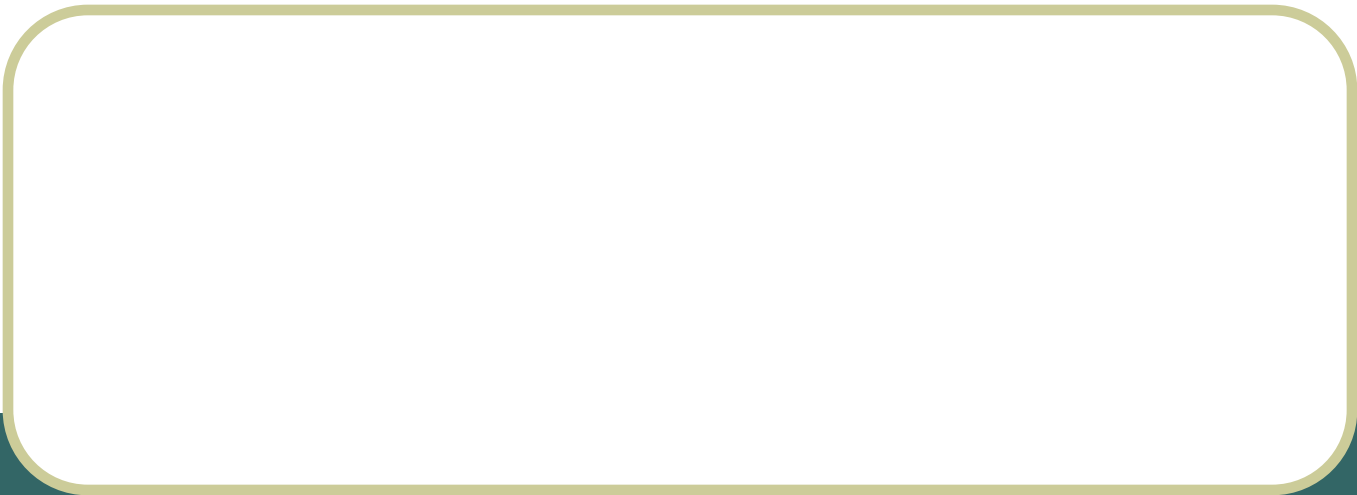


# **RNA in diagnostics**



# RNA in diagnostics

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- 1.) direct RNA diagnostics – screening of whole coding region of given gene
- 2.) gene – expression analysis:
  - differential 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 transplantation
  - differential display, PTT test, functional tests...

# RNA

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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

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- 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 (downregulation of genes and enzymatic degradation of RNA), 2) expression induction of certain genes

RNA stabilisation in sample:

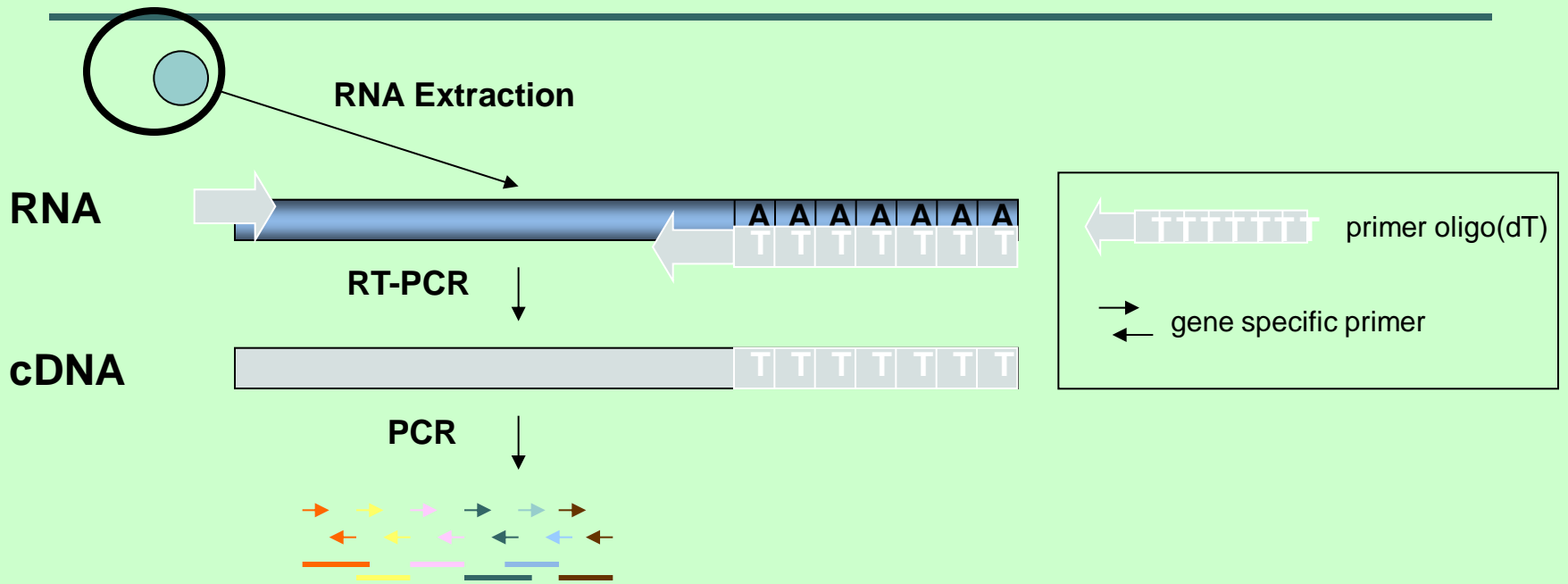
- immediately frost in liquid nitrogen and store in  $-80^{\circ}\text{C}$
- stabilisation solutions: RNAlater, PAXgene

Contamination with DNA

PCR primers overlapping border intron/exon  
digestion with DNases

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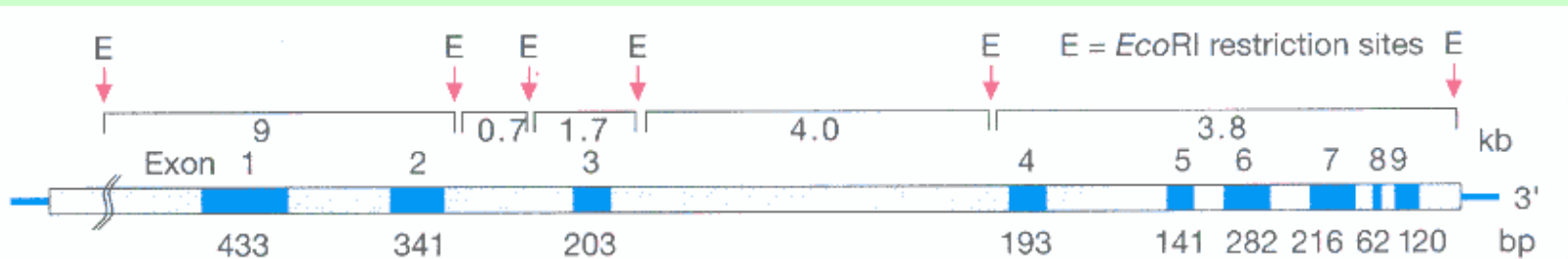
# Direct RNA diagnostics



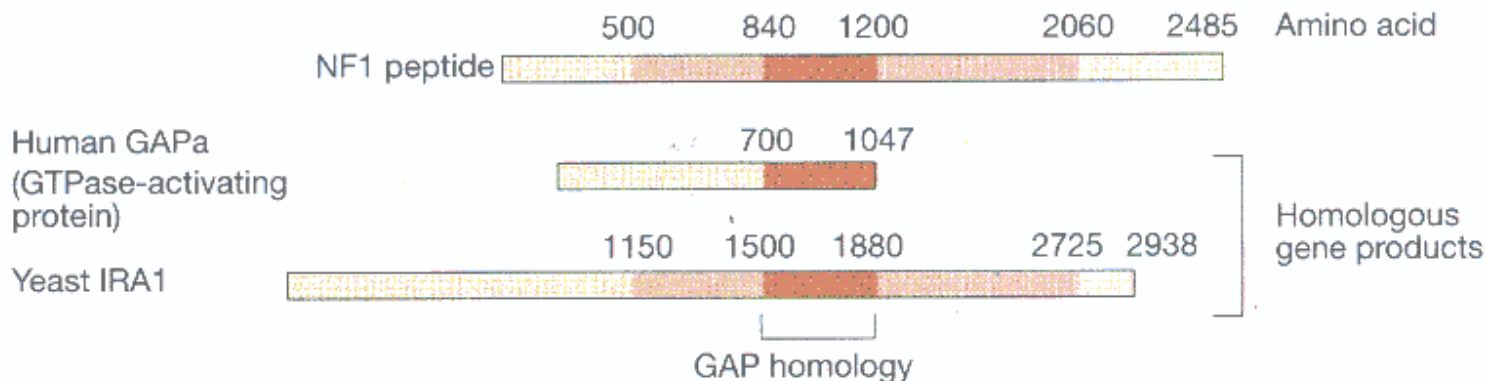
# RNA diagnostics of NF1 gene

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

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Autosomal dominant  
Frequency 1:3000  
Locus 17q

50% mutations de novo

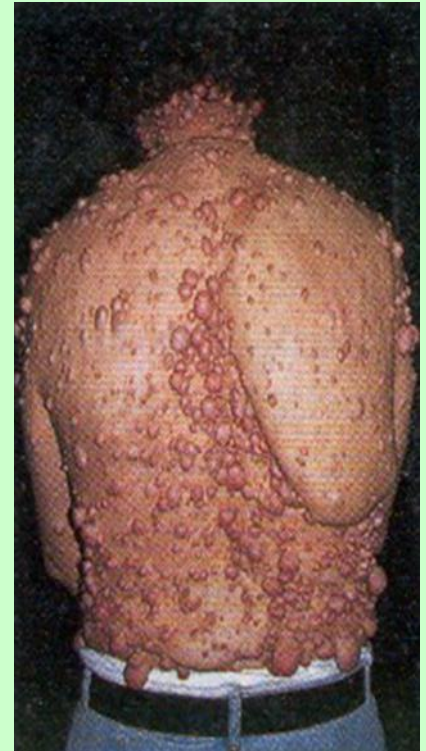
Predispositions to tumours of  
neural system



Lisch nodule



Café-au- lait spots



Neurofibromas

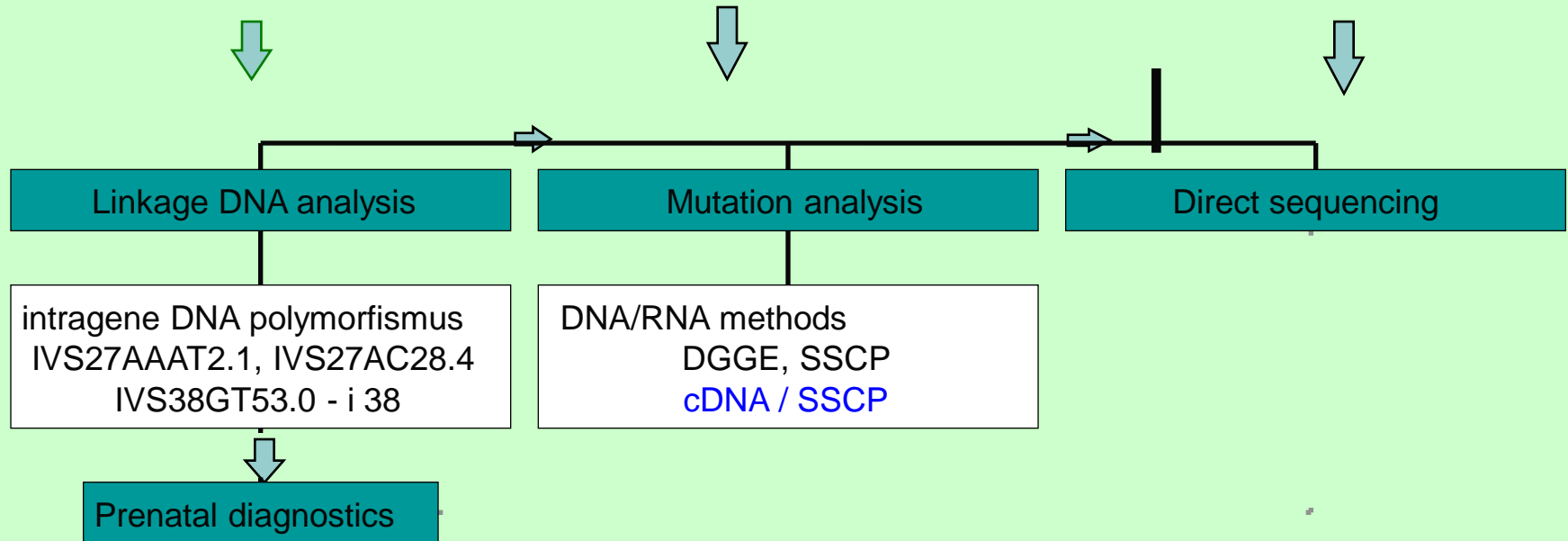
# Complications in molecular diagnostics of NF1

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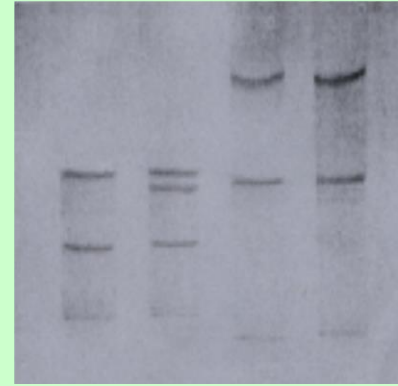
- problematic clinical diagnosis:
- up to 50 % cases *de novo*
- high mutation speed*
- length of gene* (350 kb, 60 exonů)
- absence of hot spots – need to search in whole gene
- unclear corelation between type of mutation and manifestation
- different clinical manifestation even in patients with the same mutation

# Strategy of molecular – genetic testing of NF1 patients

NF1 DNA / RNA of patient



# cDNA - SSCP analysis

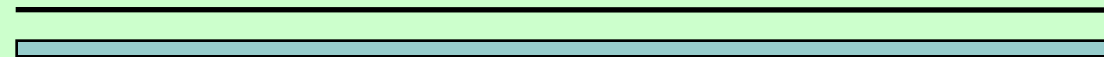


total RNA



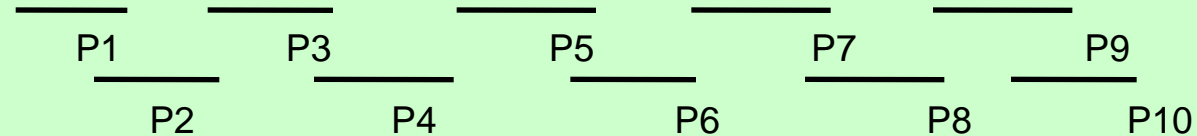
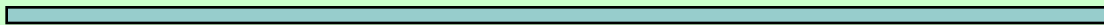
cDNA

RT ↓



PCR ↓

NF1 cDNA ( 60 exons)

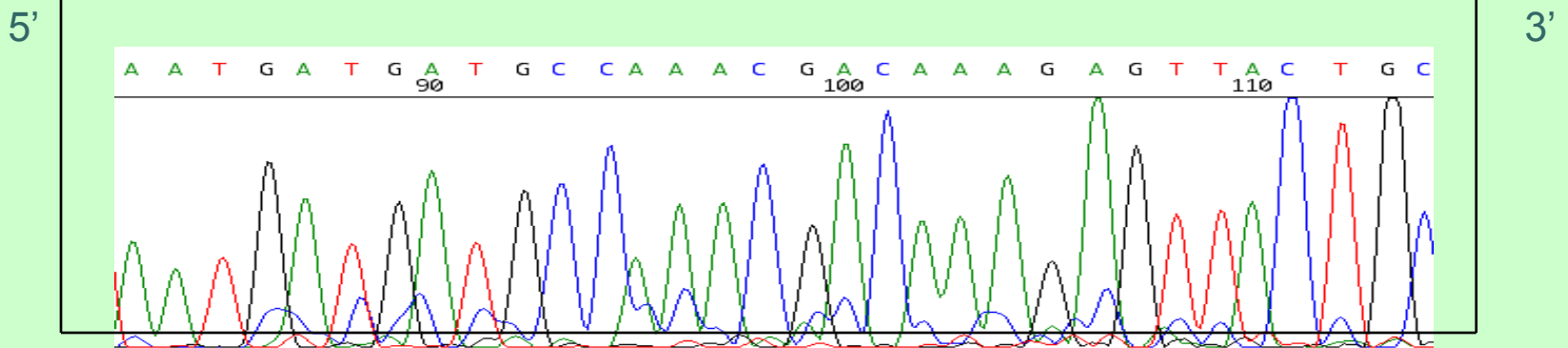


SSCP ↓

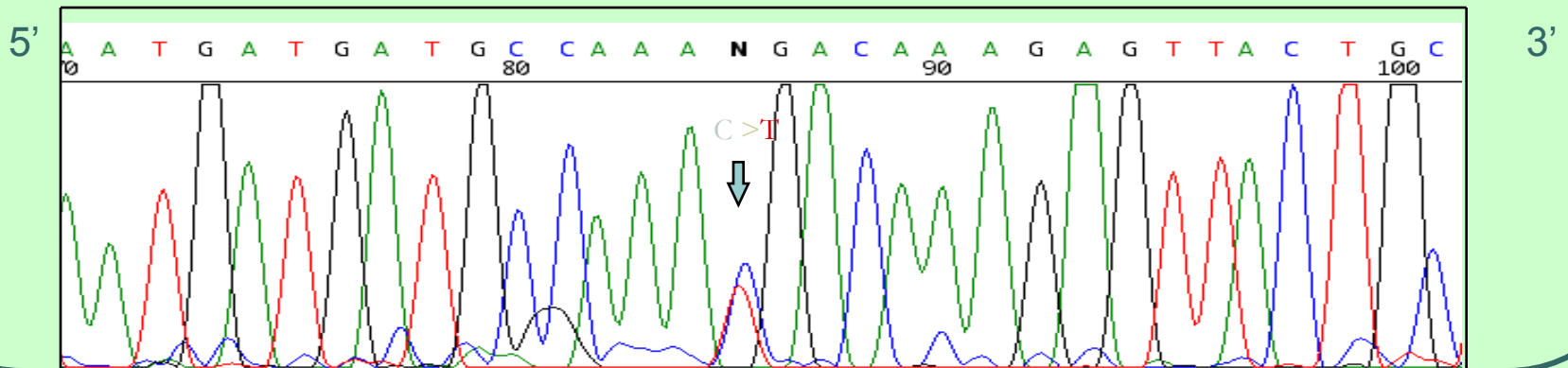
Sequencing analysis

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

standard cDNA



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



# Advantages and disadvantages of RNA diagnostics

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- Easier and faster screening of multiexonic gene (10 segments of cDNA instead of 60 exons), mRNA is without introns
- Capture of splice mutations in introns
- Capture of deletion of whole exon 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

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# **Gene expression analysis**

# Real-time PCR method

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- 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



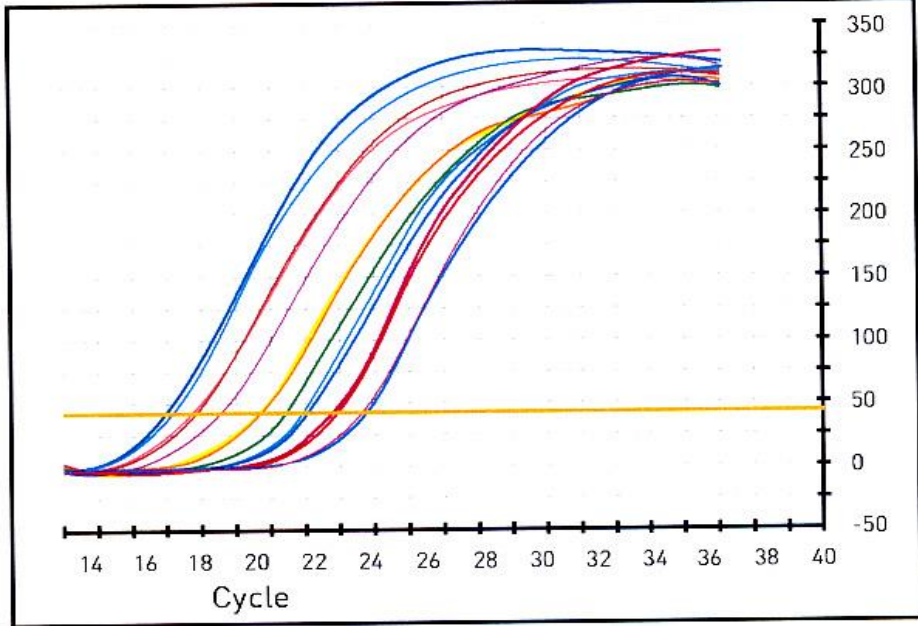
---

Determine differences in mRNA expression

- QUALITATIVE ANALYSIS  
snp – single nucleotide polymorphism detection
- QUANTITATIVE ANALYSIS  
amplicon amount detection

# Detection

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



# Expression analysis in oncology

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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

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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

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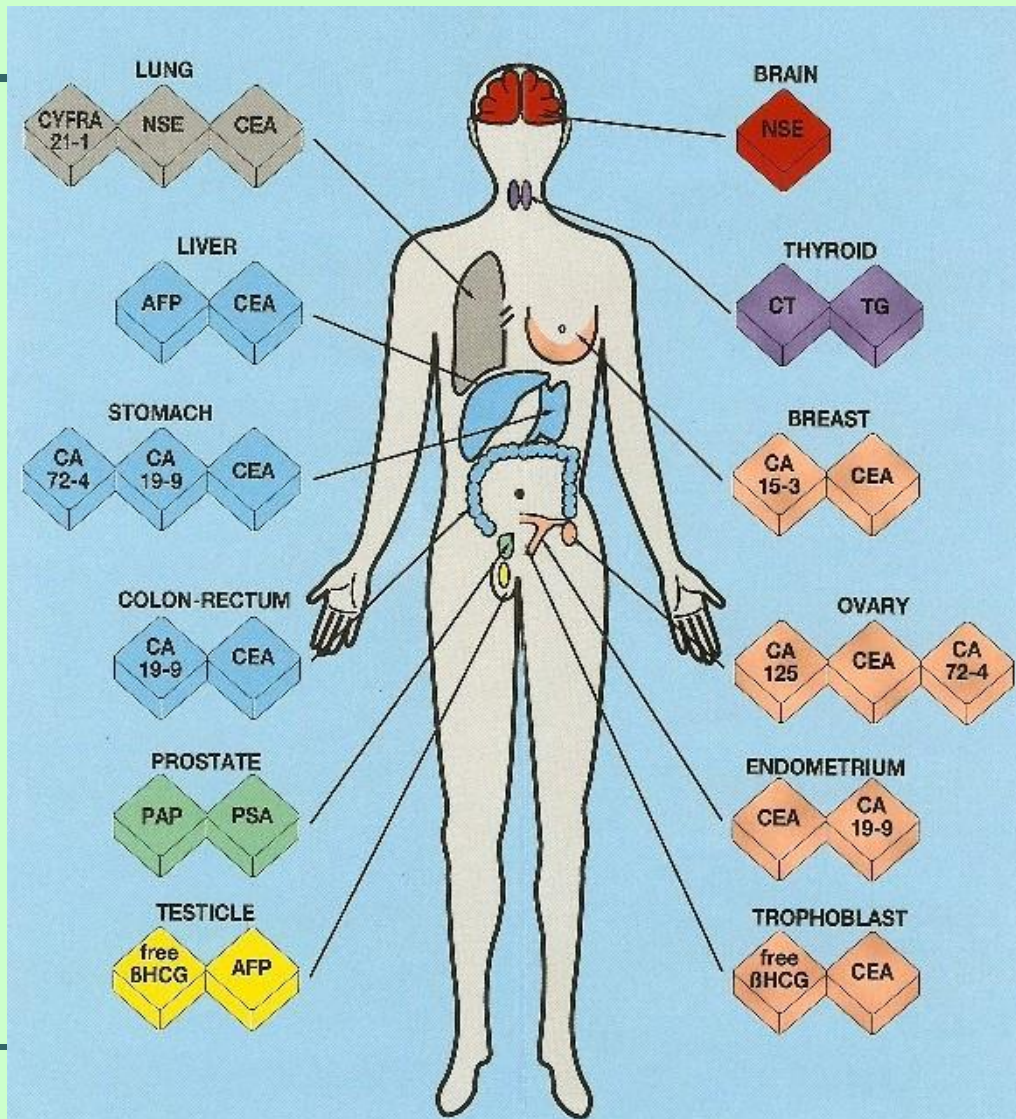
- produced only in malignant cells
- organ specific
- in biological liquids in high concentrations
- level correlates with size of tumour, stage of disease, prognosis and therapy effect
- provides evidence of residual tumour tissue

# Oncomarkes according to chemical structure or biological function

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- 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 identified 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, inflammatory 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

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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

# Human Identity Testing

# Applications:

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Forensic cases - matching suspect with evidence

Paternity testing - identifying father

Historical investigations

Missing persons investigations

Mass disasters - putting pieces back together

Military DNA “dog tag”

Convicted felon DNA databases

# Sources of Biological Evidence

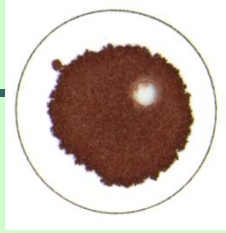
- Blood
- Semen
- Saliva
- Urine
- Hair
- Teeth
- Bone
- Tissue



**Blood Stain: Only a very small amount of blood is needed to obtain a DNA profile**

# Steps in DNA Analysis

Collection



Blood Stain

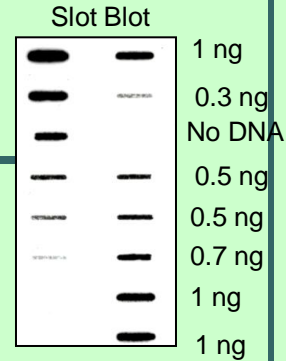


Buccal swab

Sample Collection  
& Storage



DNA  
Extraction



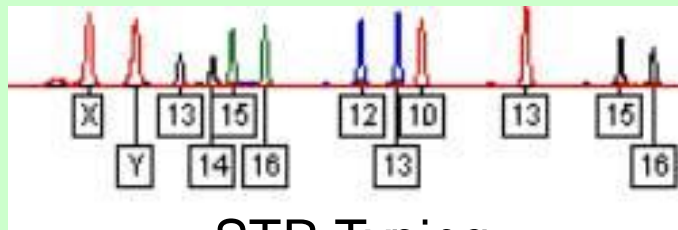
DNA  
Quantitation

Specimen Storage

Quantitation



Multiplex PCR Amplification/RFLP



STR Typing

Interpretation  
of Results

Database

Storage & Searching

Male: 13,14-15,16-12,13-10,13-15,16

Interpretation of Results



DNA Database

# What Type of Genetic Variation?

- Length Variation

short tandem repeats (**STRs**)

CTAGTCGT(**GATA**)(**GATA**)(**GATA**)GCGATCGT

Example of three alleles:

Allele 1: ...AGA...

Allele 2: ...AGAAGAAGA...

Allele 3: ...AGAAGAAGAAGAAGA...

- Sequence Variation

GCTAGTCGATGCTC(**G/A**)GCGTATGCTGTAGC

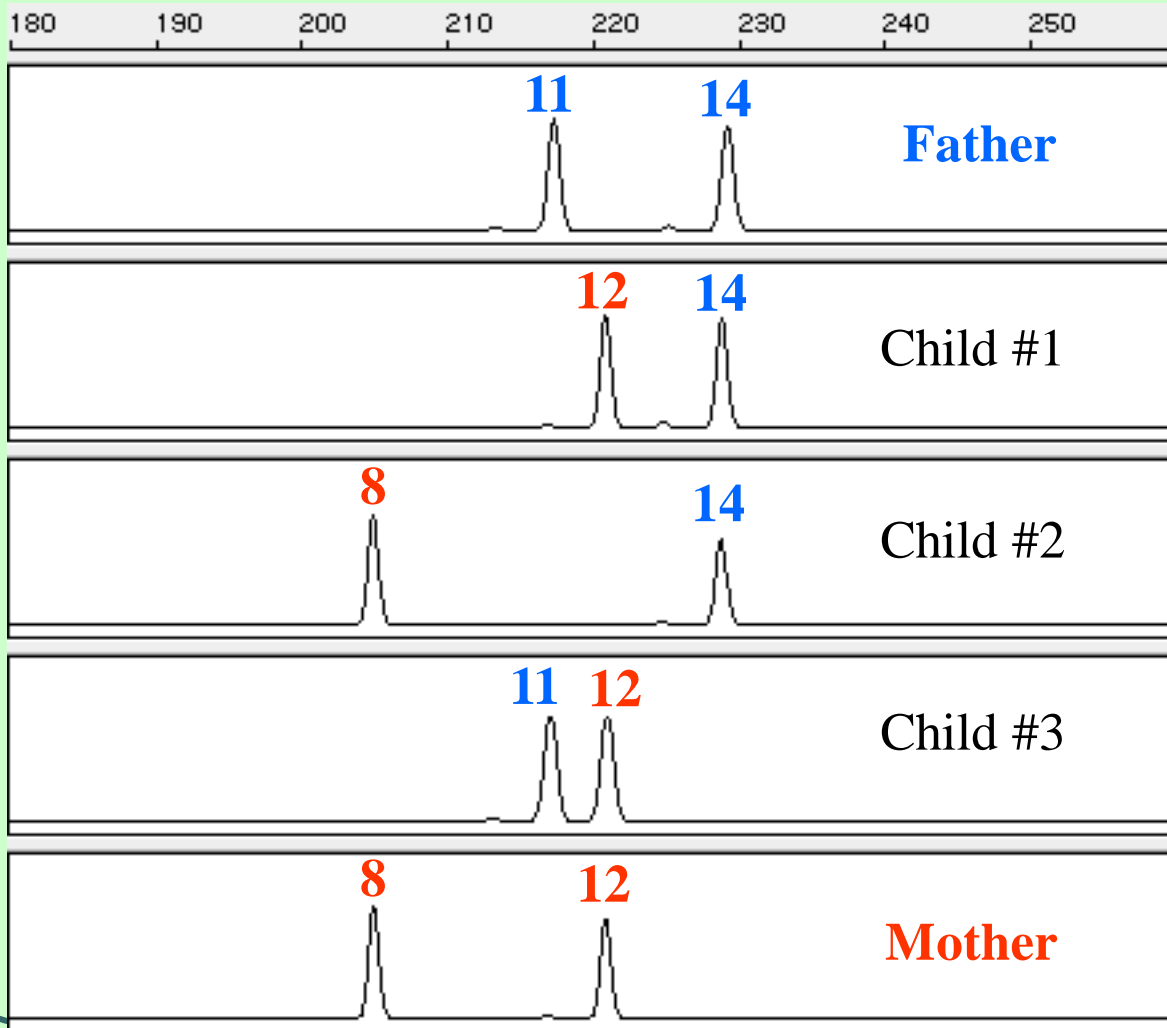
single nucleotide polymorphisms (**SNPs**)

insertions/deletions

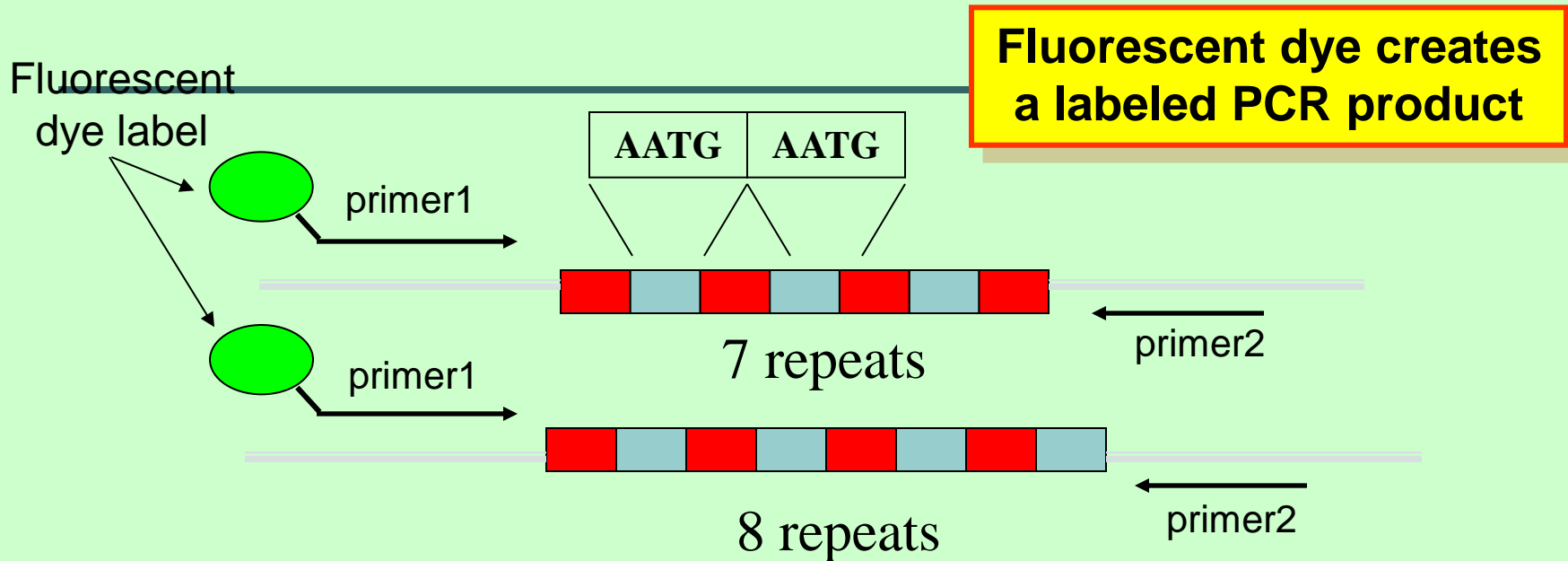
# Paternity Testing

## Family Inheritance of STR Alleles (D13S317)

PCR product size (bp)



# Short Tandem Repeats (STRs)



*the repeat region is variable between samples while the flanking regions where PCR primers bind are constant*

Homozygote = both alleles are the same length

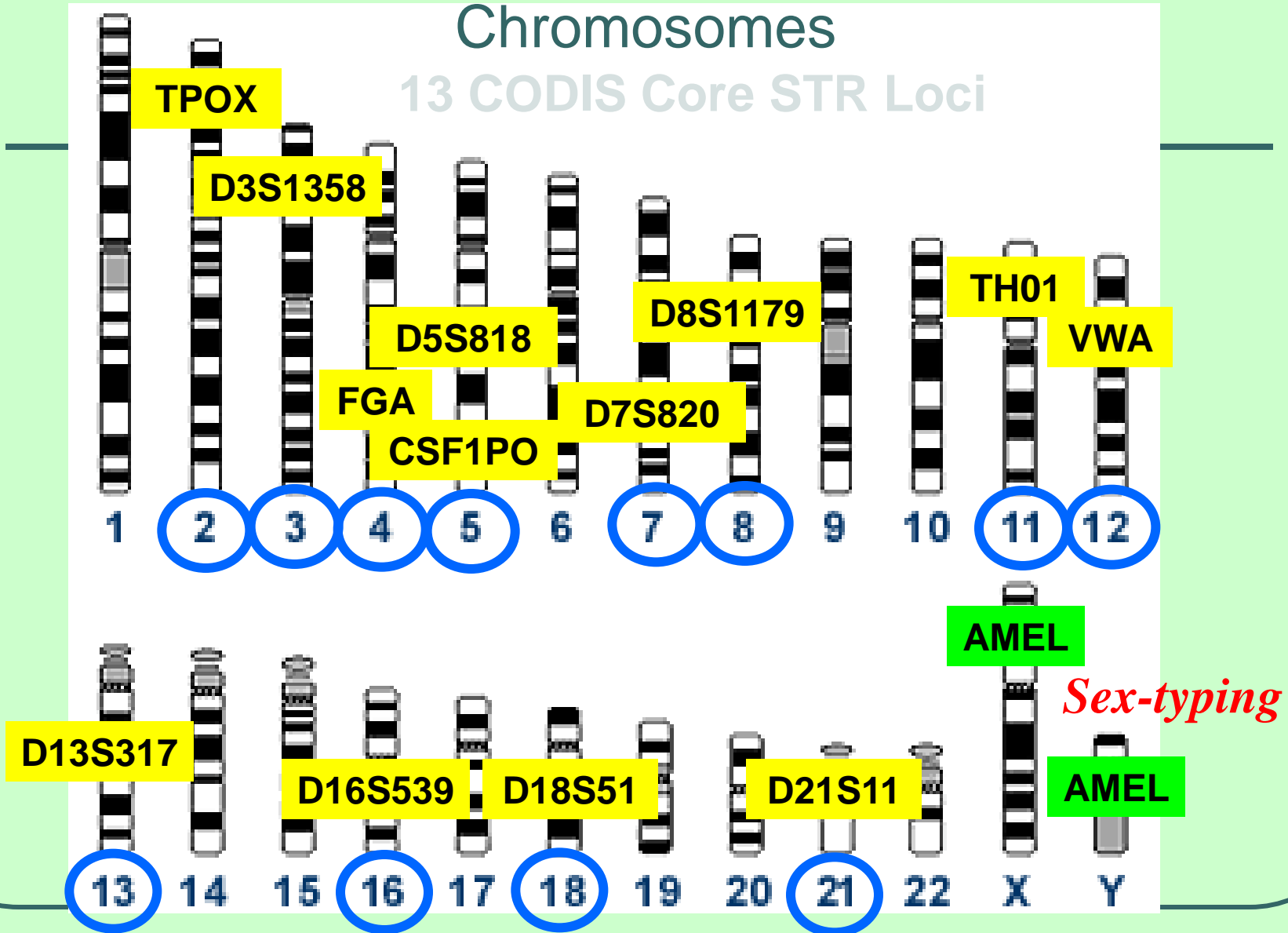
Heterozygote = alleles differ and can be resolved from one another

*Primer positions define PCR product size*



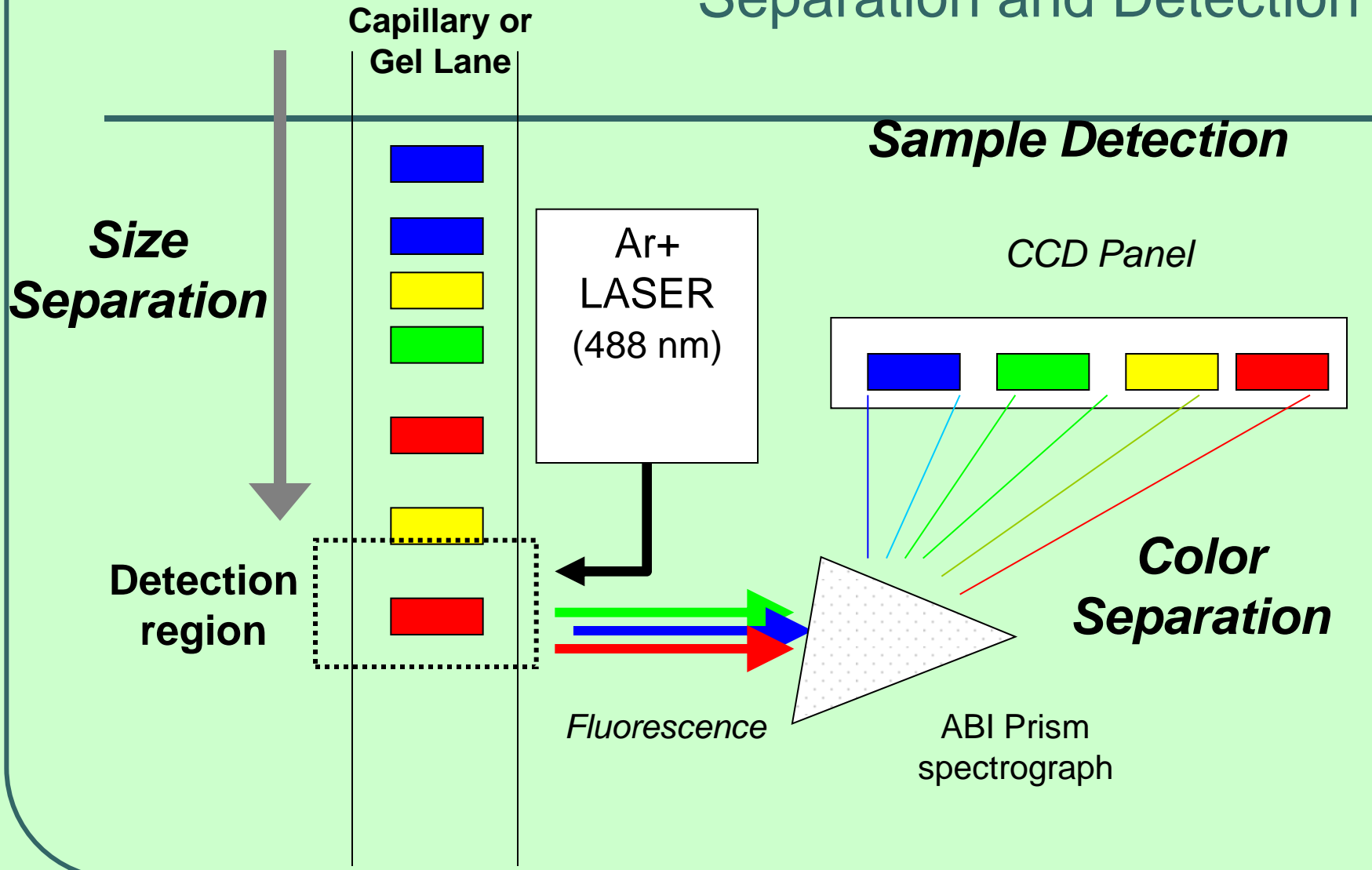
# Position of Forensic STR Markers on Human Chromosomes

13 CODIS Core STR Loci



Labeled DNA fragments  
(PCR products)

# Principles of Sample Separation and Detection



## Sample Detection

CCD Panel

Ar+  
LASER  
(488 nm)

## Color Separation

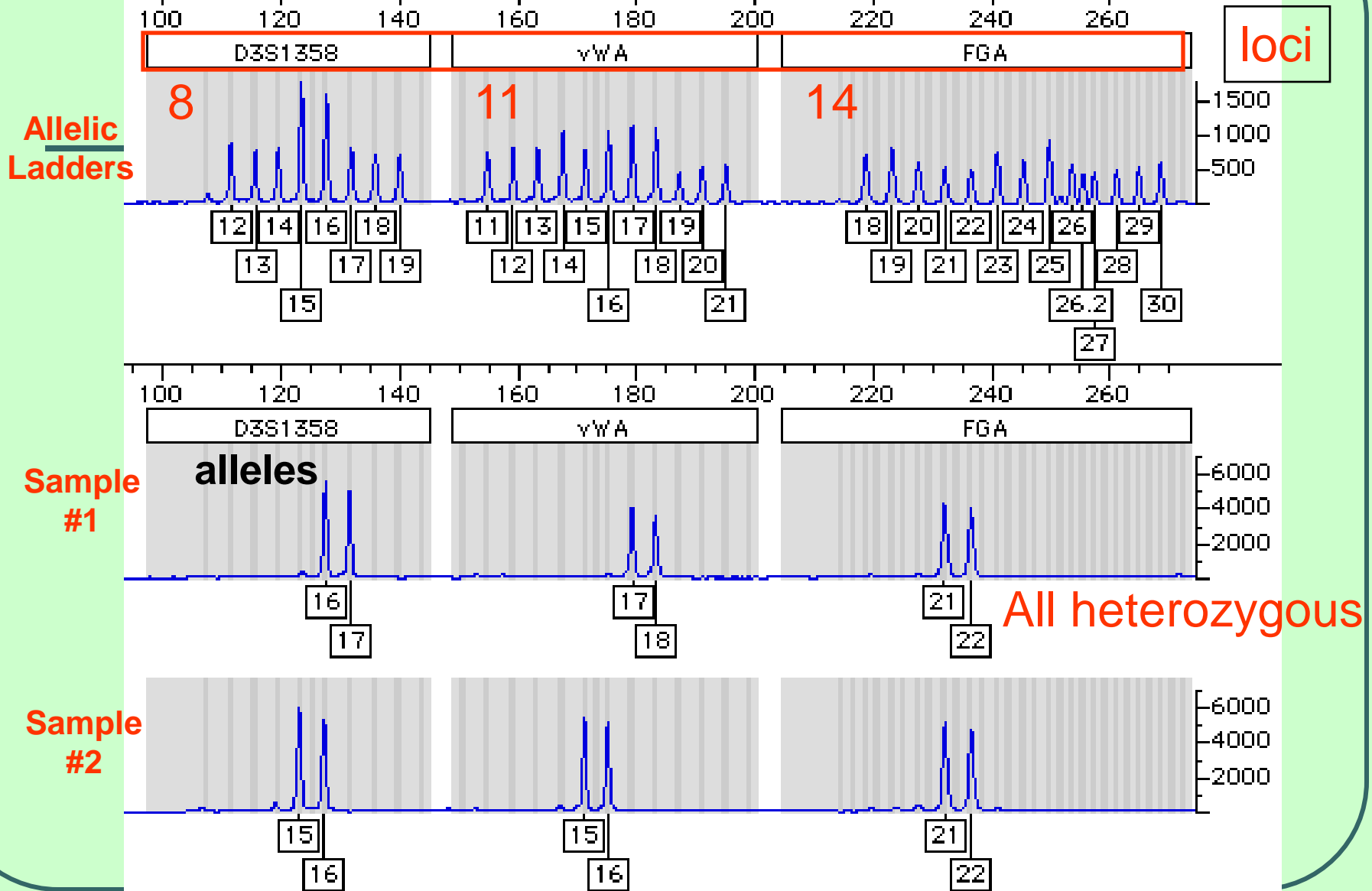
Fluorescence

ABI Prism  
spectrograph

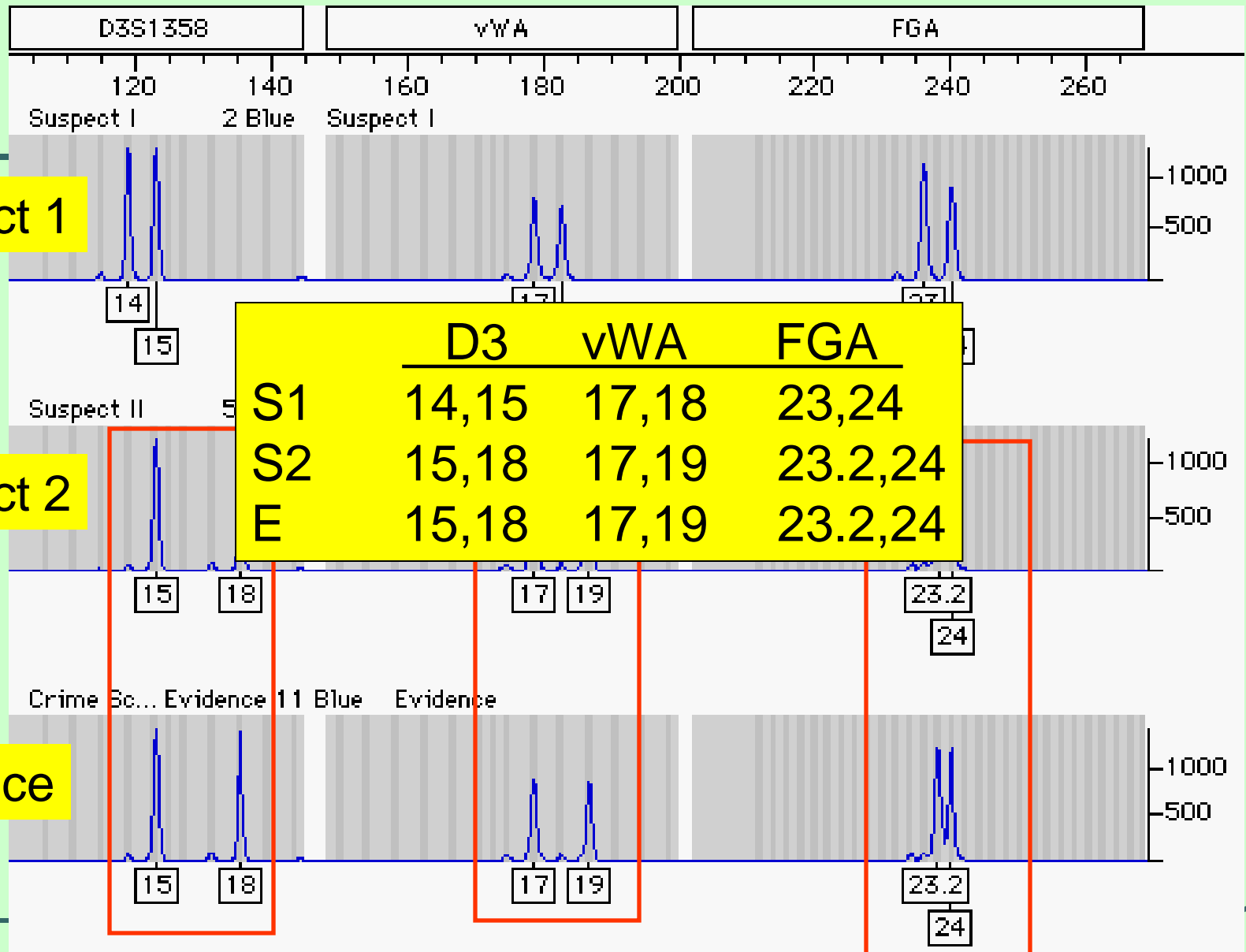
Size  
Separation

Detection  
region

# PCR Product Size (bp)



# Crime Scene - Two Suspects

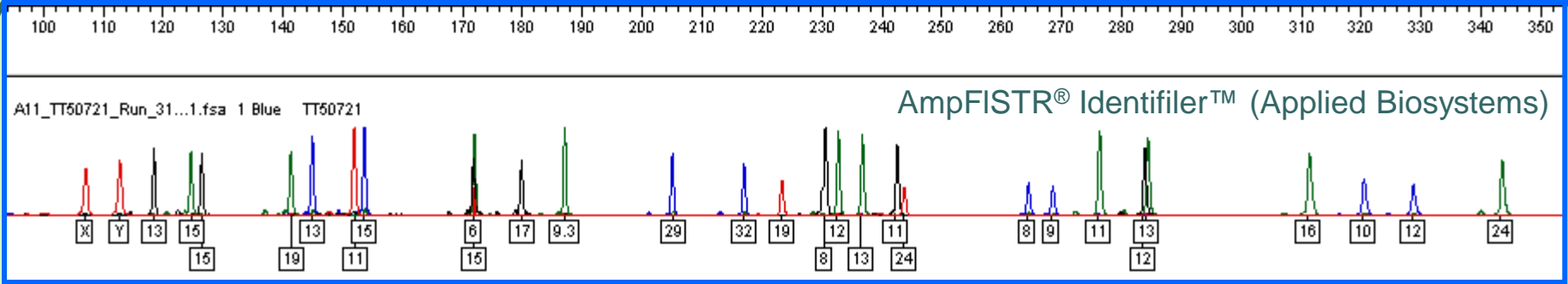


Suspect 1

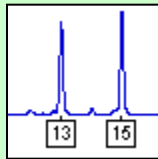
Suspect 2

Evidence

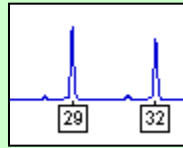
# Information is tied together with multiplex PCR and data analysis



D8S1179



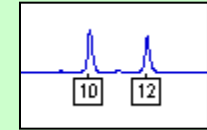
D21S11



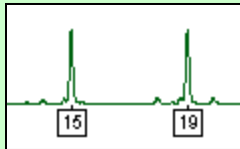
D7S820



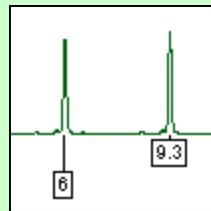
CSF1PO



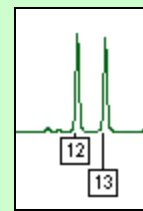
D3S1358



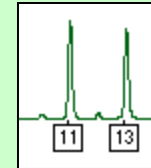
TH01



D13S317



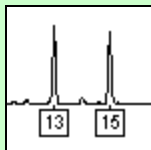
D16S539



D2S1338



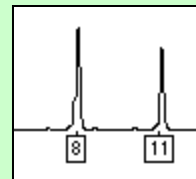
D19S433



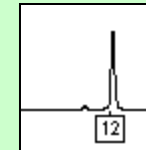
VWA



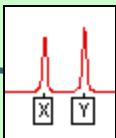
TPOX



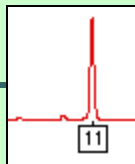
D18S51



AMEL



D5S818



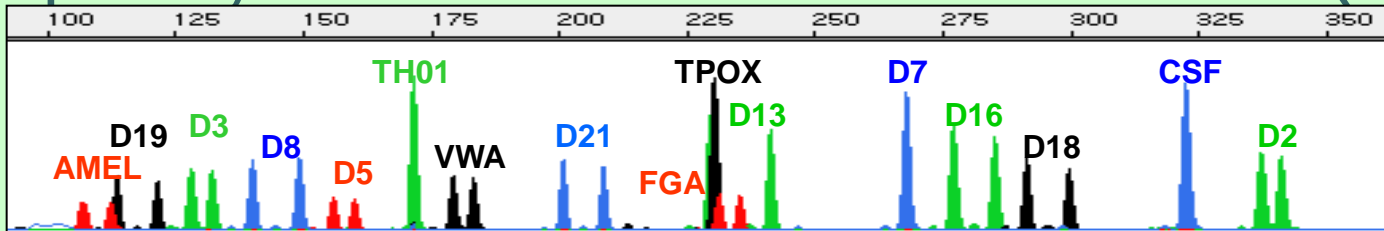
FGA



**1 integrated analysis  
vs. 16 separate runs**

# DNA Profile Frequency

AmpFISTR® Identifiler™  
(Applied Biosystems)



Locus	allele	value	allele	value	frequency, 1 in
D3S1358	16	0.2315	17	0.2118	10.20
VWA	17	0.2628	18	0.2219	8.57
FGA	21	0.1735	22	0.1888	15.26
D8S1179	12	0.1454	14	0.2015	17.07
D21S11	28	0.1658	30	0.2321	12.99
D18S51	14	0.1735	16	0.1071	26.91
D5S818	12	0.3539	13	0.1462	9.66
D13S317	11	0.3189	14	0.0357	43.92
D7S820	9	0.1478			43.28
D16S539	11	0.2723	13	0.1634	11.24
THO1	6	0.2266			18.83
TPOX	8	0.5443			3.35
CSF1PO	10	0.2537			15.09

PI = paternity index  
= 2(0.2315)(0.2118) =  
**0.0981 or 1 in 10.2**

The Random Match Probability for this profile in the FBI Caucasian population  
is **1 in 1.56 quadrillion (10<sup>15</sup>)**

# CODIS DNA Database

## Combined DNA Index System

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Used for linking serial crimes and unsolved cases  
with repeat offenders

Requires 13 core STR markers

- For independent loci, the genotype frequencies can be combined through multiplication...
- Profile Probability = Combined paternity index (CPI) =  $(P1)(P2)...(Pn) = 1$  in a very large number...

# Genetic Privacy Concerns

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- The DNA profile itself is neutral (and uninformative)—just a string of numbers like a Social Security Number
- DNA markers used in forensics were selected to be neutral and are located away from or between genes rather than being part of gene products and therefore are not generally thought to be associated with any genetic disease
- Concern is really with the DNA sample collected—would it be retained and used for any other type of testing? (e.g., Armed Forces DNA Repository can only be used for identifying combat casualties)



# PCR for DNA Profile

---

## Steps:

1. DNA sample analyzed for STR alleles present in population members
2. Analyze **population frequency**, how often combinations of alleles present
3. Population frequencies for each STR allele multiplied to estimate probability

# Combined Frequencies

STR	Allele	Frequency in population	Combined frequency
A	1	1 in 25	
B	2	1 in 100	$A1 \times B2$ (1 in 25 $\times$ 1 in 100) = 1 in 2500
C	3	1 in 320	$A1 \times B2 \times C3$ (1 in 25 $\times$ 1 in 100 $\times$ 1 in 320) = 1 in 800,000
D	4	1 in 75	$A1 \times B2 \times C3 \times D4$ (1 in 25 $\times$ 1 in 100 $\times$ 1 in 320 $\times$ 1 in 75) = 1 in 60 million