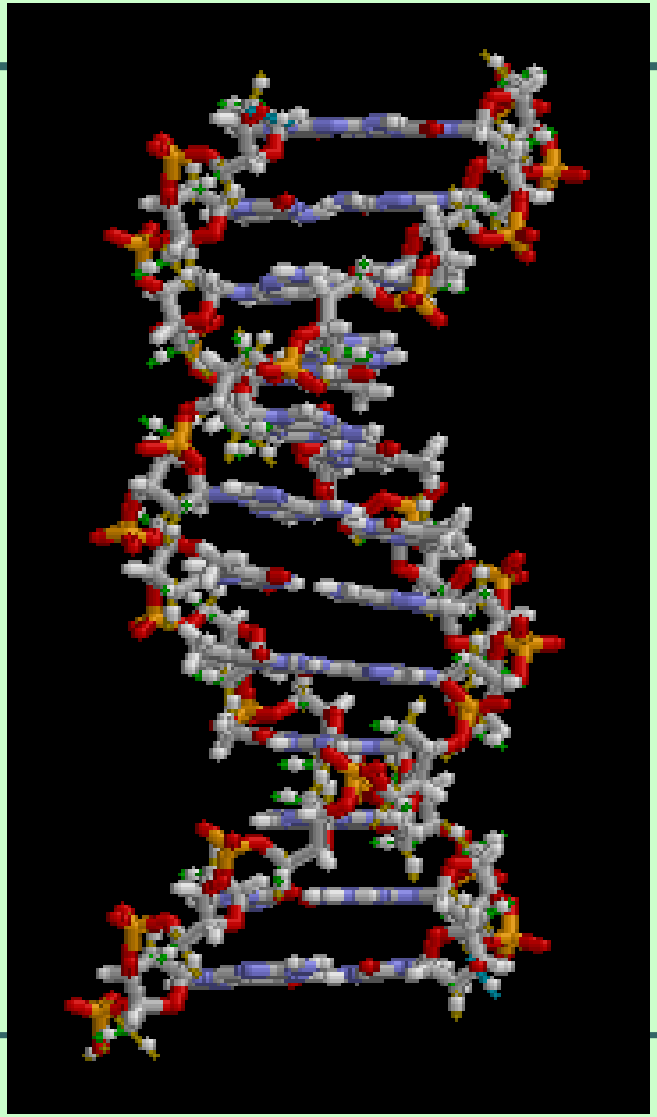
Molecular diagnostics



The human genome

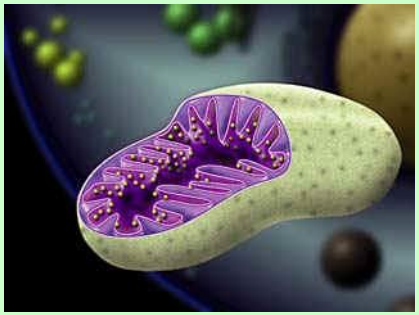
the total genetic information (DNA content) in human cells

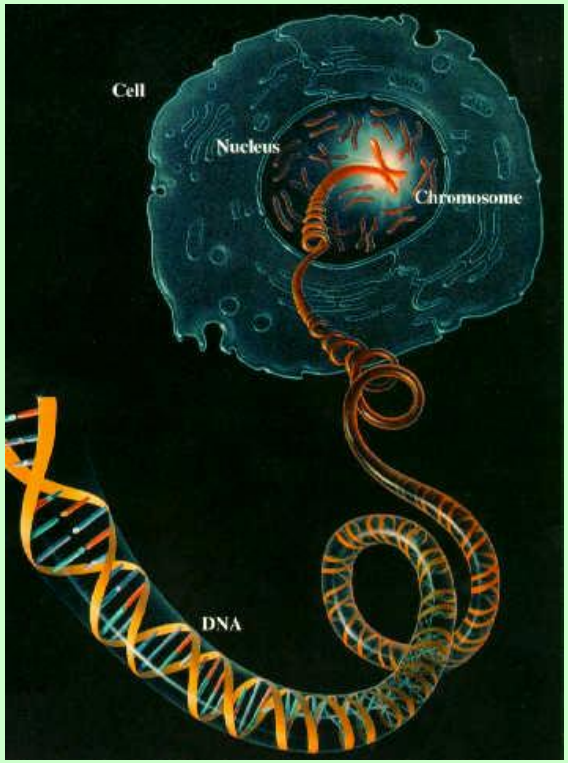
- nuclear

- mitochondrial - double-stranded DNA

is organized into one circular molecul.

Exclusively maternal inheritance



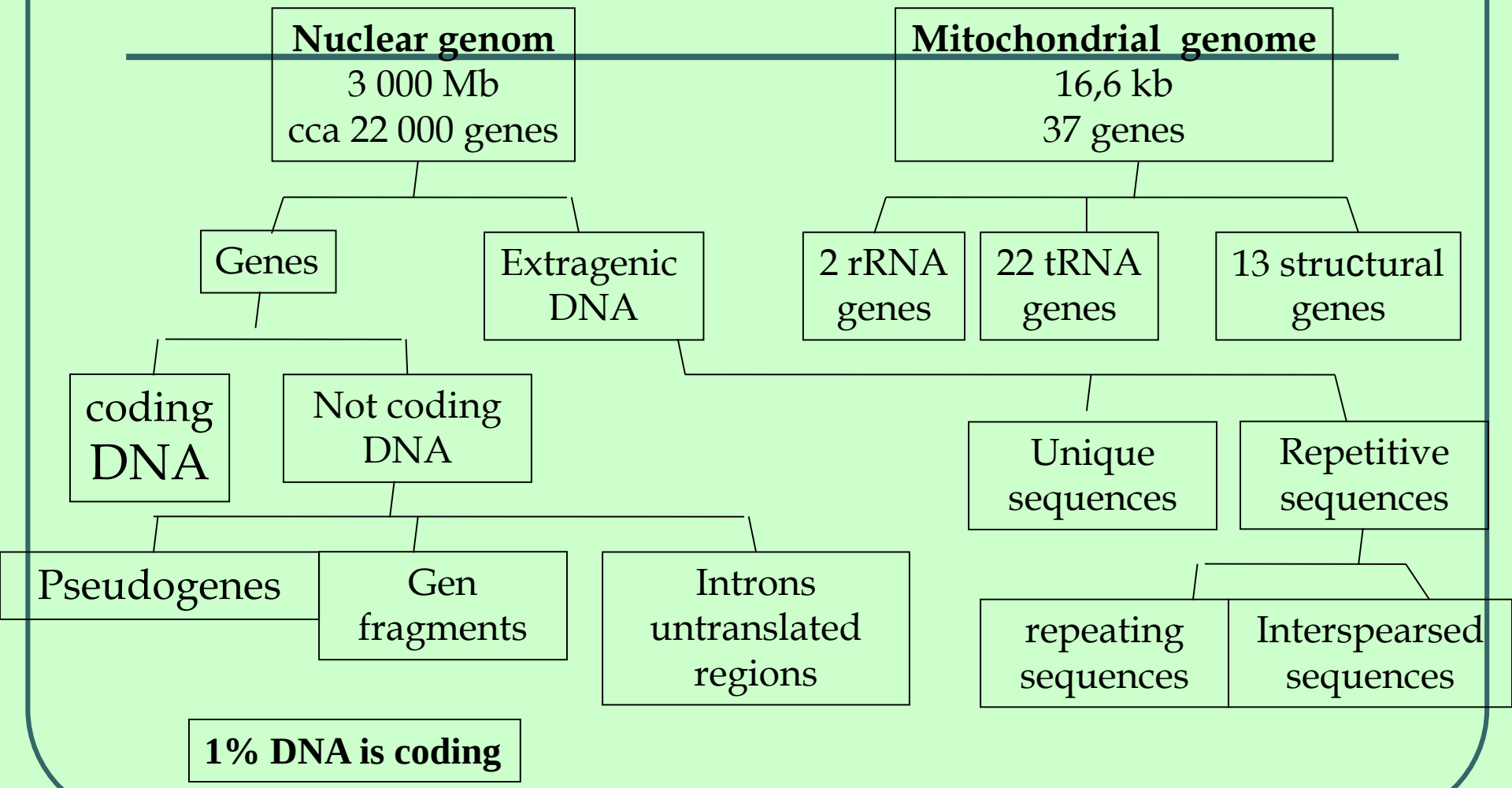


The human nuclear and mitochondrial genomes

	Nuclear genome	Mitochondrial genome
Size	3000 Mb	16.6 kb
No. of different DNA molecules	23 (in XX) or 24 (in XY) cells, all linear	One circular DNA molecule
Total no. of DNA molecules per cell	23 in haploid cells; 46 in diploid cells	Several $\times 10^3$
Associated protein	Several classes of histone and nonhistone protein	Largely free of protein
No. of genes	22 000	37
Gene density	~1/40 kb	1/0.45 kb
Repetitive DNA	Large fraction, see *Figure 7.1.*	Very little
Transcription	The great bulk of genes are transcribed individually	Continuous transcription of multiple genes
Introns	Found in most genes	Absent
% of coding DNA	1 – 2 %	~93%
Codon usage	See *Figure 1.22*	See *Figure 1.22*
Recombination	At least once for each pair of homologs at meiosis	None
Inheritance	Mendelian for sequences on X and autosomes; paternal for sequences on Y	Exclusively maternal

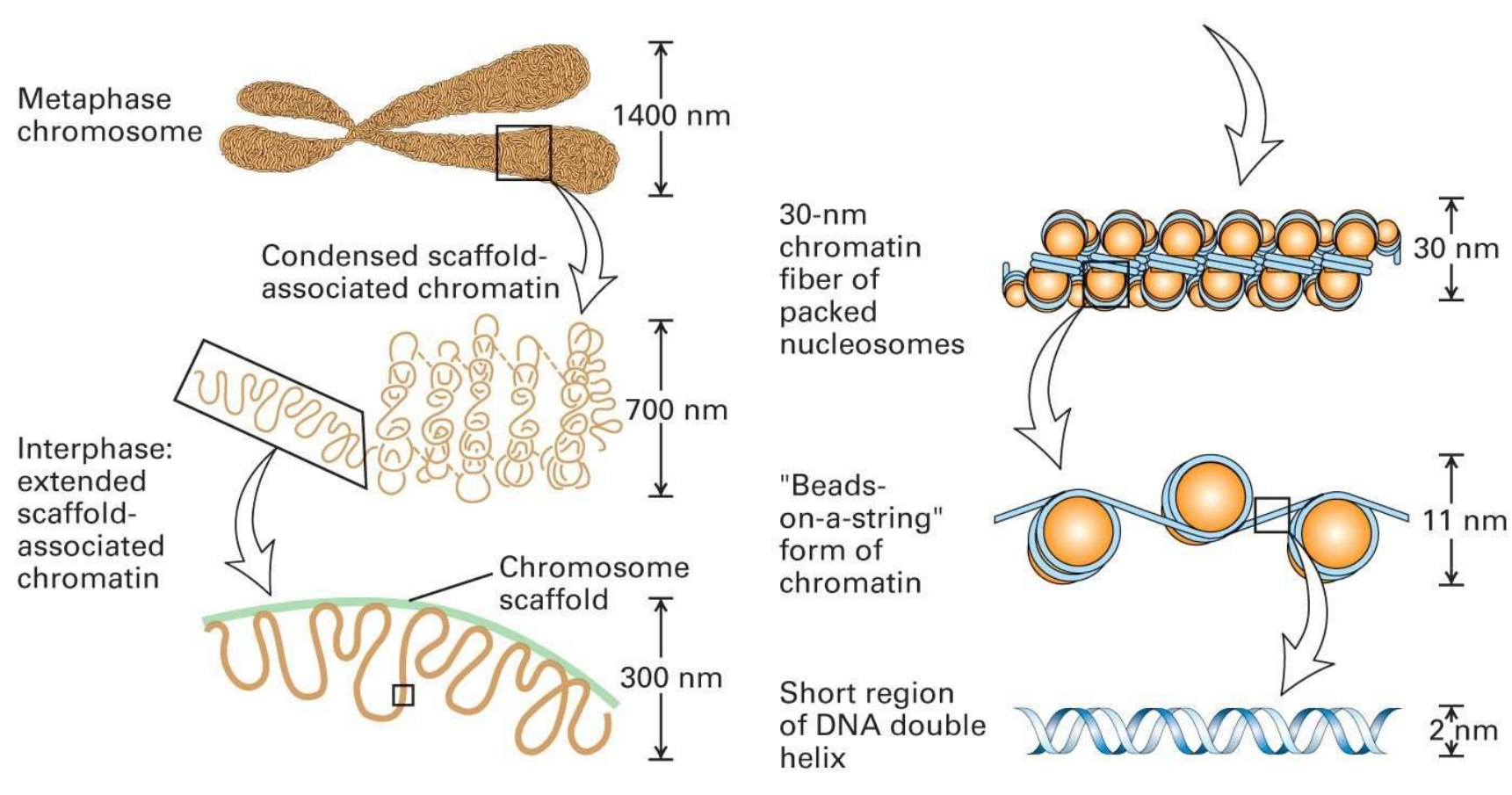
The human genome

It comprise two genomes:

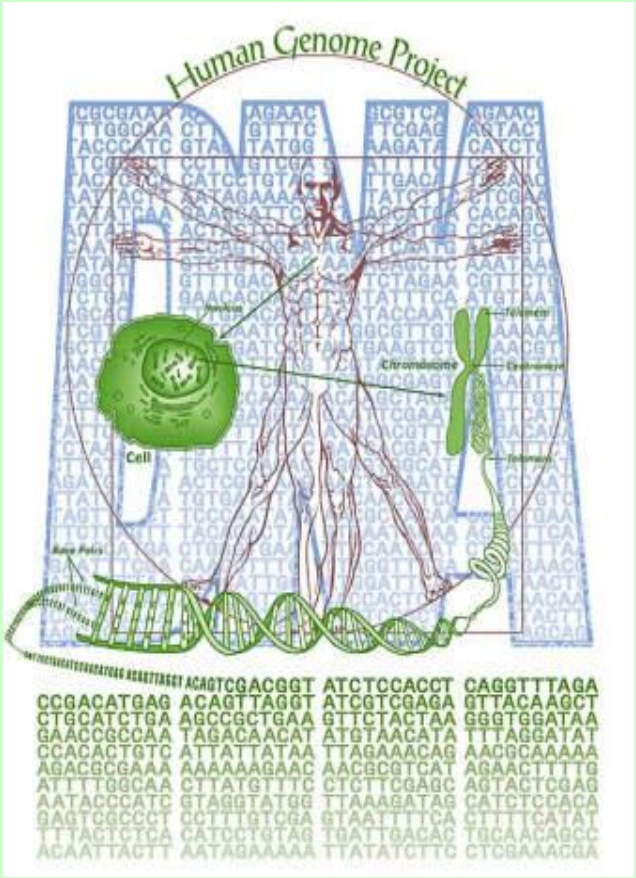


1% DNA is coding

Superstructure



Human genome project (HUGO)

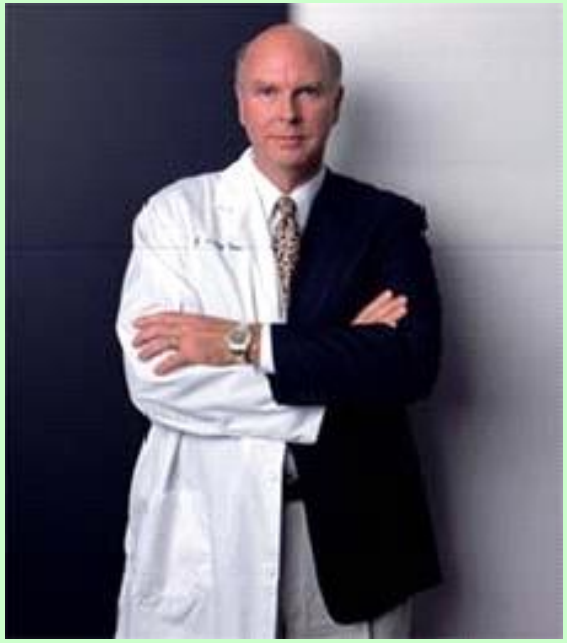


- Identify all of the genes in human DNA
- Determine the sequence of the 3 billion chemical nucleotide bases that make up human DNA
- Store this information in data bases
- Develop faster, more efficient sequencing technologies
- Develop tools for data analysis
- Address the ethical, legal, and social issues (ELSI) that ay arise form the project

Human genome project (HUGO)

- $3-billion project founded in 1990 by the United States Department of Energy and the U.S. National Institutes of Health. The international consortium comprised also geneticists in the United Kingdom, France, Germany, Japan, China and India.

A parallel project was conducted outside of government by the **Celera Corporation**



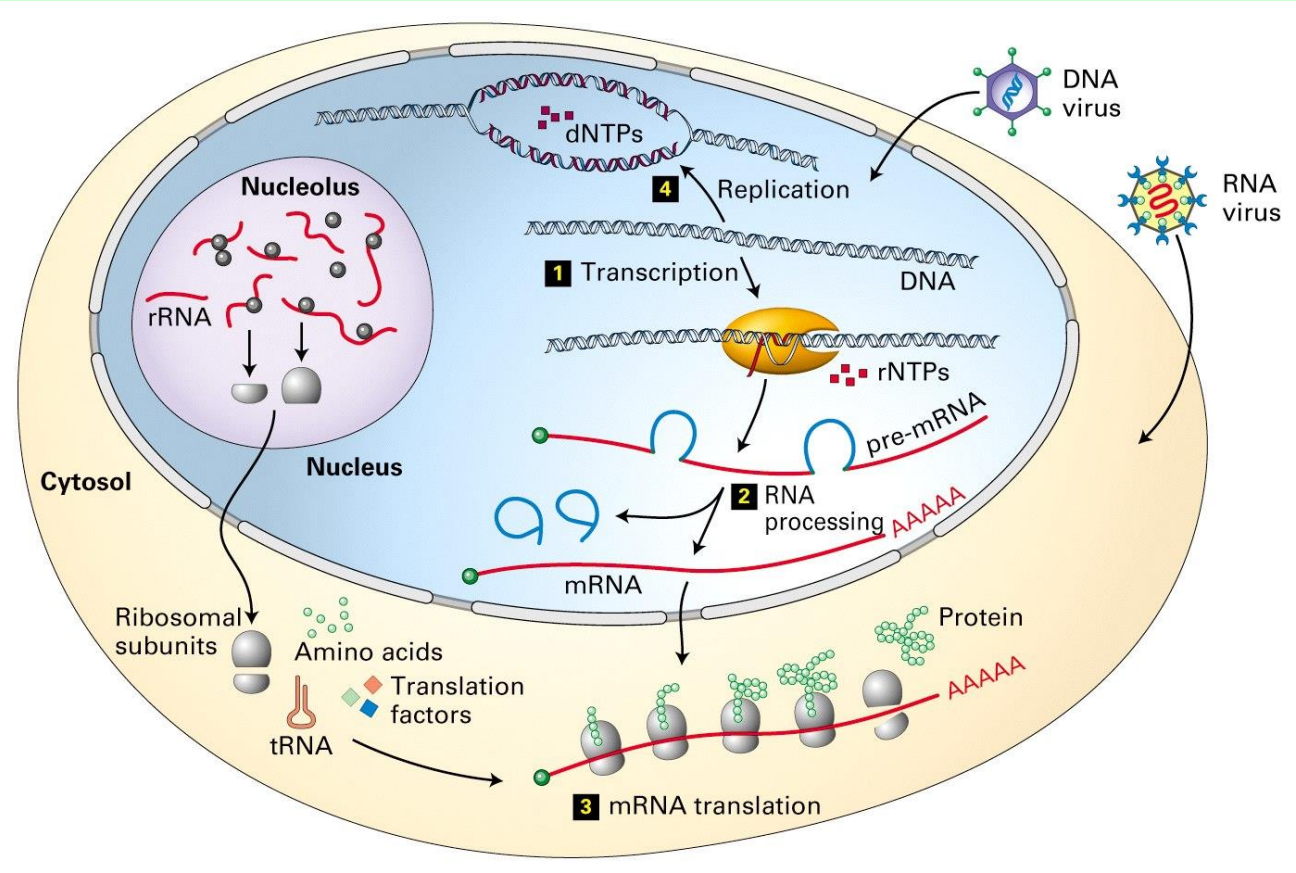
- June 6, 2000, the HGP and Celera Genomics held a joint press conference to announce that TOGETHER they had completed ~97% of the human genome

Human genome project

Key findings of Genome Project:

1. There are approx. 22,000 genes in human beings, the same range as in mice and twice that of roundworms.
   Understanding how these genes express themselves will provide clues to how diseases are caused.
2. All human races are 99.99 % alike, so racial differences are genetically insignificant.
3. Most genetic mutation occurs in the male of the species and as such are agents of change.
   They are also more likely to be responsible for genetic disorders.
4. Genomics has led to advances in genetic archaeology and has improved our understanding of how we evolved as humans and diverged from apes 25 million years ago.
   It also tells how our body works, including the mystery behind how the sense of taste works.

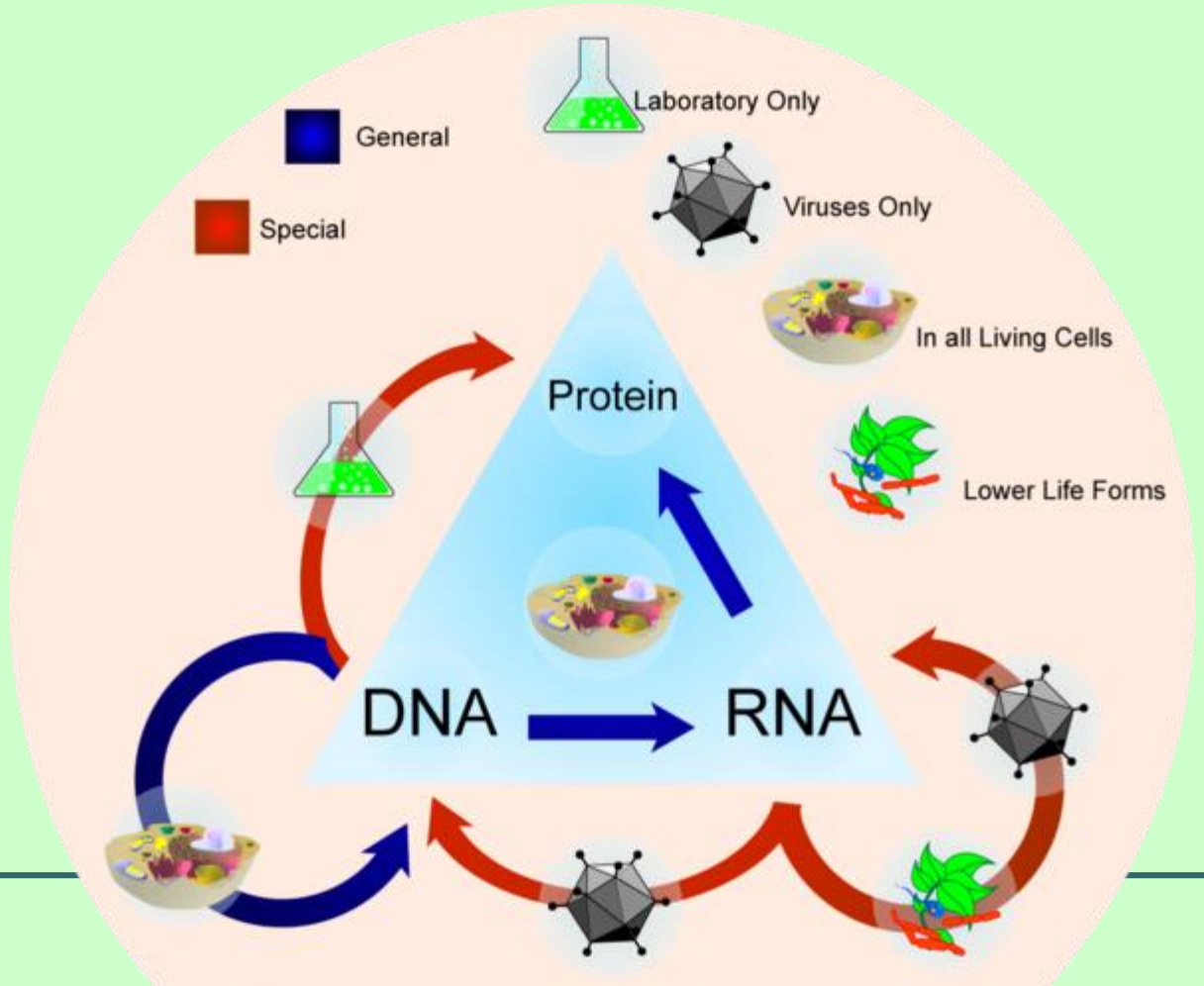
The flow of genetic information in the cell is DNA→RNA→protein



A gene is expressed in two steps

- Transcription: RNA synthesis
- Translation: Protein synthesis

The central dogma of molecular biology



The central dogma of molecular biology

the transfer of sequence information between sequential information-carrying biopolymers - DNA and RNA (both nucleic acids), and protein

The general transfers describe the normal flow of biological information:

- DNA can be copied to DNA (DNA replication),
- DNA information can be copied into mRNA, (transcription),
- proteins can be synthesized using the information in mRNA as a template (translation)

Mutations

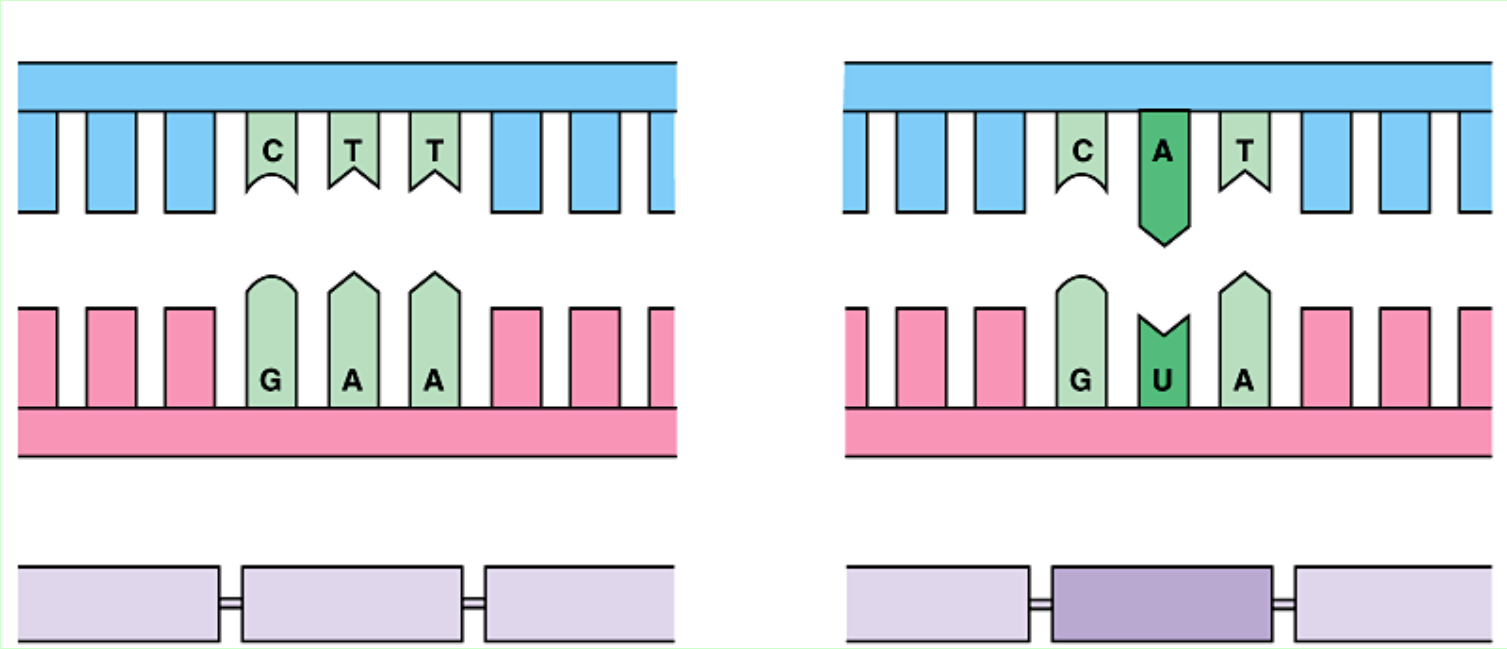
Any alteration in a gene from its natural state; may be disease causing or a benign, normal variant
Frequency less then 1 %

Mutations - positive (variability, selection)
- negative (4500 monogenic diseases, ageing)
- neutral

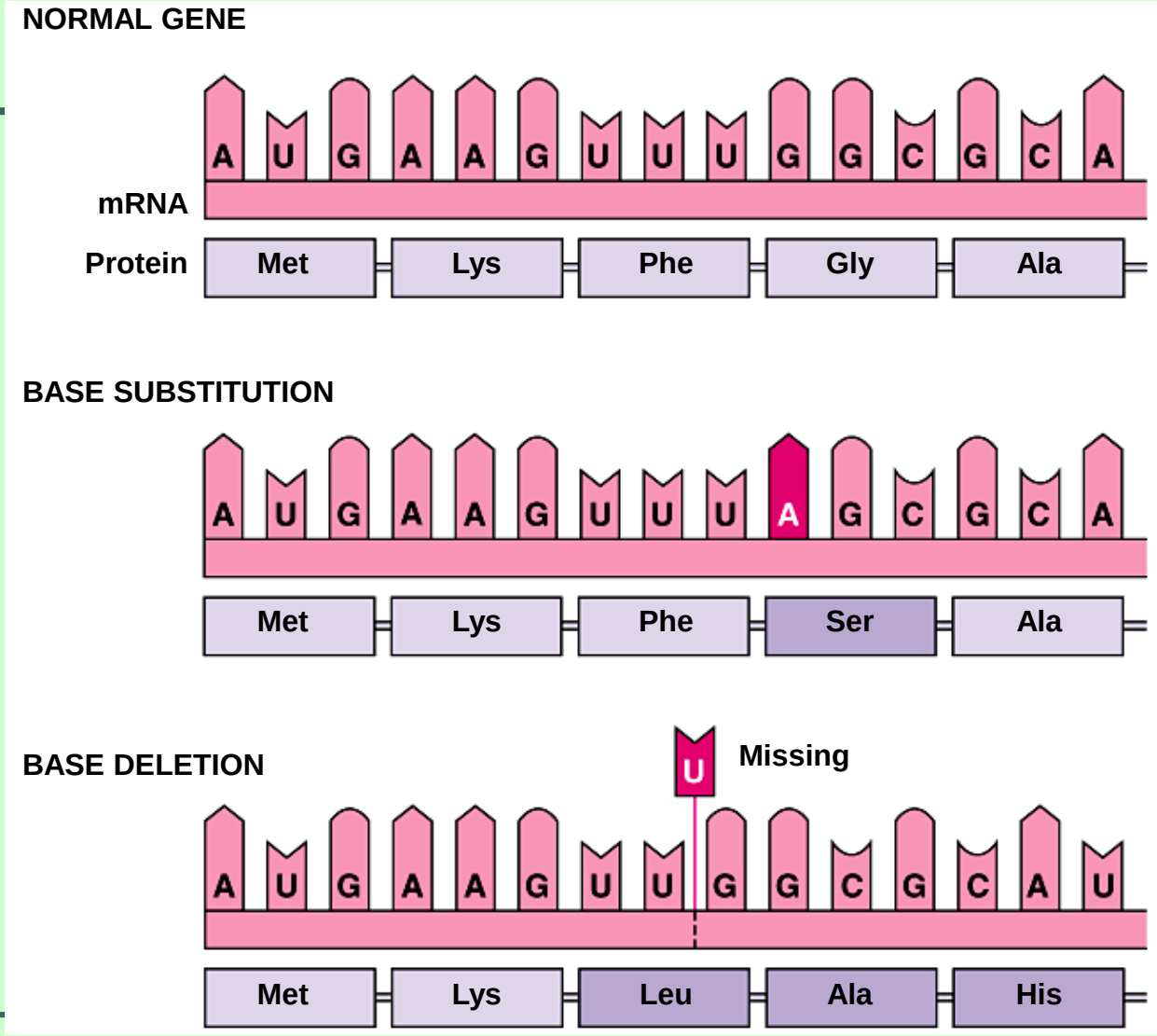
Each human: 5 – 10 patologic mutations

Mutations are changes in the DNA base sequence

These are caused by errors in DNA replication or by mutagens



Types of mutations



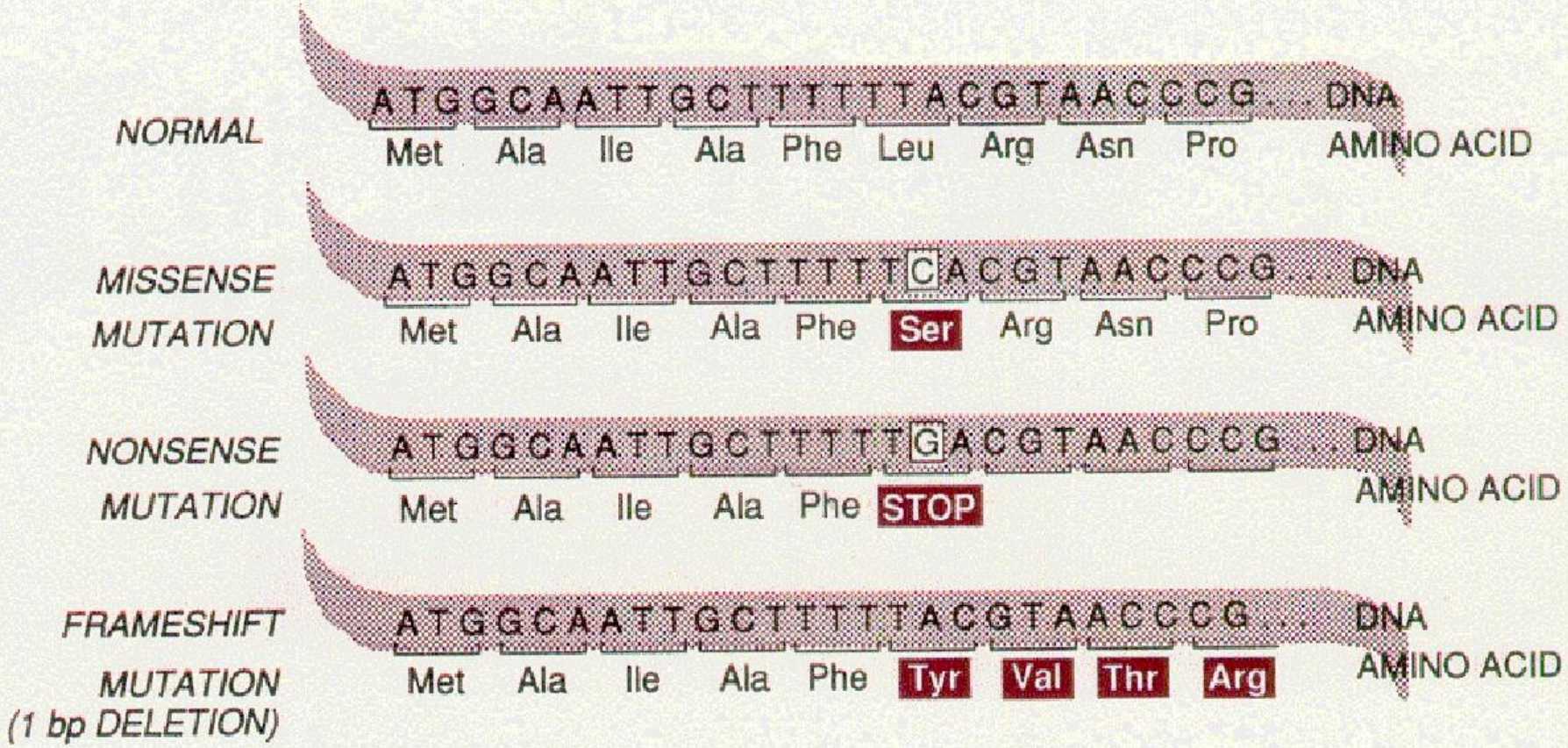


Figure 2.14. Examples of mutation. The "sense" strand DNA sequence of a coding region is shown, together with the encoded amino acid sequence. Three different mutations affecting the second nucleotide of a leucine codon are shown.

Class	Result
Single base-pair substitutions (point mutations)	
Altered structure of gene product	
Missense mutation	Single amino acid replacement in the protein
Nonsense mutation	A termination codon in the middle of the gene results in premature termination of protein synthesis
RNA-splicing mutation	The protein may be missing part or all of an exon sequence
Altered quantity of gene product	
Mutations in regulatory sequences	Transcription of the gene is altered, which can reduce or eliminate the gene product
Mutations in RNA processing and translation	The stability of the messenger RNA is altered, which may reduce the amount of gene product
Insertions or deletions	
One or two base pairs (frameshift mutations)	The addition or deletion of one or two base pairs can affect the reading frame of the gene, resulting in a grossly altered or absent gene product
Large numbers of base pairs	Large pieces of the DNA may be lost or large segments of DNA may insert into the middle of a gene, resulting in loss of function
Expansion of trinucleotide repeat sequences	Unstable trinucleotide repeats can suddenly expand in number, resulting in the alteration of production or structure of a particular gene product
Chromosomal alterations	Inversions, translocations, duplications, or gene amplification may result

Mutations in the coding sequence of a structural gene

- **Silent mutations** do not alter the amino acid sequence of the polypeptide
- **Missense mutations** - an amino acid change does occur
  - Example: Sickle-cell anemia
  - If the substituted amino acids have similar chemistry, the mutation is said to be neutral
- **Nonsense mutations** change a normal codon to a termination codon
- **Frameshift mutations** involve the addition or deletion of nucleotides in multiples of one or two
  - This shifts the reading frame so that a completely different amino acid sequence occurs downstream from the mutation

Clasification of mutations according to its effect on gene product

1. Product with lower to zero function (loss-of-function)

- typical product is enzyme
- type of mutation is frequently deletion

2. Product with abnormal function (gain-of-function)

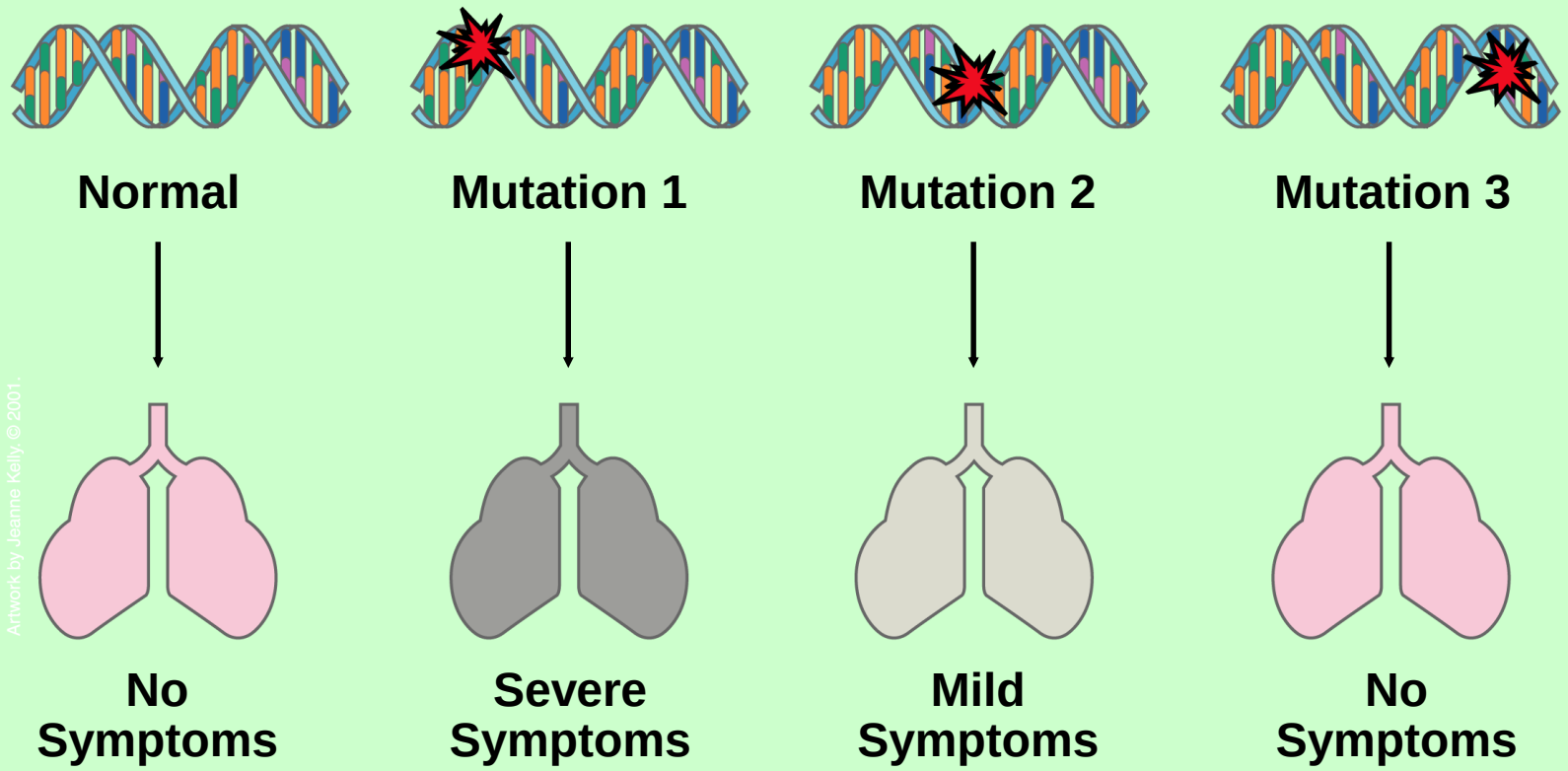
- typical product is nonenzymatic protein
- frequently in tumours (somat. mutation), rarely in monogenic diseases
- deletions do not lead to new function

Type 1 frequently recessive, type 2 dominant mutations
In some genes- both types of mutations

Disease Inheritance Is Complex

Gene Changes in Cystic Fibrosis

Mucus Production Gene



Major types of Genetic diseases

a.) chromosomal diseases

- are the result of the addition or deletion of entire chromosomes or part of chromosomes
- most major chromosome disorders are characterised by growth retardation, mental retardation and variety of somatic abnormalities
- typical examples of major chromosomal disease is Down syndrom (trisomy 21), Edwards sy (trisomy 18), Patau sy (trisomy 13)

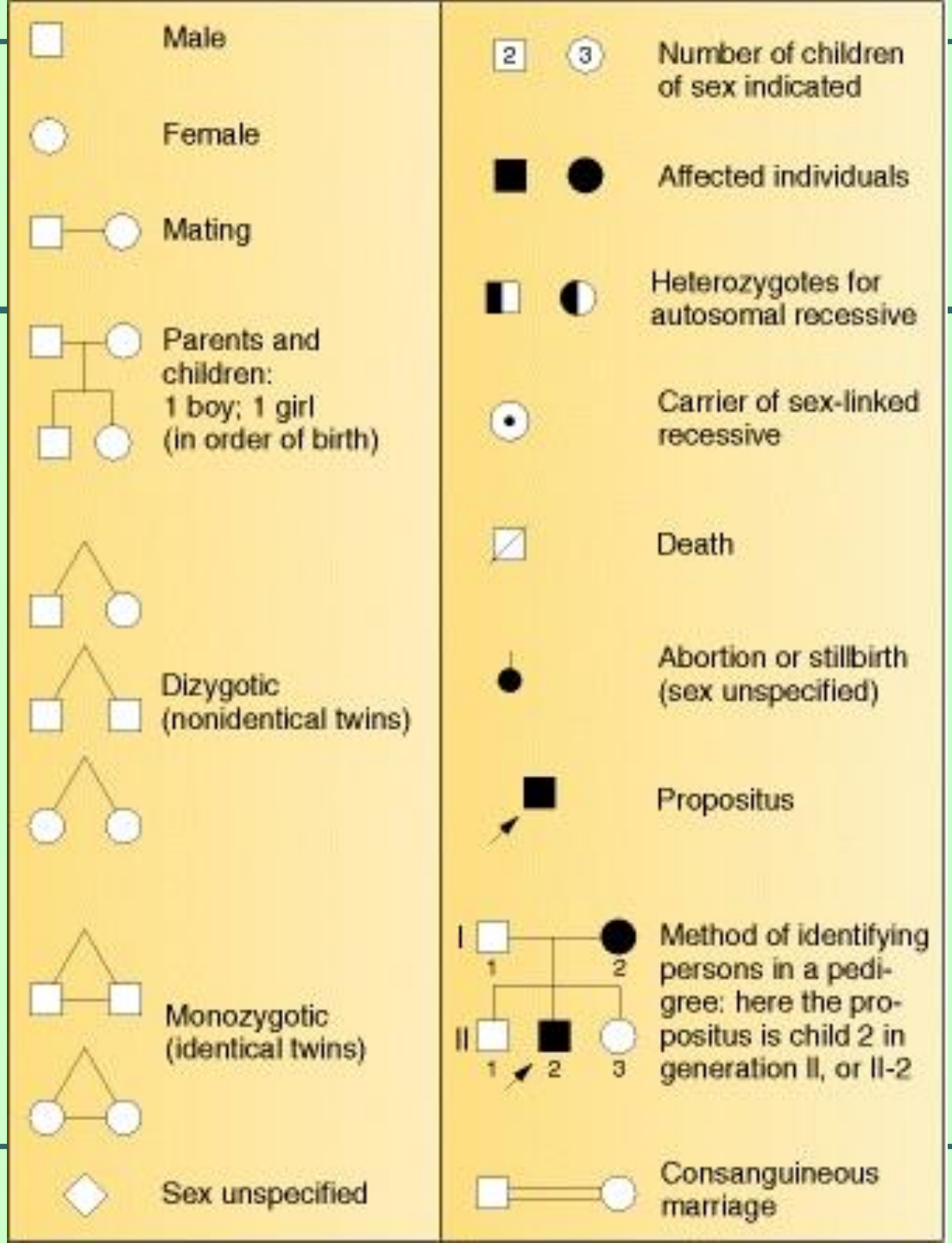
b.) monogenic diseases (single gene defects)

- only a single gene is altered (mutant) → flawed protein → manifestation (development) of a disease
- inherited in simple Mendelian fashion
- some 6000 distinct disorders are now known (sicle cell anemia, familial hypercholesterolemia, cystic fibrosis, Hemophilia A., Duchenne Muscular Dystrophy, Huntington Disease...)

c.) multifactorial diseases

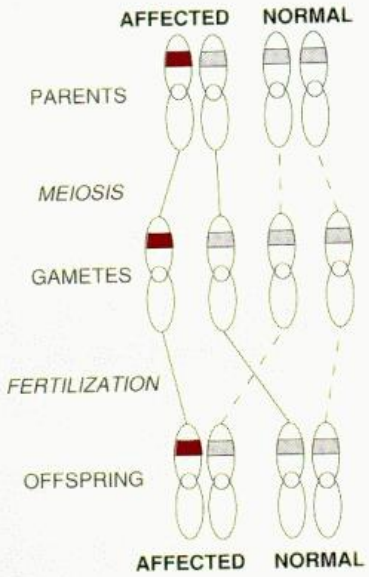
- result from the interaction of multiplex genes, each of which may have a relatively minor effect
- environmental factors contribute to the manifestation of these diseases (e.g. nutrition, exercise)
- for this group of illnesses, the contribution of the gene can be thought of as a “predisposition”
- examples: diabetes mellitus, hypertension, schizophrenia and congenital defects such as cleft lip, cleft palate and most congenital heart diseases
- very common in the population

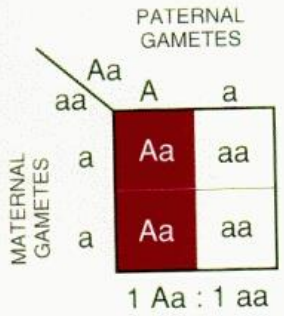
Human pedigree



Autosomal dominant inheritance process

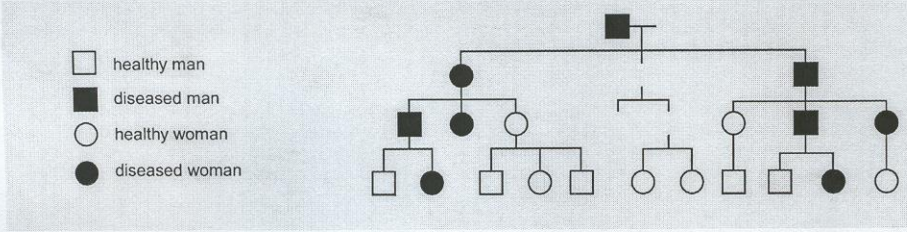
Only one of the two homologous genes is mutated and although another normal gene is present (**heterozygosity**), the illness still appears (**dominant gene effect**). If, therefore, one of the parents carries this gene, there is a 50% probability that it will be transmitted to each child. Both men and women can be affected by this. This inheritance pattern accounts for over 60% of monogenic diseases,representing by far the most common inheritance process. Obviously a mutated protein in just half the amount will have a pathological effect on the human organism in such cases. E.g. **achondorplasia**





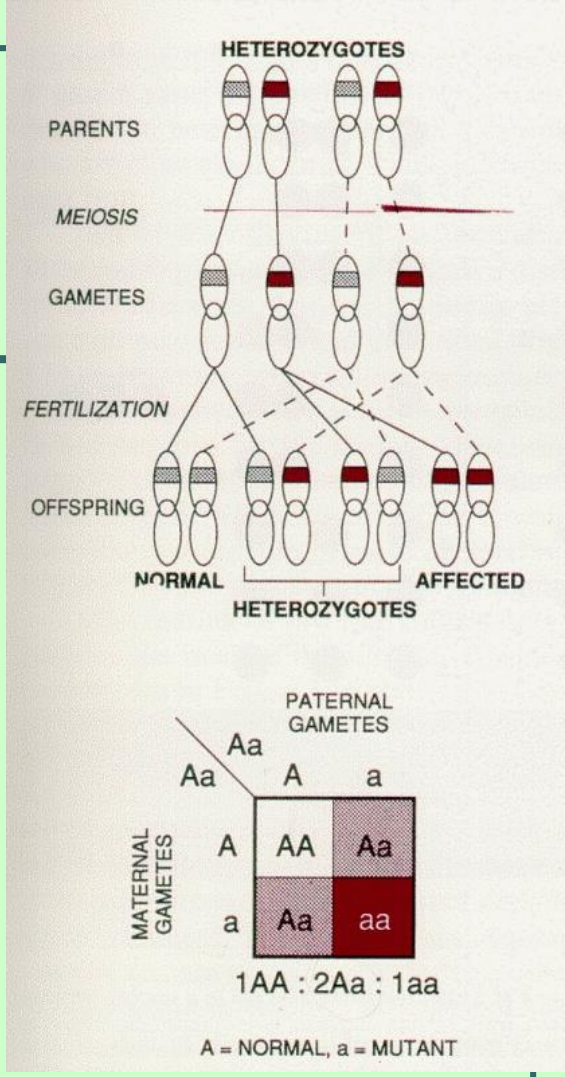
A = MUTANT, a = NORMAL

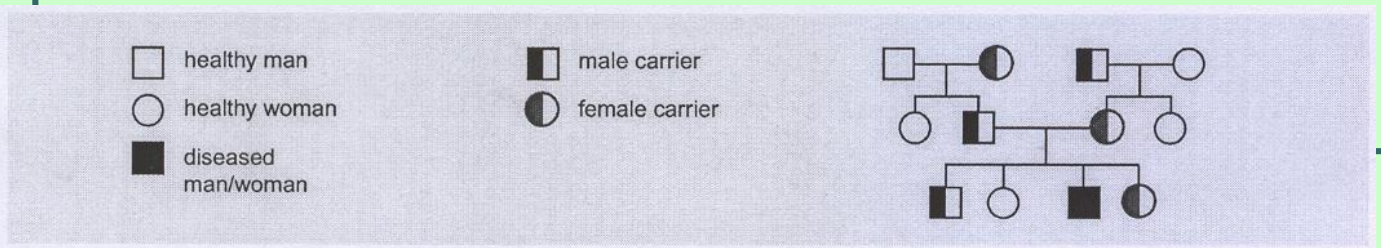
Figure 3.4. Autosomal dominant inheritance. The mating diagram *(top)* and Punnett square *(bottom)* show the outcome of a mating between a heterozygous affected individual and a homozygous normal individual. The mutant allele in the mating diagram is indicated in *red*, and affected offspring in the Punnett square are indicated in *red*.



Autosomal recessive inheritance

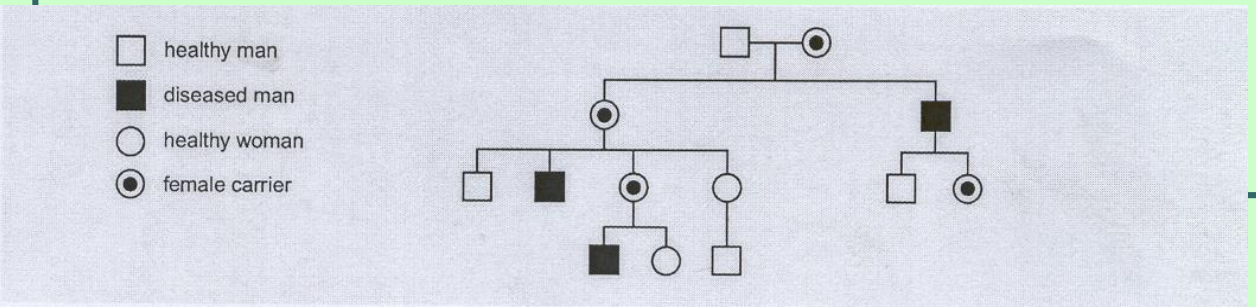
In this inheritance pattern, both homologous genes must be mutated (**homozygosity**) in order to produce an illness in the affected person. Individuals, who only receive one version of the mutated gene are called **carriers**. Both sexes can be affected. If, for example, both parents are carriers, there is a 25% chance that the child will receive both mutated genes and so develop the illness. Many metabolic diseases fall into this category (e.g. **cystic fibrosis, phenylketonuria, adrenogenital syndrome, haemochromatosis**).

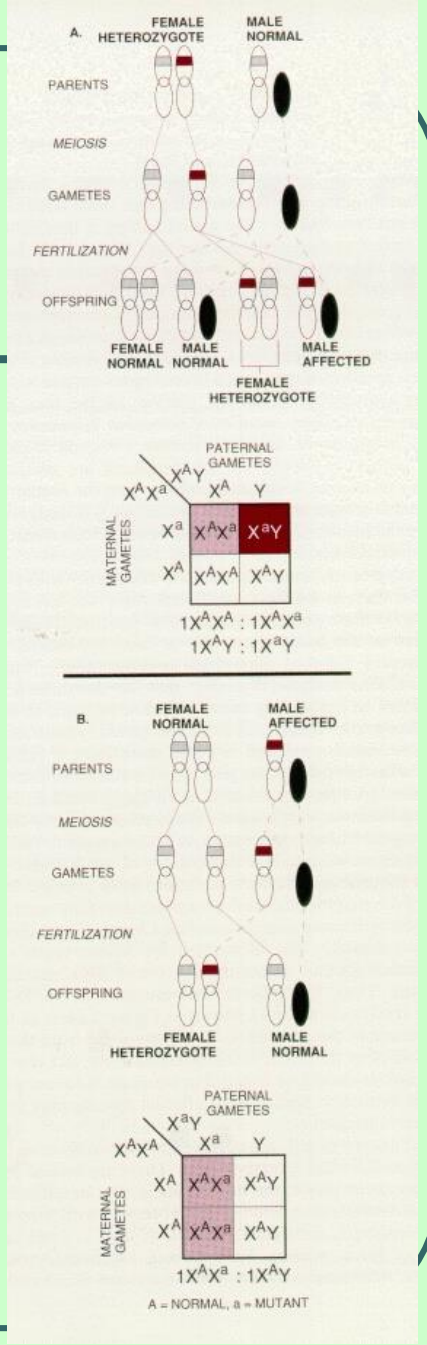




X chromosome inheritance (sex-linked inheritance)

Women have two X chromosomes. If they have a recessively acting mutated gene on one X chromosome, they are carriers for the corresponding illness. Men have only one X chromosome, since the other sex chromosome is a Y chromosome. If they have the mutated gene on the X chromosome, they will develop the illness as a rule.
If a woman is a carrier for the illness inherited by the X chromosome, there is a 50% chance that she will pass on this illness to her son. Her daughters have a 50% chance of becoming a carrier for this illness.





Identification of inherited diseases

1.) Phenotype analysis

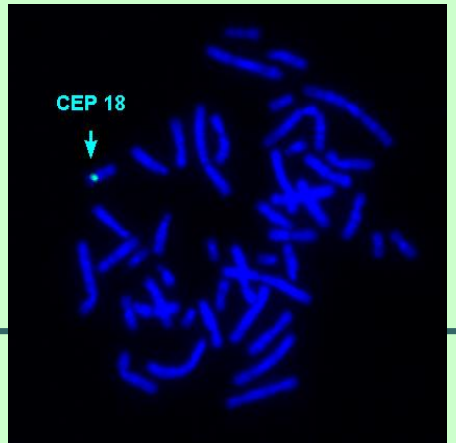
Genes are directly responsible for the production of hormones, enzymes and other proteins. Investigation procedure: Diagnostic measurement of altered or missing proteins using blood or urine analysis. This provides indirect evidence of a mutation of the gene responsible for this.

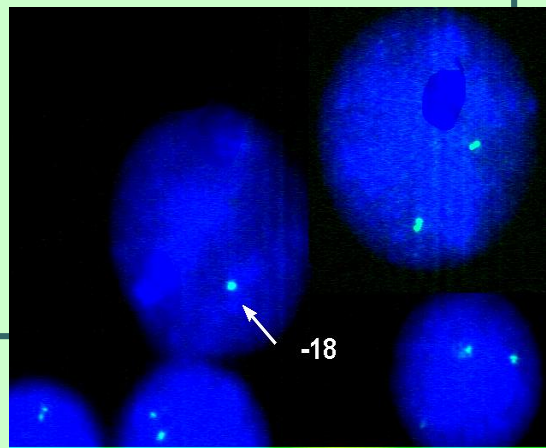
Examples: Phenylketonuria, alpha1-antitrypsin deficiency

2.) Chromosome analysis (cytogenetic investigations)

This includes microscope examinations to investigate chromosome alterations in terms of number (duplication or loss of individual chromosomes = **numeric chromosome aberration**) and in terms of structure (wrong composition, chromosome breaking = **structural chromosome aberration**). There is no detailed investigation of individual genes in such cases.

Indication: Anomalies in children (malformations, retarded development) in the context of prenatal diagnosis, tendency to miscarriages, infertility.





3.) Molecular genetics testing (DNA analysis, genome analysis DNA tests)

This provides evidence of a gene mutation responsible for producing the illness. Here it is determined whether the sequence of the DNA bases (**nucleotide sequence**) has changed within the affected

DNA/RNA diagnosis of genetic diseases

Not all mutation test use DNA. Testing RNA by **RT-PCR** has advantages when screening genes with many exons (NF1 gene, DMD gene...) or seeking splicing mutations.

Very important in molecular genetic testing is using **a protein-based functional assay**, which may classify the products into two simple groups: *functional* and *nonfunctional* – essential question in most diagnostics

Limitations of DNA analysis

monogenic and also polygenic diseases sometimes do not occur in both twins, even though the genetic information is the same in identical twins. This is due to several factors:

Penetrance: not every pathogenic mutation leads to the manifestation of a disease in the lifetime of a person.

Expressivity on the other hand describes quantitative differences in the manifestation of the disease/symptoms. Sometimes, the two concepts are difficult to separate, when, for example, a disease is so weakly manifested that it can no longer be diagnosed.

The age at which the disease manifests itself can vary strongly. An example of this is Huntington's chorea. Differences in the onset of diseases are sometimes explained by so-called **dynamic mutations**. In passing on to the next generation, the disease-inducing mutation can lead to an earlier onset of the illness (**anticipation**) involving the extension of a mutated sequence of bases.

In many cases, genetic information is manifested in a different way when it is inherited from the mother than when it is inherited from the father. Here one speaks of **imprinting**.

Molecular genetics testing (DNA analysis, genome analysis DNA tests)

A.) Direct testing
– DNA from a patient is tested to see whether or not it carries a given pathogenic mutation

B.) Indirect testing (gene tracking)
- linked markers are used in family studies to discover whether or not the consultand inherited the disease-carrying chromosome from a parent

A.) Direct testing

• provides evidence of a gene mutation responsible for producing the illness. It is determined whether the sequence of the DNA bases (**nucleotide sequence**) has changed
• to see wheter the DNA of tested person has a gene normal or mutant

Detection of mutation in relevant gene **always confirms the clinical diagnosis**
we must know
which **gene** to examine
the relevant „**normal**“ (wild type) **sequence**

Mutation testing methods can be divided into two groups:

1. Mutation detection methods (scoring) – test the DNA for the presence or absence of one specific mutation. Searching for known mutations

2. Mutation screening methods (scanning) – screen a sample for any deviation from the standard sequence.

1. Mutation detection methods – test a DNA for the presence or absence of one specific mutation

searching for *known* sequence change is **possible for**:

- diseases where all affected people in the population have **one particular mutation**
- most affected people in the population have **one** of limited number of **specific mutations**
- diagnosis **within a family** - once mutation is characterized, other family members need to be tested for that particular mutation

2.Mutation screening methods - screen a sample for any deviation from the standard sequence

The mutation screening is possible for diseases where a good proportion of patients carry independent mutations.

Testing for unknown mutations in laboratory suffer **two limitations**:

methods are quite **laborious** and **expensive** for use in diagnostic service, which needs to produce answers quickly

detect differences between the patient´s sequence and published normal sequence (not distinguish between pathogenic and nonpathogenic changes.)

Polymerase chain reaction (PCR)

To amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA

Kery Mullis – 1983 discovered the PCR procedure, for which he was awarded the Nobel prize

PCR

selective amplification of specific target DNA sequence within heterogeneous collection of DNA (total genomic DNA or complex cDNA) requires:

-sequence information from the target sequence for construction two oligonucleotide primer sequences (15 – 30 nucleotides long)

-denatured genomic DNA

-heat stable DNA polymerase

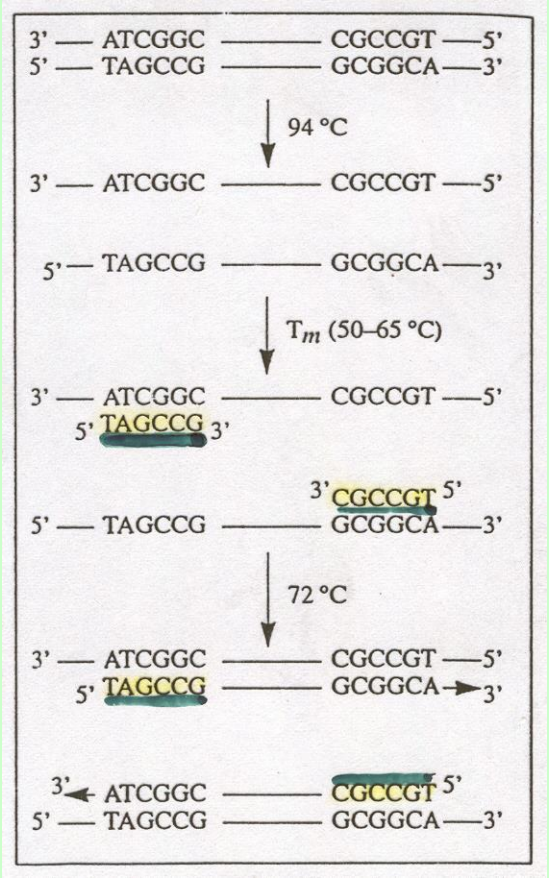
-DNA precursors (four deoxynucleotide triphosphates dATP, dCTP, dGTP and dTTP)

PCR involves sequential cycles composed of three steps:

- **Denaturation (typically at about 93 – 95^{o} C)**

- **Reannealling (at temperatures usually from about 50 ^{o} – 70^{o}C, depending on T_m of the expected duplex**

- **DNA synthesis – typically at about 70 –75^{o}**



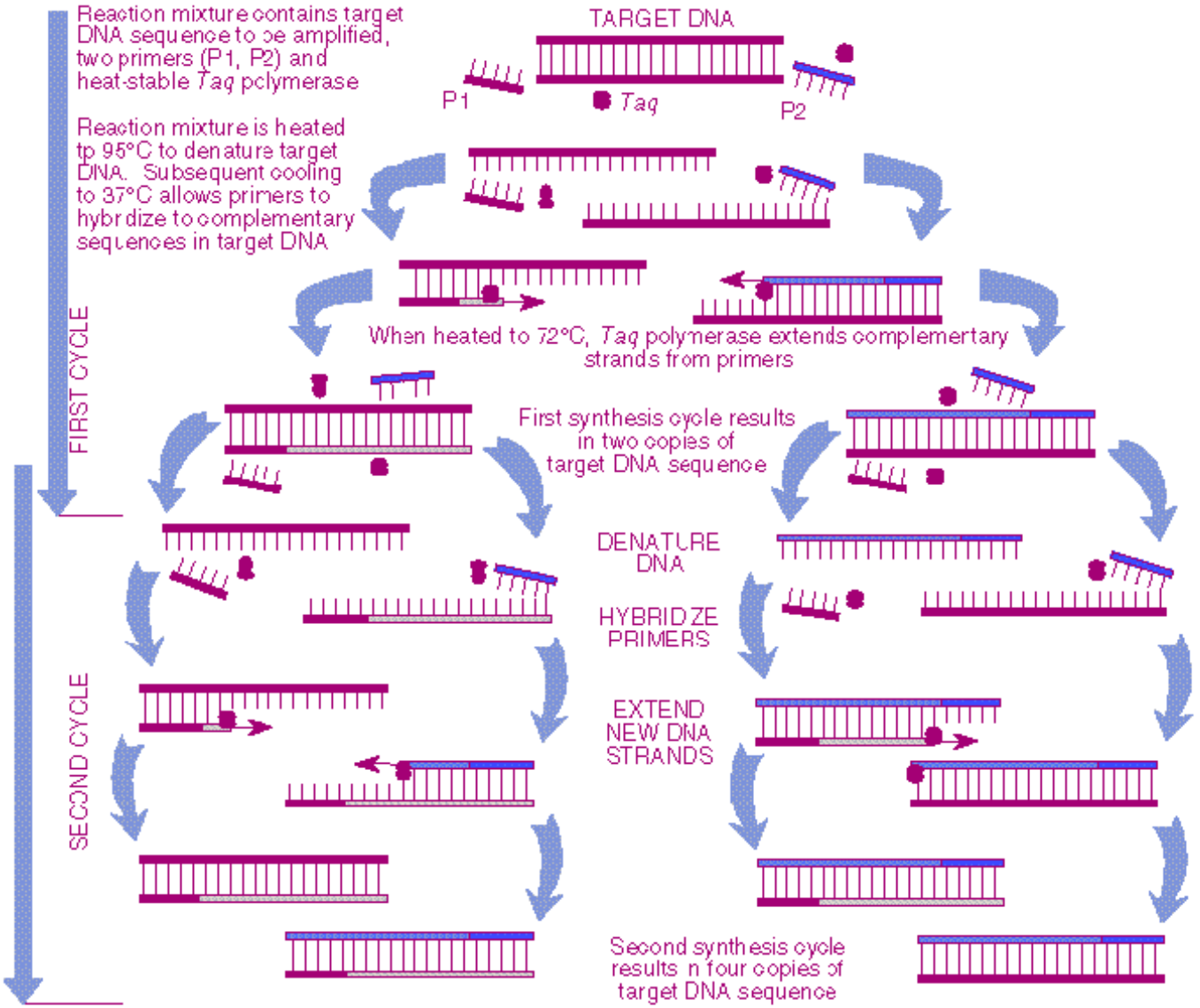
Senzitivity of PCR allows us to use a wide range of samples:

- **blood samples**
- **monthwashes or buccal scrapes**
- **chorionic villus biopsy samples**
- **amniocentesis speciments**
- **ome or two cells** (removed from eight-cell stage embgryos)
- **hair, semen**
- **archived pathological specimens**

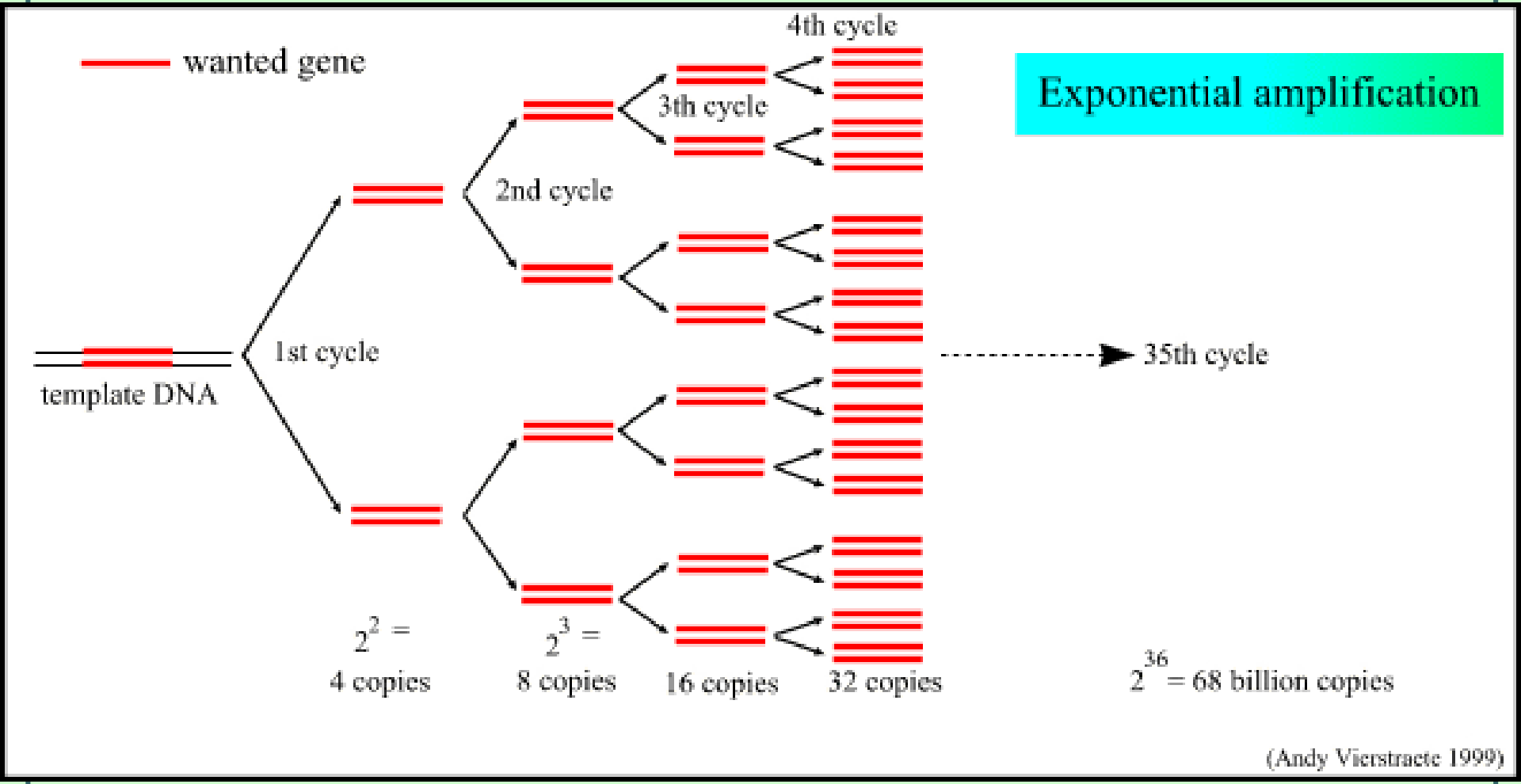
Guthrie cards (spot of dried blood)

ORNL-DWG 91M-17476

DNA Amplification Using Polymerase Chain Reaction



Source: *DNA Science*, see Fig. 13.



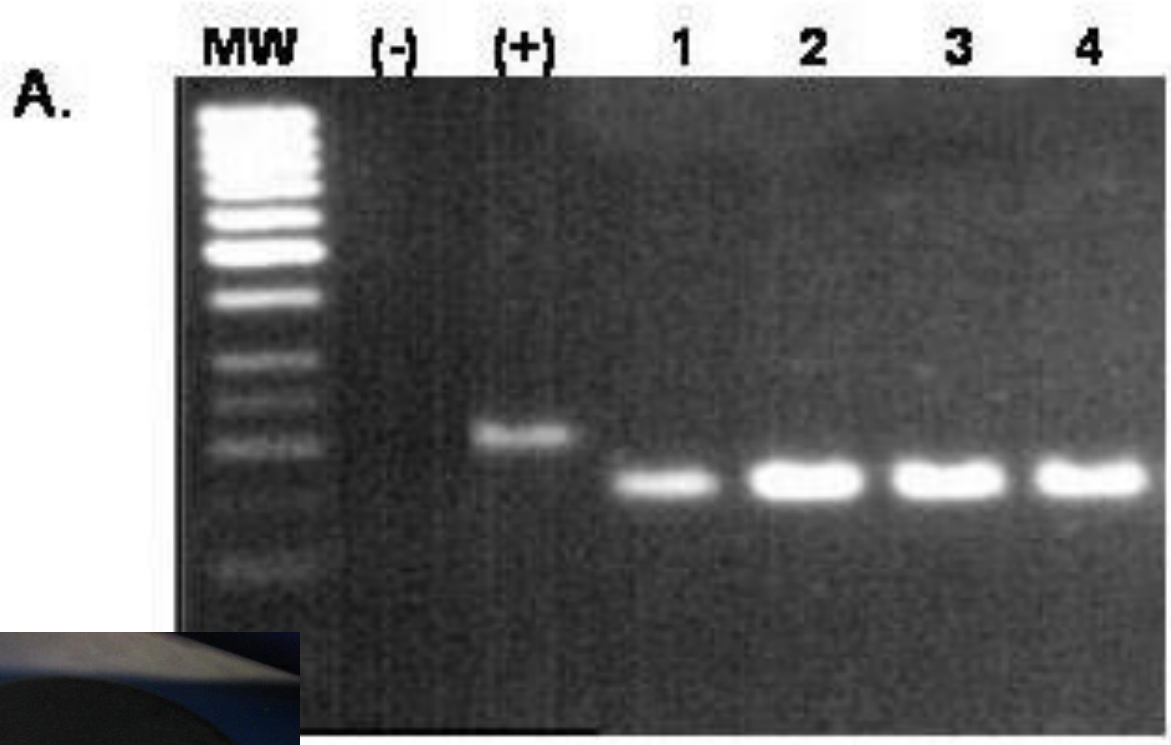
Exponential amplification

(Andy Vierstraete 1999)

Electrophoresis

- to separate and visualize DNA or RNA fragments by size and reactivity
- migration of DNA in electric field
- ethidium bromide

- Agarose electrophoresis
- Polyacrylamide gel electrophoresis (PAGE)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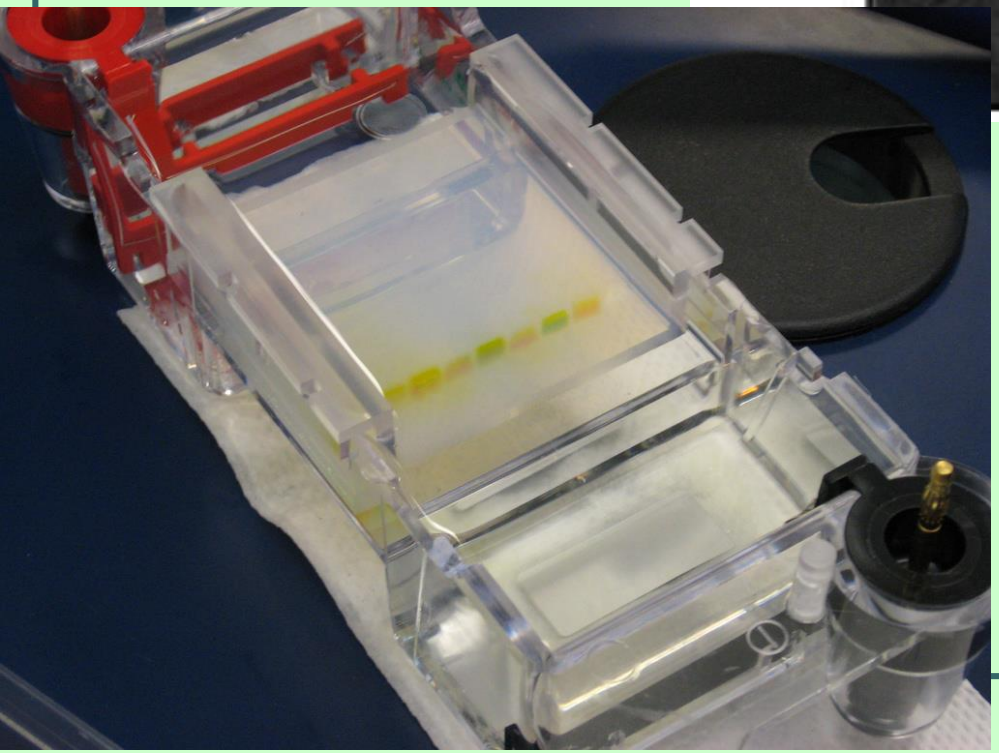
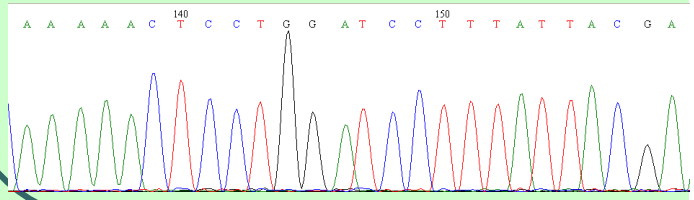


- **sequence analysis:** (synonym: sequencing) Process by which the nucleotide sequence is determined for a segment of DNA



Step 1: Amplification.

A. The segment of DNA to be sequenced is PCR-amplified using normal nucleotides (i.e., dATP, dTTP, dGTP, and dCTP) and fluorescently labeled, dideoxy nucleotides (i.e., ddATP, ddTTP, ddGTP, ddCTP). (Dideoxy nulceotides arrest chain elongation.)

PCR amplification

Segment of DNA

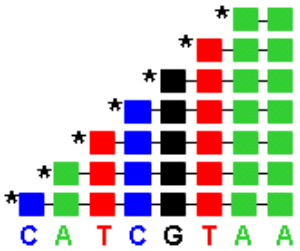
B. The DNA segment is copied when normal nucleotides are incorporated. Copying ceases when a dideoxy nucleotide is incorporated. By this process, many different-sized fluorescently labeled DNA fragments are produced.

Key: Nucleotides used in PCR reaction

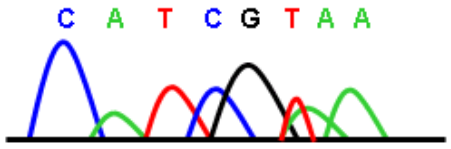
Normal nucleotides	Fluorescently labeled, dideoxy nucleotides
dATP	ddATP
dTTP	ddTTP
dGTP	ddGTP
dCTP	ddCTP

DNA fragments

Step 2: Sequence determination. The fragments are sorted by length. A sequencing machine reads the fluorescent wavelengths to determine which nucleotide is at the end of each fragment.

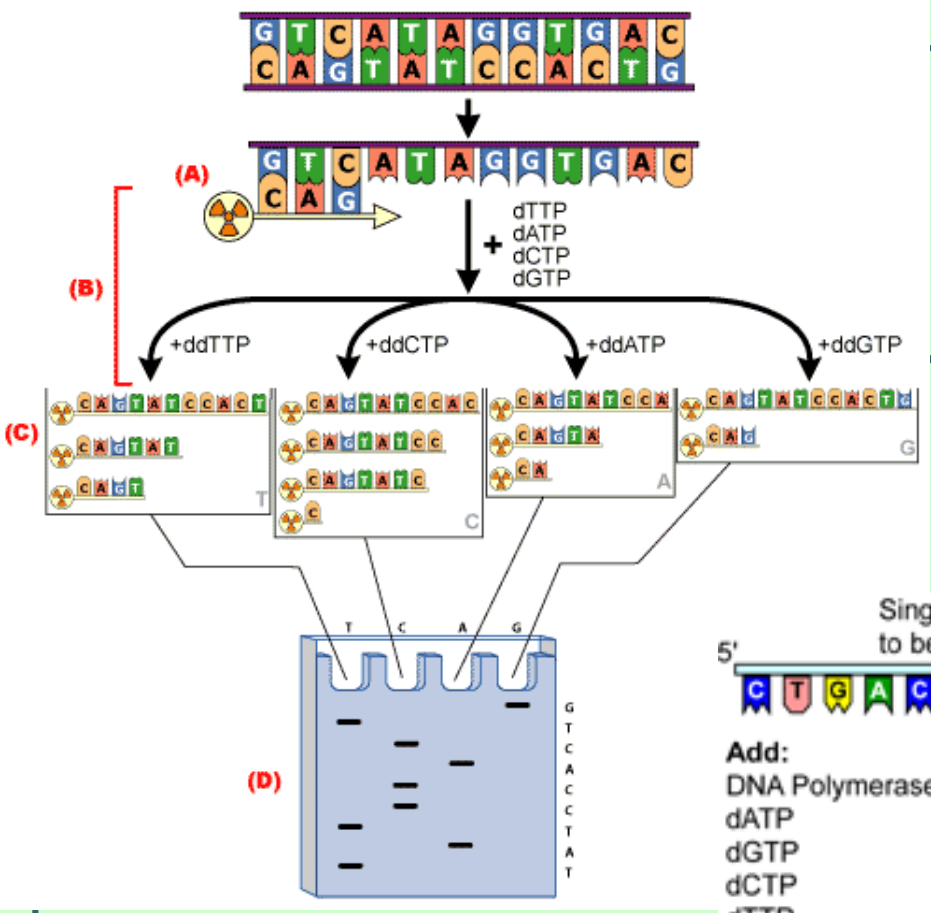


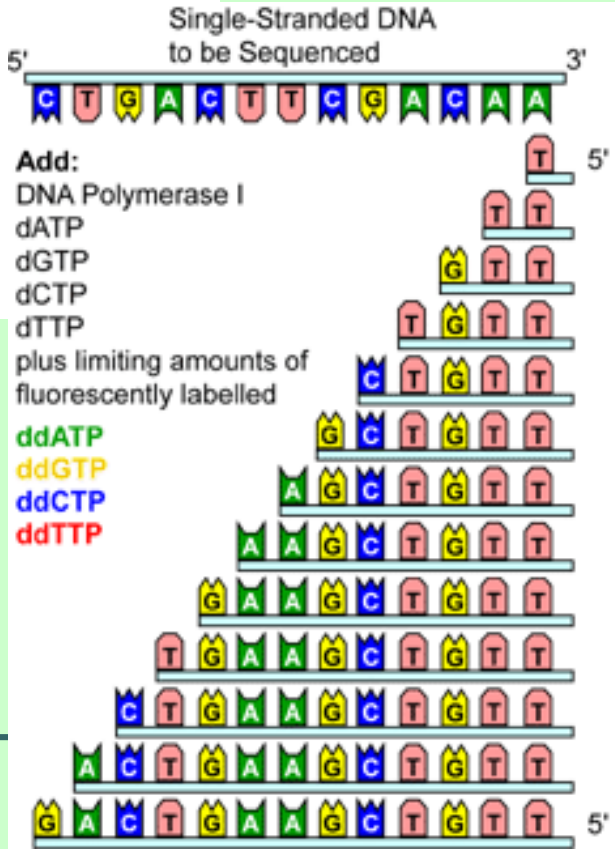
Step 3: Sequence reporting. Sequence data are typically displayed on an electropherogram as colored peaks. Each peak represents a nucleotide, corresponding to the letter above it.



Electropherogram

Wavelength	Nucleotide
Red	T
Green	A
Blue	C
Black	G


(A)
dTTP
dATP
dCTP
dGTP
(B)
+ddTTP
+ddCTP
+ddATP
+ddGTP
(C)
T
C
A
G
(D)
T C A G
G T C A C C T A T


Single-Stranded DNA
to be Sequenced
5'
3'
Add:
DNA Polymerase I
dATP
dGTP
dCTP
dTTP
plus limiting amounts of
fluorescently labelled
ddATP
ddGTP
ddCTP
ddTTP

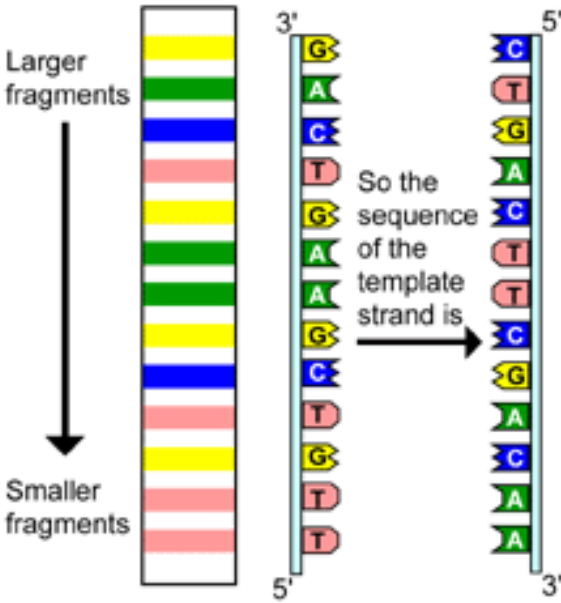
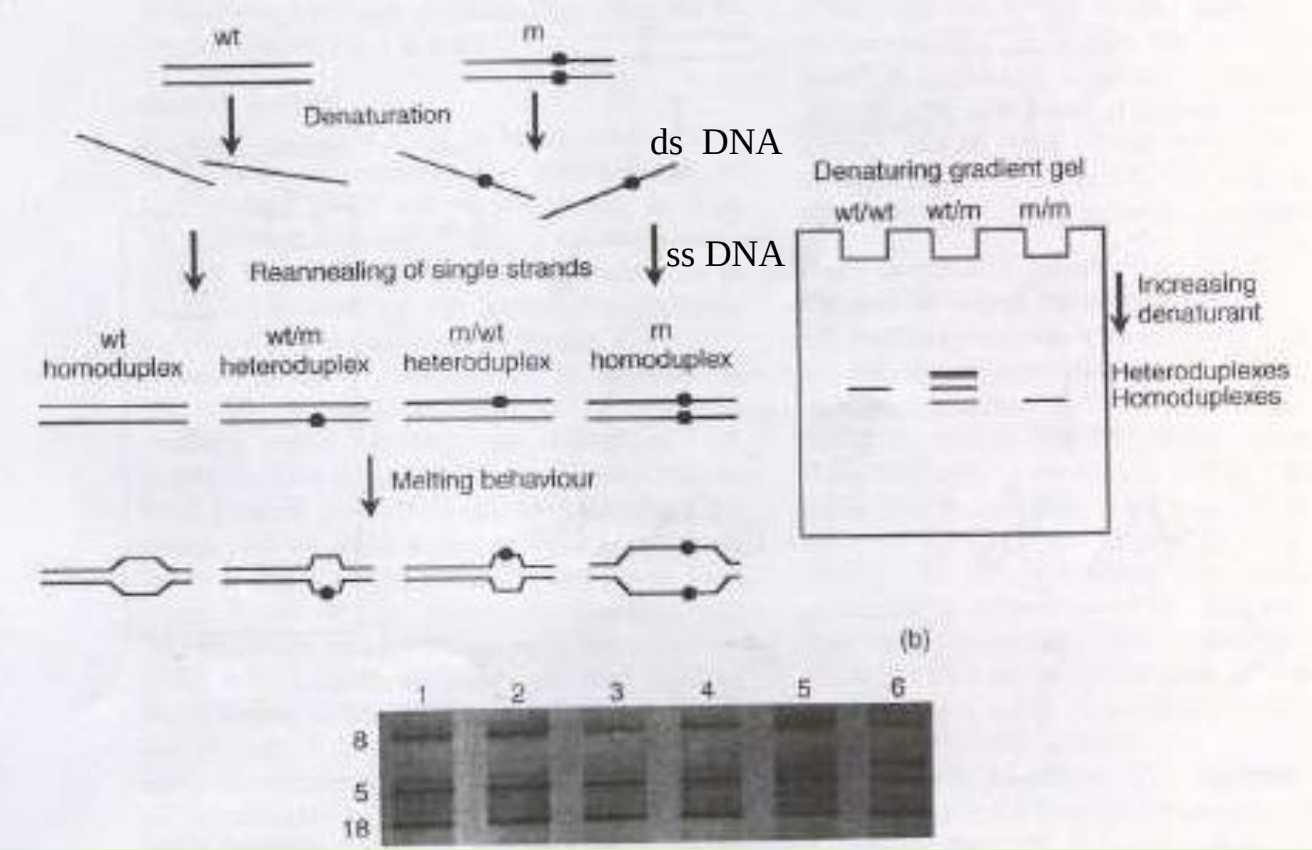

Larger
fragments
Smaller
fragments
3'
5'
So the
sequence
of the
template
strand is
5'
3'

Table 16.1: Methods for scanning a gene for point mutations

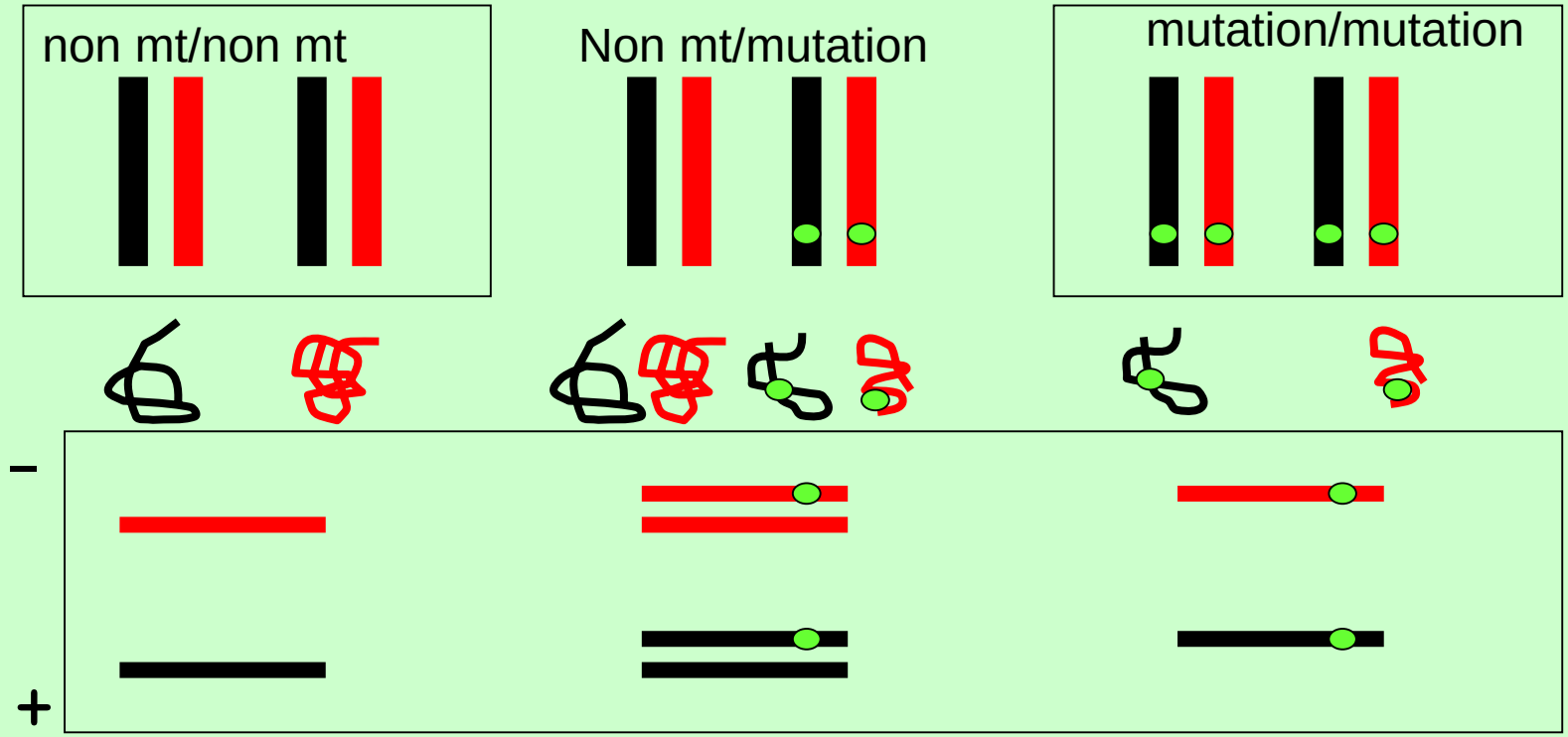
Method	Advantages	Disadvantages
Sequencing	Detects all changes Mutations fully characterized	Laborious Generates excessive information
Heteroduplex gel mobility	Very simple	Short sequences only (<200 bp) Limited sensitivity Does not reveal position of change
Single-strand conformation polymorphism (SSCP) analysis	Simple	Short sequences only (<200 bp) Does not reveal position of change
Denaturing gradient gel electrophoresis (DGGE)	High sensitivity	Choice of primers is critical GC-clamped primers expensive Does not reveal position of change
Mismatch cleavage (i) chemical (ii) enzymic	High sensitivity Shows position of change	Toxic chemicals Experimentally difficult
Protein truncation test (PTT)	High sensitivity for chain terminating mutations Shows position of change	Chain terminating mutations only Expensive Experimentally difficult Best with RNA

denaturating gel gradient electrophoresis (DGGE)



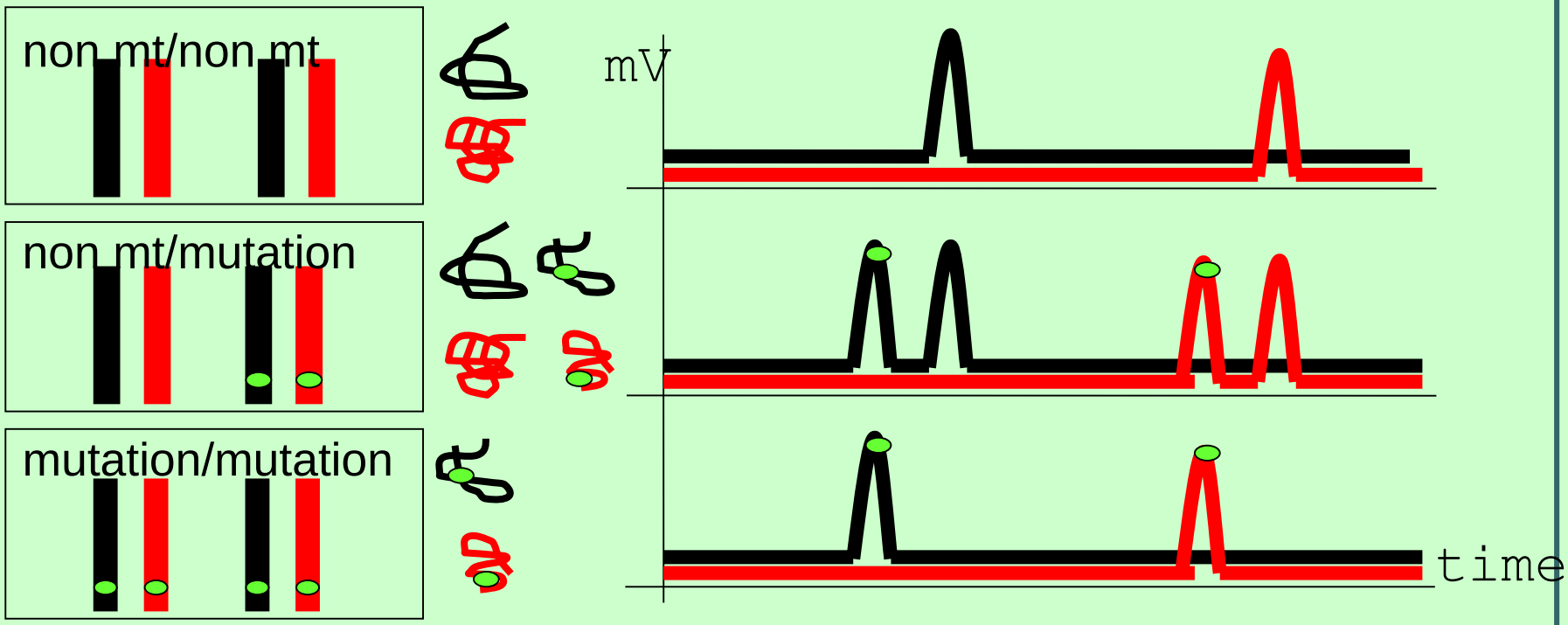
DGGE: the sequence-specific denaturation characteristics in a chemical gradient (in the gel) lead to partial separation of strands. This in turn leads to differential mobility and results in a single band per variant

SSCP in gel (Single-strand conformation polymorphism)

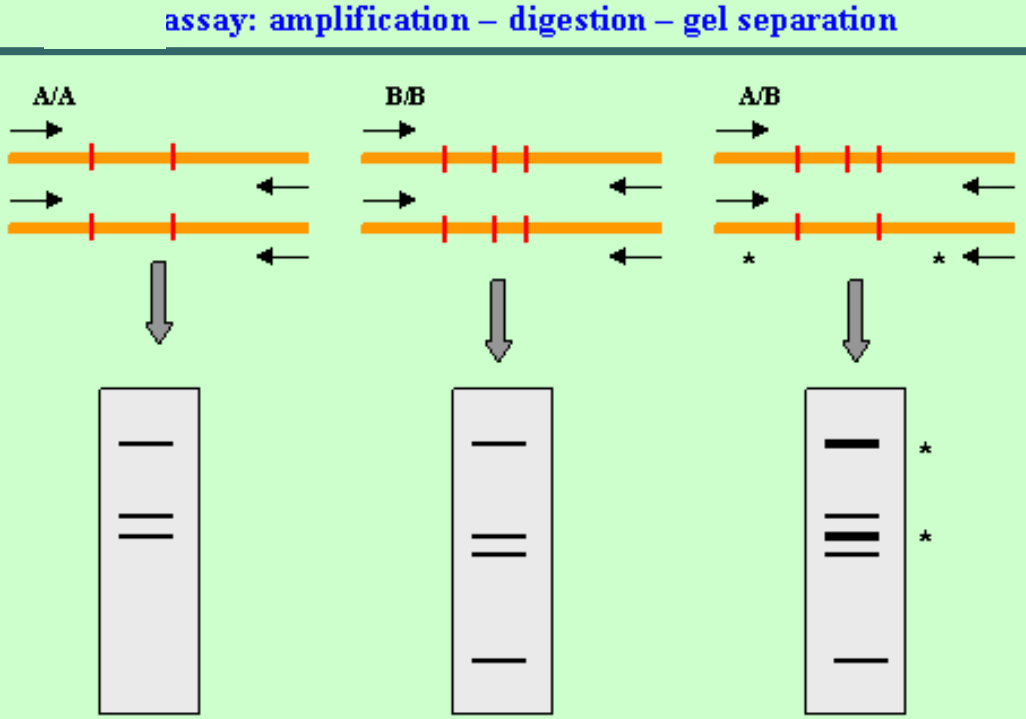


SSCP: after denaturation, single strands form a sequence-specific structure. This structure leads to differential mobility in a non-denaturing matrix and two bands per variant

SSCP in capillary



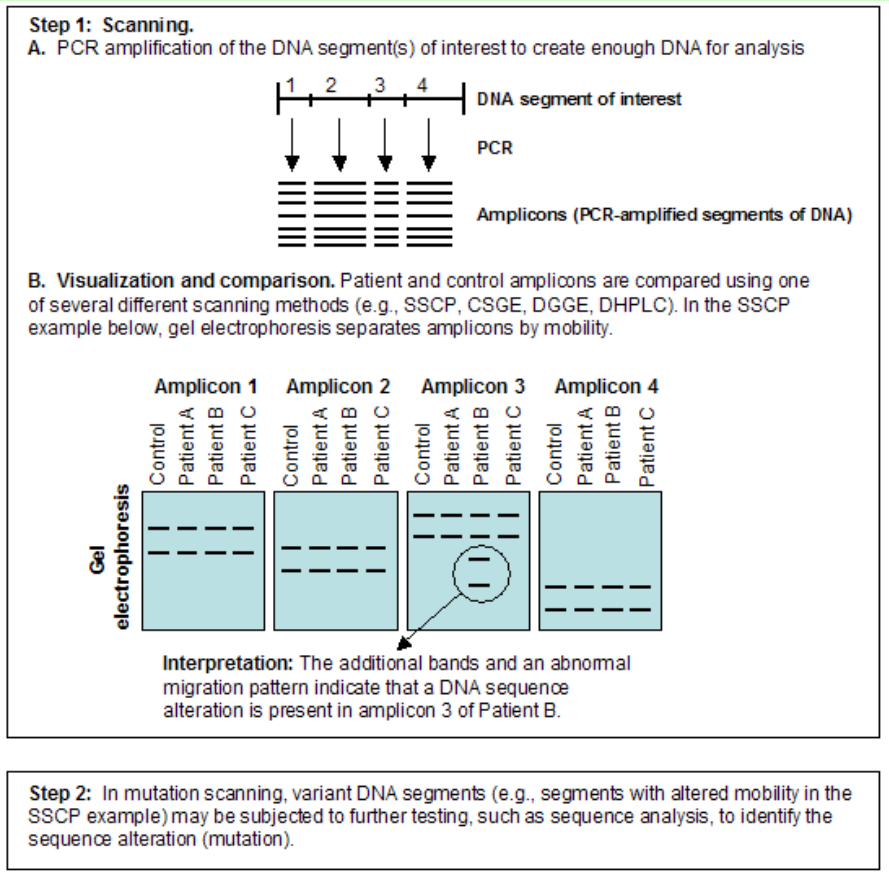
RFLP



- Unique sequence primers are used to amplify a mapped DNA sequence from two related individuals, A/A and B/B, and from the heterozygote A/B. In the case of the heterozygote A/B, two different PCR products will be obtained, one which is cleaved three times and one which is cleaved twice.

mutation scanning (synonym: mutation screening):

A process by which a segment of DNA is screened via one of a variety of methods to identify variant gene region(s). Variant regions are further analyzed (by sequence analysis or mutation analysis) to identify the sequence alteration



Some Clinical Implications

- Mutation scanning is used when mutations are distributed throughout a gene, when most families have different mutations, and when sequence analysis would be excessively time-consuming due to the size of a given gene.
- Mutation scanning may cover the entire gene or select regions.
- The sequence alteration identified in a segment of DNA may be a benign variant (polymorphism), a disease-causing mutation, or an alteration of undetermined significance.

Types of sequence alterations that may be detected:

- Pathogenic sequence alteration reported in the literature
- Sequence alteration predicted to be pathogenic but not reported in the literature
- Unknown sequence alteration of unpredictable clinical significance
- Sequence alteration predicted to be benign but not reported in the literature
- Benign sequence alteration reported in the literature

Possibilities if a sequence alteration is not detected

Patient does not have a mutation in the tested gene (e.g., a sequence alteration exists in another gene at another locus)

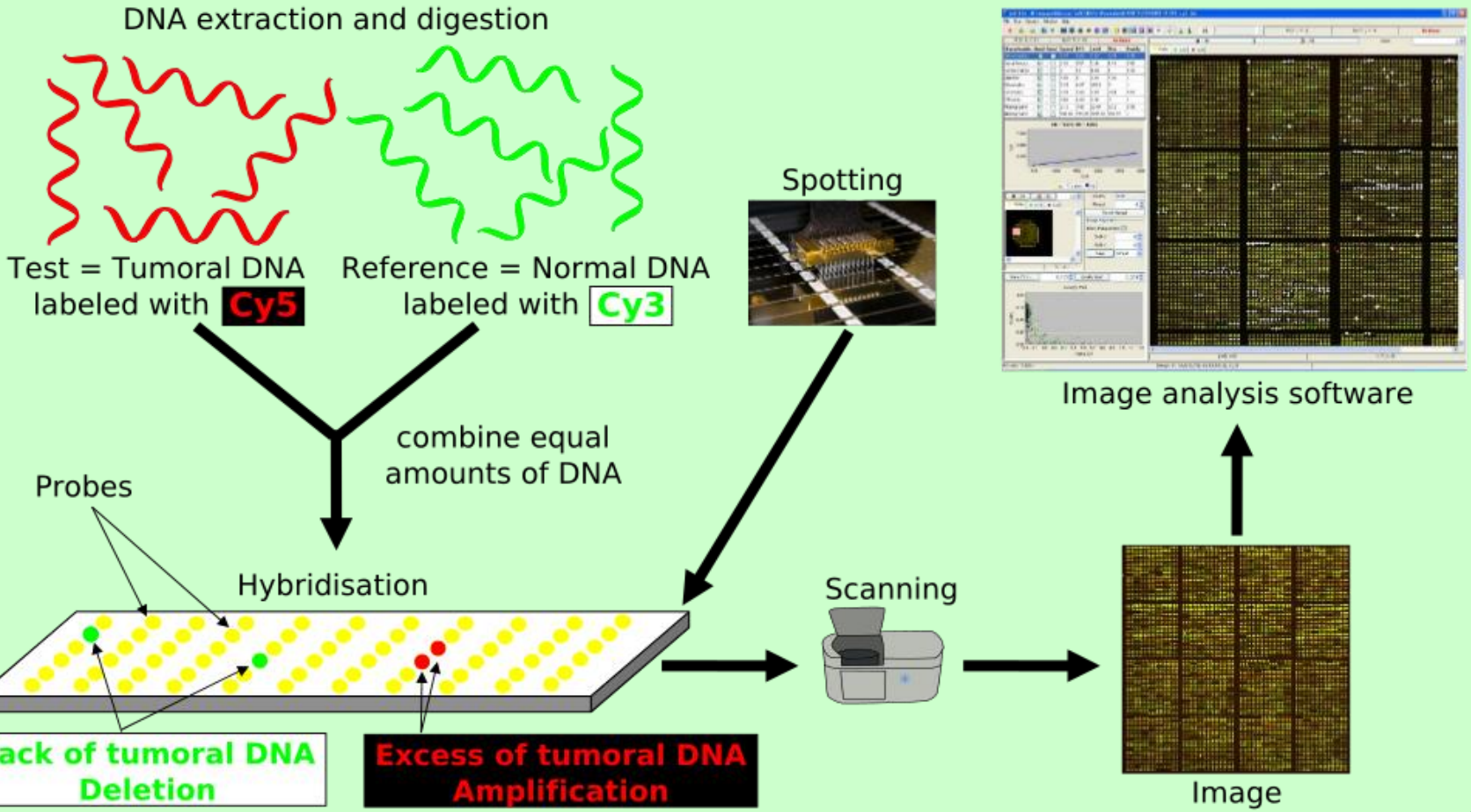
Patient has a sequence alteration that cannot be detected by sequence analysis (e.g., a large deletion)

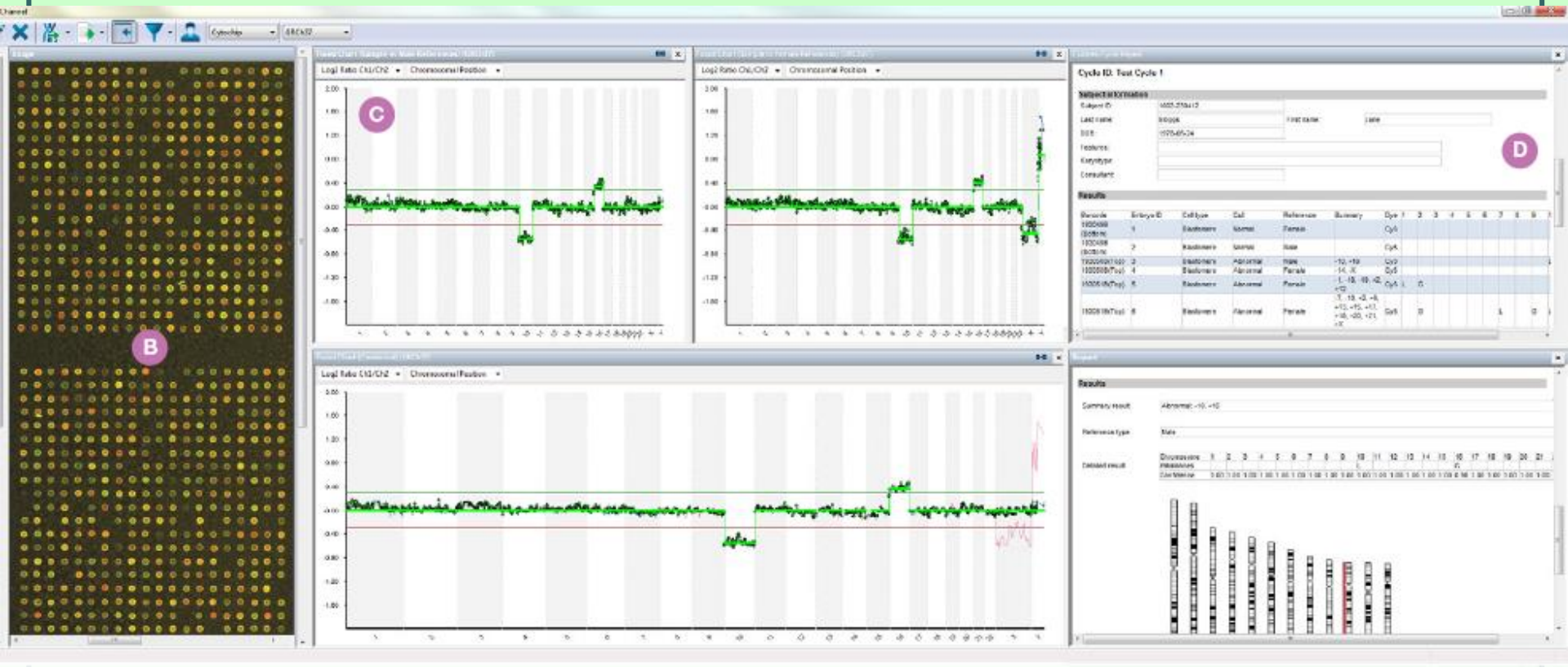
Patient has a sequence alteration in a region of the gene (e.g., an intron or regulatory region) not covered by the laboratory's test

array CGH (aCGH)

- for analysing copy number variations (CNVs) in the DNA of a test sample compared to a reference sample,
- compare two genomic DNA samples arising from two sources
- used for: genomic abnormalities in cancer, submicroscopic aberrations, preimplantation genetic diagnosis

- inability to detect structural chromosomal aberrations without copy number changes, such as mosaicism, balanced chromosomal translocations and inversions


DNA extraction and digestion
Test = Tumoral DNA
labeled with Cy5
Reference = Normal DNA
labeled with Cy3
combine equal
amounts of DNA
Probes
Hybridisation
Lack of tumoral DNA
Deletion
Excess of tumoral DNA
Amplification
Spotting
Scanning
Image
Image analysis software

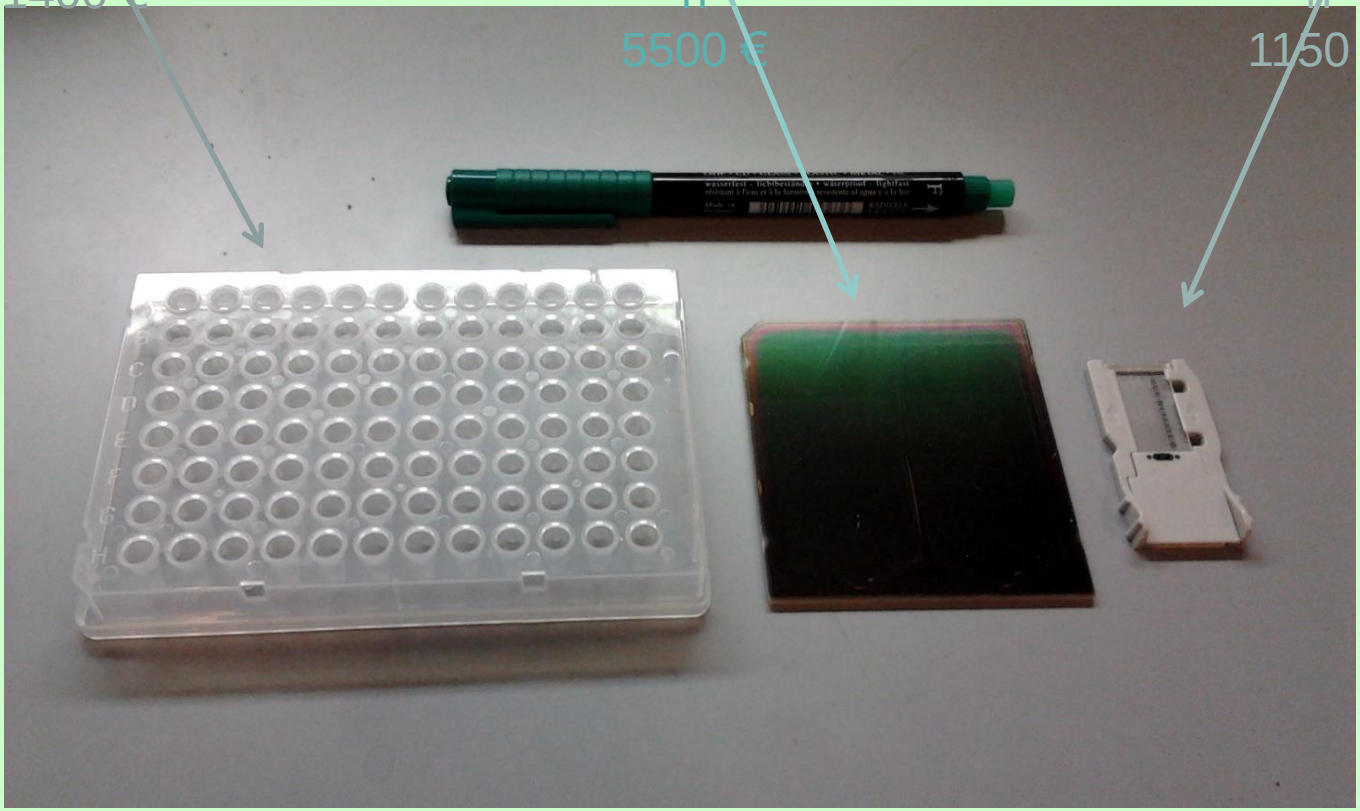

B
C
D

Next generation sequencing (NGS)

- Four main technologies
- All massively parallel sequencing
- – Sequencing by synthesis
  - Sanger/Dideoxy chain termination
  - Pyrosequencing (Roche/454)
  - Reversible terminator (Illumina)
  - Ion torrent (Life Technologies)
  - Zero Mode Waveguide (Pacific Biosciences) - 3rd generation sequencing
- – Sequencing by ligation
  - SOLiD (Applied Biosystems)
- – Direct reading of DNA sequence - 3rd generation sequencing
  - Nanopore sequencing
  - Electron microscope

Sequencing Matrices

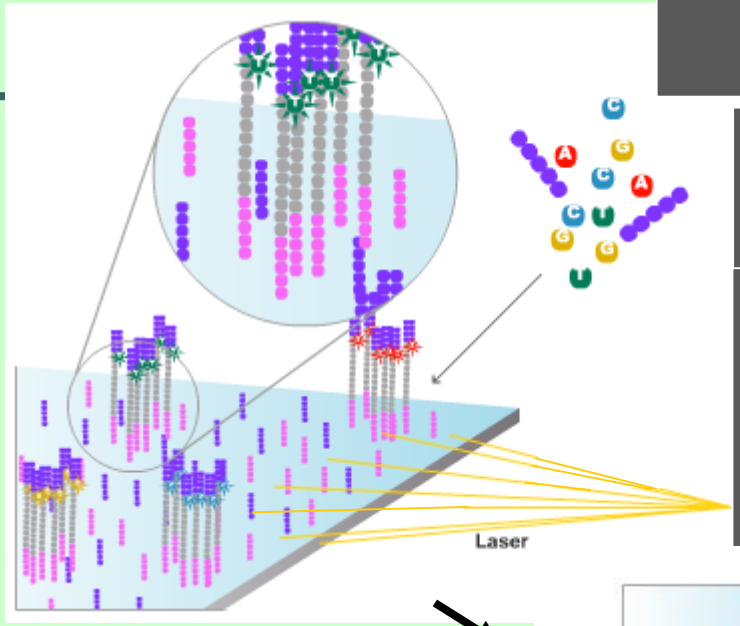
Sanger, 96-well, 8 capillaries	Pyrosequencing, 2 regions	Revers. terminator, MiSeq
96 x 600 bp / 24 h	1,000,000 x 600 bp / 20 h	10,000,000 x 250 bp / 40 h
1400 €	5500 €	1150 €



Reversible Terminator (HiSeq, MiSeq, NextSeq)

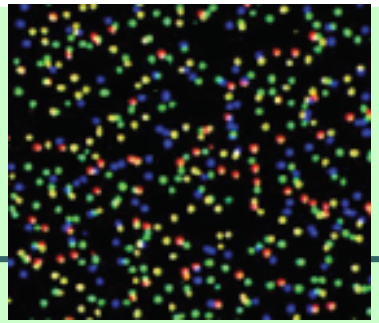
Sequencing DNA clusters one base at a time

A mix of sequencing primers (complementary to one of the adapter sequences), DNA polymerase and differentially fluorescent labelled reversible chain terminator dNTPs (A, C, T and G) are added to flow cell



Depending on the first nucleotide in the cluster, a specific fluorescent reversible chain terminator dNTP is incorporated leading to a stop in DNA synthesis!

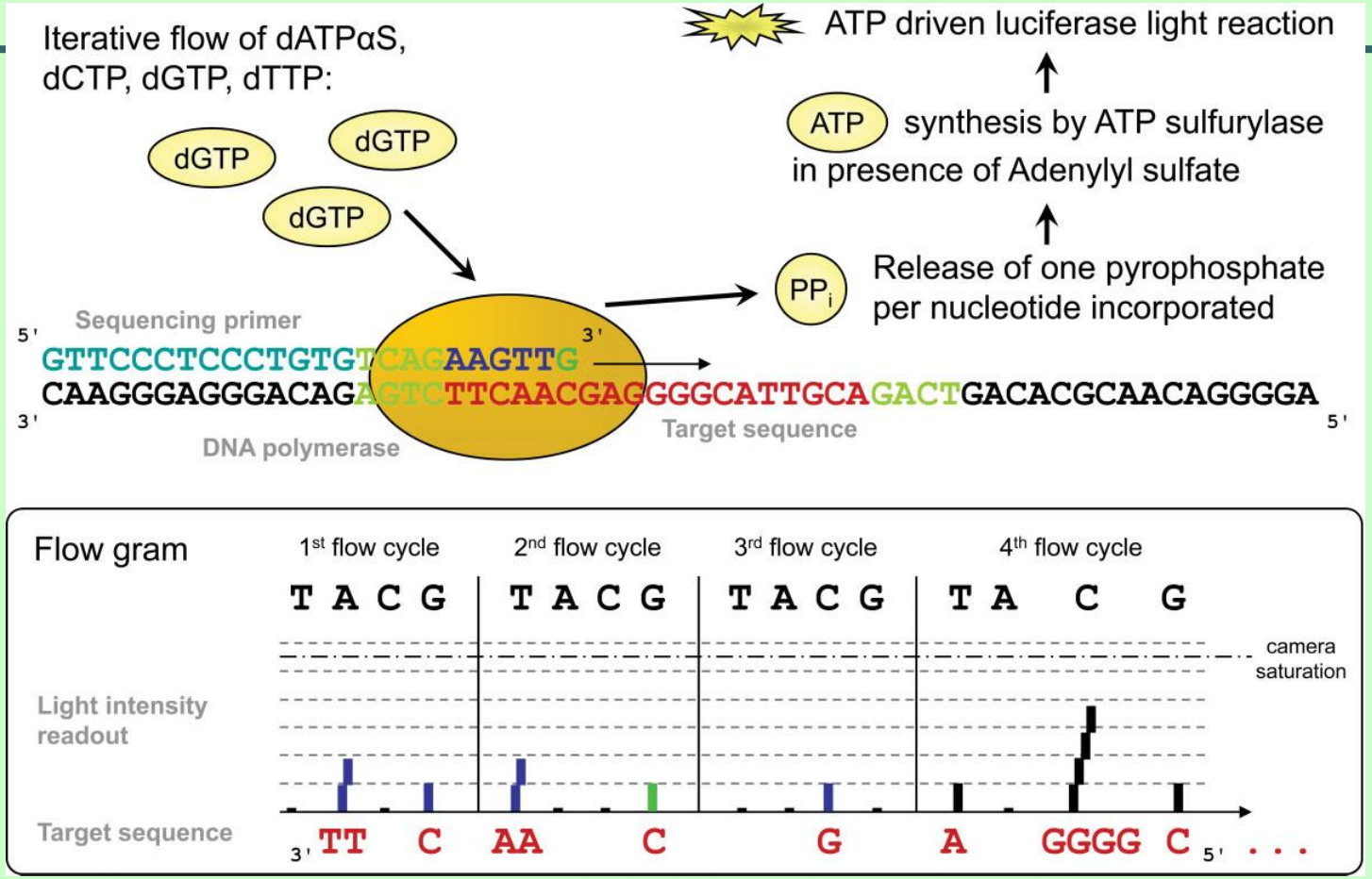
After washing unincorporated nucleotides away, a laser excites the flow cell and detects which of the four fluorescent chain terminator dNTPs were incorporated in each cluster on the flow cell. *i.e.* decodes the first sequenced base



Once an image recording what was the first nucleotide to be incorporated in each cluster has been taken, both the fluorescent dyes and the blocking group that prevents extension of the DNA are removed (hence 'reversible chain terminator dNTPs) and the cycle is repeated

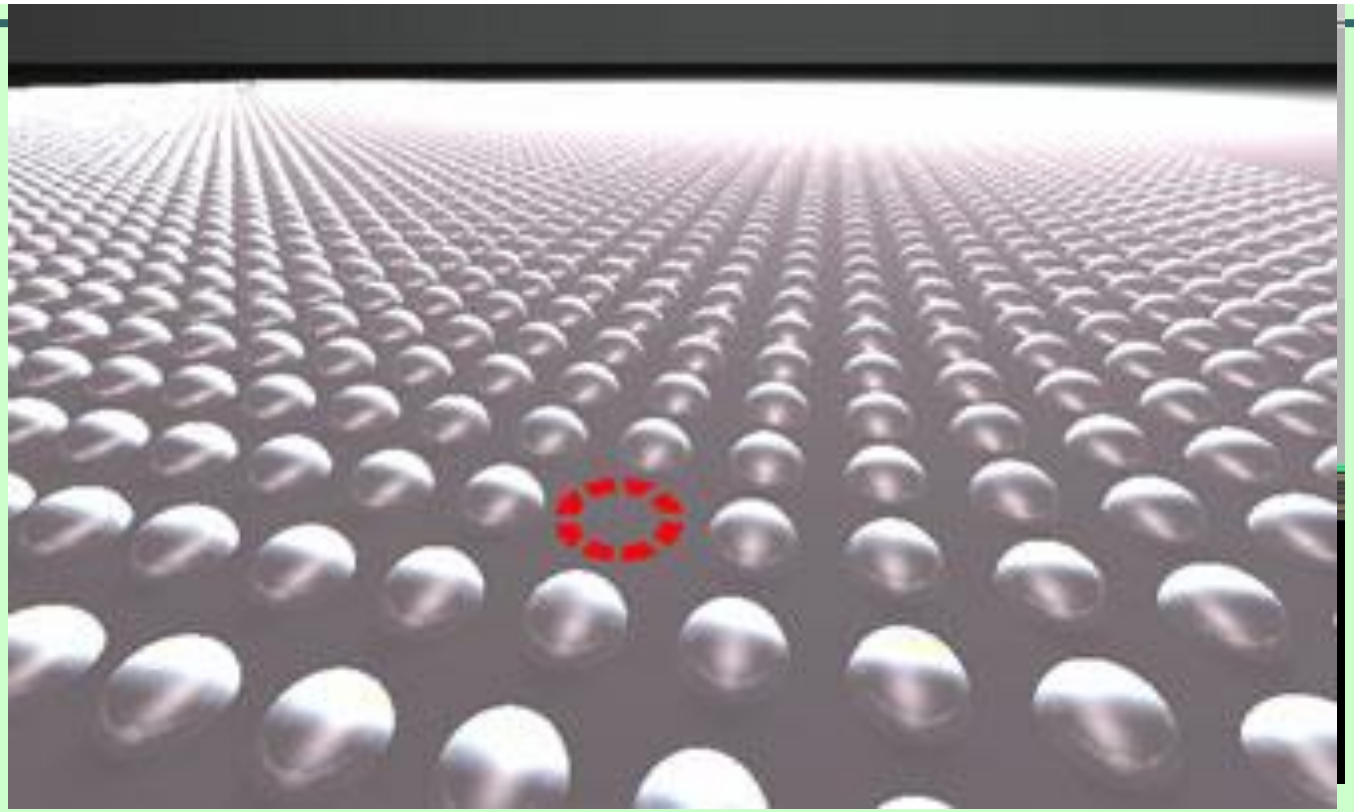
Pyrosequencing (GS FLX, GS Junior)

Sequencing by synthesis



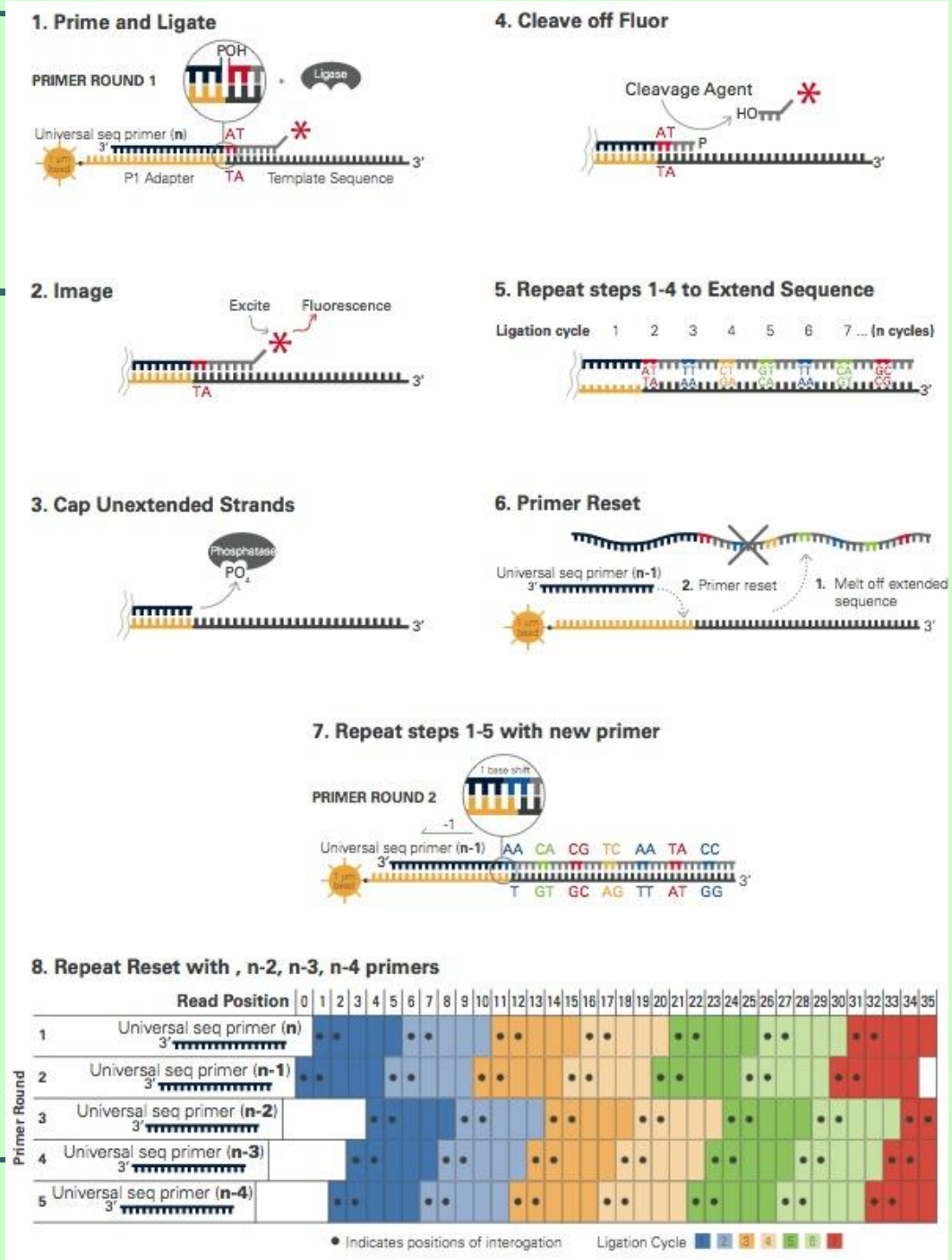
Ion torrent sequencing

Sequencing by synthesis



At each time, a chip is flooded with a single nucleotide. If the nucleotide matches the sequence, H+ is released and pH is changed. If it does not match the sequence, pH is not changed. Change in the pH is measured.

Oligo Ligation Detection (SOLiD)



Zero Mode Waveguide (Single molecule real time seq)

3rd generation sequencing

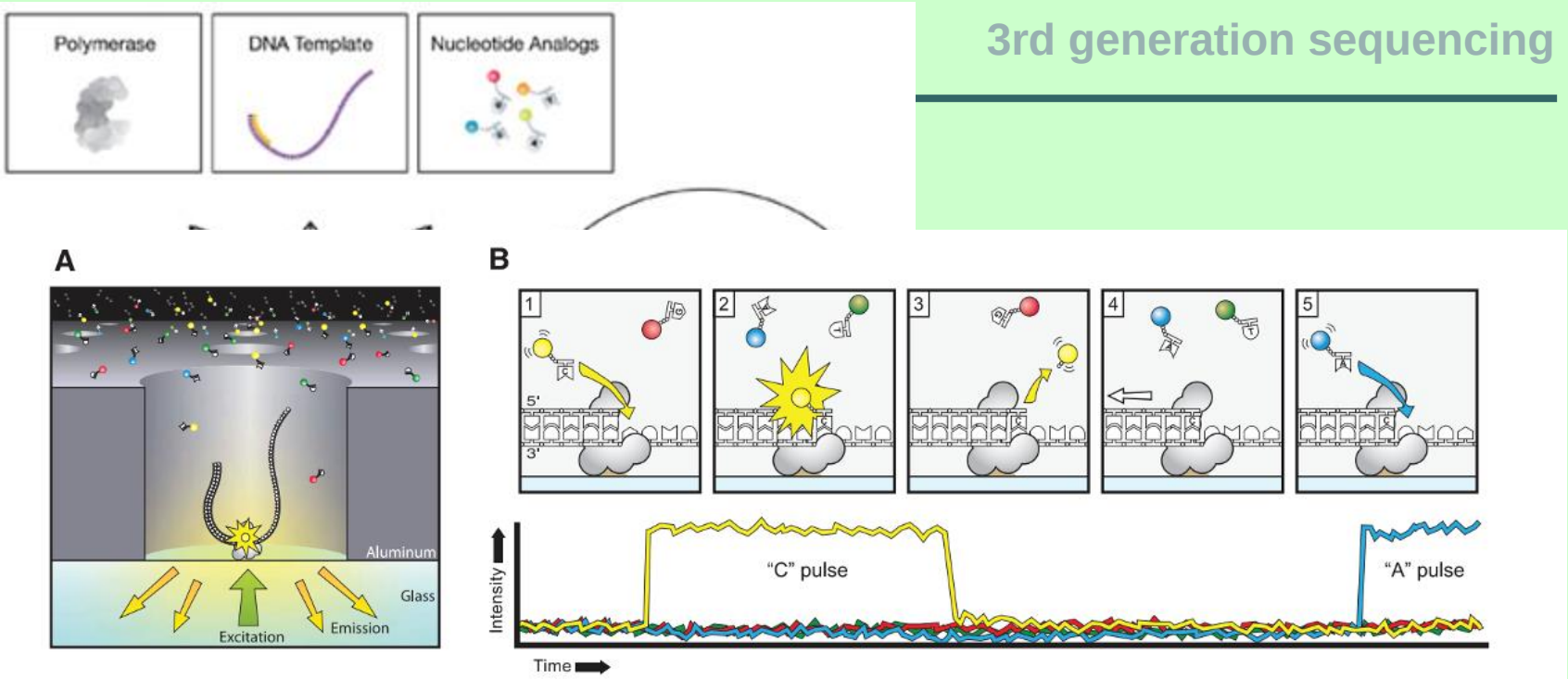
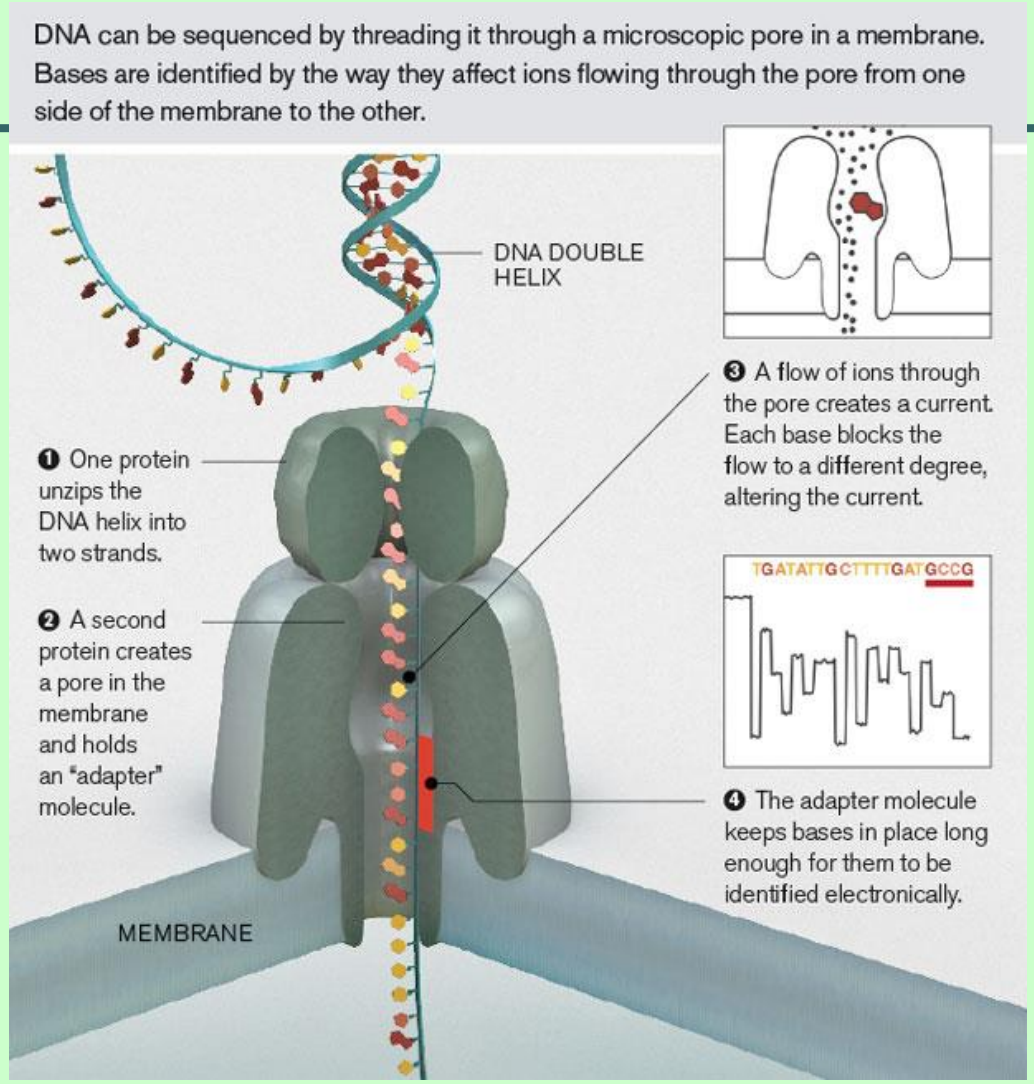


Fig. 1. Principle of single-molecule, real-time DNA sequencing. (**A**) Experimental geometry. A single molecule of DNA template-bound Φ29 DNA polymerase is immobilized at the bottom of a ZMW, which is illuminated from below by laser light. The ZMW nanostructure provides excitation confinement in the zeptoliter (10^{-21} liter) regime, enabling detection of individual phospholinked nucleotide substrates against the bulk solution background as they are incorporated into the DNA strand by the polymerase. (**B**) Schematic event sequence of the phospholinked dNTP incorporation cycle, with a corresponding expected time trace of detected fluorescence intensity from the ZMW. (1) A phospholinked nucleotide forms a cognate association with the template in the polymerase active site, (2) causing an elevation of the fluorescence output on the corresponding color channel. (3) Phosphodiester bond formation liberates the dye-linker-pyrophosphate product, which diffuses out of the ZMW, thus ending the fluorescence pulse. (4) The polymerase translocates to the next position, and (5) the next cognate nucleotide binds the active site beginning the subsequent pulse.

Nanopore sequencing (direct reading)



B.) Indirect testing

historically first type of DNA diagnostic method
most of the mendelian diseases went through a phase of gene tracking and moved on to direct test once the genes were cloned
with some diseases, even though the gene has been cloned, mutations are hard to find
mutations are scattered widely over a large gene
the existence of homologous pseudogenes
the lack of mutational hot spots

never confirm clinical diagnosis!

linkage analysis: (synonym: **indirect DNA analysis**) Testing DNA sequence polymorphisms (normal variants) that are near or within a gene of interest to track within a family the inheritance of a disease-causing mutation in a given gene

DNA sequence polymorphisms

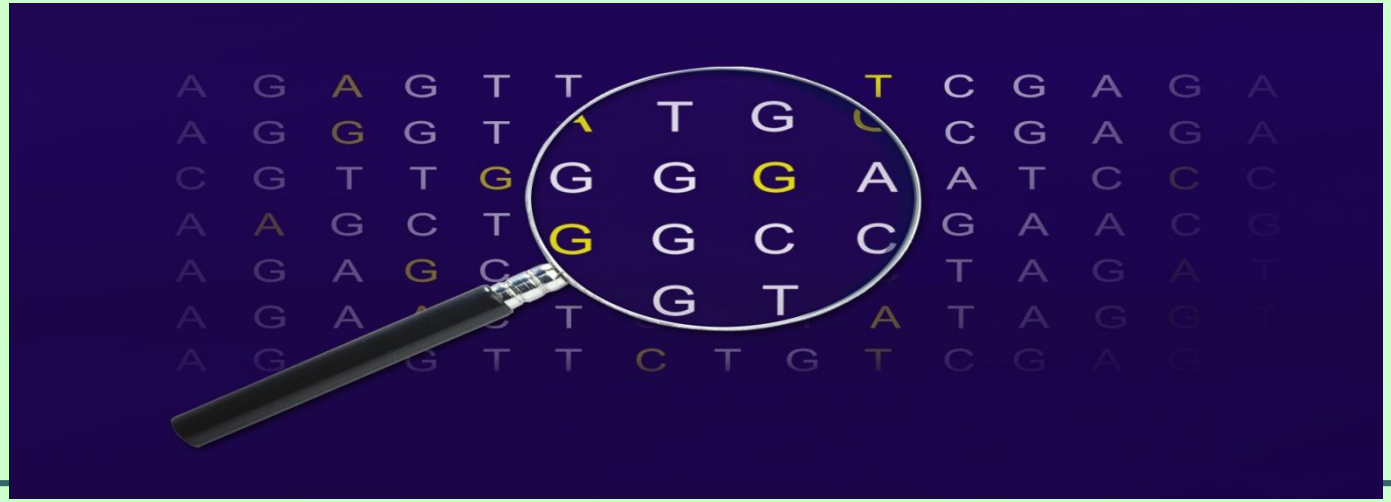
- Single nucleotid polymorphismus (SNP) – substitution of bases. In genome approx. 30 mil. SNP

- Minisatellite (VNTR) consist of repetitive, generally GC-rich, variant repeats (> 6bp) that range in length from 10 to over 100bp, these variant repeats are tandemly intermingled

- Microsatelite – Short Tandem Repeats (STR) consist of short sequence typically from 2 to 6 nucleotides long tandemly repeated several times (2 – 100x), and characterised by many alleles

Use of polymorphic regions

- Identification of persons/samples DNA
- paternity testing (VNTR, STR)
- Undirect diagnostics of monogenic diseases
- Searching of new genes
- SNP and multifactorial diseases



The three steps of linkage analysis

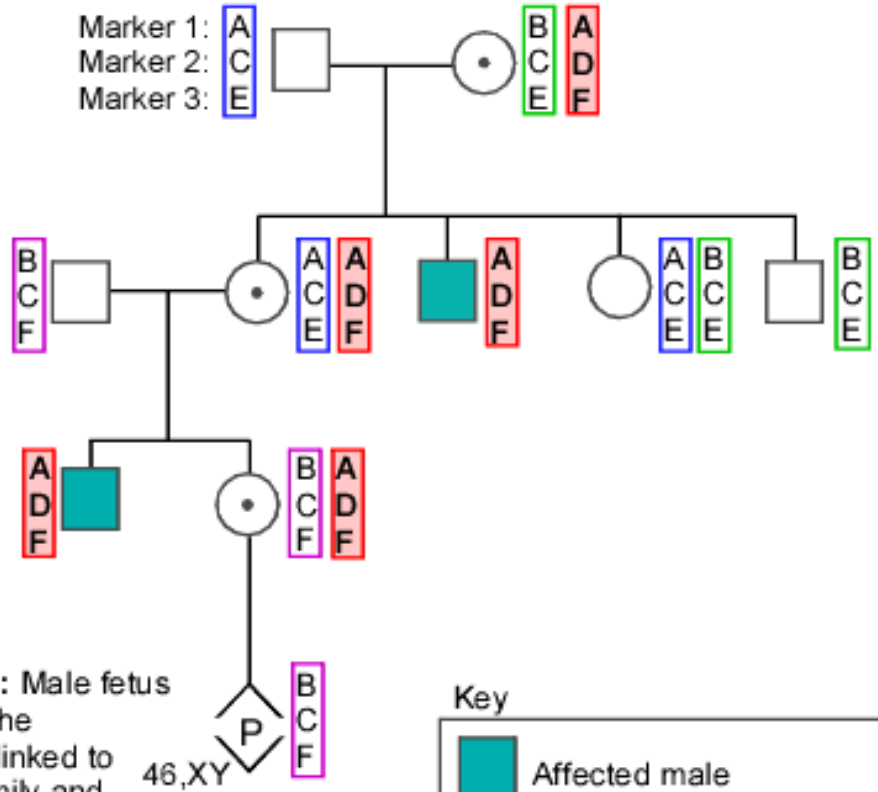
- **Establish haplotypes:** Multiple DNA markers lying on either side of (flanking) or within (intragenic) a gene-region of interest are tested to determine the set of markers (haplotypes) of each family member.

- **Establish phase:** The haplotypes are compared between family members whose genetic status is known (e.g., affected, unaffected) in order to establish the haplotype associated with the disease-causing allele.

- **Determine genetic status:** Once the disease-associated haplotype is established, it is possible to determine the genetic status of at-risk family members.

Example: Linkage analysis for prenatal diagnosis of an X-linked disorder.

- **Establish haplotype:** In this example, three markers on the X chromosome are tested. At marker one, the two possible variations are: **A** and **B**; at marker two: **C** and **D**; and at marker three: E and F. The sets of letters (i.e., haplotypes) next to each individual represent the varients they have for each variant. Women have two X chromosomes and thus have two haplotypes of X-chromosome markers; men have a single X chromosome and thus have only one haplotype of X-chromosome markers.
- **Establish phase:** It can be inferred that the haplotype **ADF** is linked to the mutated allele on the X chromosome.

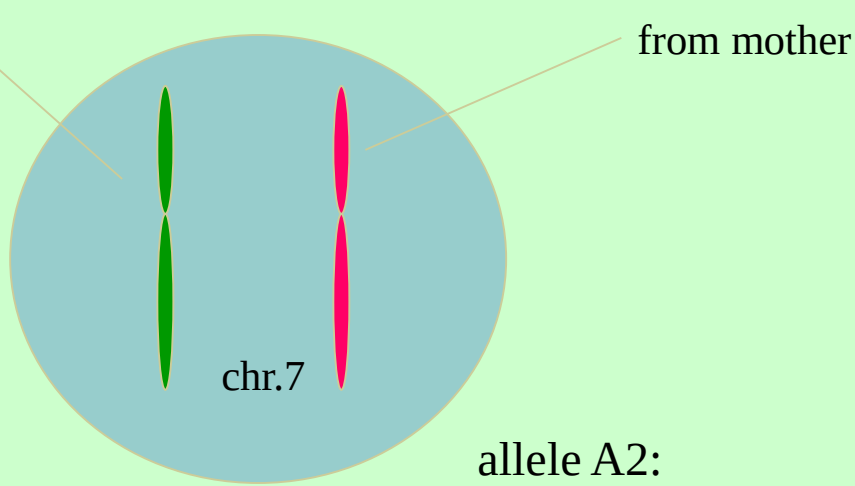


- **Determine genetic status:** Male fetus inherited from the mother the X-chromosome haplotype linked to the normal allele in this family and, therefore, is predicted to be unaffected.

Indirect DNA analysis

gene CFTR - intron 8 - polymorphic site (CA)n

GTATCACACACATTCGG

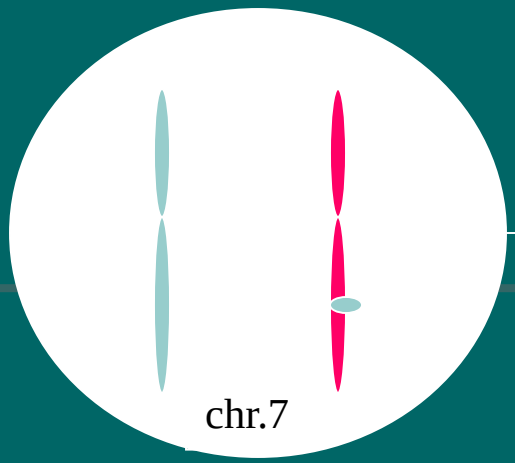


allele A1:
------ GTATCACATTCGG----

the lenght of this allele is 130 bp

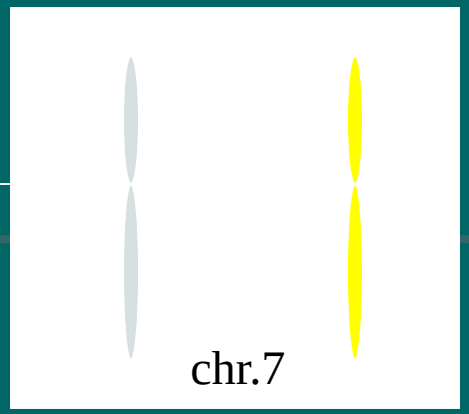
allele A2:
-----GTATCACACACATTCGG---

the lenght of this allele is 134 bp



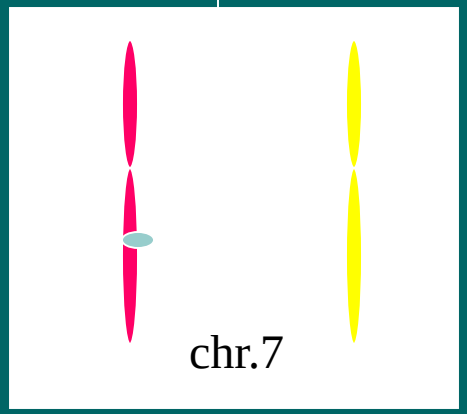
dF508 / non

A1 / A3



non / ?

A1 / A1



dF508 / ?

A1 / A1

non / non

A1 / A3

non informative

mutation in CFTRgene

Linkage analysis is often used when *direct DNA analysis is not possible* because the gene of interest is unknown or a mutation within that gene cannot be detected in a specific family.

In most instances, the haplotype itself has no significance*; it has meaning only in the context of a family study*.

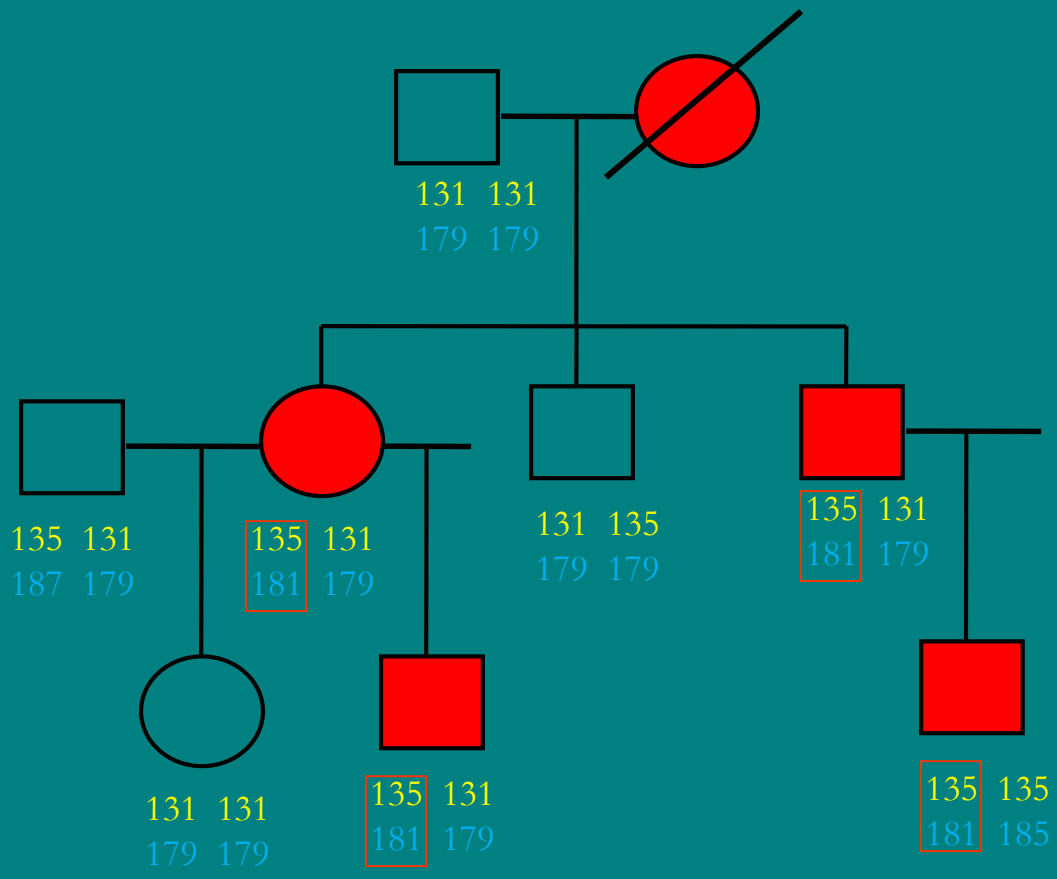
The accuracy of linkage analysis is dependent on:

- The accuracy of *the clinical diagnosis* in affected family member(s).
- The *distance between the disease-causing mutation and the markers*. Linkage analysis may yield false positive or false negative results if recombination of markers between maternally and paternally-inherited chromosomes occurs during gamete formation. The risk of recombination is proportional to the distance between the disease-causing mutation and the markers. The *risk of recombination* is lowest if *intragenic markers* are used.

The *informativeness of genetic markers* in the patient's family. If the DNA sequence for a given variant differs on the maternally-inherited and paternally-inherited chromosomes, that marker is **informative**. If the DNA sequence for a given variant does not differ on the two chromosomes, that marker is not **informative**.

Indirect diagnosis – Neurofibromatosis type 1

Autosomal dominant



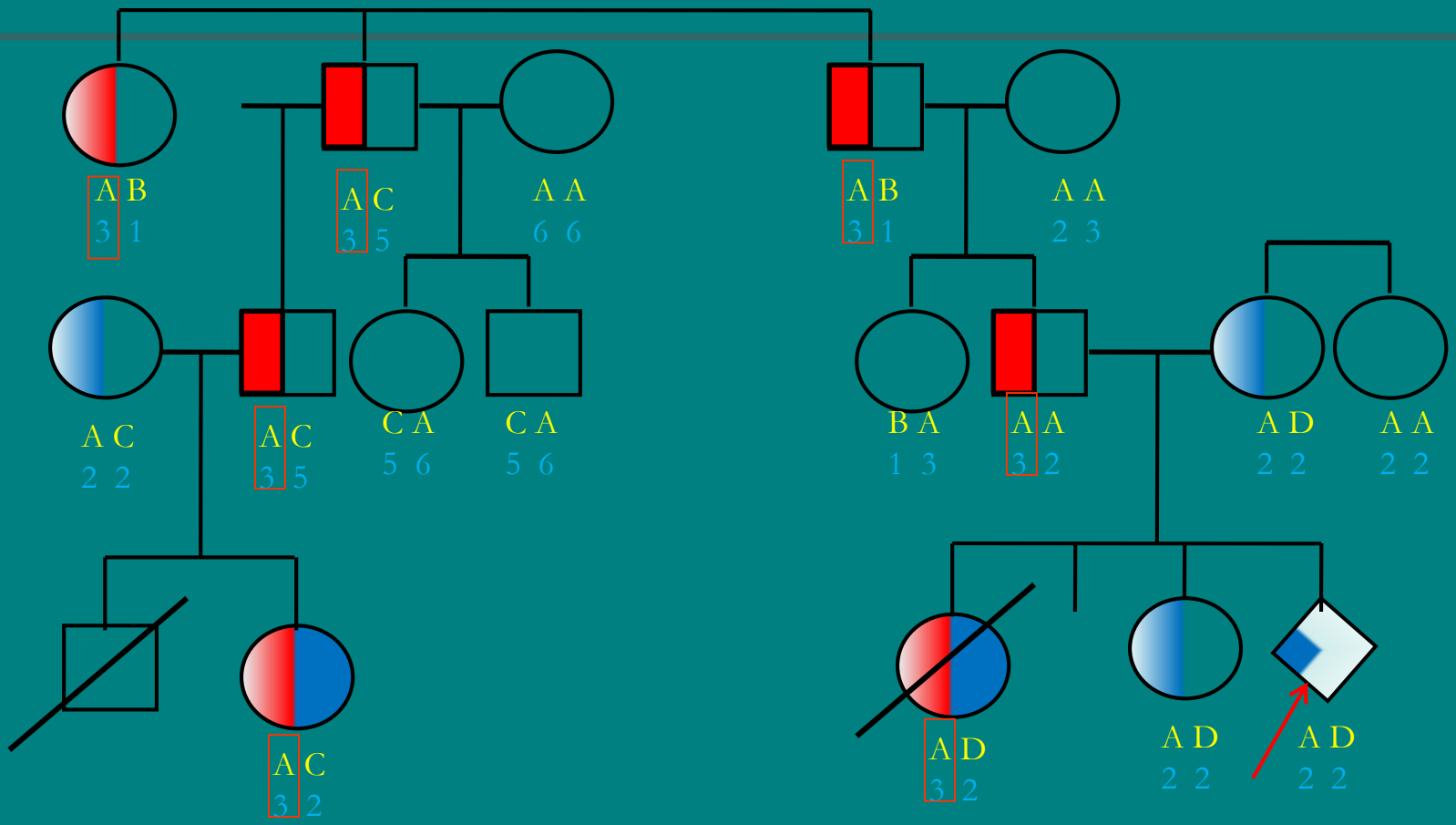
Polymorfic systems
GXAlu / i27b
IVS38GT /i38

unknown mutation

haplotype in assotiation with unknown mutation

Indirect diagnosis – cystic fibrosis

Autosomal recessive



F508del

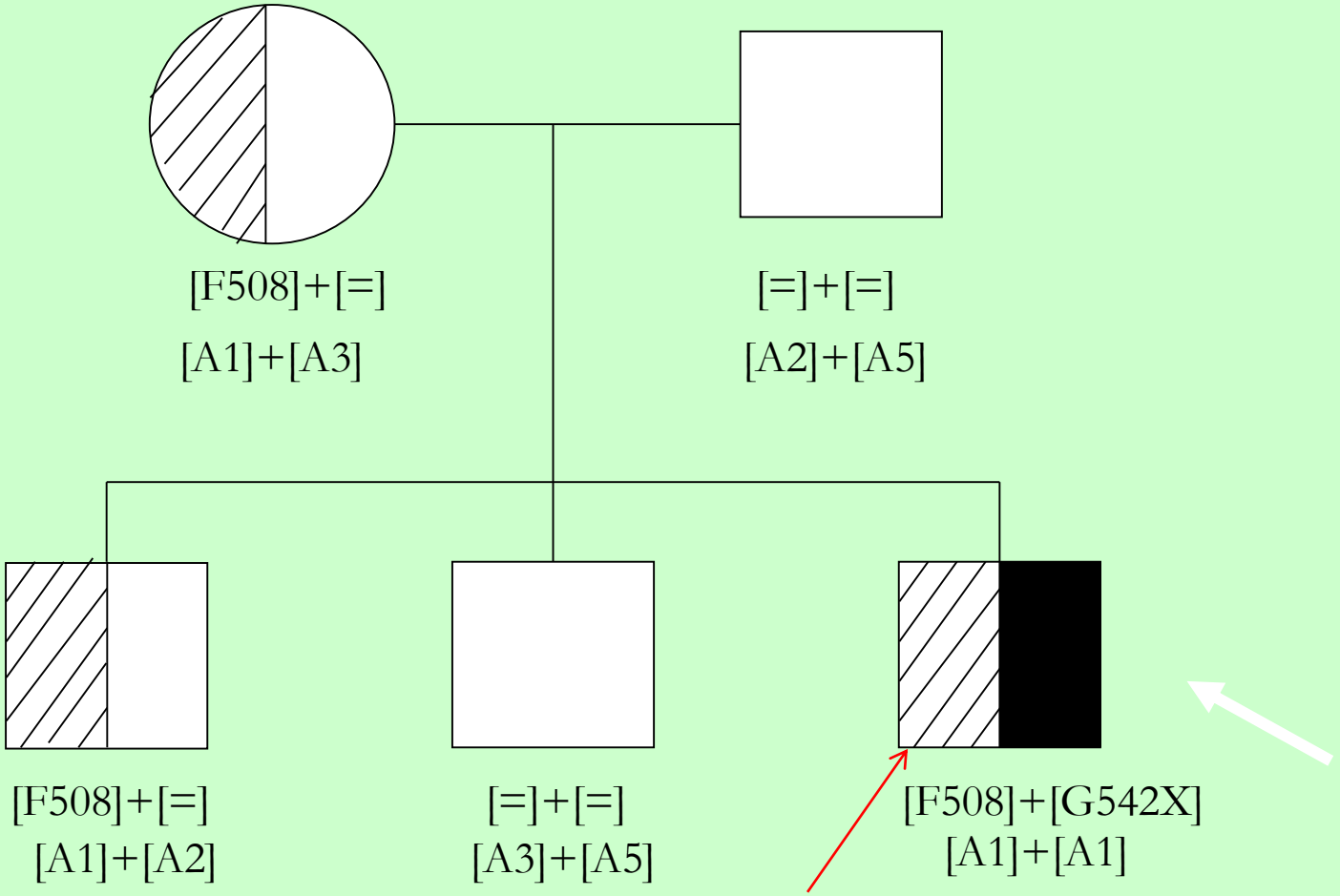
unknown mutation

Polymorfic systems IVS17BTA alleles 1 -6

IVS8BTA alleles A - D

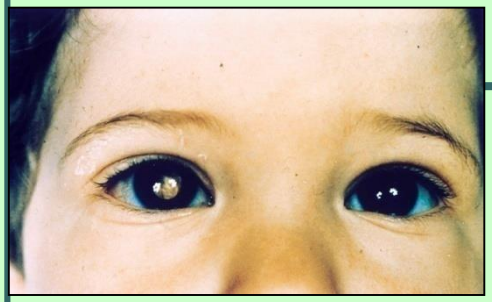
haplotype in assotiation with unknown mutation

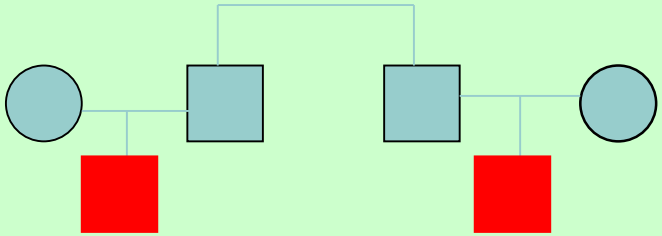
Indirect diagnosis – cystic fibrosis



de novo mutation

Retinoblastoma





RB1

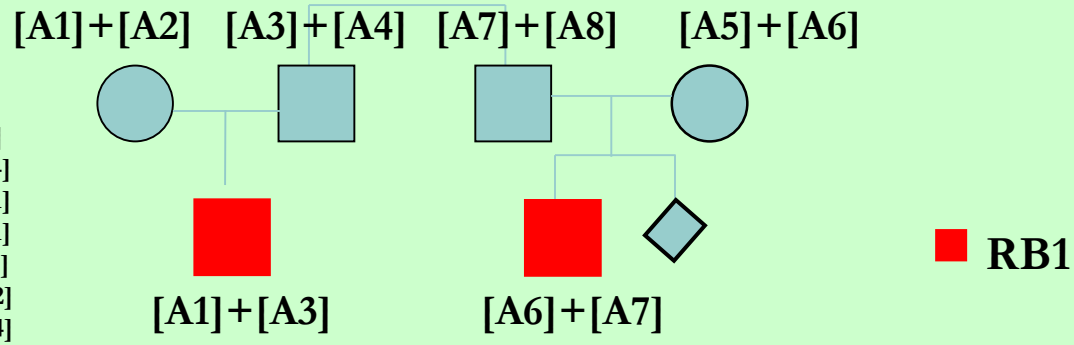
Mutation analysis of Rb1 was done

Pathology in Rb1 gene was not detected

Retinoblastoma - Indirect diagnostics

Polymorfic markers

- **extragene (DS13S 1307, DS13S 272, DS13S 164)**
- **intragene (Rb1.20B)**



A1: DS 13S 1307 [141] DS 13S 272 [133] DS13S 164 [179] Rb1.20B [3]
A2: DS 13S 1307 [151] DS 13S 272 [133] DS13S 164 [188] Rb1.20B [4]
A3: DS 13S 1307 [139] DS 13S 272 [127] DS13S 164 [179] Rb1.20B [1]
A4: DS 13S 1307 [139] DS 13S 272 [133] DS13S 164 [186] Rb1.20B [1]
A5: DS 13S 1307 [139] DS 13S 272 [131] DS13S 164 [188] Rb1.20B [1]
A6: DS 13S 1307 [126] DS 13S 272 [133] DS13S 164 [188] Rb1.20B [2]
A7: DS 13S 1307 [126] DS 13S 272 [129] DS13S 164 [188] Rb1.20B [4]
A8: DS 13S 1307 [139] DS 13S 272 [127] DS13S 164 [178] Rb1.20B [5]

Haplotype with pathology cannot be established

Explanation:

- occurance of mutation in another system of cell division and growth regulation
- nonhereditary form of retinoblastoma in both cousins