Molecular diagnostics

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Aplications of Molecular Genetic Testing

Genetic testing, through interrogation of DNA, RNA, can provide critical information for the detection of heritable disease genotypes for a number of different applications

- **Diagnostic testing**
  Testing for a gene pathological variant in symptomatic individuals as a diagnostic aid
  Testing requires genetic counseling.
- **Newborn screening**
  Testing is used to screen populations to identify prevalent genetic pathological variant in asymptomatic infants.
  The purpose of the screening is to identify affected babies early in life to allow for appropriate intervention before irreversible damage occurs.
- **Presymptomatic testing**
  Testing for a pathogenic variant in asymptomatic individuals in order to predict or asses the risk of disease in the future.
  These applications include testing for diseases in which lifestyle changes, increased medical surveillance, or medical intervention might be beneficial if the pathological variant is known.
  Testing requires extensive pretest and posttest counseling.
- **Carrier screening**
  Testing for a pathological variant in an autosomal recesive disorder in asymptomatic individuals for the purpose of family planning and genetic counseling to determine probability of disease in childern.
  Test requires extensive pretest and posttest counseling.
- **Prenatal diagnosis**
  Testing fetal cells/tissues for mutations to determine if a fetus is affected with a disease early in the pregnancy.

Diagnostic testing

Classification System for sequence variant identified by genetic testing

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Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results

Sharon E. Plon[1,*,#], Diana M. Eccles[2,*], Douglas Easton[3], William D. Foulkes[4], Maurizio Genuardi[5], Marc S. Greenblatt[6], Frans B.L. Hogervorst[7], Nicoline Hoogerbrugge[8], Amanda B. Spurdle[9], and Sean Tavtigian[10] for the IARC Unclassified Genetic Variants Working Group[†]

Class	Description	Probability of being Pathogenic
5	Definitely Pathogenic	>0.99
4	Likely Pathogenic	0.95–0.99
3	Uncertain	0.05–0.949
2	Likely Not Pathogenic or of Little Clinical Significance	0.001–0.049
1	Not Pathogenic or of No Clinical Significance	<0.001

5 classes linked to validated quantitative measures of causality/ pathogenicity

Class	Clinical Testing	Surveillance Recommendations if At-Risk Relative is Positive	Research Testing of Family Members
5	Test at-risk relatives for variant	Full high-risk surveillance guidelines	Not indicated
4	Test at-risk relatives for variant *	Full high-risk surveillance guidelines	May be helpful to further classify variant
3	Do not use for predictive testing in at-risk relatives *	Based on family history (and other risk factors)	May be helpful to further classify variant
2	Do not use for predictive testing in at-risk relatives *	Treat as "no mutation detected" for this disorder	May be helpful to further classify variant
1	Do not use for predictive testing in at-risk relatives *	Treat as "no mutation detected" for this disorder	Not indicated

All 5 classes are linked to clinical recommendations

Newborn screening

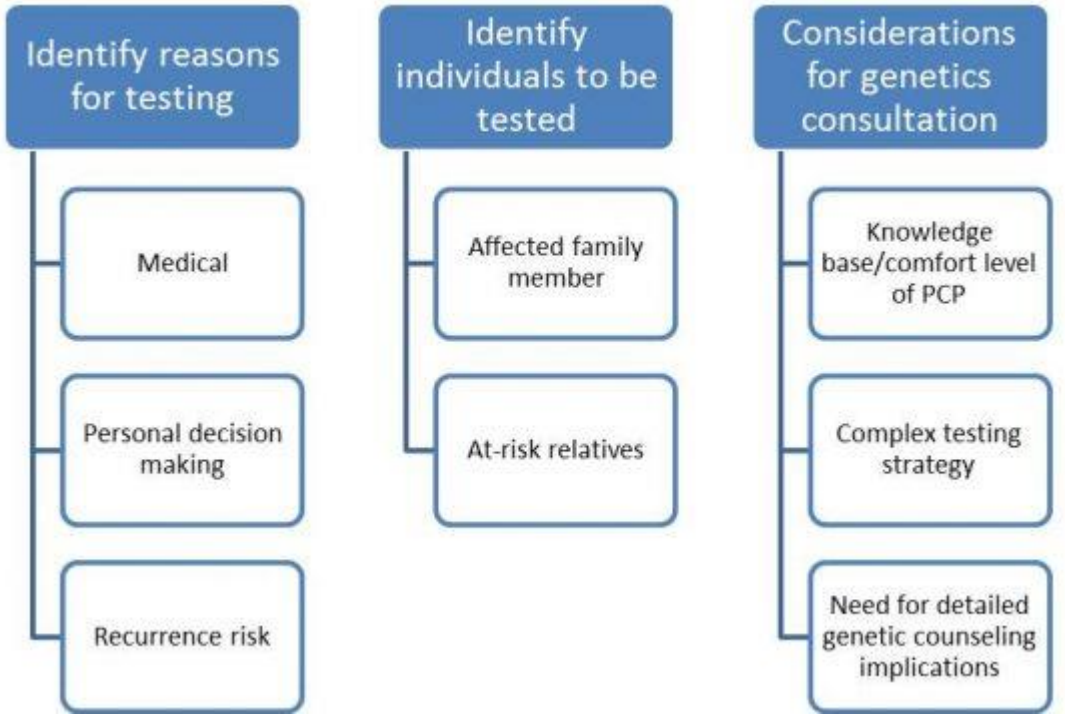
- Newborn infant has screening tests before leaving the hospital.
- There may be different tests depending on the country where you live
- Tests on a few drops of blood from pricking the baby's heel.
- The tests look for inherited disorders.

These tests look for serious medical conditions.
If not treated, some of these conditions can cause lifelong health problems.
Others can cause early death.
With early diagnosis, treatment can begin right away,
before serious problems can occur or become permanent.

Presymptomatic (predictive) testing

This is used in a clinical setting for late-onset conditions such as Huntington disease and familiar cancers.
Ideally the family pathogenic variant will have been identified through testing an affected individual.

Predictive testing needs to be done within the context of detailed written protocols that define in advance the response to each possible outcome and allow individuals to have adequate information on which to base their decision about whether to proceed to testing.



Carrier screening

This is used for autosomal and X-linked recessive conditions (and also for balanced chromosomal abnormalities.

It would normally be carried out at the request of the patient, and for a reason beyond mere curiosity.

Might be recommended in various situations including

1. If one or both partners have a family history of disease
2. If one or both partners are members of a population or ethnic group with a higher incidence of the disease
3. If partners are seeking preconception or prenatal testing
4. General population screening

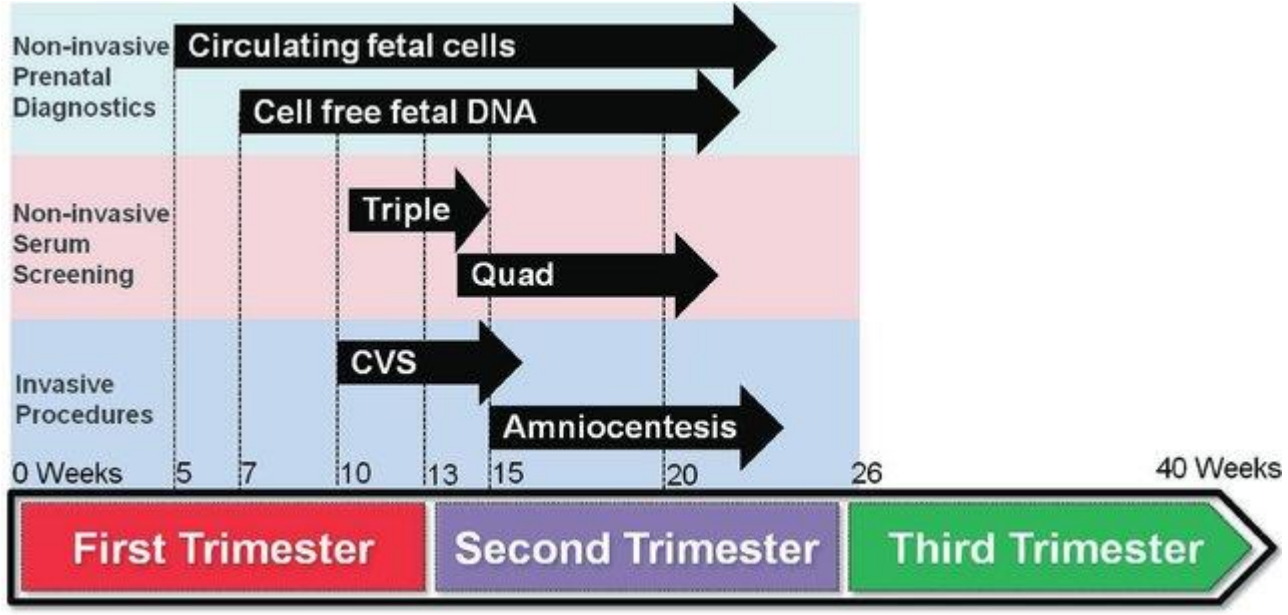
Counseling should always be part of the process, though when testing is done as a part of a population screening program, Pre-test counseling is likely to be quite minimal.
Children should not be tested unless there is an immediate benefit to the child.

Prenatal diagnosis

Prenatal diagnosis means diagnosis before birth. It's a way to see if developing baby has a problem.
These tests help find genetic disorders before birth.
Some parents have increased risk of having a baby with a genetic disorder or other problem.
Knowing about problems before the baby is born may help parents. They may be able to make better decisions about health care for their infant. Certain problems can be treated before the baby is born.
Other problems may need special treatment right after delivery.
In some cases, parents may even decide not to continue the pregnancy.

Prenatal diagnosis

This can be carried out at several stages of pregnancy



A comparative schematic of prenatal diagnostic techniques and their applicability with respect to the progress of pregnancy:

- conventional invasive procedures (amniocentesis, chorionic villi sampling); Testing can be done at 11 to 13 wk gestation by chorionic villus sampling (CVS) or at 14-20 wk by amniocentesis.
- serum screening techniques (triple and quad screens - ultrasonography);
- non-invasive prenatal diagnostics (cell-free fetal DNA sampling methods).

Prenatal diagnosis

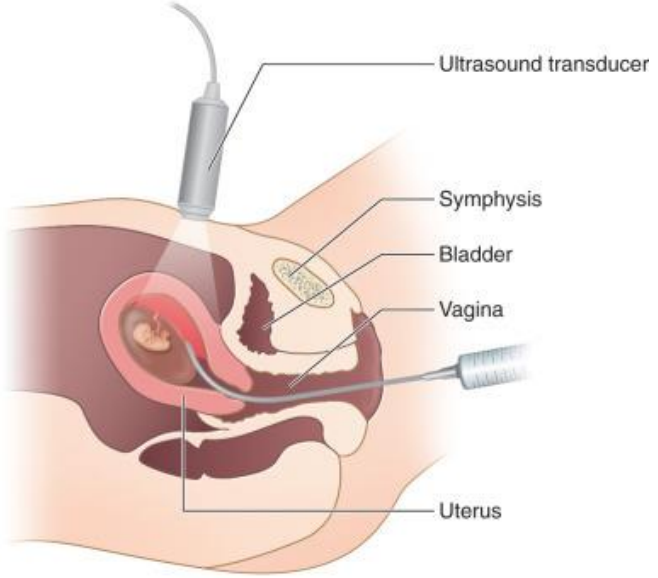
Chorion villus biopsy (CVS)

- is a prenatal test that involves taking a sample of tissue from the placenta
- is usually performed between 11 and 13 weeks of gestation under ultrasound guidance.
- carries around a 2% additional risk of causing a miscarriage.

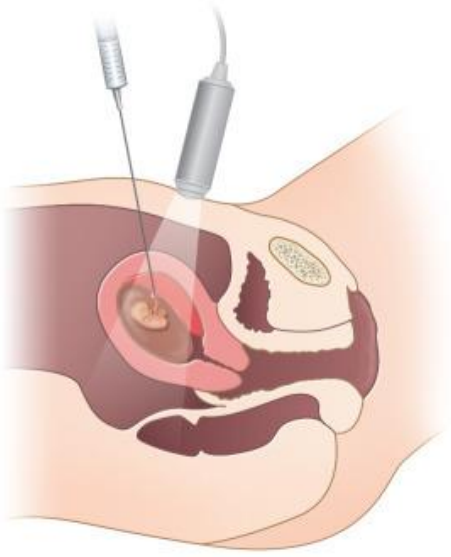
There are two types of CVS procedures:

- Transcervical - *in this procedure, a catheter is inserted through the cervix into the placenta to obtain the tissue sample* (A).
- Transabdominal - *in this procedure, a needle is inserted through the abdomen and uterus into the placenta to obtain the tissue sample* (B).

A



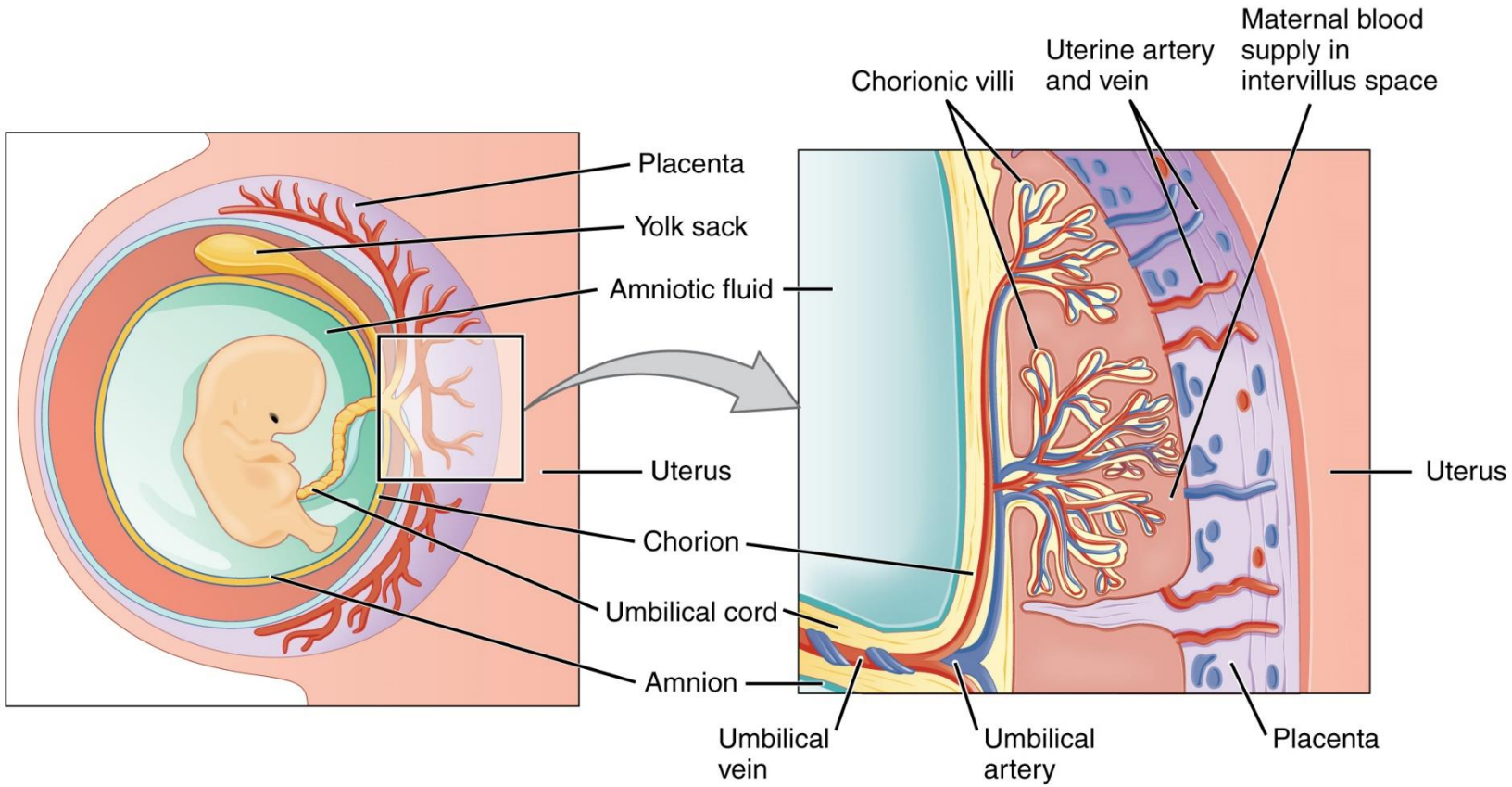
B



The chorion is the outermost of the fetal membranes, and sampling instrument should not penetrate the amniotic cavity. Once removed, material needs expert dissection under microscope to pick fetal material free of contaminating maternal tissue.

Prenatal diagnosis

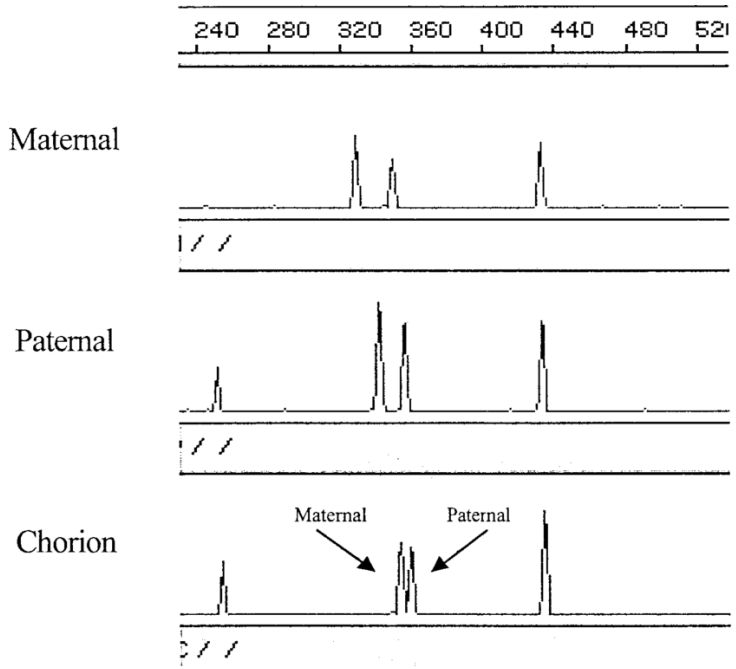
Chorionic villi are derived from the trophoblast, the extraembryonic part of the blastocyst.



Prenatal diagnosis

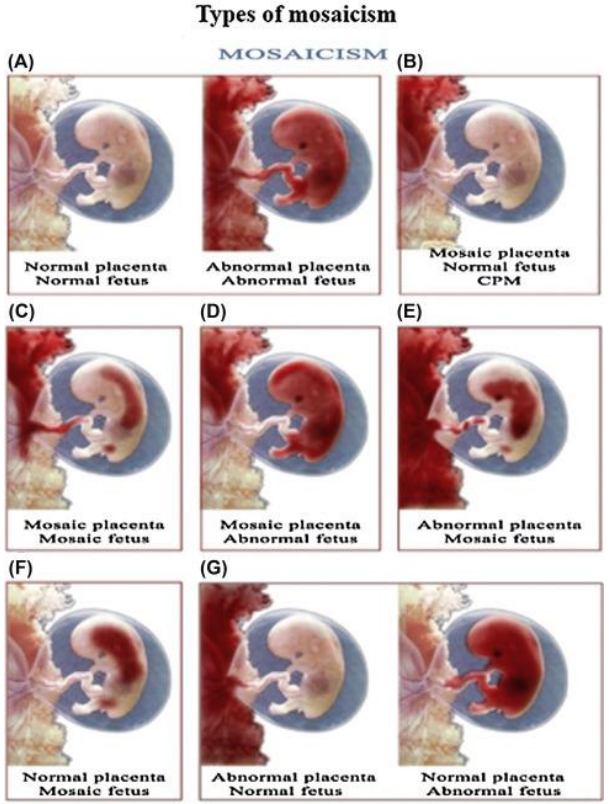
Chorionic villi can be used for

- DNA extraction for molecular diagnostics
  For DNA testing, results should always be compared to a control sample of the mother´s blood DNA, to ensure that the test result reflects the fetal genotype.



Electrophoretogram of the quantitative fluorescent polymerase chain reaction (QF-PCR) products of maternal, paternal, and chorionic samples, tested using the amelogenin X/Y (AMXY) and D21S1414 (STR) markers. Note, in the chorionic biopsy the maternal X and the paternal chromosome Y marker. The paternal chromosome 21 marker is also present in chorionic sample.

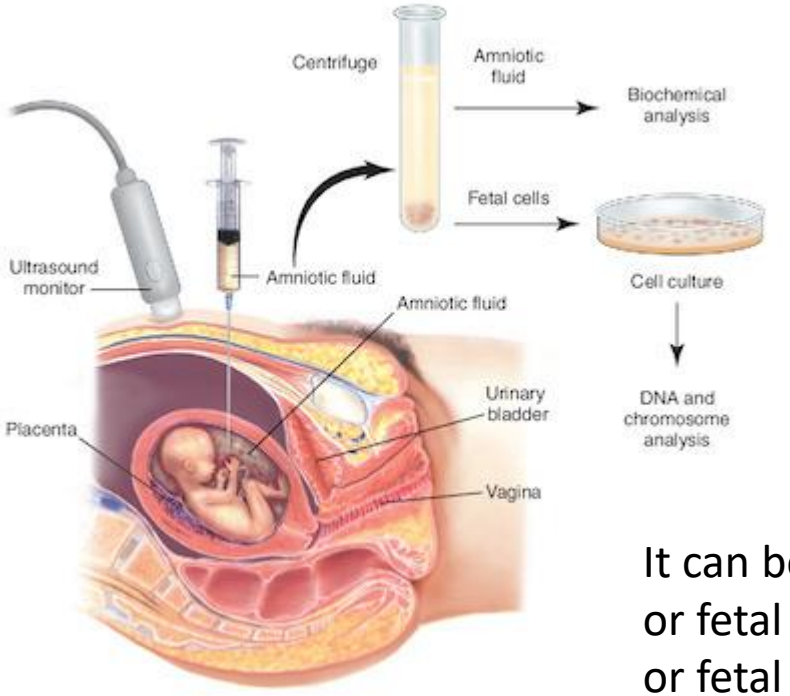
- Rapid cytogenetic analysis of dividing cells already present.
  Such short-term cultures need to be confirmed with long-term cultures.
  Mosaicism detected in villi is difficult to interpret: retrospectively it often turns out to have been confined to the placenta



Prenatal diagnosis

Amniocentesis(AMC)

- is a prenatal test that involves taking a sample of amniotic fluid
- amniotic fluid consists mainly of fetal urine and waschings from the lungs.
- is usually performed between 14 and 20 weeks of gestation under ultrasound guidance.
- carries around a 0,5 – 1,0 % additional risk of causing a miscarriage.



It can be analyzed biochemically,
or fetal cells can be isolated from fluid and cultured for cytogenetic analysis
or fetal DNA can be isolated from fluid for molecular analysis

Prenatal diagnosis

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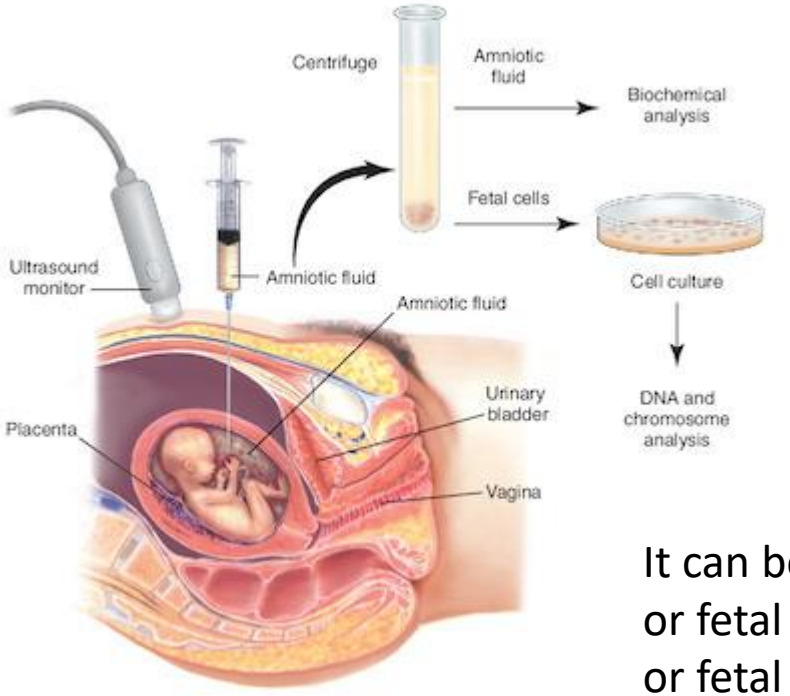

Biochemical analyses include testing for AFP, a protein produced by the fetal liver: a high level indicates that fetus has open lesion, most likely an open neural tube defect, but possibly an abdominal wall defect.

It can be analyzed biochemically,
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Prenatal diagnosis

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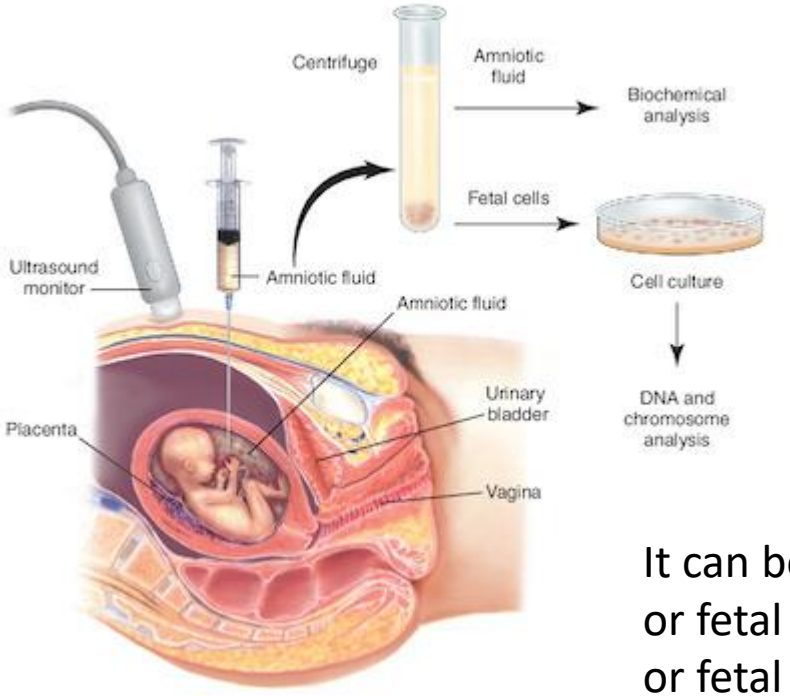


Culture of cells from amniotic fluid for cytogenetic analysis to detect specific chromosomal abnormalities, takes around 2 weeks to obtain quality preparations. Newer technique that does not require cell culture is QF-PCR and array-CGH.

It can be analyzed biochemically,
or fetal cells can be isolated from fluid and cultured for cytogenetic analysis
or fetal DNA can be isolated from fluid for molecular analysis

Prenatal diagnosis

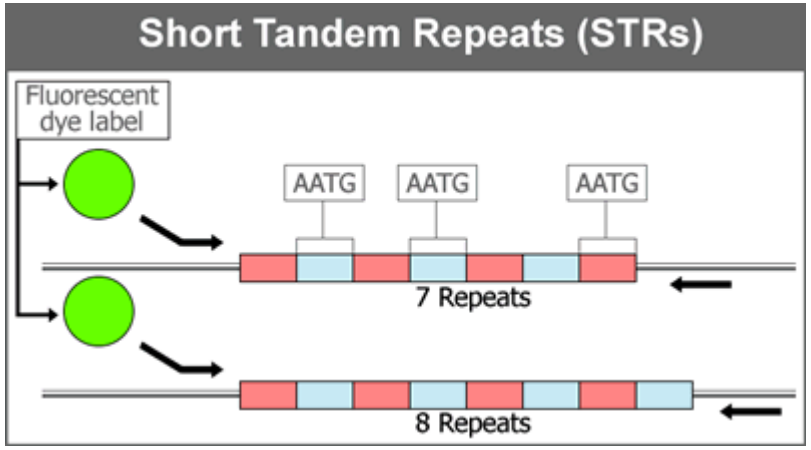
QF-PCR
Quantitative PCR with fluorescence-labeled primers

Is widely used as a rapid prenatal test for the common chromosomal trisomies
Autosomal chromosome aneuploid pregnancies that survive to term, namely, trisomies 13, 18, and 21, X and account for 90% of chromosome abnormalities with a severe phenotype identified in prenatal samples.
They are traditionally detected by full karyotype analysis of cultured cells.
The average reporting time for a prenatal karyotype analysis is approximately 14 days,
and in recent years, there has been increasing demand for more rapid prenatal results with respect to the common chromosome aneuploidies, to relieve maternal anxiety and facilitate options in pregnancy.
The rapid tests that have been developed negate the requirement for cultured cells,
instead directly testing cells from the amniotic fluid or chorionic villus sample,
with the aim of generating results within 24 h of sample receipt.
A quantitative fluorescence (QF)-PCR-based approach is now widely used
and reported as a clinical diagnostic service.
It may be used as a stand-alone test or as an adjunct test to full karyotype or array CGH analysis,
which scan for other chromosome abnormalities not detected by the QF-PCR assay.

Prenatal diagnosis

QF-PCR
Quantitative PCR with fluorescence-labeled primers

QF-PCR uses fluorescent labelled primers to amplify STR regions from DNA, by PCR.
Short tandem repeats (STR, microsatelite) are highly polymorphic sequences found in the human DNA.



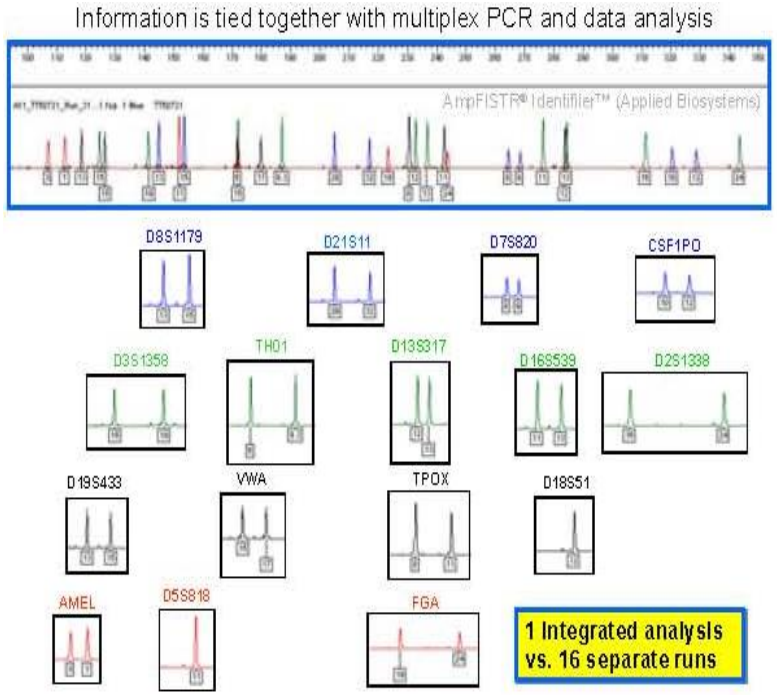
The amount of fluorescently labelled amplicons are measured after fragment length separation in capillary electrophoresis.
QF-PCR is a quantitative method that determines the presence of different alleles
which means the determination of chromosomal copy number

Prenatal diagnosis

QF-PCR

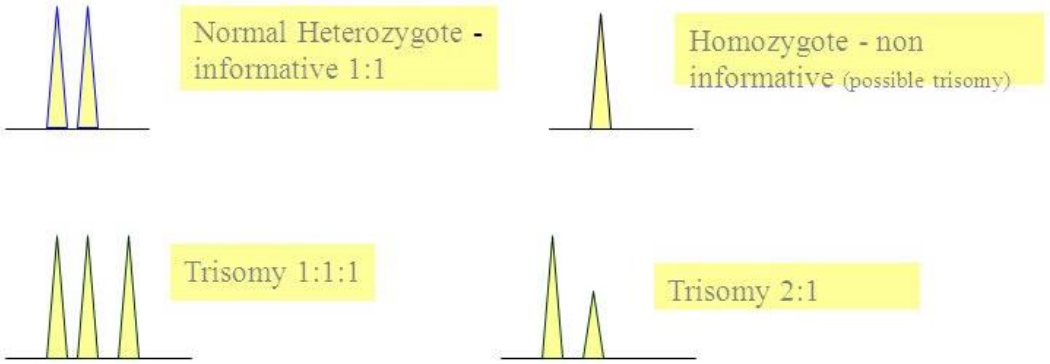
Quantitative PCR with fluorescence-labeled primers

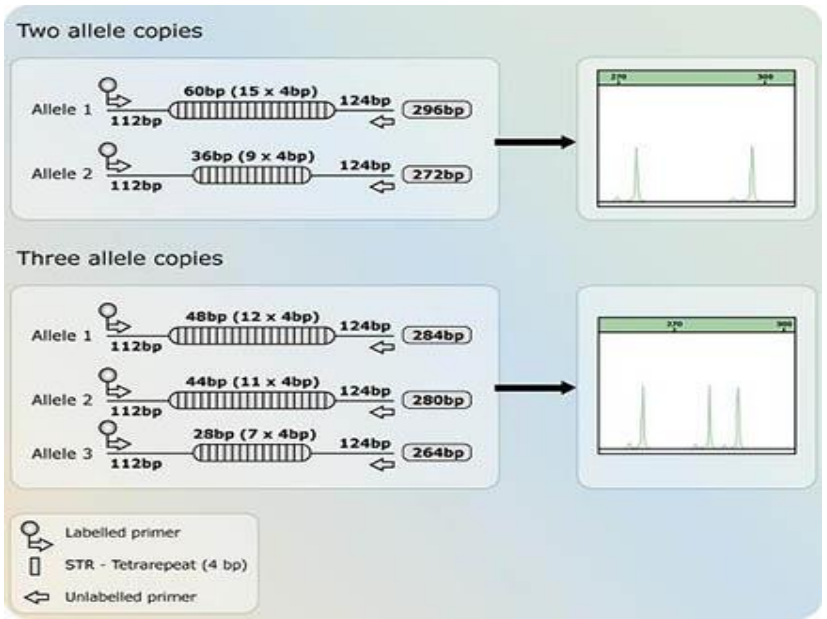
It depends on using a gene analyzer to compare the relative amounts of product
from multiplexed series of microsatelite markers from chromosomes 13, 18, 21, X and Y.
Typically, seven loci from each chromosome would be amplified.
Primers are designed and labeled so that the PCR products from each locus are clearly identifiable by their size and color.



Prenatal diagnosis

QF-PCR
Quantitative PCR with fluorescence-labeled primers





Because microsatelite alleles vary slightly in length, different alleles of the same microsatelite often give peak at slihtly different position.
Thus a locus may amplify as

- *two peaks (2 alleles with with different number of repeats – different length, heterozygous status)*

or

- *one larger peak (2 alleles with with the same number of repeats – the same length, homozygous status)*

If there is a trisomy the three alleles of each locus on that chromosome may

- *give three small peaks,*

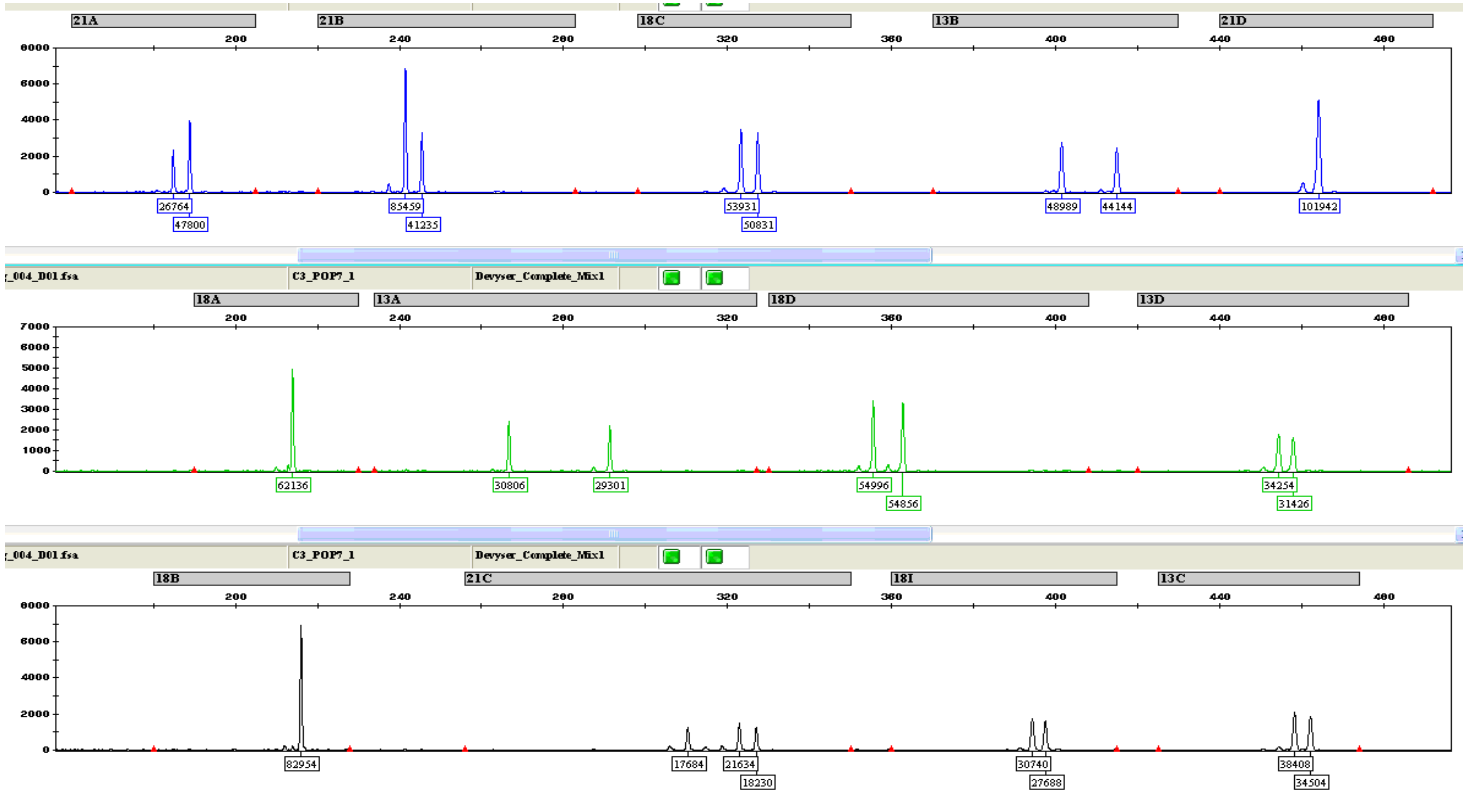
or

- *two peaks in a 2:1 size ratio*

or sometimes a single large peak – an uninformative result for that locus

Prenatal diagnosis

QF-PCR
Quantitative PCR with fluorescence-labeled primers



Typical electrophoretogram (Mix 1) showing the profile of a trisomic sample (47, XY, +21)

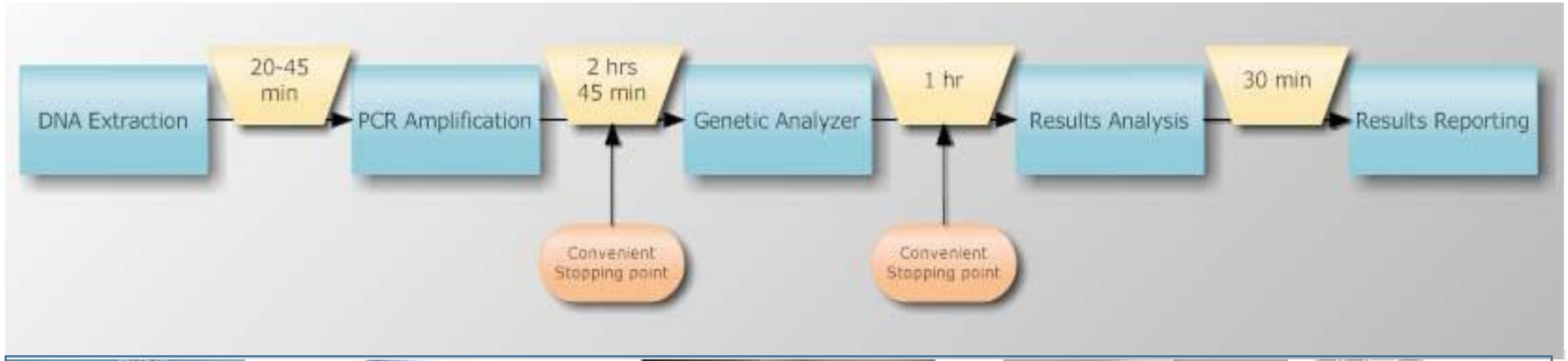
Prenatal diagnosis

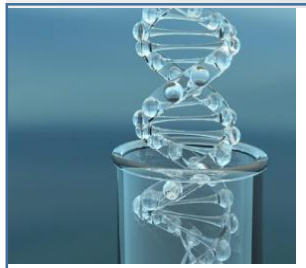
QF-PCR

Quantitative PCR with fluorescence-labeled primers

QF PCR Work flow

1. DNA extraction from e.g. amniotic fluid
2. PCR amplification
3. Capillary electroforesis
4. Result analysis, diagnosis





Prenatal diagnosis

Non- Invasive Prenatal Testing (NIPT)

Analysis of cell-free fetal DNA (cffDNA) in maternal blood

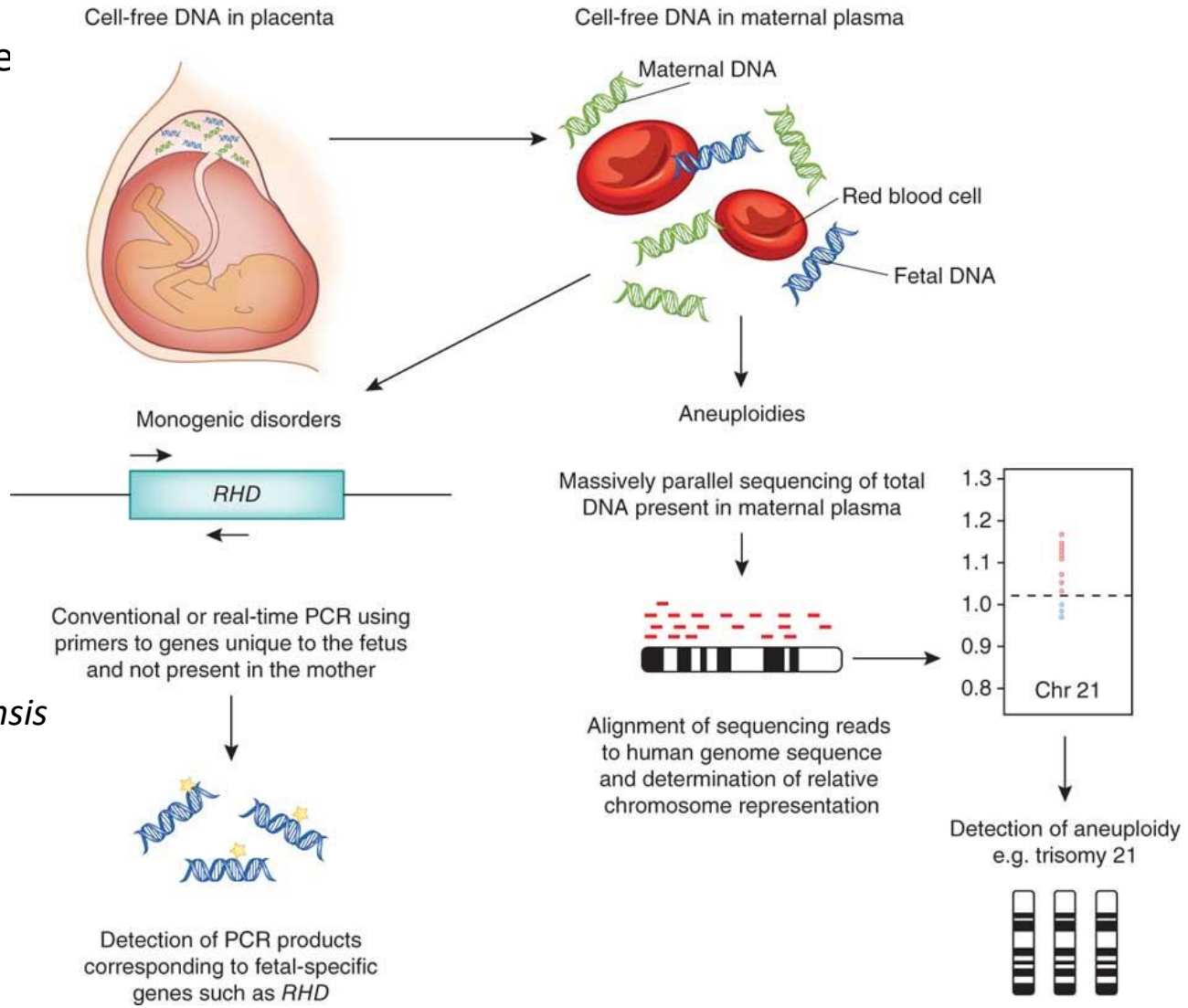
Many researchers have attempted to develop noninvasive prenatal testing (NIPT) methods in order to investigate the genetic status of the fetus.
The aim is to avoid invasive procedures such as chorionic villus and amniotic fluid sampling, which result in a significant risk for pregnancy loss.
The discovery of cell-free fetal DNA circulating in the maternal blood has great potential for the development of NIPT methodologies.

Methodologies developed are mainly based on counting DNA sequences
via next-generation sequencing
high resolution real-time PCR
and microarray-based methodologies.

Prenatal diagnosis

Non- Invasive Prenatal Testing (NIPT)

Analysis of cell-fre



NIPT strategies have been applied for

- fetal sexing,
- the determination of the fetal rhesus status,
- Inherited and *de novo* monogenic disease,
- fetal aneuploidy investigation,

this is currently regarded as a screening test,
with positive results needing confirmation by amniocentensis

Molecular genetic testing

Can be used a direct or indirect analysis

Direct testing

detects the specific disease-causing pathogenic variants or foreign DNA sequence

Indirect testing (gene tracking)

This type of testing is commonly referred to as linkage analysis.
Polymorphic markers closely associated with the disease-causing gene are used
to assess whether an individual has inherited the pathogenic allele of the disease-causing gene responsible for the disease phenotype.

Linkage analysis is based on tracking the inheritance of polymorphic markers in a family with a genetic disease.
If the markers and the disease-associated gene are in proximity, then the likelihood of a recombination event occurring between them is minimal. Thus, coinheritance of the markers and the disease-associated gene is likely.
The advantage of linkage analysis is that the gene of interest need only be mapped to a chromosomal location.
Limitations to this technology include significant labor and turnaround-times, the need to analyze samples from many family members, and the possibility of having to use numerous markers to obtain informative data.

Direct testing

provides evidence of a pathological variant responsible for producing the illness.
It is determined whether the sequence of the DNA (nucleotide sequence) has changed.

These assays require that pathological variant and/or the gene sequence of interest is known.

Sequence changes testing methods can be divided into two groups:

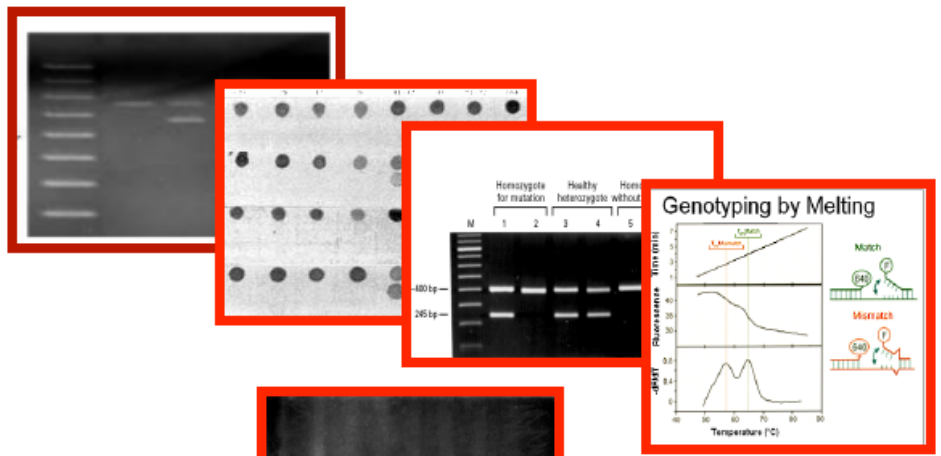
1. Scoring – methods for detecting specific sequence changes

2. Scanning – methods for scannig a gene for any sequence change

Nucleotide variants in DNA sequence - methods for detecting

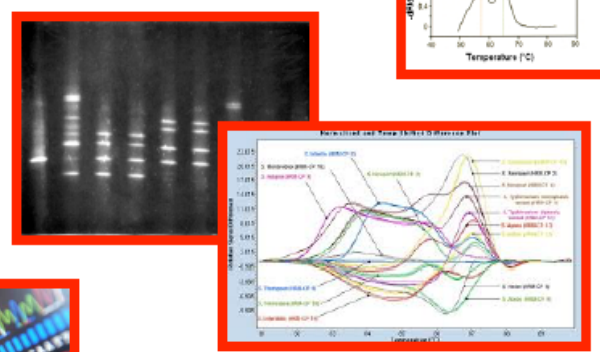
Targeted methods

- Reverse dot blot hybridization
- ARMS-PCR
- Restriction enzyme (RE)-PCR
- Real-time PCR (with probes)



Gene scanning methods

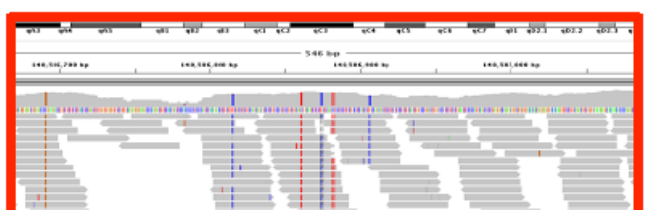
- DGGE
- High resolution melting analysis (HRMA)



Generic methods

- Sanger sequencing (Automated)

MASSIVELY PARALLEL SEQUENCING/ NEXT GENERATION SEQUENCING!!



Nucleotide variants in DNA sequence - methods for detecting

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- Reverse dot blot hybridization
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← scoring

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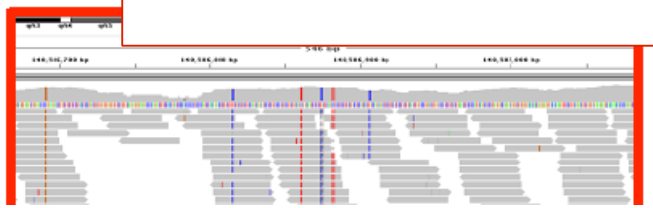


MASSIVELY PARALLEL SEQUENCING/ NEXT GENERATION SEQUENCING!!

Scoring - methods for detecting specific sequence changes

Searching for known sequence change is possible for:

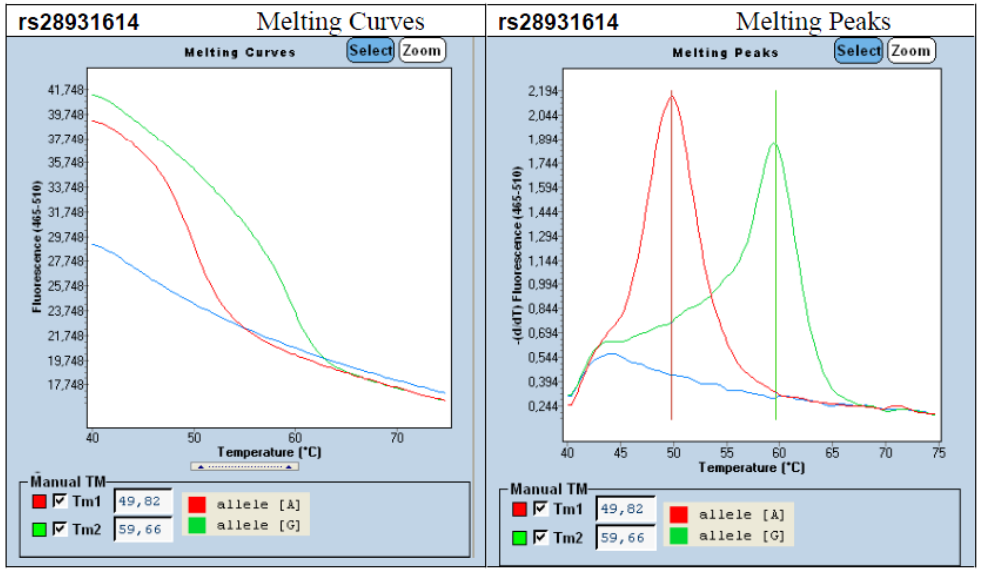
- The disease in question may always be caused by exactly the same sequence change
  ***An example is* Achondroplasia**

Achondroplasia

is the most common of the skeletal dysplasias that result in marked short stature (dwarfism)

arise from a change in the same base pair of *FGFR3*, that are autosomal dominant
around 98% of persons with achondroplasia have a c.1138G>A gene change,
and 1% have a c.1138G>C mutation

targeted mutation analysis is the routinely employed molecular test



method of genotyping by melting point analysis
on High Resolution Melting (HRM)
on the LightCycler® 480 System platform
using the LightSNiP assay.

Scoring - methods for detecting specific sequence changes

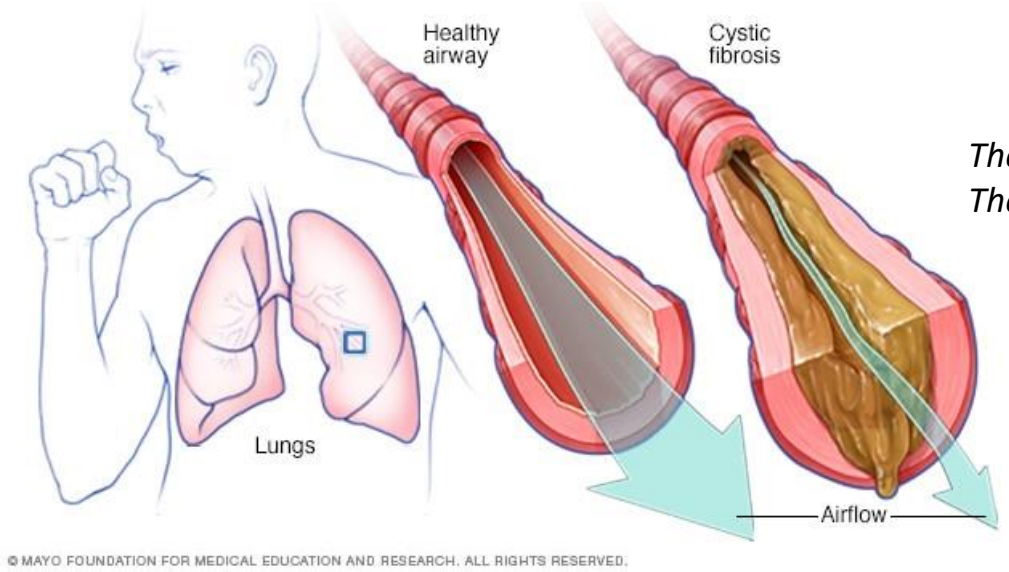
Searching for known sequence change is possible for:

- The disease in question may always be caused by exactly the same sequence change
  An example is **Achondroplasia**

- A disease may be caused by various different sequence changes, but one or a few variants may be so frequent in a particular population that it is worth first checking for these befor going on to a more general search.
  An example is Cystic Fibrosis*; over 2000 different variants have been reported, but 65% of all pathogenic variants of CFTR gene in Europeans are one particular deletion of 3 nucleotides p. F508del*

Cystic fibrosis

In people with CF, patogenic variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause the CFTR protein to become dysfunctional.
When the protein is not working correctly, it's unable to help move chloride -- a component of salt -- to the cell surface.
Without the chloride to attract water to the cell surface, the mucus in various organs becomes thick and sticky.



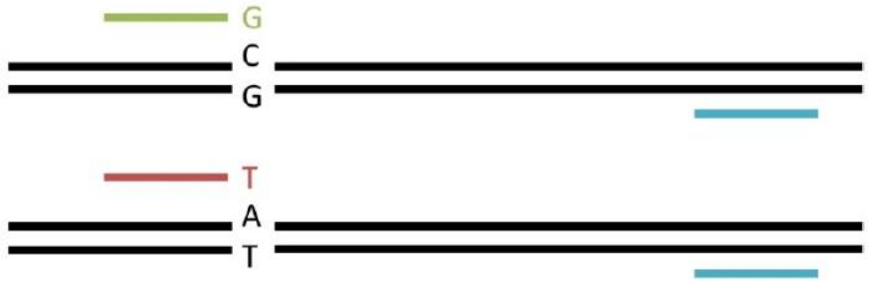
The airways fill with thick, sticky mucus, making it difficult to breathe.
The thick mucus is also an ideal breeding ground for bacteria and fungi.

Cystic fibrosis

Most genetic tests only screen for the most common CF mutations: **Allele-specific polymerase chain reaction**

→ **rozděleno do 2 PCR**

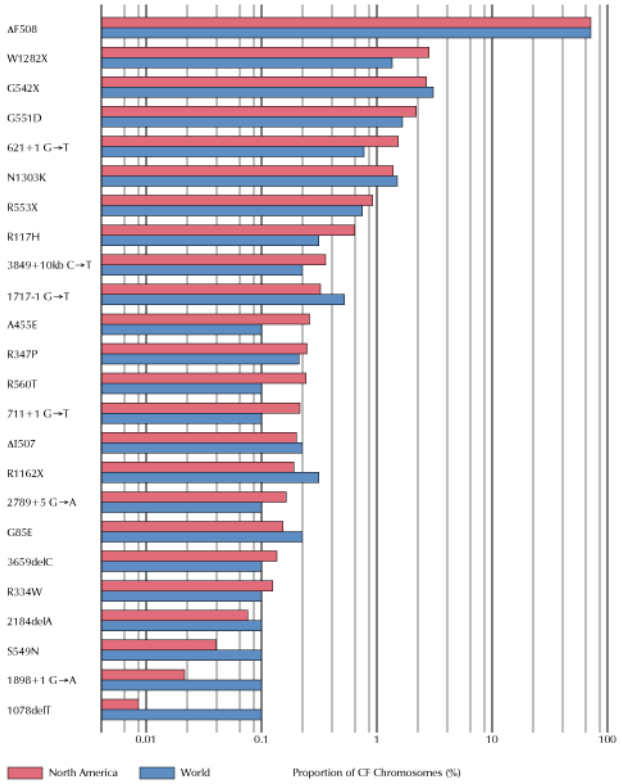
1. F-WT+R
2. F-MT+R

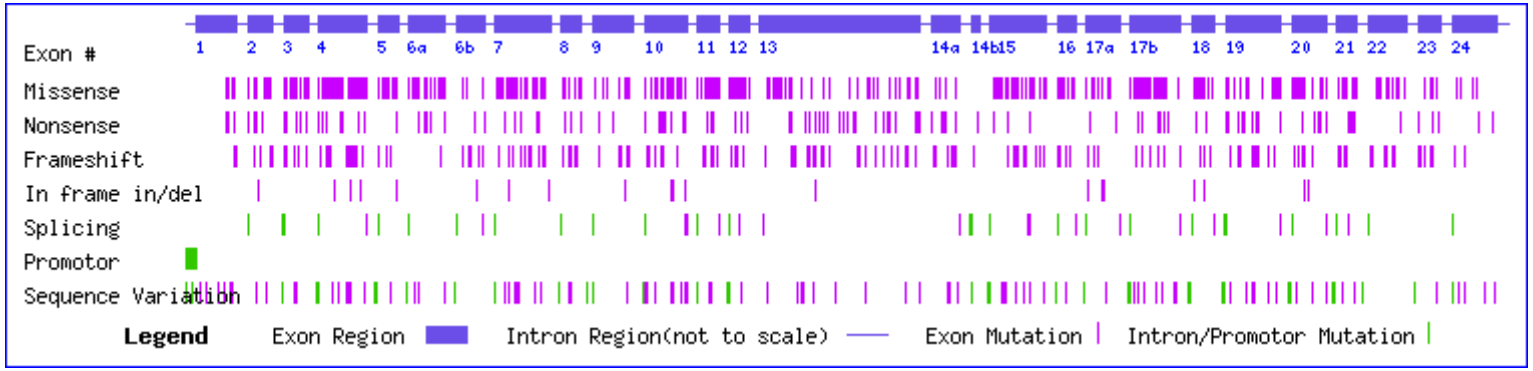


Allele-specific polymerase chain reaction (AS-PCR) is a technique based on allele-specific primers, which can be used to analyze single nucleotide polymorphism.
The *allele-specific PCR* is also called the (amplification refractory mutation system) ARMS-PCR corresponding to the use of two different primers for two different alleles.
One is the mutant set of primers which are refractory (resistant) to the normal PCR, and the other is the normal set of primers, which are refractory to the mutant PCR reaction.
The 3’ ends of these primers are modified such that one set of the primer can amplify the normal allele while others amplify the mutant allele.
This mismatch allows the primer to amplify a single allele.

Cystic fibrosis

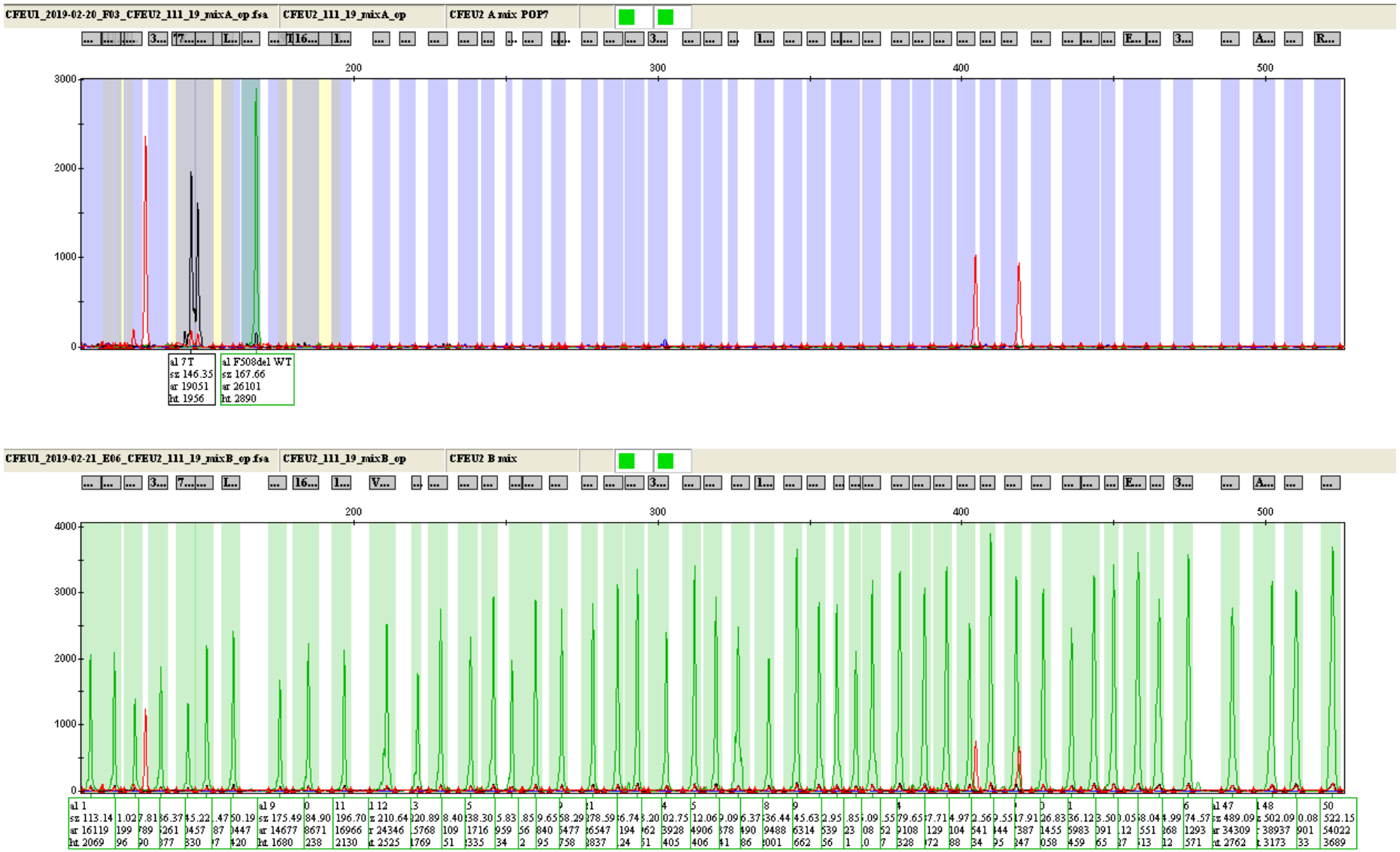
Although over 2000 different variants in the CFTR gene have been described in cystic fibrosis patients, most of these have been seen in only one or a very few cases.
A small number of patologiocal variants are relatively common.
Mutation testing in cystic fibrosis therefore starts by checking for these common mutation.





Elucigene® CF-EU 2v1

MT + WT pro F508del (+STR)



WT - F508del (+STR)

typical electropherogram obtained through ARMS analysis

Scoring - methods for detecting specific sequence changes

Searching for known sequence change is possible for:

- The disease in question may always be caused by exactly the same sequence change
  ***An example is* Achondroplasia**

- A disease may be caused by various different sequence changes, but one or a few variants may be so frequent in a particular population that it is worth first checking for these befor going on to a more general search.
  An example is Cystic Fibrosis*; over 2000 different variants have been reported, but 65% of all pathogenic variants of CFTR gene in Europeans are one particular deletion of 3 nucleotides p. F508del*

- The test may be to check somebody for a family pathogenic variant that has already been identified and characterized in other family members

- The test may be to check samples from haelthy controls to make sure a variant found in a patients is not a non-pathogenic variant present in normal population

Scanning- methods for scanning a gene for any sequence changes

A dignostic laboratory often needs to check every exon of candidate gene in a patient to look for patogenic variants.

Given the average sizes of exons and introns (145 bp and 3365 bp, respectively)
this usually means PCR amplifying and **sequencing** each exon individually.

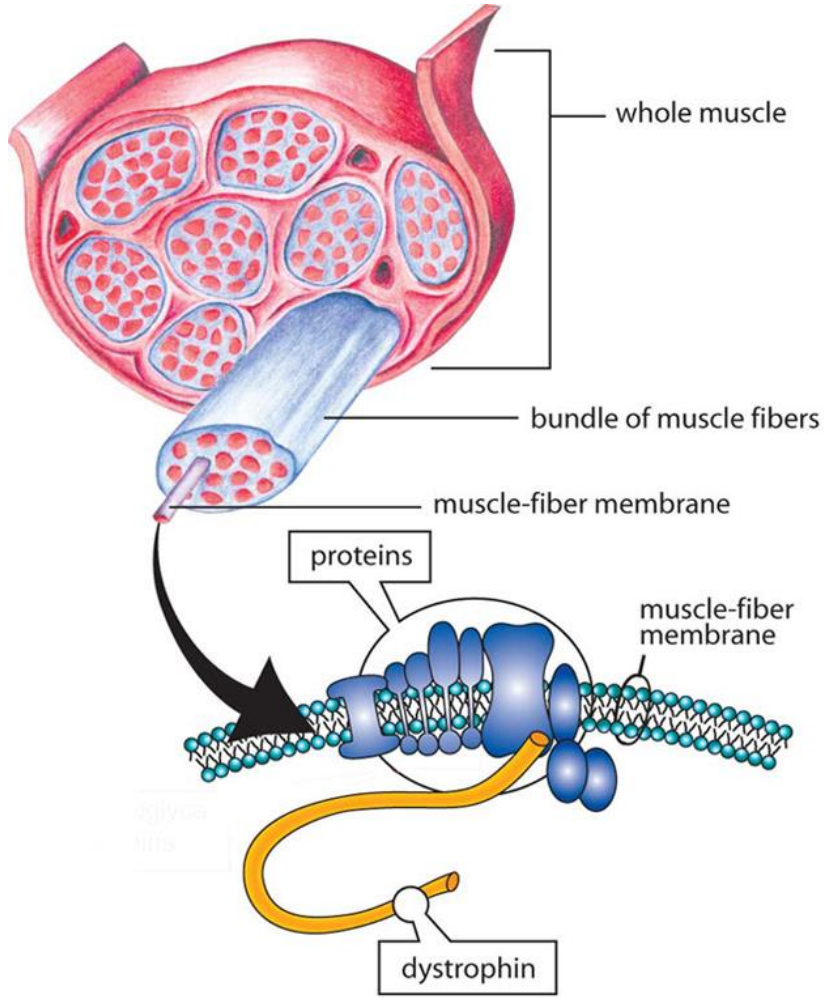
Various methods were developed to save sequencing costs by scanning each exon quickly and cheaply to eliminate those that apparently contained no variants (SSCP, DGGE, dHPLC).

The cost of sequencing has now fallen to the point that this approach is seldom used, except for scanning a gene to check for deletions or duplications of whole exon
An example Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD)

a genetic disorder characterized by progressive muscle degeneration and weakness due to the alterations
DMD occurs because the mutated *DMD* gene fails to produce any functional protein called *dystrophin*
that helps keep muscle cells intact.

The other dystrophinopathy is Becker muscular dystrophy (BMD, a mild form of DMD)
Individuals with BMD genetic mutations make dystrophin that is partially functional, which protects their muscles from degenerating as badly or as quickly as in DMD.



Muscles are made up of bundles of fibers (cells).
A group of interdependent proteins along the membrane surrounding each fiber helps to keep muscle cells working properly.
When one of these proteins, dystrophin, is absent, the result is Duchenne muscular dystrophy (DMD);
poor or inadequate dystrophin results in Becker muscular dystrophy (BMD).

Duchenne muscular dystrophy (DMD)

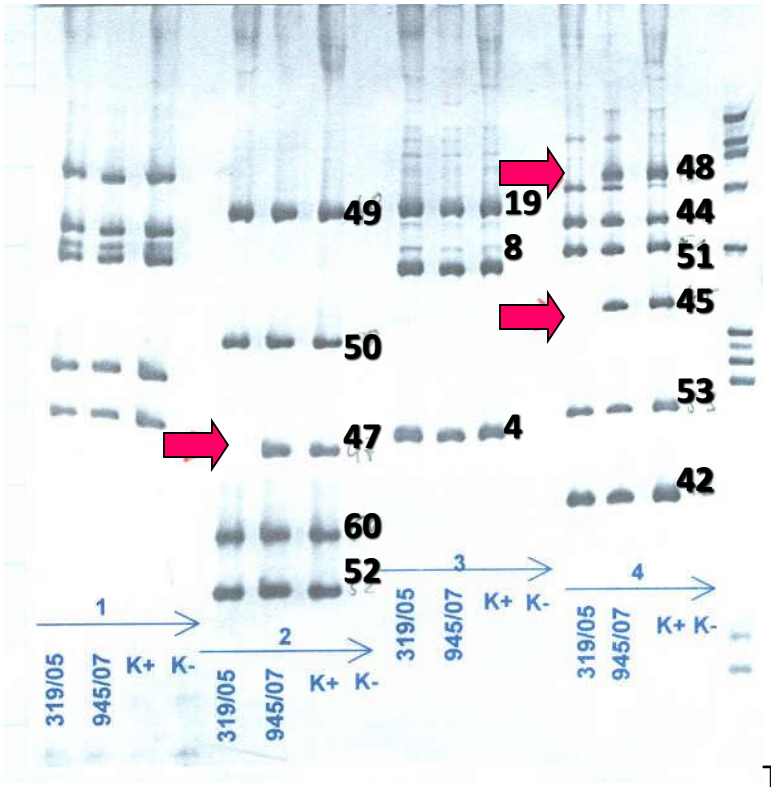
has an X-linked recessive inheritance pattern
and is passed on by the mother, who is referred to as a *carrier*.

The dystrophin gene is the largest gene yet identified in humans and is located in the short arm of the X chromosome, in the Xp21.2 locus (a locus is the position of a gene on a chromosome).

The majority (60 - 70 %) of mutations of the dystrophin gene are deletions of one or more complete exons.

These deletions are relatively easy to detect in an affected male.

Duchenne muscular dystrophy (DMD)



confirmed DMD:
deletion DMD exons 45, 47, 48

4 sets multiplex PCR

Tube 1	
Pm	535 pb
exon 3	410 pb
exon 43	357 pb
exon 13	238 pb
exon 6	207 pb

Tube 2	
exon 49	439 pb
exon 50	271 pb
exon 47	181 pb
exon 60	139 pb
exon 52	113 pb

Tube 3	
exon 19	459 pb
exon 8	360 pb
exon 4	196 pb

Tube 4	
exon 48	506 pb
exon 44	426 pb
exon 51	388 pb
exon 45	307 pb
exon 53	212 pb
exon 42	155 pb

To check for partial deletions, individual exons of the gene are amplified by PCR.
Primers are designed to match sequences in the introns flanking an exon,
so that the product contains the complete exon and some intronic sequence.
Each exon gives a different sized product by varying the amount of intron included.
A series of PCR reactions can be performed in one operation (multiplexed).
The mix of products from all PCR amplifications is run on an electroforetic gel.

Duchenne muscular dystrophy (DMD)

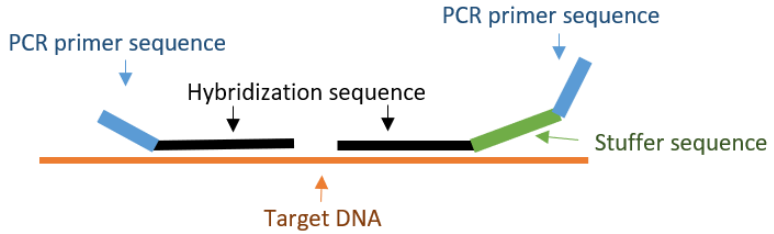
Carrier testing in women is more difficult than testing a boy because a carrier would be hetorozygous and every exons of the dystrophin gene would amplify from her normal chromosome.
A quantitative test is required - MLPA

Duchenne muscular dystrophy (DMD)

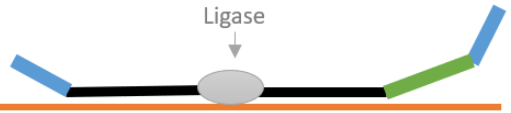
Multiplex ligation-dependent probe amplification (**MLPA**) is a variation of the multiplex PCR that permits amplification of multiple targets with only a single primer pair.
It detects copy number changes at the molecular level, and software programs are used for analysis.

MLPA consists of the following steps:
1.Denaturation
2.Hybridization
3.Ligation
4.Amplification (by PCR)
5.Fragment Separation and Data Analysis

1 – Denaturation; 2 – Hybridization

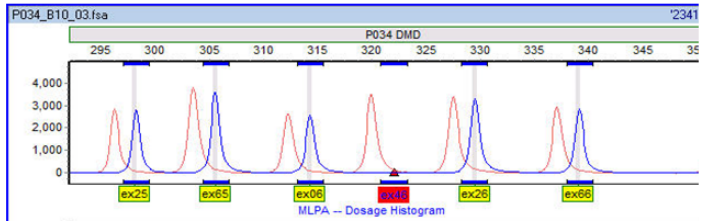


3 – Ligation



4 – Amplification

5 – Fragment separation and Data analysis



1 - Denaturation and 2 – Hybridization

Denaturation involves separation of the annealed DNA strands, so that double-stranded DNA becomes single-stranded.
Hybridization involves hybridizing the DNA sample to specific probes. Because it is a multiplex technique, you can analyze each sample by up to 60 probes simultaneously, thus targeting different sites!
These probes have a primer sequence that binds to the PCR-primer in the amplification process.
All different probes will have the same primer binding sequence.
Additionally, the probes also have a hybridization sequence complementary to the target site that will allow the probe to bind to the DNA. Both probes will hybridize on adjacent sites on the DNA strand.
One of the probes from the pair contains a stuffer sequence, which is different in length for each target site.
The length of the stuffer sequence changes between different probes, allowing multiplexing.
So, you can expect each amplification product to have a unique length!

3-Ligation

The ligation step will bind the two probes together. In this step, a specific enzyme called DNA ligase is used.
It binds the probes that are already hybridized on adjacent sites of the DNA strand at the target site.
The ligase used in MLPA protocols is ligase-65, an NAD-dependent ligase enzyme.
Both probes contain the binding sites for PCR-primers. This means, if we were to use the probes as a single molecule, we would obtain an amplification product, even without the DNA target site, thus giving us non-specific amplification.
The enzyme ligase is extremely specific: if there are any mismatches between the probe and the target site, the ligase will not be able to bind the probes and no amplification would occur.
Consequently, MLPA detects specific point mutations, and even distinguishes between pseudogenes and the real target gene.

4-Amplification

The next step is amplification, which is essentially a polymerase-chain reaction (PCR). For the PCR step, a polymerase, dNTPs, and a forward and reverse primer are added. Since all of the probes have the same PCR-primer sequence, it will only be necessary to add one pair of universal primers to study all of our targets. The forward primer is fluorescently labelled, allowing visualization and quantitation during analysis.

5-Fragment Separation and Data Analysis

After amplification, the fragments are separated by capillary electrophoresis. Capillary electrophoresis separates fragments based on their length, and shows different length fragments as peak patterns, called an electropherogram. Each amplicon has a different known size, due to the stuffer sequence on each specific probe, and therefore each amplicon can be quantified during data analysis.

The data obtained by capillary electrophoresis will be the input for the analysis. MRC- Holland provides a free software for data analysis – Coffalyser.

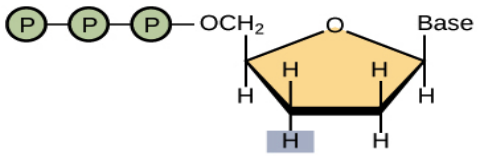
By comparing each sample to a set of reference samples, we can obtain a probe ratio. This probe ratio will inform us of how many copy numbers a gene has. Since most human genes are diploid, if the sample presents two copies, the ratio will be 1.0; i.e. the sample probes have obtained the same number of genes as the reference sample.

However, if the ratio is 0.5 there was only one copy of the gene in the individual, which probably means a heterozygous deletion of the target gene. If, on the other hand, the ratio is 1.5, there is, probably, a heterozygous duplication of a gene. MRC-Holland offers many different kits that may have the solution for your problems.

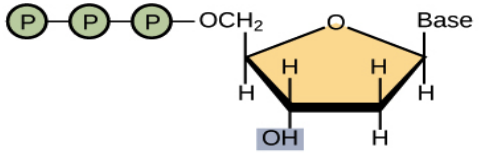
DNA sequencing

is the process of determining the sequence of nucleotide bases

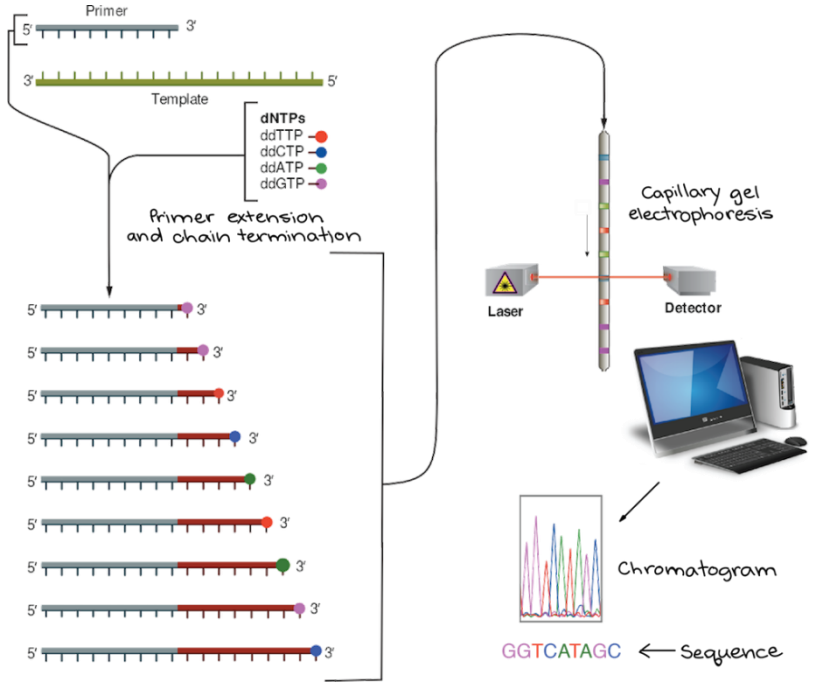
For almost all of past 30 years all DNA sequencing has been based on a single basic technic, dideoxy or Sanger sequencing.



Dideoxynucleotide (ddNTP)



Deoxynucleotide (dNTP)



DNA sequencing

is the process of determining the sequence of nucleotide bases

Sanger sequencing are performed in an automated fashion via sequencing machine.
Each method follows three basic steps.

1. DNA Sequence For Chain Termination PCR

The DNA sequence of interest is used as a template for a special type of PCR called chain-termination PCR. Chain-termination PCR works just like standard PCR, but with one major difference: the addition of modified nucleotides (dNTPs) called dideoxyribonucleotides (ddNTPs). In the extension step of standard PCR, DNA polymerase adds dNTPs to a growing DNA strand by catalyzing the formation of a phosphodiester bond between the free 3’-OH group of the last nucleotide and the 5’-phosphate of the next.
In chain-termination PCR, the user mixes a low ratio of chain-terminating ddNTPs in with the normal dNTPs in the PCR reaction. ddNTPs lack the 3'-OH group required for phosphodiester bond formation; therefore, when DNA polymerase incorporates a ddNTP at random, extension ceases. The result of chain-termination PCR is millions to billions of oligonucleotide copies of the DNA sequence of interest, terminated at a random lengths (n) by 5’-ddNTPs.
In automated Sanger sequencing, all ddNTPs are mixed in a single reaction, and each of the four dNTPs has a unique fluorescent label.

2. Size Separation by Gel Electrophoresis

In the second step, the chain-terminated oligonucleotides are separated by size via gel electrophoresis. In gel electrophoresis, DNA samples are loaded into one end of a gel matrix, and an electric current is applied; DNA is negatively charged, so the oligonucleotides will be pulled toward the positive electrode on the opposite side of the gel.
Because all DNA fragments have the same charge per unit of mass, the speed at which the oligonucleotides move will be determined only by size. The smaller a fragment is, the less friction it will experience as it moves through the gel, and the faster it will move. In result, the oligonucleotides will be arranged from smallest to largest, reading the gel from bottom to t
In automated Sanger sequencing, all oligonucleotides are run in a single capillary gel electrophoresis within the sequencing machine.

3. Gel Analysis & Determination of DNA Sequence

The last step simply involves reading the gel to determine the sequence of the input DNA. Because DNA polymerase only synthesizes DNA in the 5’ to 3’ direction starting at a provided primer, each terminal ddNTP will correspond to a specific nucleotide in the original sequence (e.g., the shortest fragment must terminate at the first nucleotide from the 5’ end, the second-shortest fragment must terminate at the second nucleotide from the 5’ end, etc.) Therefore, by reading the gel bands from smallest to largest, we can determine the 5’ to 3’ sequence of the original DNA strand.
In automated Sanger sequencing, a computer reads each band of the capillary gel, in order, using fluorescence to call the identity of each terminal ddNTP.
In short, a laser excites the fluorescent tags in each band, and a computer detects the resulting light emitted. Because each of the four ddNTPs is tagged with a different fluorescent label, the light emitted can be directly tied to the identity of the terminal ddNTP. The output is called a chromatogram, which shows the fluorescent peak of each nucleotide along the length of the template DNA.

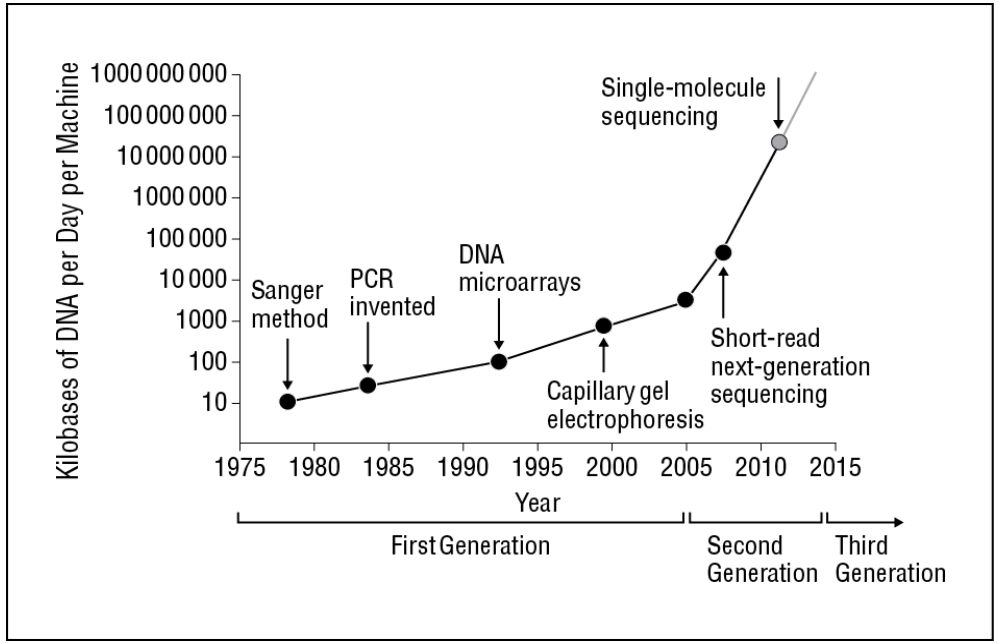
DNA sequencing

is the process of determining the sequence of nucleotide bases

Several revolutionary new sequencing technologies have burst onto the scene.

Collectively called „next generation sequencing“ or „massively parallel sequencing

Development of new sequencing methods allowed faster and more economical genomic research. With these technologies, it is now possible to determine the complete sequence of human genome in a short time period and at a relatively low cost.

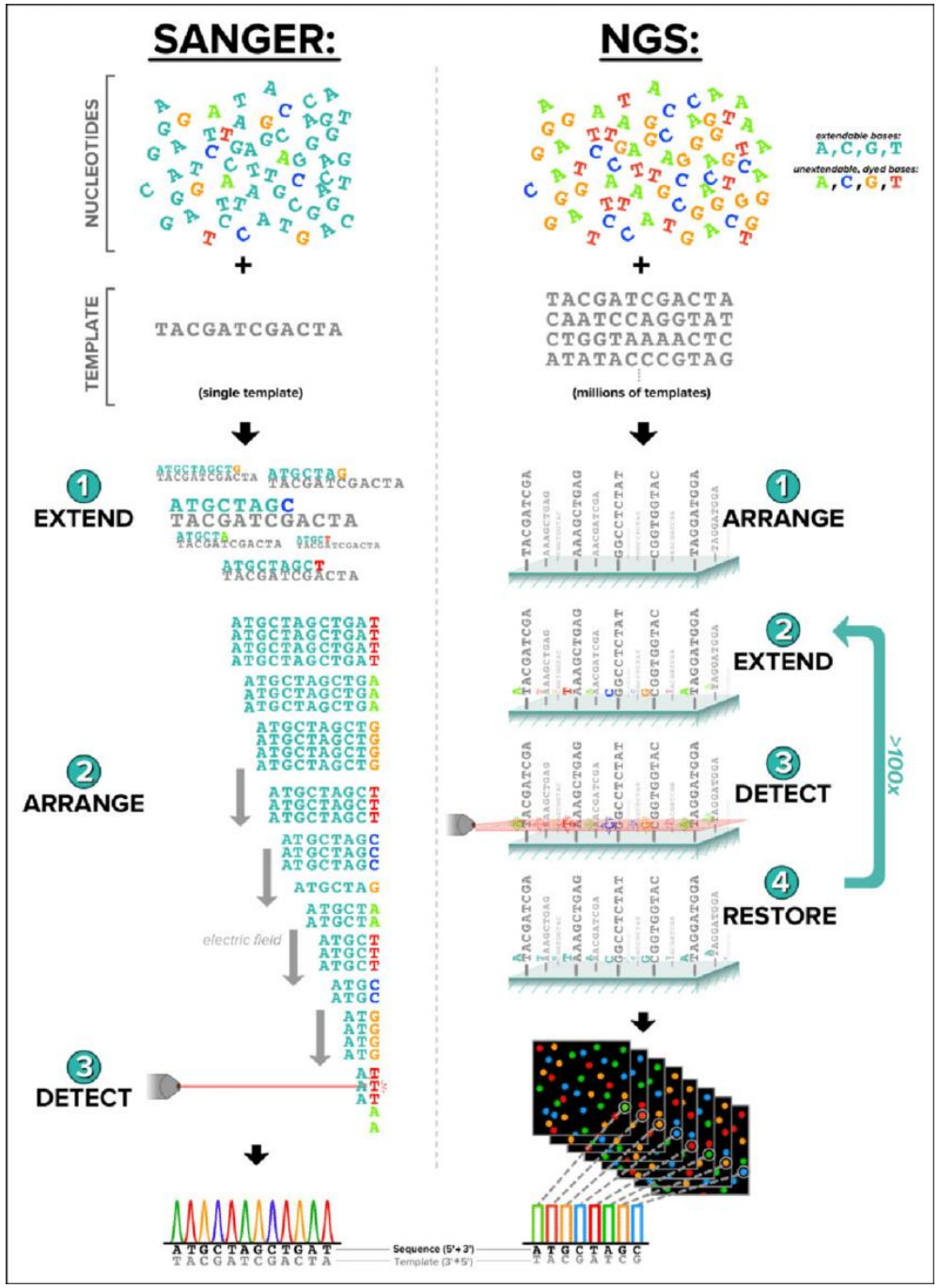


DNA sequencing

Basic features and performances of NGS platforms.

NGS platforms/company/max output per run	Read length per run (bp)	No. reads per run	Time (h or days)	Cost per 106 bases	Raw error rate (%)	Platform cost (USD approx.)	Chemistry
First generation							
Sanger/Life Technologies/84 kb	800	1	2 h	2400	0.3	95,000	Dideoxy terminator
Second generation							
454 GS FLX+/Roche/0.7 Gb	700	1×10^6	24/48 h	10	1	500,000	Pyrosequencing
GS Junior/Roche/70 Mb	500	1×10^5	18 h	9		100,000	Pyrosequencing
HiSeq/Illumina/1500 Gb	2x150	5×10^9	27/240 h	0.1	0.8	750,000	Reversible terminators
MiSeq/Illumina/15 Gb	2x300	3×10^8	27 h	0.13	0.8	125,000	Reversible terminators
SOLiD/Life Technologies/120 Gb	50	1×10^9	14 days	0.13	0.01	350,000	Ligation
Retrovolocity/BGI/3000 Gb	50	1×10^9	14 days	0.01	0.01	12×10^6	Nanoball/ligation
Ion Proton/Life Technologies/100 Gb	200	6×10^7	2–5 h	1	1.7	215,000	Proton detection
Ion PGM/Life Technologies/2 Gb	200	5×10^6	2–5 h	1	1.7	80,000	Proton detection
Third generation							
SMRT/Pac Bio/1 Gb	>10,000	1×10^6	1–2 h	2	12.9	750,000	Real-time SMS
Heliscope/Helicos/25 Gb	35	7×10^9	8 days	0.01	0.2	1.35×10^6	Real-time SMS
Nanopore/Oxford Nanopore Technologies/1 Gb	>5000	6×10^4	48/72 h	<1	34	1000	Real-time SMS
Electron microscopy/ZS	7200		14 h	<0.01		1×10^6	Real-time SMS
Genia nanopore (http://www.geniachip.com)							Real-time SMS

DNA sequencing



Next generation sequencing“ or „massively parallel sequencing

NGS has the potential to find causal mutations, including de novo, novel and familial mutations, associated with genetic diseases and, due the variable phenotypic presentations of the disorder, vastly improve molecular diagnosis. First generation DNA sequencing with chain-terminating inhibitors invented by Sanger in 1977, led to many genetic discoveries and has been widely used for over 30 years in research and diagnostic laboratories. Although considered a major technological breakthrough, and still finding utility today for variant verification, the technique has limitations, in particular when examining large regions of the genome.

More recently NGS has begun to replace Sanger sequencing due its ability to sequence large numbers of genes, the whole exome (protein-coding regions) or entire genome at once.

Thus applications of NGS include targeted gene panels, whole exome sequencing (WES) and whole genome sequencing (WGS).

Custom gene panel testing allows for screening of multiple potentially clinically relevant genes and for more flexibility in phenotype–genotype correlations than required when testing individual genes.

WES focusses on the protein coding regions in the genome, comprising approximately 1–2% of the genome, attributable to ~85% of disease related mutations.

In contrast, WGS provides information on the entire genome (both coding and non-coding regions), providing additional information on mutations in regulatory regions, as well as copy number variations with higher efficiency than WES.

The ~99% of the genome not included as exome sequence also contains untranslated regions which may have a regulatory role (e.g., non-coding RNAs or transcription binding sites) along with potential protein coding sites yet to be annotated as genes

The impact of variants found in non-coding regions are not currently well understood, however it is feasible that a single or a combination of variants could have a significant impact on the pathology.

This is most evident for non-coding variants that may influence expression levels or mRNA splicing, affecting protein abundance or isoforms.