RNA in diagnostics

RNA in diagnostics

- 1.) direct RNA diagnostics – screening of whole coding region of given gene

- 2.) gene – expression analysis:
  – diferential diagnostics of some tumours
  – detection of circulation tumour cells in blood and bone marrow
  – monitoring of course of therapy and detection of residual disease
  – control of graft before autologous transplatation
  – differential display, PTT test, functional tests...

RNA

Mammalian cell:

- 10 - 30 pg total RNA
  - rRNA (28S,18S, 5S) 80-85%
  - tRNA, snRNA 15-20%
  - mRNA 1-5%

  360 000 mRNA molecules/cell,

  12 000 different transcripts

  typical length of 1 transcript cca 2kb

RNA Unstability

- presence of ribonucleases (RNases) in cell
- RNase
  - very stable
  - do not need cofactors
  - efficient in low concentrations
  - difficult inactivation
  - contamination with RNases : human skin
    dust particles (bacterias, fungi)

- isolation and analysis of RNA : special approach and methods

Stabilisation and storage of RNA

•gene-expression analysis: analysed RNA must represent *in vivo* expression of sample
•Complications - 1) reduction mRNA (downregulatoin of genes and enzymatic degradation of RNA), 2) expression induction of certain genes

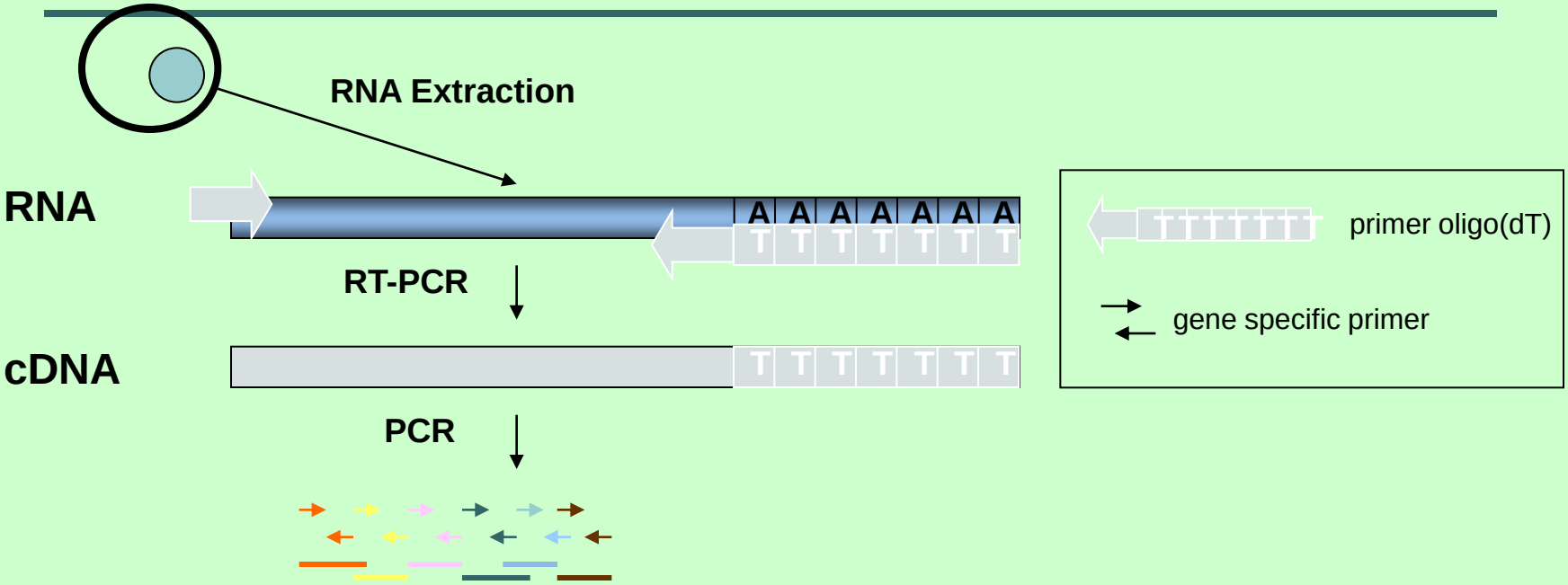
RNA stabilisation in sample:

- immediately frost in liquid nitrogen and store in -80°C
- stabilisation solutions: RNAlater, PAXgene

Contamination with DNA

PCR primers overlapping border intron/exon
digestion with DNases

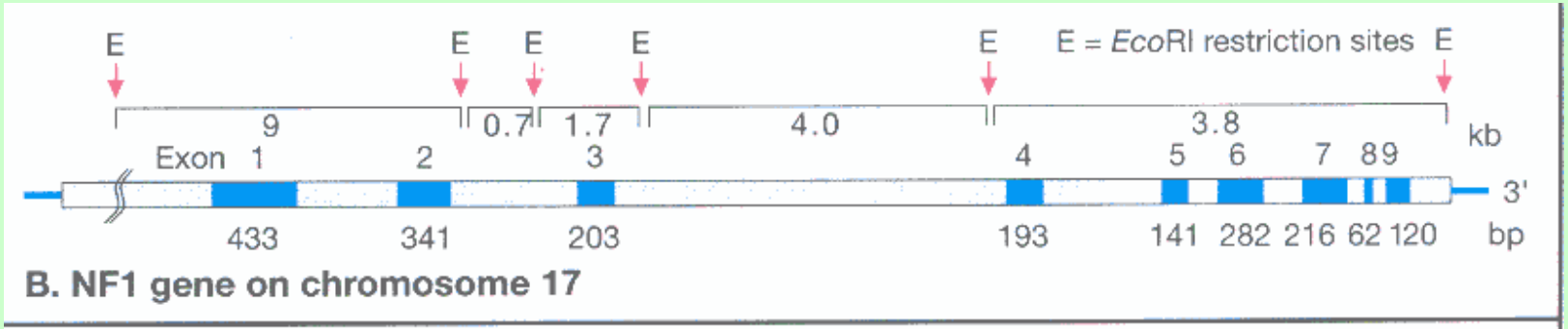
Direct RNA diagnostics


RNA Extraction
RNA
A A A A A A A
T T T T T T T
RT-PCR
cDNA
T T T T T T T
PCR
primer oligo(dT)
gene specific primer

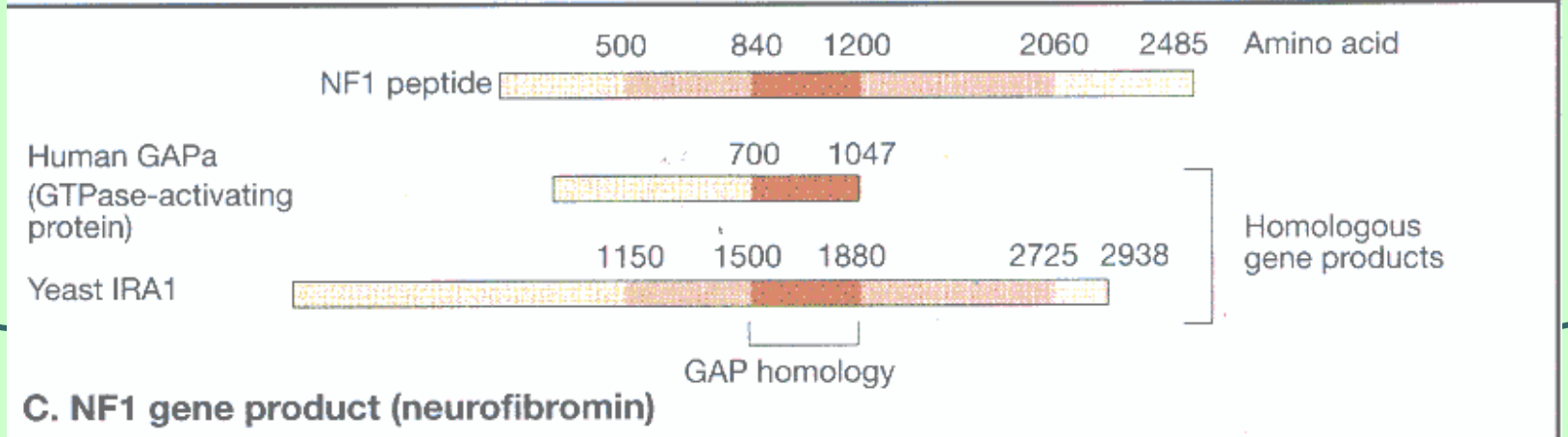
RNA diagnostics of NF1 gene

NF1 gene: 350 kb, 60 exons, 11 - 13 kb mRNA

- protein neurofibromin: 2818 aminoacids, probably tumour supresor



B. NF1 gene on chromosome 17



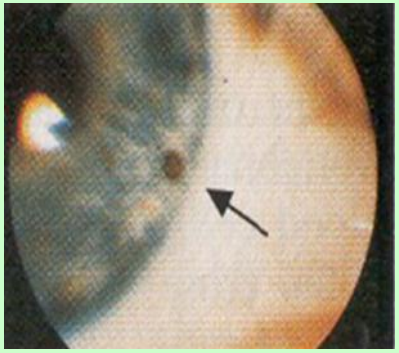
C. NF1 gene product (neurofibromin)

Neurofibromatosis type 1
von Recklinhausen disease

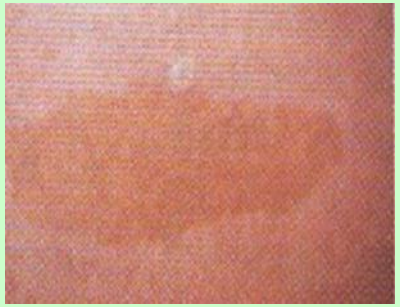
Autosomal dominant
Frequency 1:3000
Locus 17q

50% mutations de novo

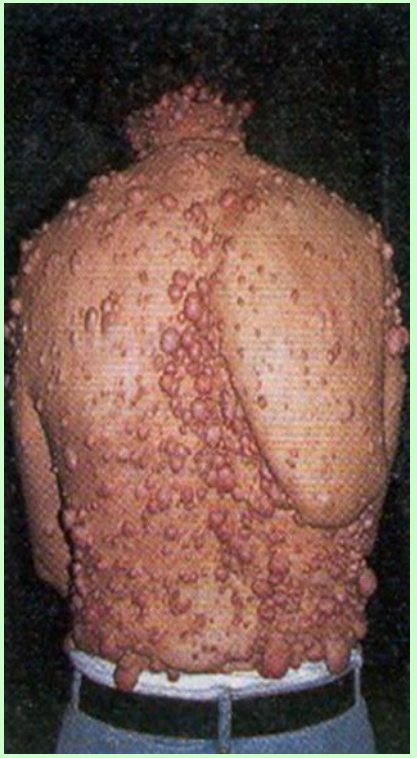
Predispositions to tumours of neural system



Lisch nodule



Café-au- lait spots



Neurofibromas

Complications in molecular diagnostics of NF1

-problematic clinical diagnosis:

-up to 50 % cases *de novo*

-high mutation speed

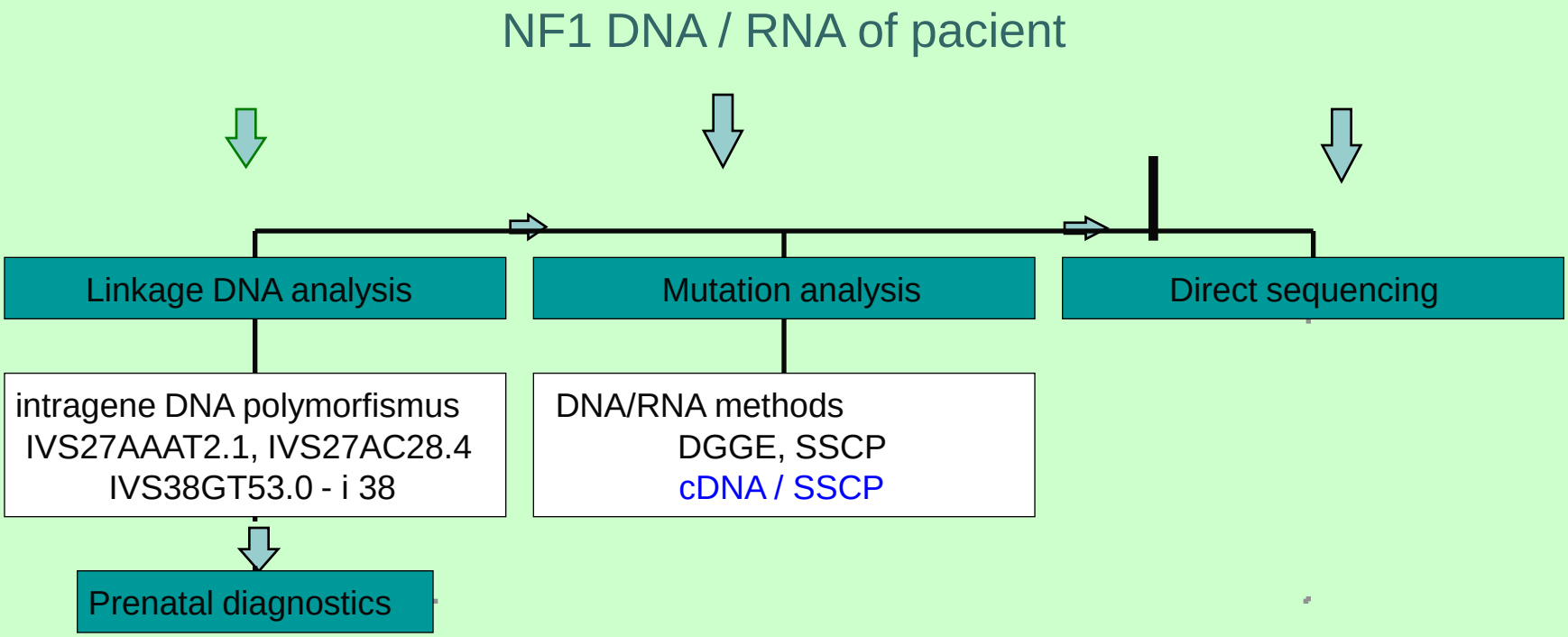
-length of gene (350 kb, 60 exonů)

-absence of hot spots – need to search in whole gene

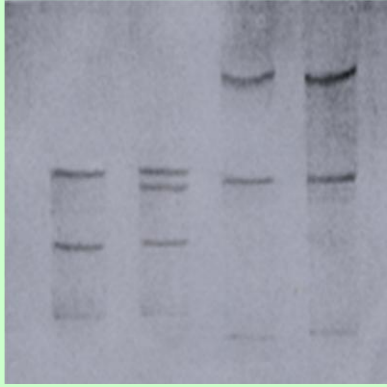
-unclear corelation between type of mutation and manifestation

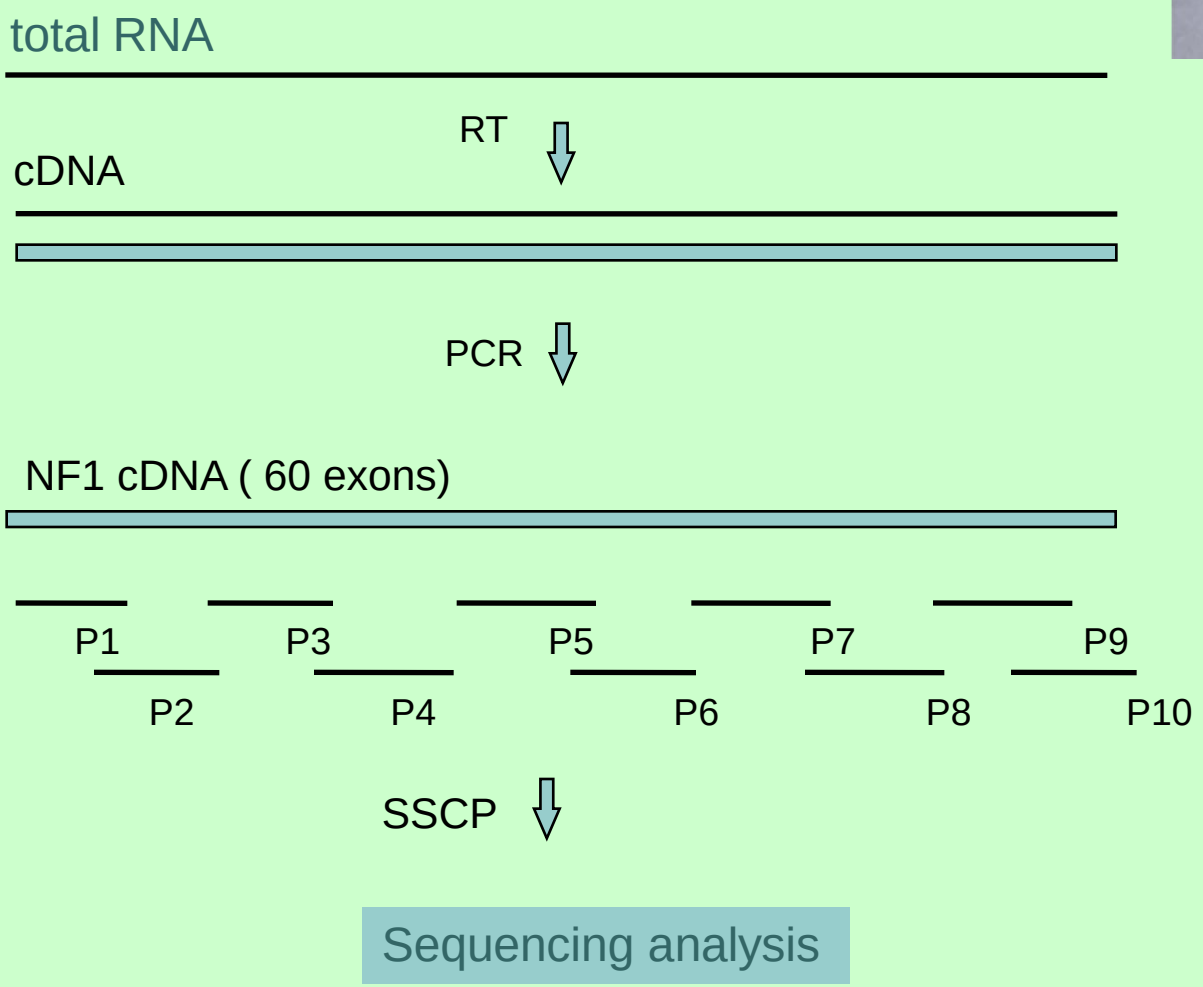
-different clinical manifestation even in patients with the same mutation

Strategy of molecular – genetic testing of NF1 patients



cDNA - SSCP analysis



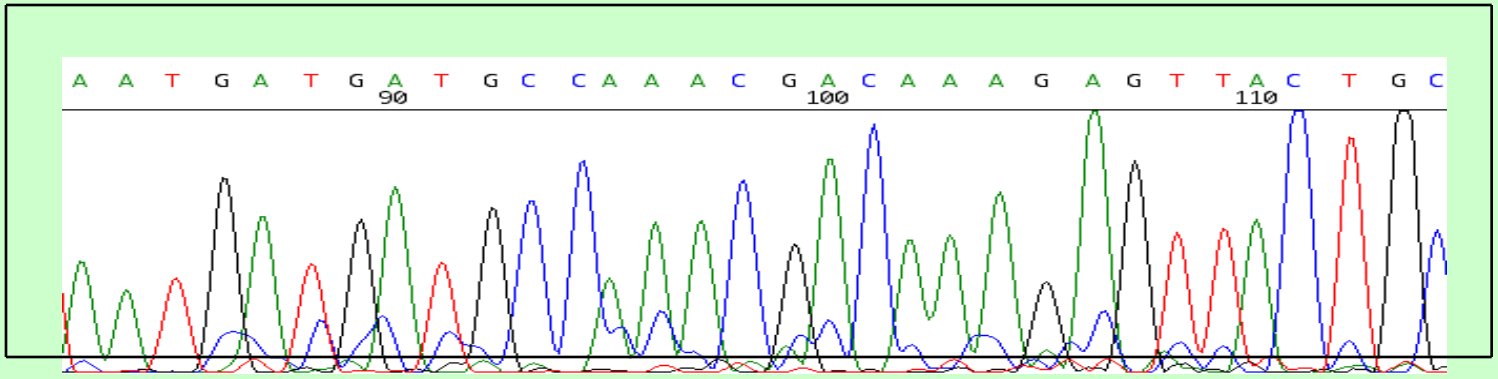


Sequencing analysis

Sequencing of cDNA segment P7 of NF1 gene (exons 28 -32/33)

standard cDNA

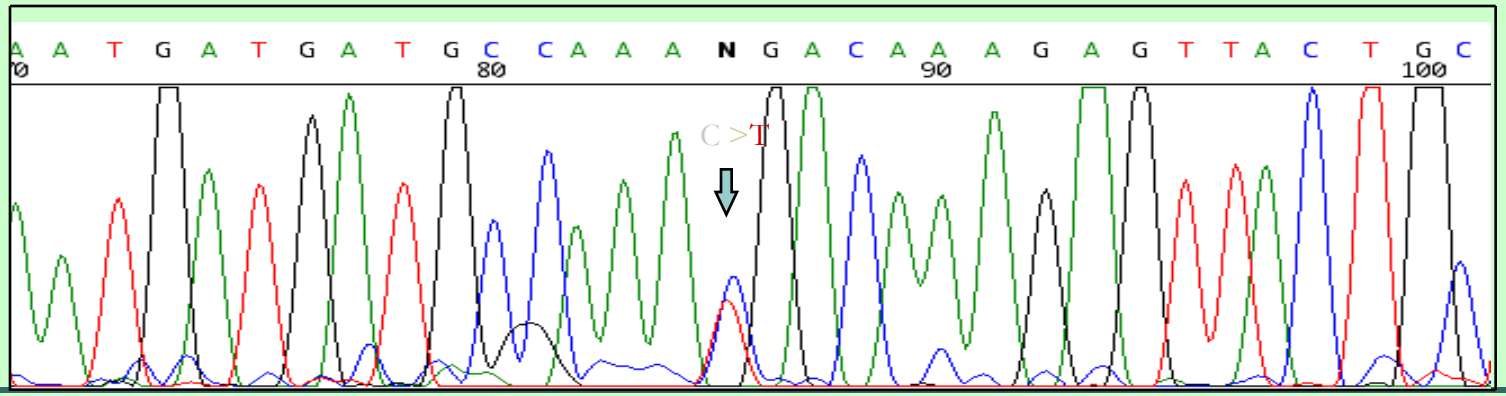
5’



3’

cDNA of NF1 pacient, mt C5839T (Arg > STOP)

5’



3’

Advantages and disadvantages of RNA diagnostics

- Easier and faster screening of multiexonic gene (10 segments of cDNA instead of 60 exons), mRNA is without introns

- Capture of splice mutations in introne s

- Capture of deletion of whole exonu on one allele

- cheaper

- More difficult taking blood for RNA isolation

- Lower stability of RNA

- Longer segments – problems with electroforetic separation and sequencing

- Unclear effect of mutation on phenotype level

Gene expression analysis

Real-time PCR method

- Real-Time PCR combines DNA amplification with real time amplified product detection in a single tube

- Reliable
- Precise
- Fast
- Universal

- Variability of used probes
- Possibility of detection of more mutation during analysis

- Measurement of fluorescence

Determine differences in mRNA expression

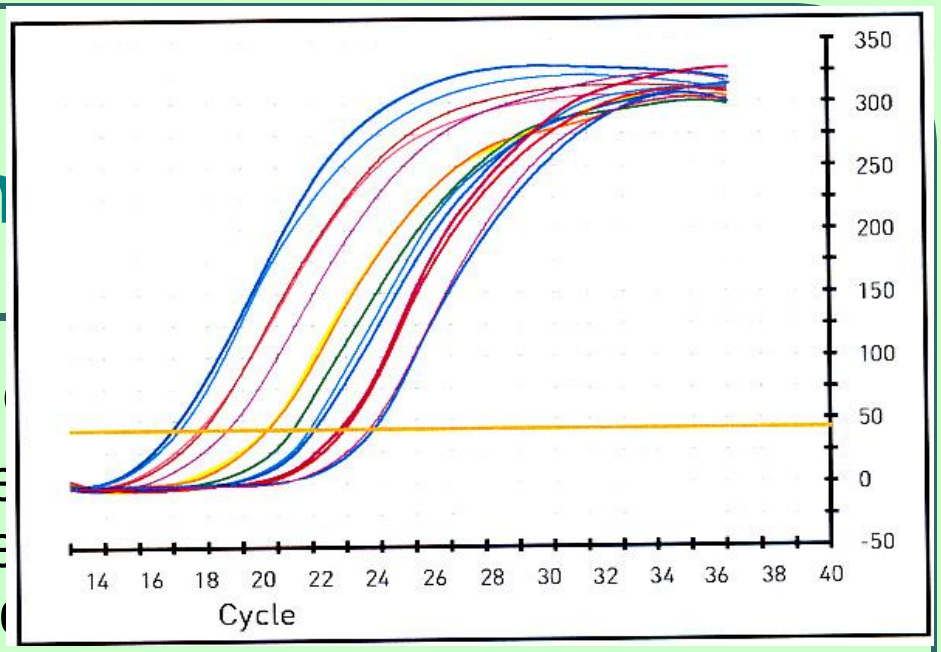
- QUALITATIVE ANALYSIS

  snp – single ncleotide polymorphism detection

- QUANTITATIVE ANALYSIS

  amplicon amount detection

Detection



- Two types of Real-Time P
- 1. Specific Sequence Dete
  a specific sequence of inte
  products. Can be used to
  (TaqMan)
- 2. Non-Specific Detection- Detects any dsDNA produced during the reaction (SYBR green)

Expression analysis in oncology

Tumour cells have different gene expression profile than healthy cells

Change in amount and spectrum of expressed mRNA

Relative quantification of expression relative to housekeeping gene
Housekeeping gene: control gene which is constant in the samples

Oncomarkers

A tumor marker is a substance found in the blood, urine, or body tissues that can be elevated in cancer. There are many different tumor markers, each indicative of a particular disease process, and they are used in oncology to help detect the presence of cancer.

From substances produced by normal cells they differ qualitatively – normal cells do not produce them, or quantitatively – they are produced by both types of cells

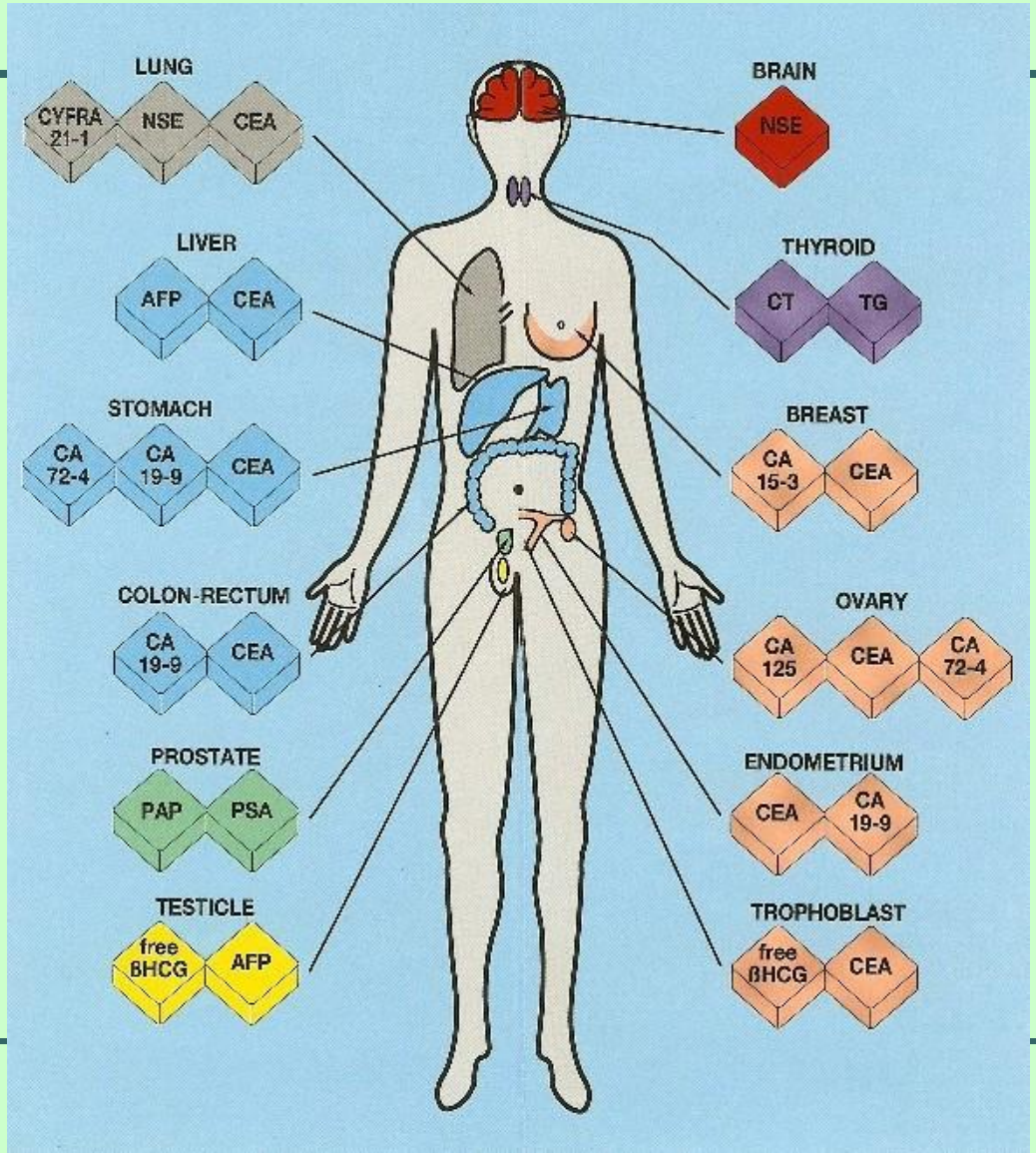
Ideal Oncomarker

- produced only in malignant cells
- organ specific
- in biological liquids in high concentrations
- level correlates with size of tumour, stage of disease, prognosis and therapy effect
- provides evidence of residual tumour tissue

Oncomarkes according to chemical structure or biological function

- oncofetal antigens
- enzymes
- hormones
- intracellular oncomarkers
- other non specified substances

Onkomarkers: indication according to organ



- **Prostate-Specific Antigen:** PSA is prostate-specific, not cancer-specific. A variety of conditions can raise PSA levels: prostatitis (prostate inflammation), benign prostatic hypertrophy (prostate enlargement), and prostate cancer. PSA levels can also be influenced by a number of other things.

- **Carcinoembryonic Antigen:** Although CEA was first indentified in colon cancer, an abnormal CEA blood level is specific neither for colon cancer nor for malignancy in general. Elevated CEA levels are found in a variety of cancers other than colonic, including pancreatic, gastric, lung, and breast. It is also detected in benign conditions including cirrhosis, inflamatory bowel disease, chronic lung disease, and pancreatitis. The CEA was found to be elevated in up to 19 percent of smokers and in 3 percent of a healthy control population.

Thus, the test for oncomarker cannot substitute for a pathological diagnosis.

Detection of minimal residual disease

Detection of presence of isolated tumour cells in blood, bone marrow and lymphatic system – possible precursors of metastases

Imunohistochemistry – sensitivity 1 : 10 000
Flow cytometry – sensitivity 1 : 100 000
PCR – sensitivity 1: 1 000 000
Real- time PCR – sensitivity up 1 : 10 000 000