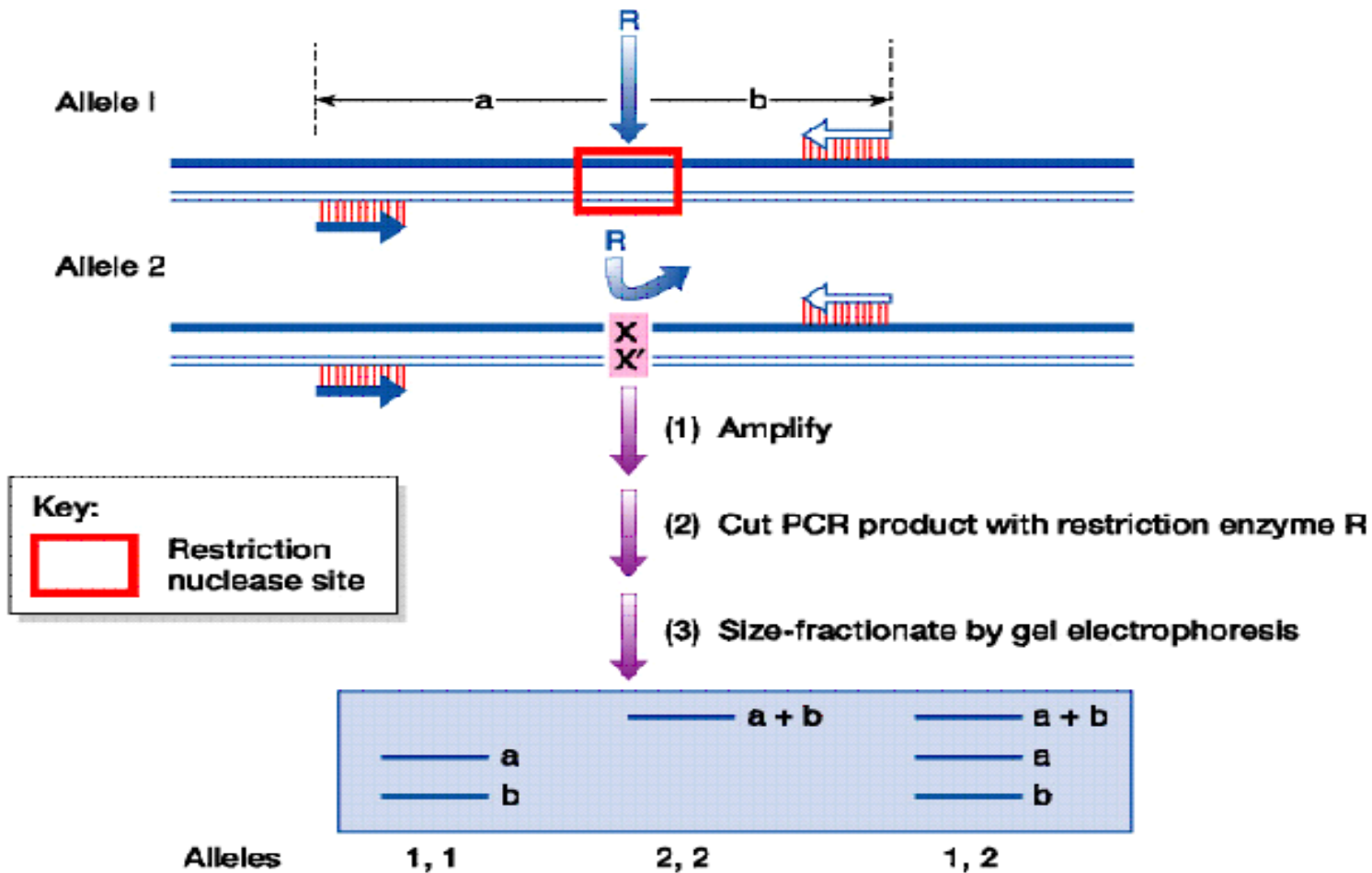
 some mutation sites do not correspond to any

HaeIII
```
5' ...G G C C... 3'
3' ...C C G G... 5'
```

BamHI
```
5' ...G G A T C C... 3'
3' ...C C T A G G... 5'
```

-> Rare endonucleases are difficult to work with, and often of a poor quality
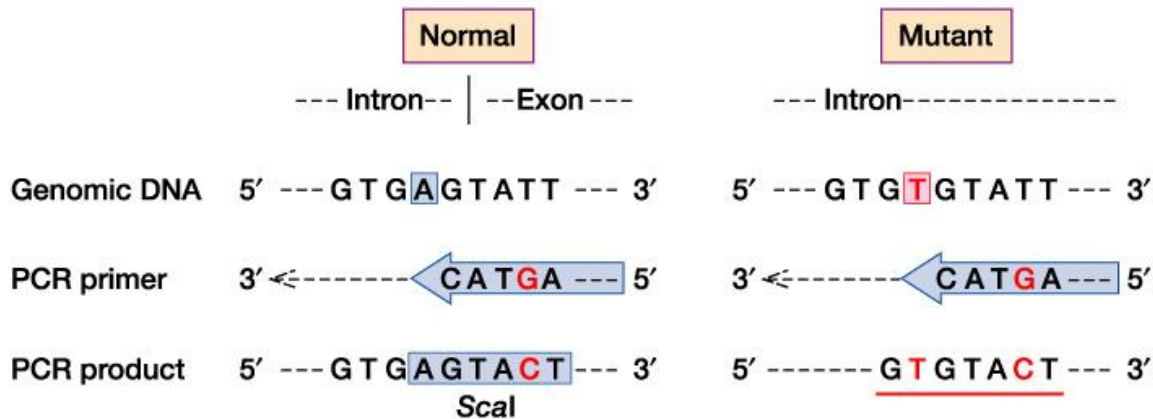
DNA Diagnostic Systems

PCR based methods

Restriction fragment length polymorphism (RFLP)
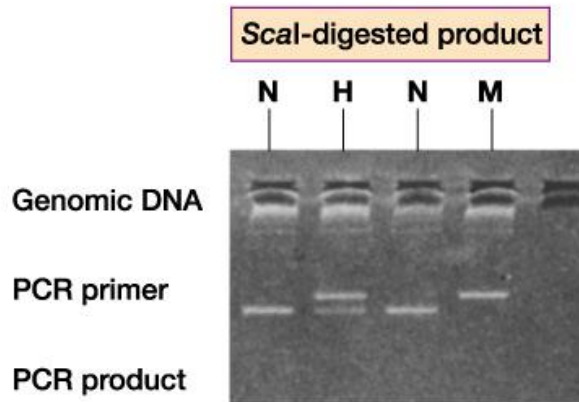
DNA Diagnostic Systems

PCR based methods

Restriction fragment length polymorphism (RFLP)

Modified method:

Diagnostic restriction site introduced artificially

by purposedly mismatched PCR primer

Example: Diagnosis of sickle cell anemia

Figure 18-8 Human Molecular Genetics, 3/e. (© Garland Science 2004)

DNA Diagnostic Systems

PCR based methods

Random Amplified Polymorphic DNA (RAPD)

- RAPD is often used to **show relatedness** among DNA populations.

- In this procedure arbitrary (**random**) primers are used during PCR to produce a fingerprint of the DNA.

- A single primer is used which must anneal in 2 places on the DNA template and region between the primers will be amplified.

- The primers (8-10nt) are likely to anneal in **many places** on the template DNA and will produce a **variety of sizes** of amplified products.

- Amplified products are separated by agarose gel electrophoresis and visualized.

- If the samples have similar genetic make up then the pattern of bands on the gel will be similar and vice versa.

- This procedure is widely used to differentiate between different cultivars/varieties of the same plant.

- Issues to consider when using this procedure include reproducibility, quality of DNA, and several primers may have to be used.
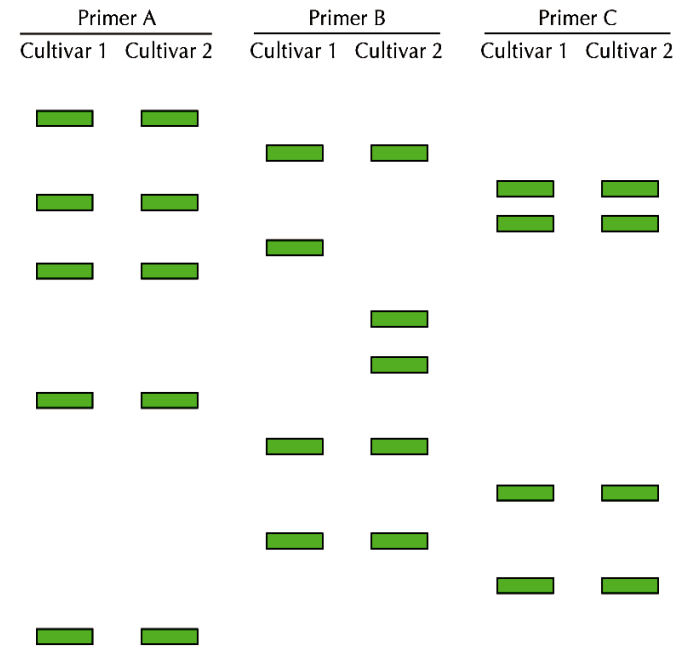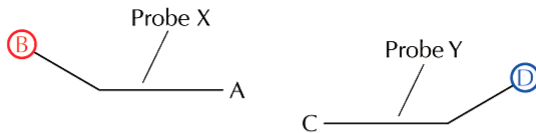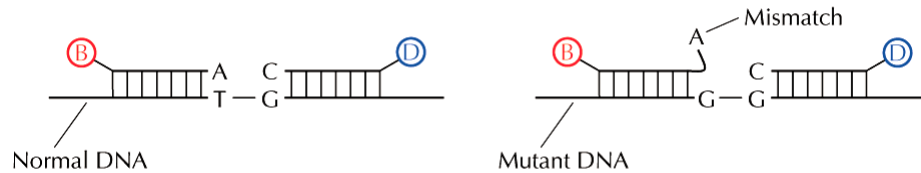
DNA Diagnostic Systems

PCR based methods

Oligonucleotide Ligation Assay (OLA)

- Many diseases are caused by a single nucleotide (nt) change in the wild type gene.
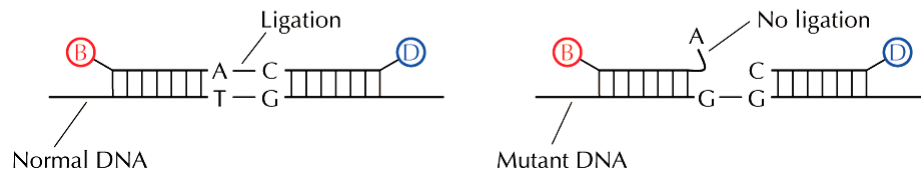- A single nt change can be detected by PCR/OLA

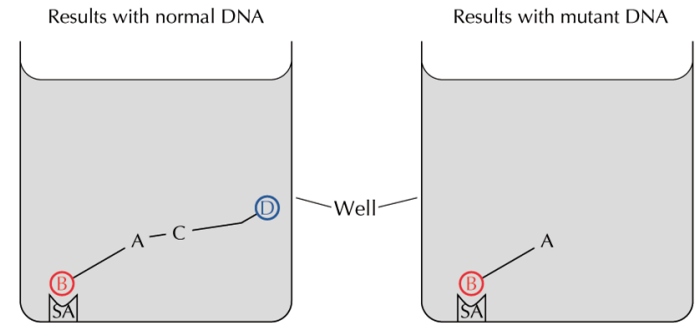**A** Synthesize a pair of oligonucleotide probes

B Hybridize probes to PCR-amplified DNA

Normal DNA

Mutant DNA

C Add ligase to hybridized DNA

Normal DNA

Mutant 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
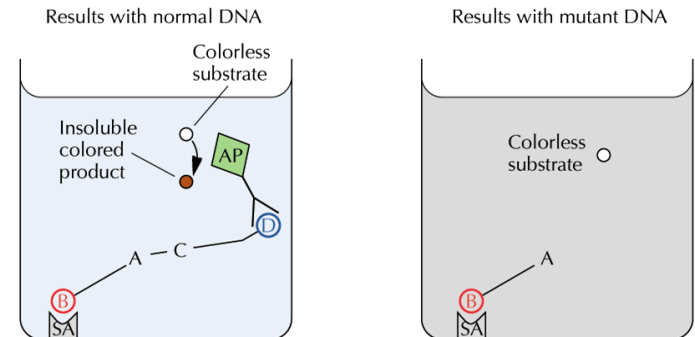
Bacterial Biosensors

- Bacterial sensors can be used to test for environmental pollutants.
- Bacteria with bioluminescent are good candidates for pollutant sensors.
- In the presence of pollutants, the bioluminescent decreases.
- The structural genes (*luxCDABD*) encodes the enzyme for bioluminescent was cloned into the soil bacteria *Pseudomonas fluorescens*.
- The cells that luminescence to the greatest extent and grew as well as the wild type were tested as pollutant sensors.
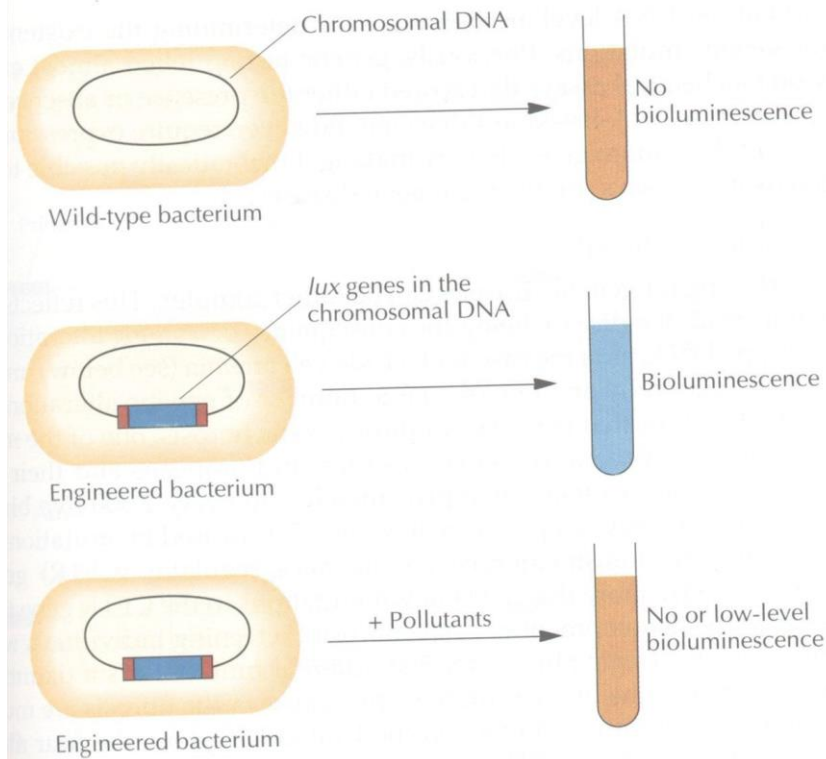

- To screen water samples for pollutants (metal or organic) a suspension of *P. fluorescens* was mixed with the solution to be tested.
- After a 15 min incubation the luminescence of the suspension was measured.
- When the solution contained low to moderate levels of pollutants the bioluminescence was inhibited.
- The procedure is rapid, simple, cheap and a good screen for pollutants.

Nanogenomics

Nano Genomics and nanoproteomics allow the study and comparison of the huge number of genes and proteins involved in the cell cycle progression of human T lymphocytes and in its transformation in lymphoma. Nano Genomics has, however, many pitfalls that only functional proteomics, called nucleic acid programmable protein array (NAPPA), is capable of overcoming by probing with unique sensitivity native in situ protein-protein interactions. This allows identification of the key proteins involved in the control of cancer and proliferation in the light of recent label-free NAPPA approaches based on nanotechnologies. Bioinformatics in combination with label free NAPPA, anodic porous alumina (APA) and DNA analyser (DNASER) microarrays appear capable of providing the long-range framework for the basic molecular understanding of cancer and cell cycle progression.
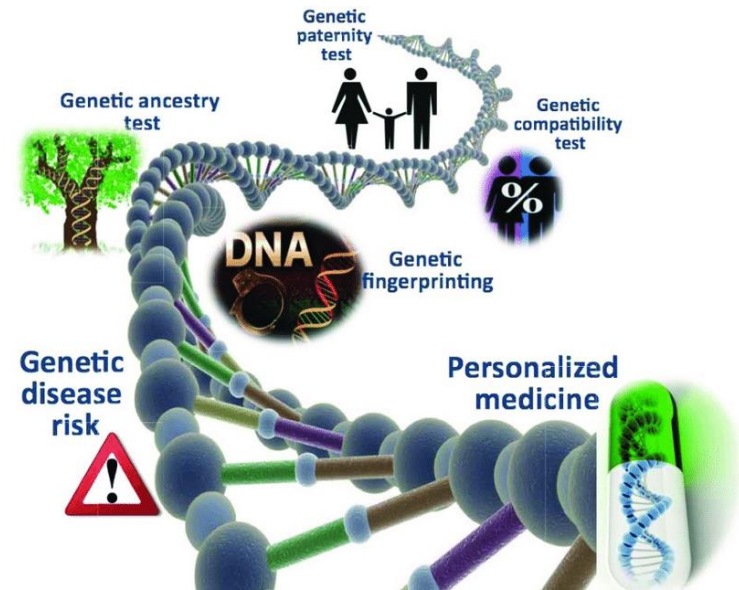
Cluster analysis

Performed with FuzMe software. The determination of key leader genes associated with cell cycle progression and with human organ transplants was recently successfully carried out by utilizing raw microarray data and non/statistical bioinformatics based on the identification of 'key genes' not as those mostly changing their expression, but as those having the strongest interconnections.

Expression genes are identified in the datasets of both normal T lymphocytes and the lymphoma genes as obtained from microarrays of lymphoma and normal T-cells for up-regulated and down-regulated entries by means of cluster analysis.

Cluster analysis or **clustering** is the task of grouping a set of objects in such a way that objects in the same group (called a **cluster**) are more similar (in some sense) to each other than to those in other groups (clusters).

It is a main task of exploratory data mining, and a common technique for statistical data analysis, used in many fields, including pattern recognition, image analysis, information retrieval, data compression, computer graphics and machine learning.
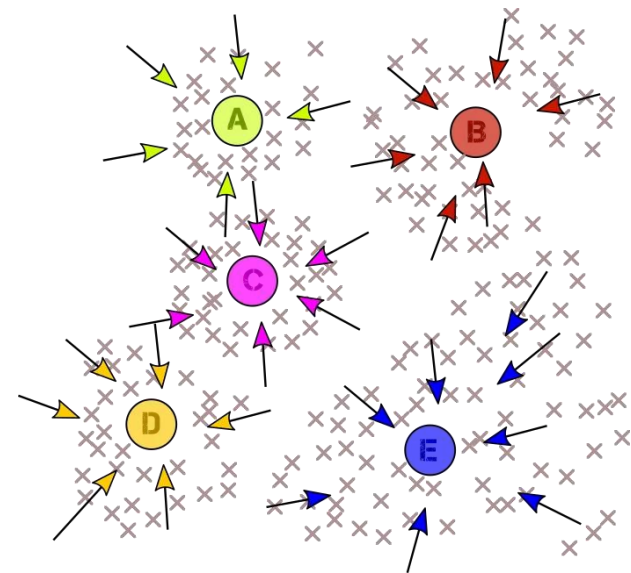
Nanorobotics in medicine

Overview of the existing and emerging nanorobotic applications across specialties of medicine.

| Specialty	Brief Description
Microbiology	Use of magnetotactic bacteria to transport and navigate nanorobots
Hematology	Circulating "respirocyte" nanorobots to deliver oxygen and return remove waste products from periphery
Hematology	Circulating "clottocyte "nanorobot with hemostatic functions
Hematology	Phagocytic "microbivores" with customizable antigen binding sites for targeting of pathogens
Dentistry	Dental anesthesia and sensitive teeth through nanorobot penetrating dentinal tubules for occlusion or administration of targeted analgesic
Dentistry	Enhancement of the success rate of root canal procedures by providing visualization of root
Dentistry	Improved daily dental hygiene and teeth cosmetics by replacement of enamel layers
Neurosurgery	Single axon manipulation and transection with use nanoknife
Neurosurgery	Circulating nanorobot for the monitoring of intracranial aneurysm development and progression
Oncology	Screening nanorobot circulating and monitoring for detection of neoplasia
Oncology	Direct drug delivery to cancerous tissue to limit systemic toxicity and increase effectiveness
Oncology	Mapping of margins of tumor to improve resection during surgery
Vascular	Screening for atherosclerosis, cancer, aneurysms, and more
Vascular	Localization of bleeding site for assisting embolization
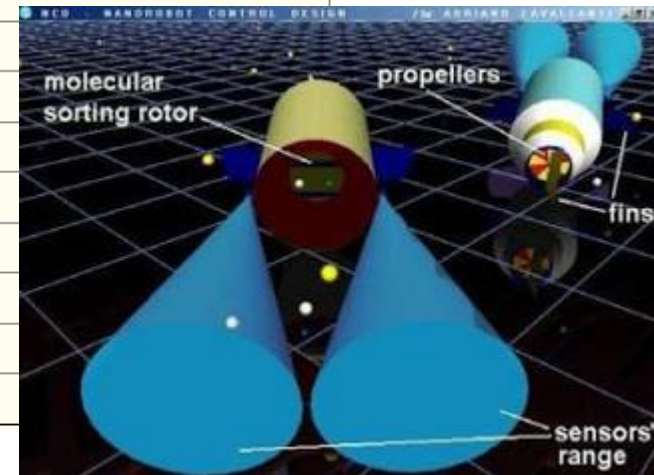
Microbiology

Magnetospirillum

An effective strategy for enabling propulsion of microrobots and nanorobots is coupling them to magnetotactic bacteria such as Magnetococcus, Magnetospirillum magnetotacticum or Magnetospirillum magneticum. The largest component of these nanorobots integrated into magnetotactic bacteria would be the bacterial cell component. The smallest known species of magnetotactic bacteria is the marine magnetotactic spirillum, which is 0.5 μm (500 nanometers), just above the upper limit of the NNI's definition of the nanoscale. However, the marine magnetotactic spirillum's usefulness is limited by their speed, and magnetotactic cocci are more useful for intravascular function.

The magnetotactic bacteria can be guided in the desired direction using the application of magnetic fields. The components of the magnetotactic bacteria that are responsive to the magnetic field are called magnetosomes. Magnetosomes are prokaryotic pseudo-organelles with about 15-20 magnetite crystals, each about 50 nm in diameter, contained within an invagination of the prokaryotic cell membrane. Magnetite crystals are composed of Fe_3O_4, a common iron oxide. Magnetotactic cocci have been found to travel in consistent and predictable patterns following established geomagnetic lines.
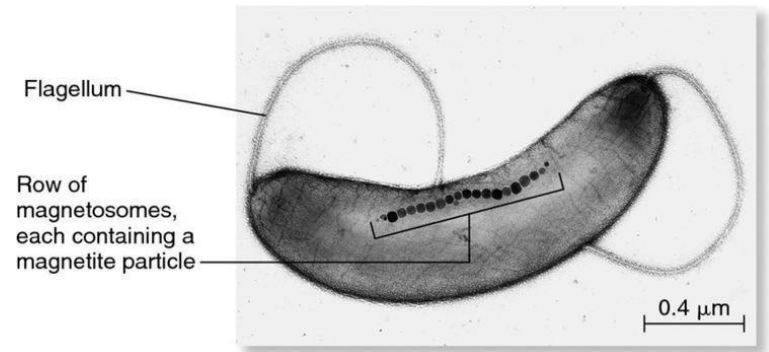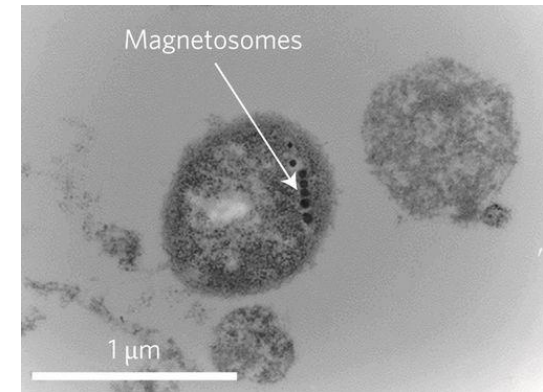

Magnetococcus

Hematology

The powering of the nanorobots can be done by metabolising local glucose and oxygen for energy. In a clinical environment, another option would be externally supplied acoustic energy. Other sources of energy within the body can also be used to supply the necessary energy for the devices. They will have simple onboard computers capable of performing around 1000 or fewer computations per second. This is because their computing needs are simple. Communication with the device can be achieved by broadcast-type acoustic signalling.

A navigational network may be installed in the body, with stationkeeping navigational elements providing high positional accuracy to all passing nanorobots that interrogate them, wanting to know their location. This will enable the physician to keep track of the various devices in the body. These nanorobots will be able to distinguish between different cell types by checking their surface antigens (they are different for each type of cell). This is accomplished by the use of chemotactic sensors keyed to the specific antigens on the target cells.

When the task of the nanorobots is completed, they can be retrieved by allowing them to exfuse themselves via the usual human excretory channels. They can also be removed by active scavenger systems. This feature is design-dependent.
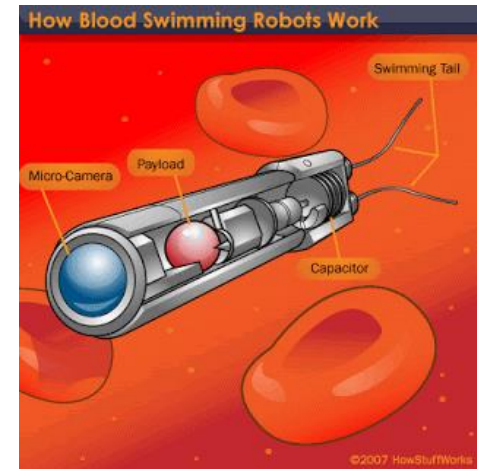
Dentistry
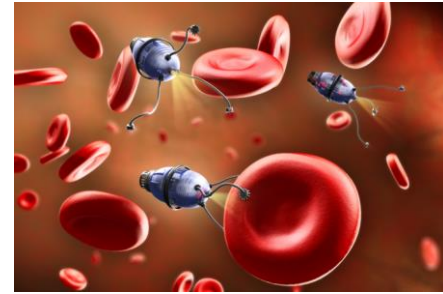
Some of the most promising uses of microscopic robots (in addition to nanoparticles) in dentistry and orthodontics are:

- Anaesthetics – Nanobots are delivered to the patient's gingiva via a colloidal suspension and then controlled by the dentist once they have settled in the pulp. The dentist instructs the robots to shut down all nerve sensation.
- The treatment of oral cancer – Otherwise known as Brachytherapy, tiny metal coated beads called nanoshells are able to selectively destroy tumor cells while leaving healthy tissue intact.
- Adhesives – Nanoparticles are mixed in a nano solution which is highly homogenous, therefore ensuring a perfect mixture of bonding material with improved strength. Nanoparticles are currently used in the resin-based reconstruction procedure.
- Sterilization – For use in the sterilization of instruments; there are many commercially available nano solutions. They purport to be hypoallergenic, environmentally friendly, non-corrosive, and non-staining while killing a broad spectrum of organisms.
- Bone replacement – Since successful bone generation is dependent upon the available surface area of the tooth, bone growth can be expedited with nanoscale placements of material That greatly improve the surface topography of the tooth.
- Permanent cure for sensitivity – Based on the same idea as the clinical application of anesthesia, nanobots can be selectively placed in dental tubules to permanently block them from causing ongoing sensitivity.
- "Dentifribots" as a dentifrice to destroy bacteria – These are delivered by a toothpaste or mouthwash and are so effective at targeting all subgingival surfaces to destroy pathogenic bacteria, they make dental floss seem prehistoric. The "dentifribot" is technically a mechanical structure that destroys itself after ingestion.

Current practicing and future pathologists are called on to actively incorporate molecular knowledge into their diagnostic armamentarium and deeply transform laboratory frameworks and pathology educational training programs.
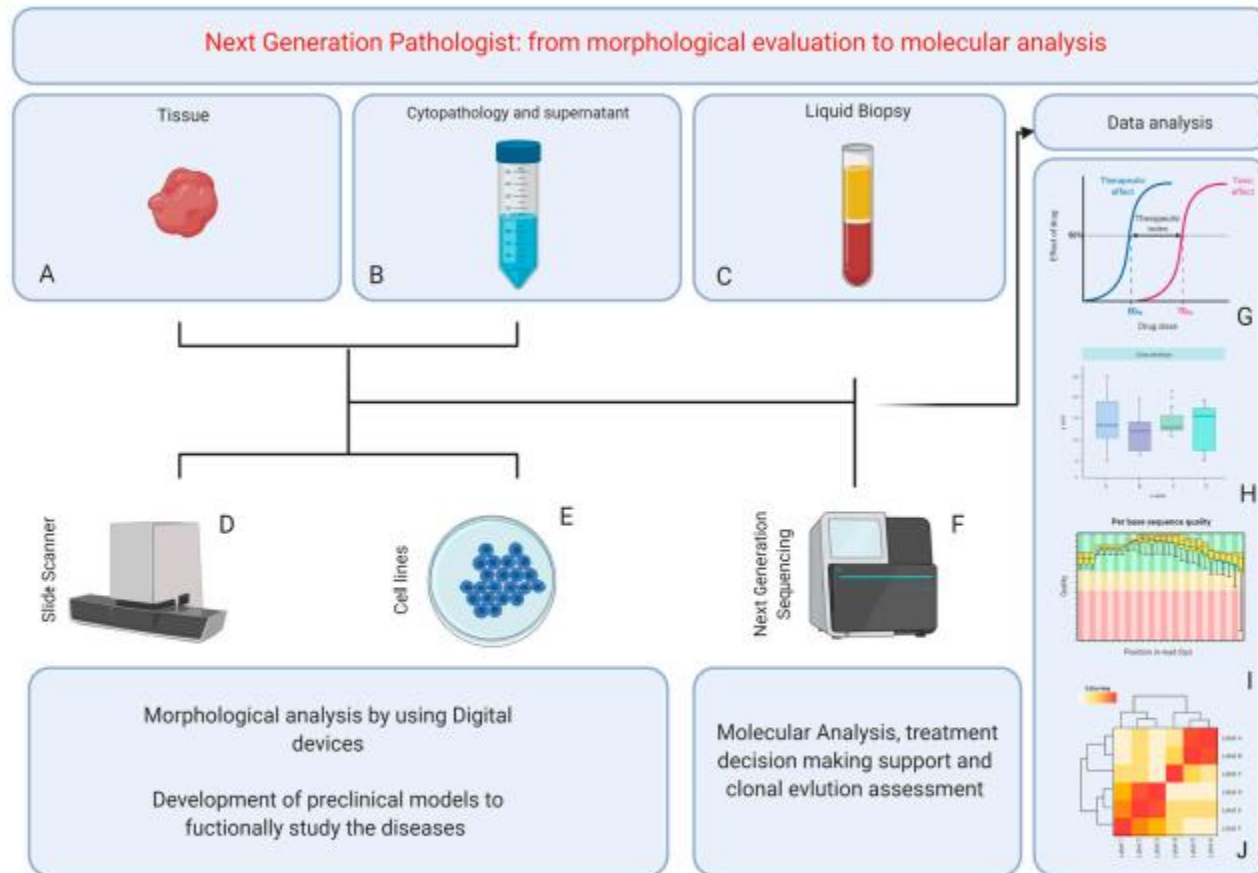
Figure 1. Schematic representation of next-generation pathologist area of expertise, from tissue management and analysis (**A**), including cytological samples and body fluids (**B,C**), to fully morpho-molecular characterization (**D–F**) of patients. (**G–J**): schematic representation of integrative data analysis among drug dose–response (**G**), cell line expression profile (**H**) and next-generation results on DNA (**I**) and RNA (**J**). Credit by Biorender.

QUESTIONS OR COMMENTS?