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

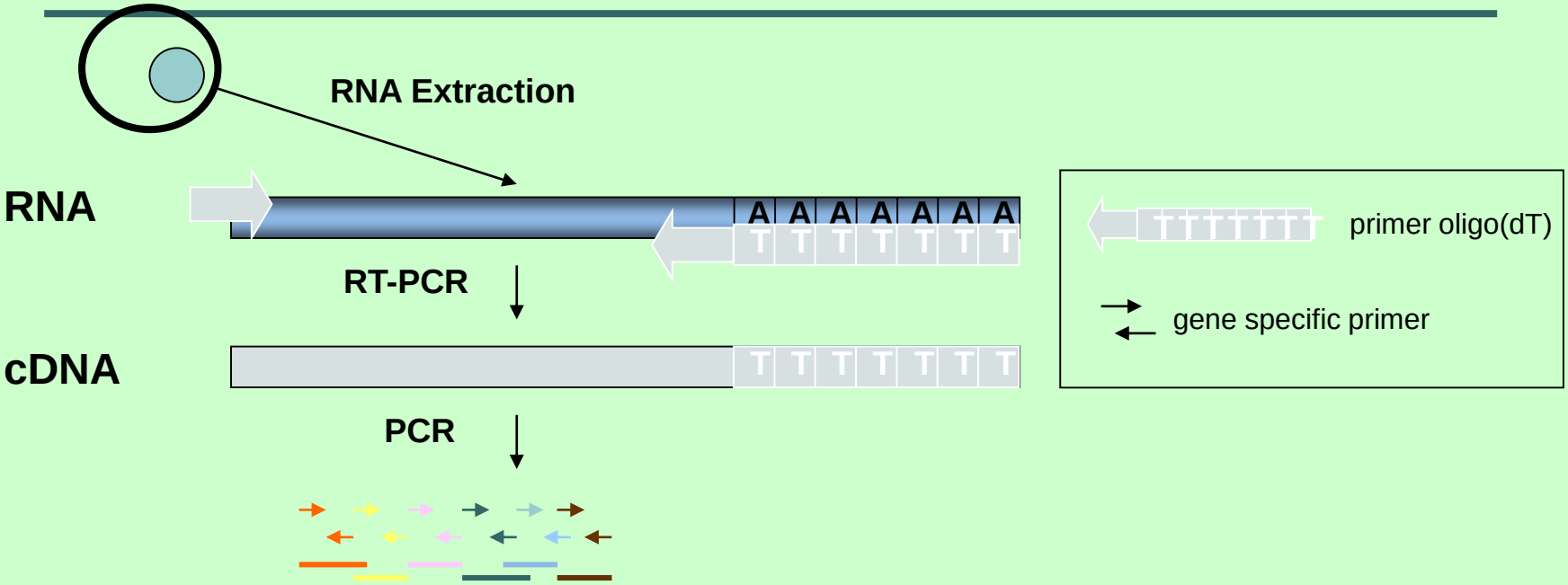
RNA stabilisation in sample:

- 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

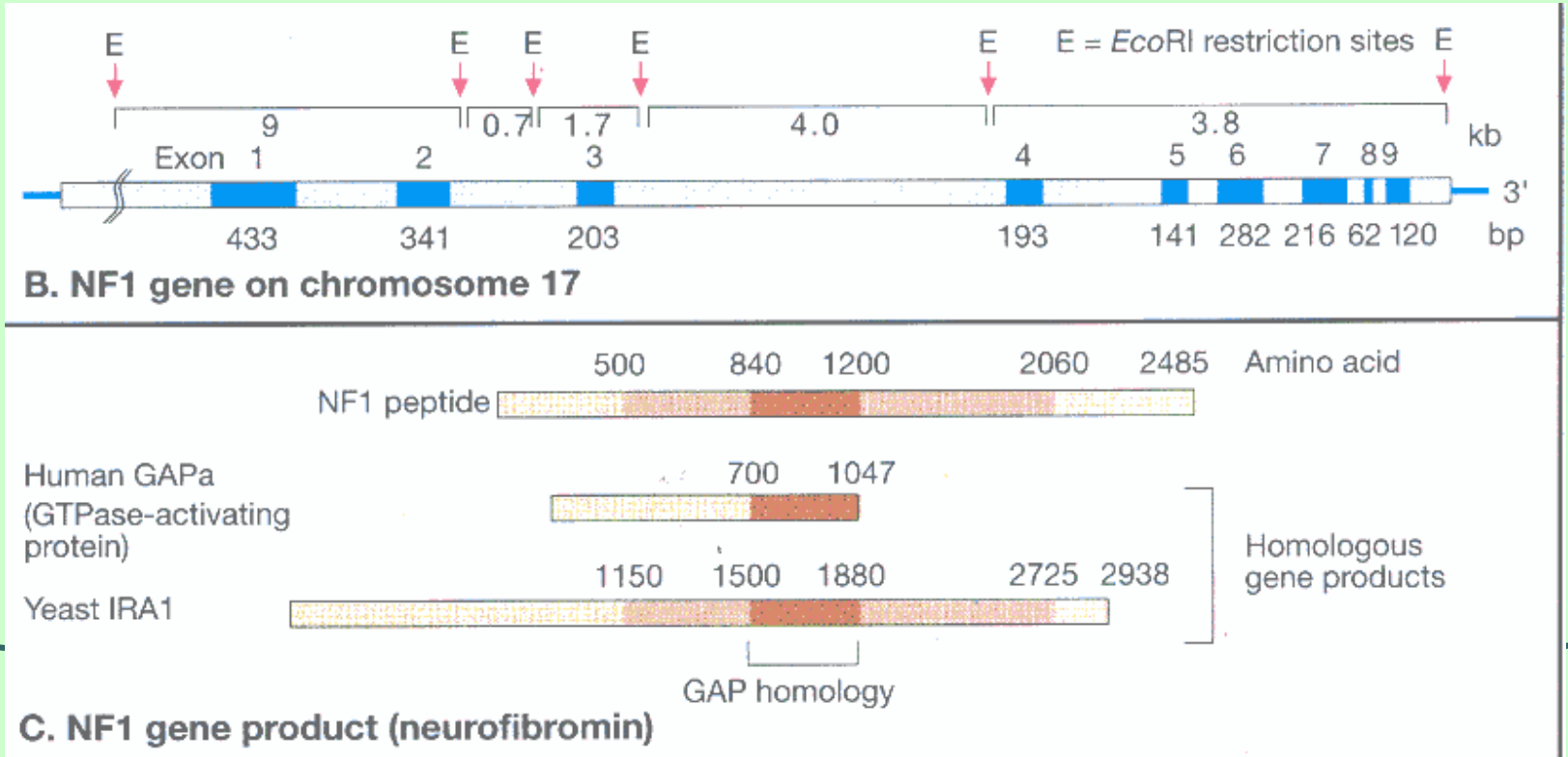
Direct RNA diagnostics


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

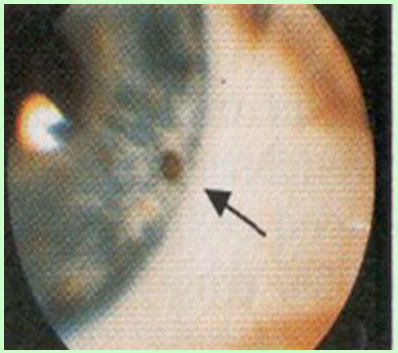


Neurofibromatosis type 1
von Recklinhausen disease

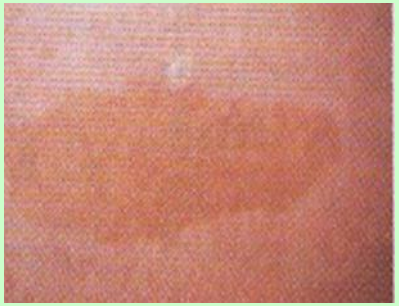
Autosomal dominant
Frequency 1:3000
Locus 17q

50% mutations de novo

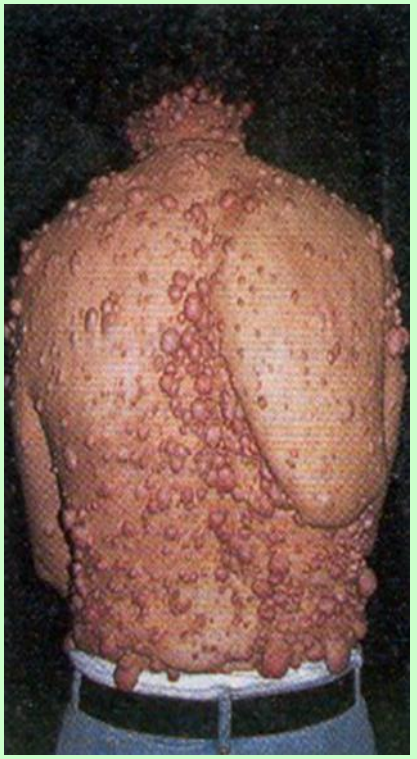
Predispositions to tumours of neural system



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

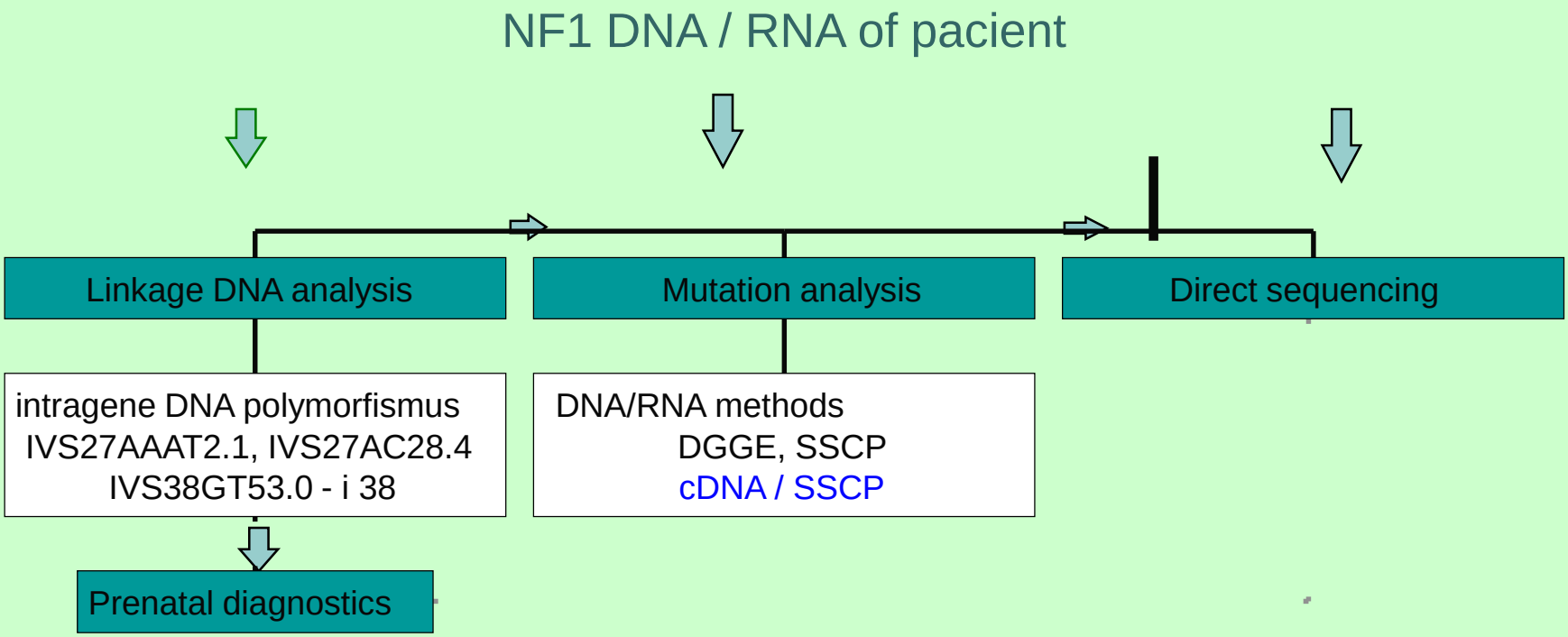
-length of gene (350 kb, 60 exons)

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

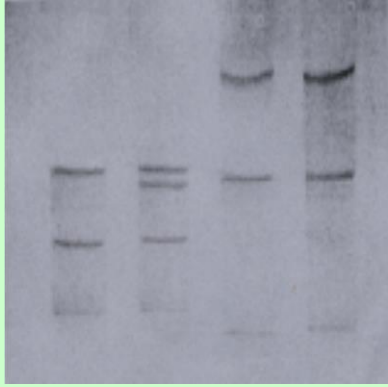
-unclear corelation between type of mutation and manifestation

-different clinical manifestation even in patients with the same mutation

Strategy of molecular – genetic testing of NF1 patients



cDNA - SSCP analysis



total RNA

RT

cDNA

PCR

NF1 cDNA (60 exons)

P1 P3 P5 P7 P9

P2 P4 P6 P8 P10

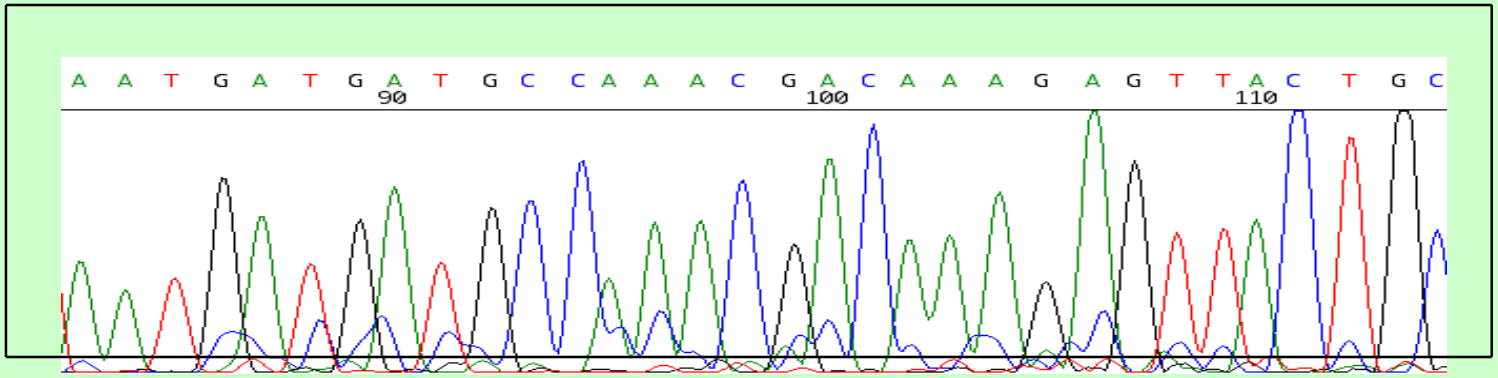
SSCP

Sequencing analysis

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

standard cDNA

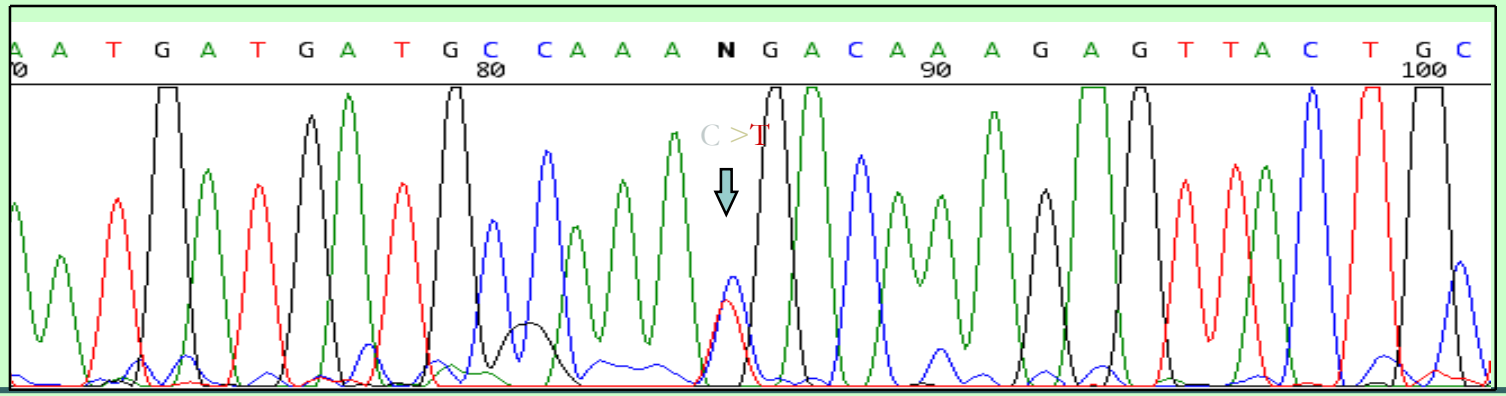
5’



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

- Capture or deletion of whole exone 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

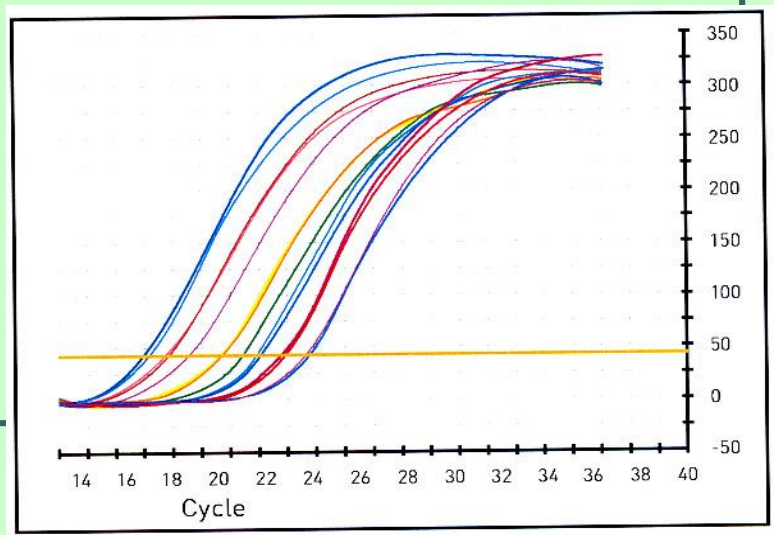
  snp – single ncleotide polymorphism detection

- QUANTITATIVE ANALYSIS

  amplicon amount detection

Detection methods

- Two types of Real-Time PCR detection chemistries:
- 1. Specific Sequence Detection- Distinguishes between a specific sequence of interest and non-specific products. Can be used to detect different alleles (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

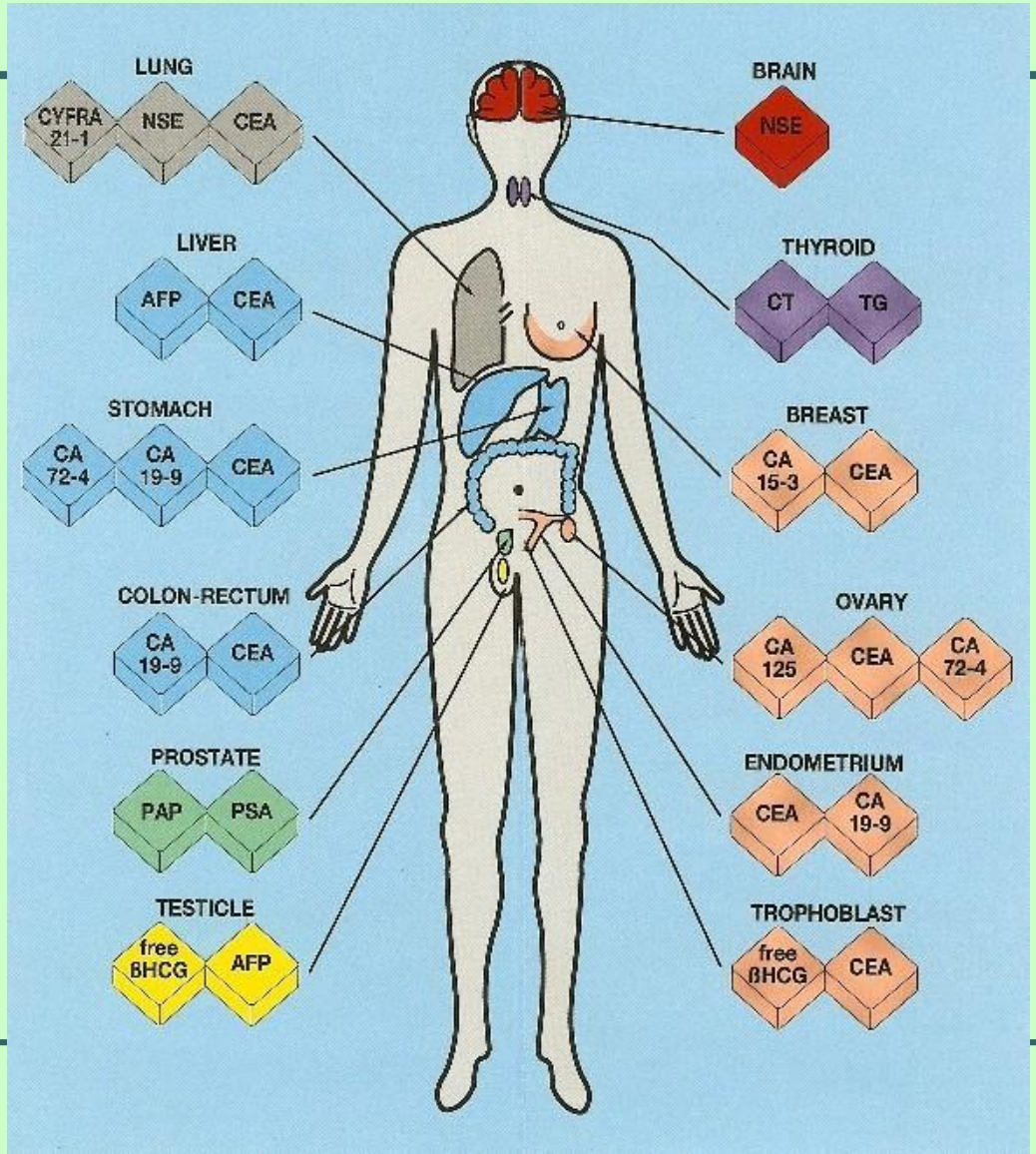
Ideal Oncomarker

- 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

Human Identity Testing

Applications:

Forensic cases - matching suspect with evidence

Paternity testing - identifying father

Historical investigations

Missing persons investigations

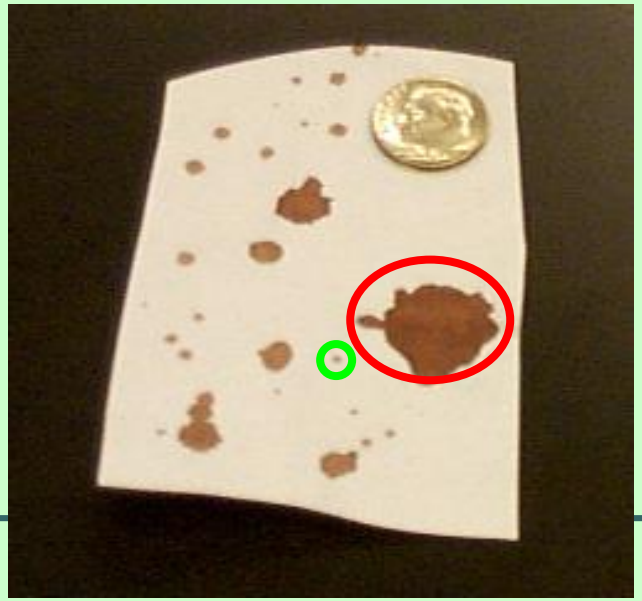
Mass disasters - putting pieces back together

Military DNA “dog tag”

Convicted felon DNA databases

Sources of Biological Evidence

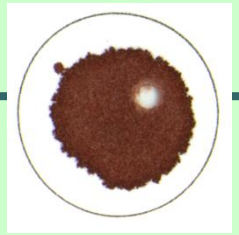
- Blood
- Semen
- Saliva
- Urine
- Hair
- Teeth
- Bone
- Tissue



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

Steps in DNA Analysis

- Collection
- Specimen Storage
- Extraction
- Quantitation
- Genotyping
- Interpretation of Results
- Database
  Storage & Searching

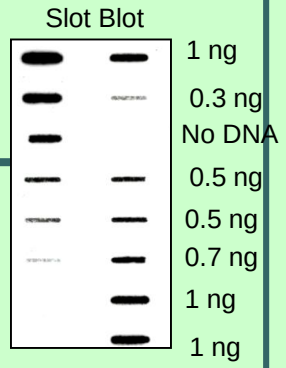


Blood Stain

Buccal swab

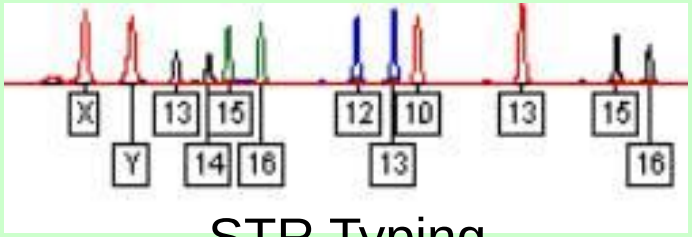
Sample Collection & Storage

DNA Extraction



DNA Quantitation

Multiplex PCR Amplification/RFLP



STR Typing

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

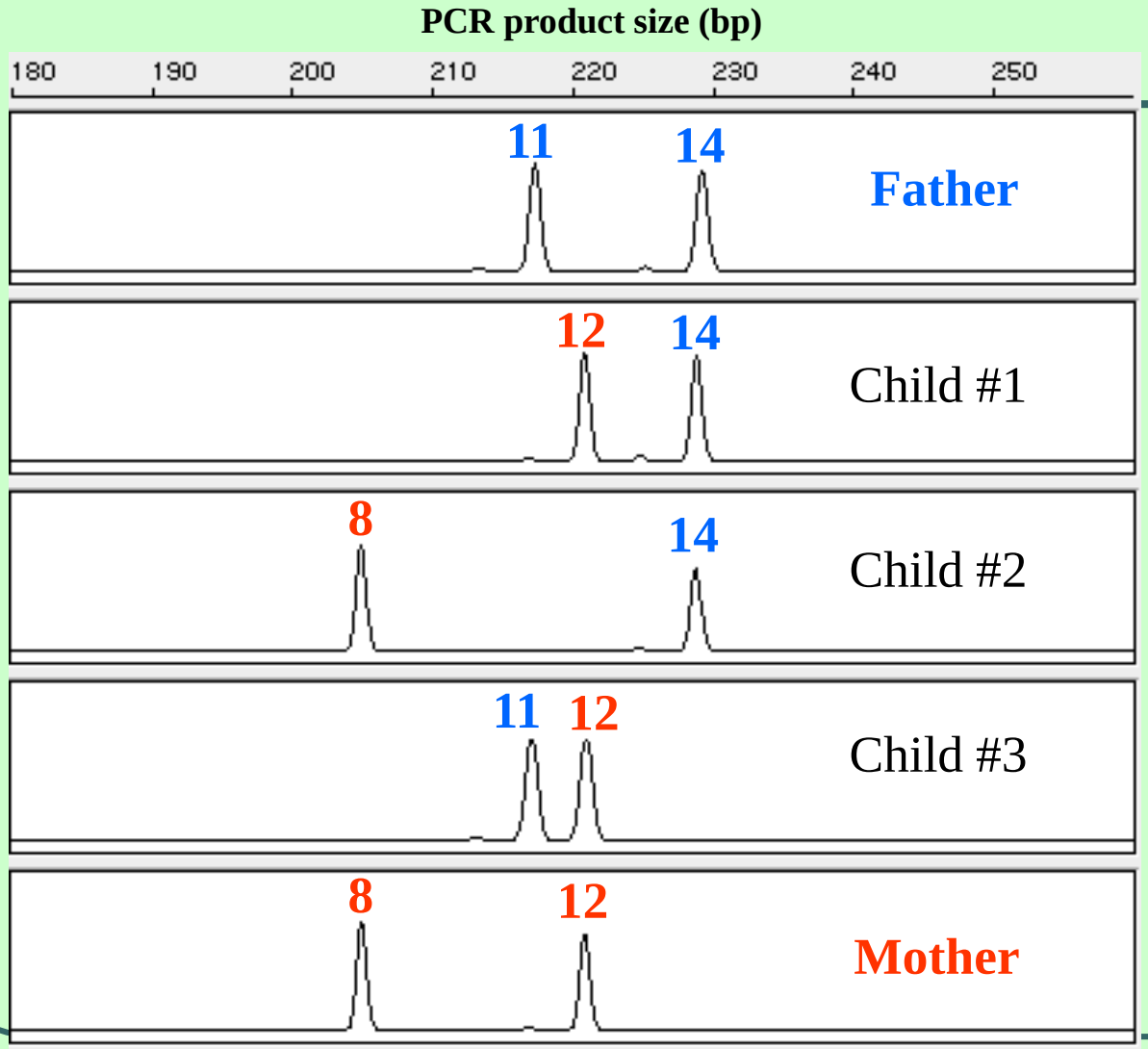
GCTAGTCGATGCTC**(G/A)**GCGTATGCTGTAGC

single nucleotide polymorphisms (SNPs)

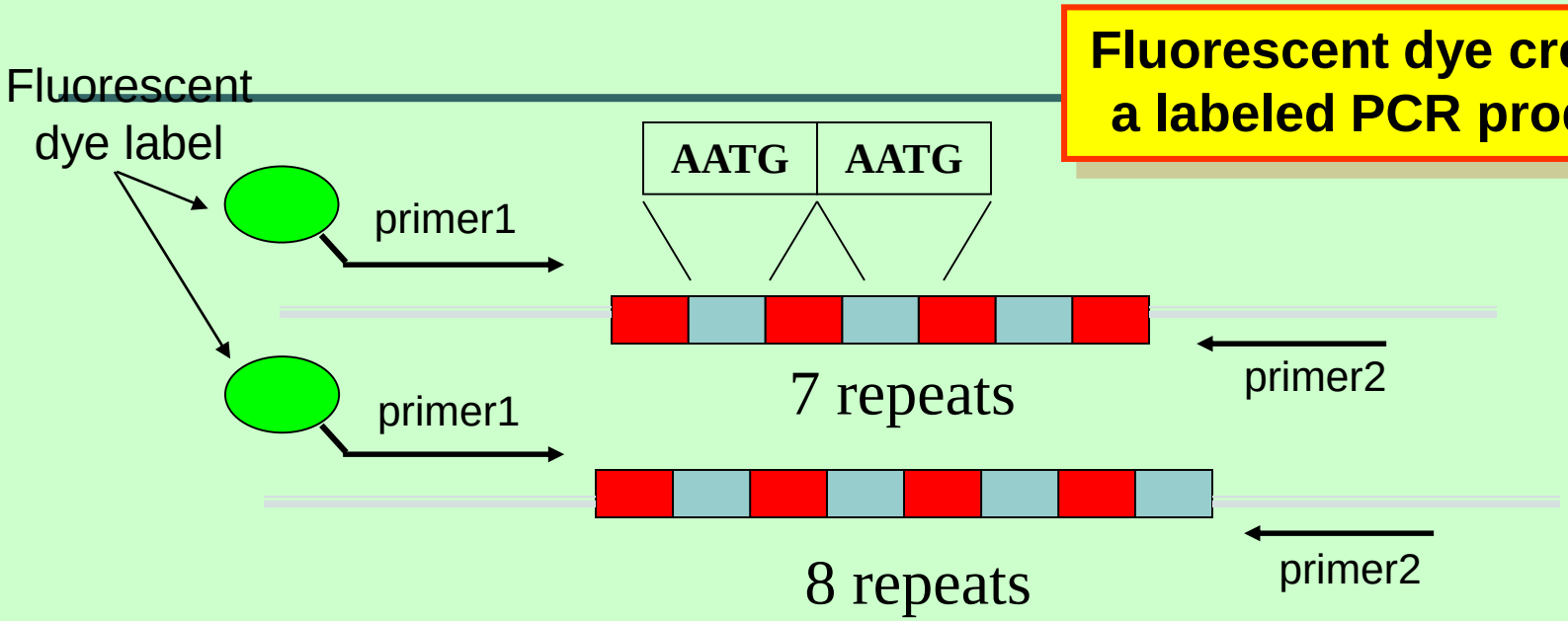
insertions/deletions

Paternity Testing

Family Inheritance of STR Alleles (D13S317)



Short Tandem Repeats (STRs)



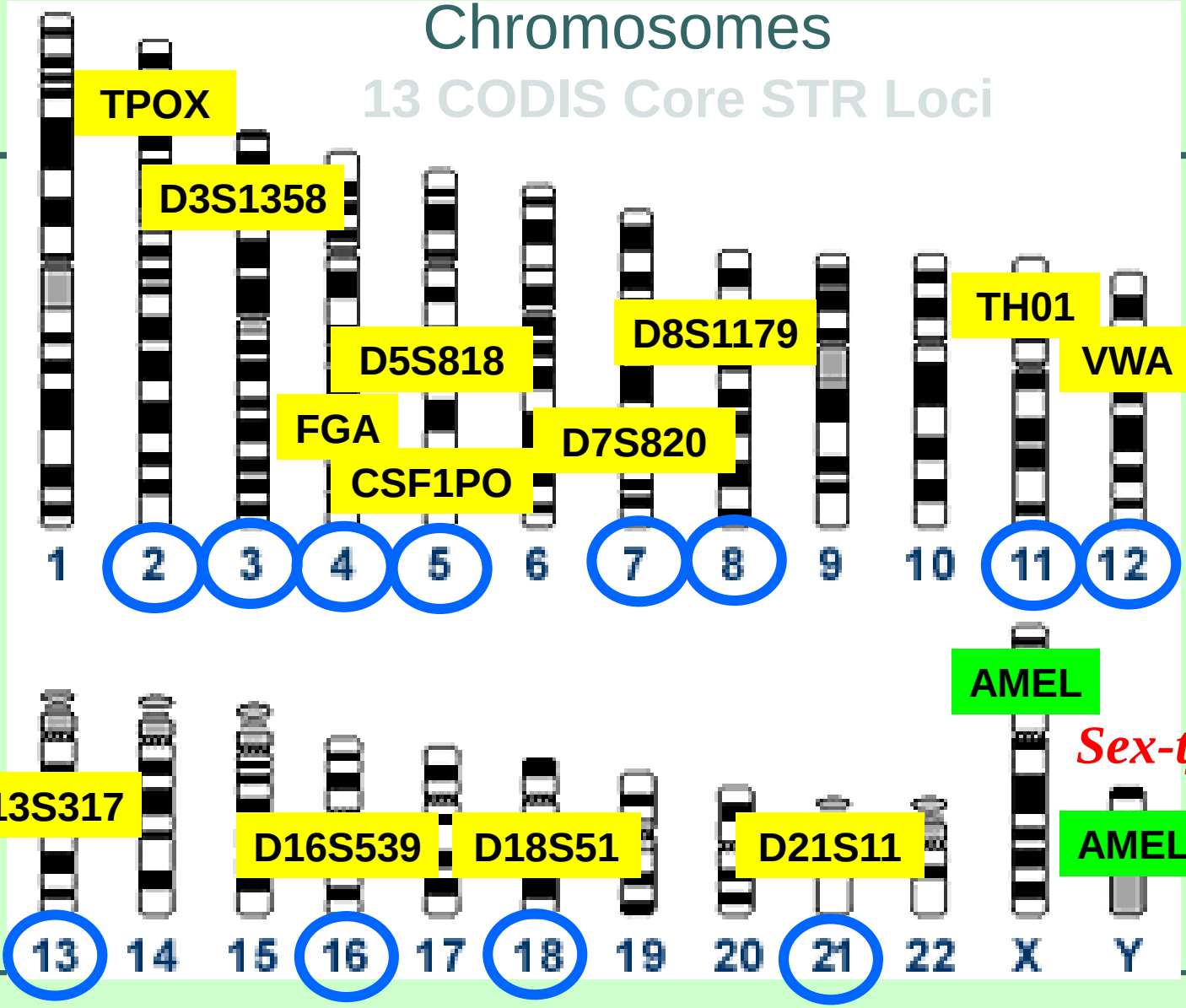
the repeat region is variable between samples while the flanking regions where PCR primers bind are constant

Homozygote = both alleles are the same length

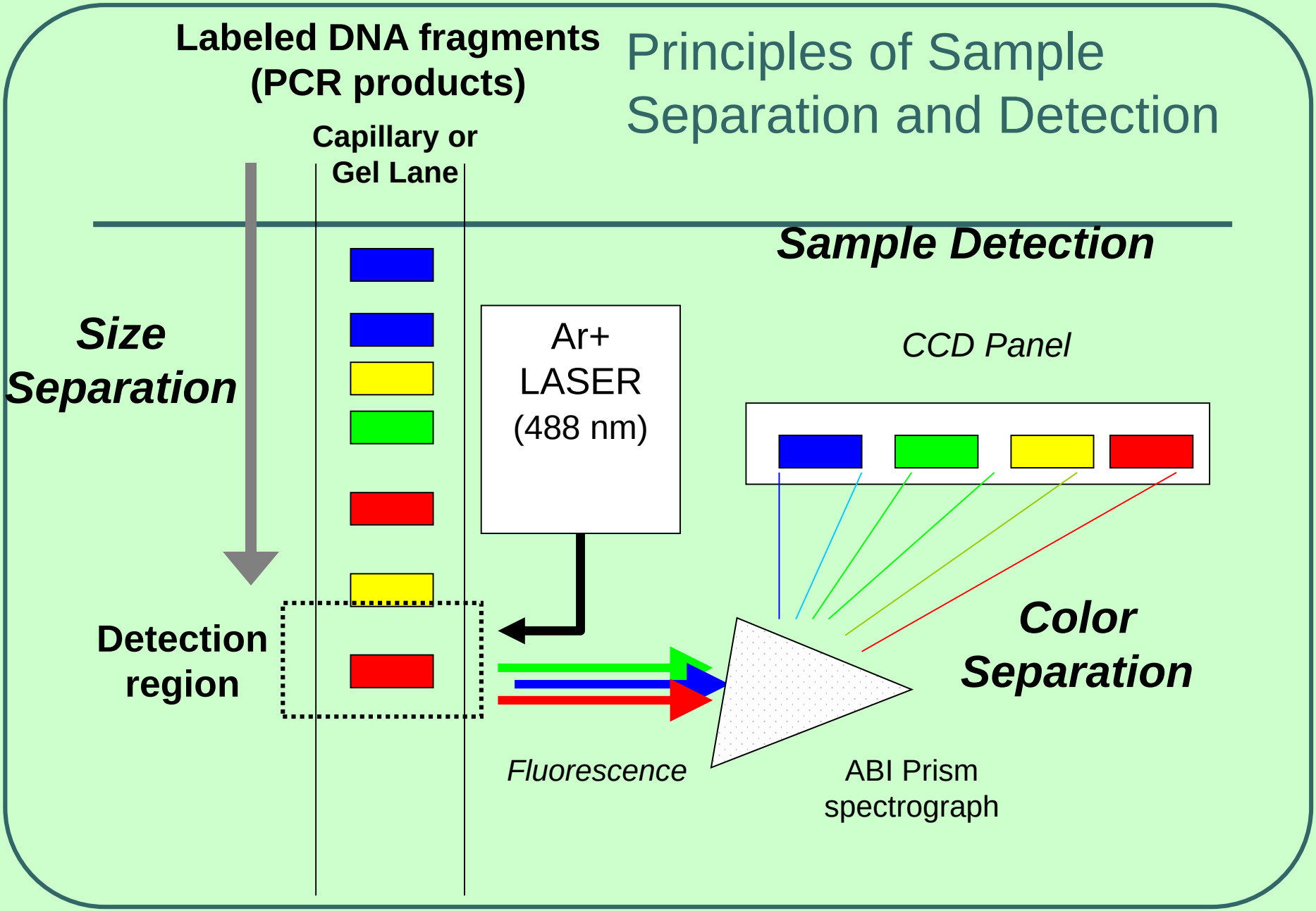
Heterozygote = alleles differ and can be resolved from one another

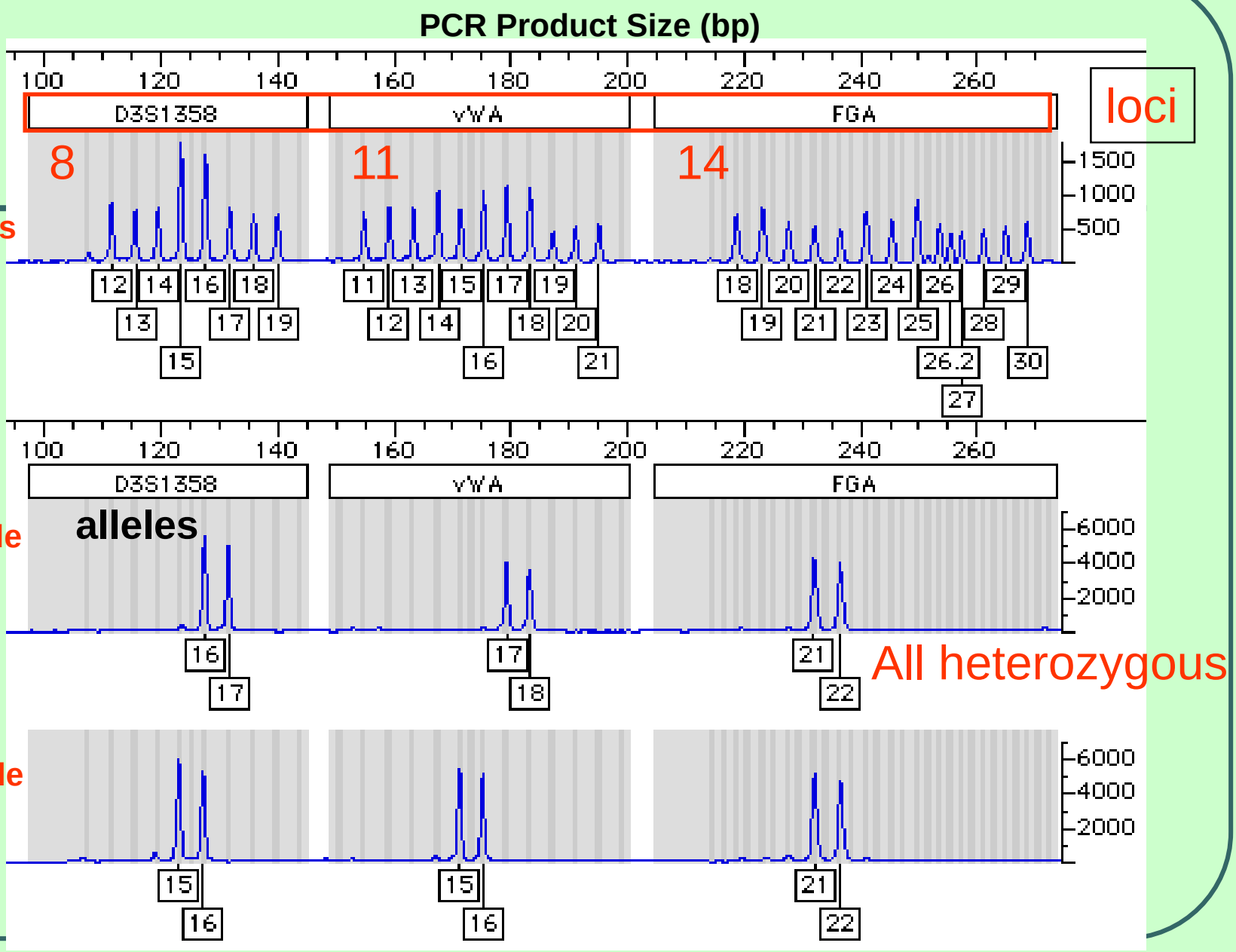
Primer positions define PCR product size

Position of Forensic STR Markers on Human Chromosomes

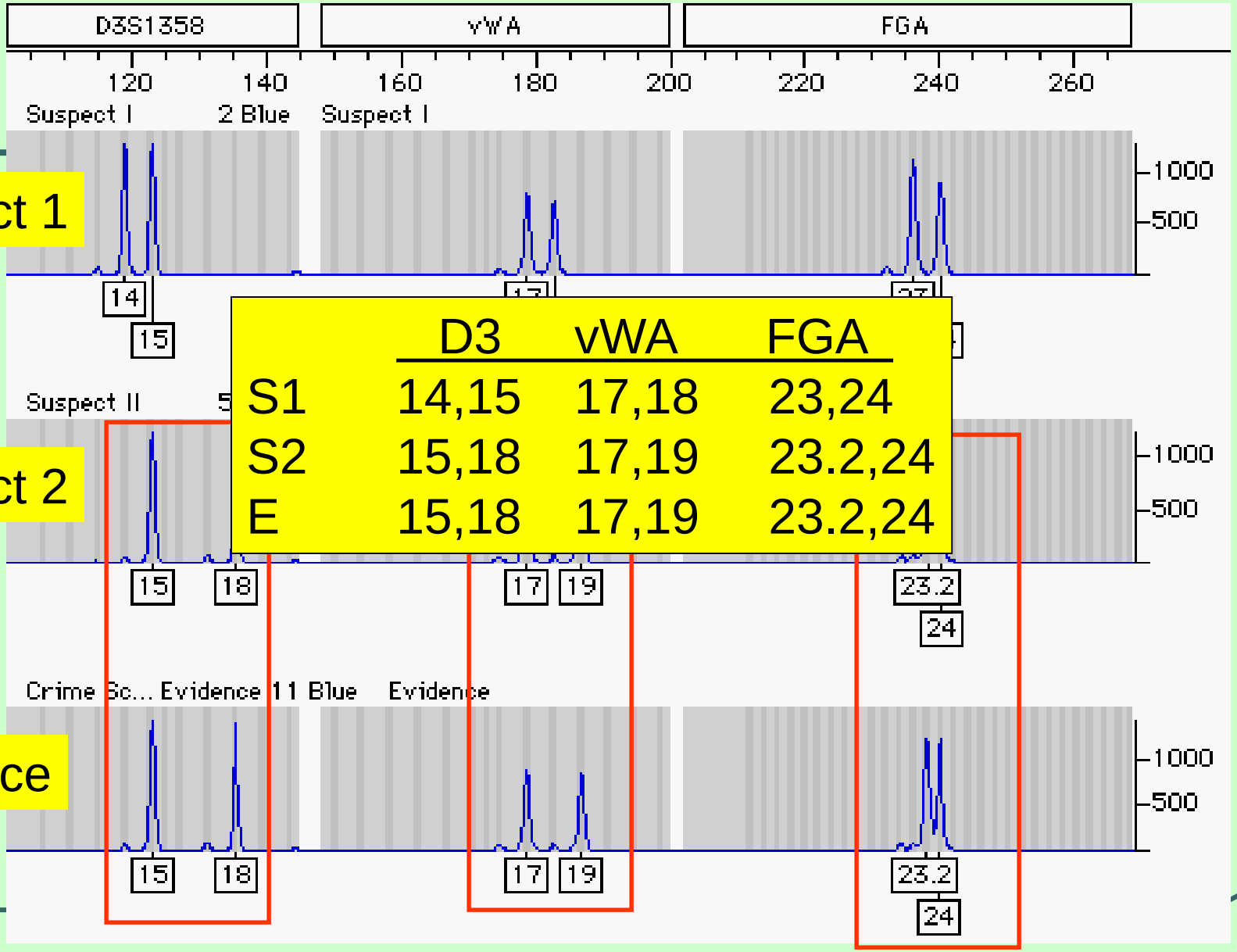


Principles of Sample Separation and Detection




PCR Product Size (bp)
100 120 140 160 180 200 220 240 260
D3S1358
vWA
FGA
loci
Allelic Ladders
8
11
14
1500
1000
500
12 14 16 18
13 17 19
15
11 13 15 17 19
12 14 18 20
16 21
18 20 22 24 26 29
19 21 23 25 28
26.2 30
27
Sample #1
alleles
6000
4000
2000
16 17
17 18
21 22
All heterozygous
Sample #2
15 16
15 16
21 22

Crime Scene - Two Suspects



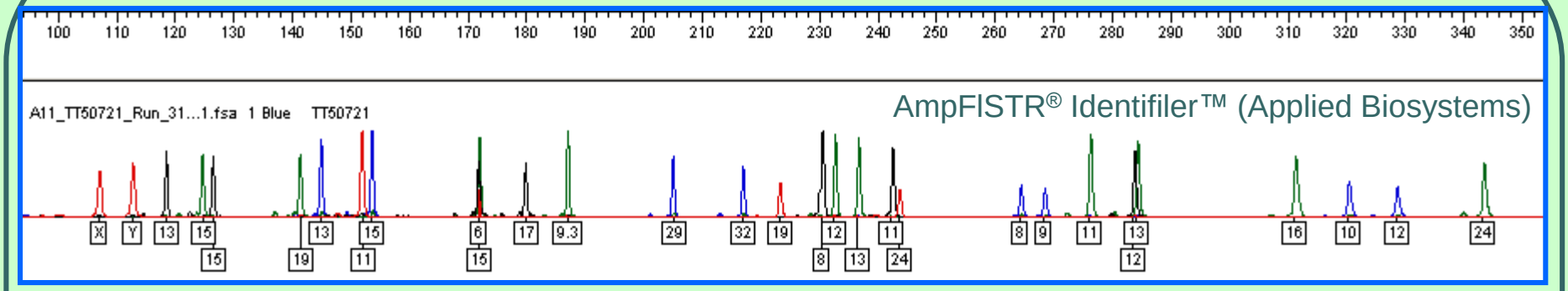
Suspect 1

Suspect 2

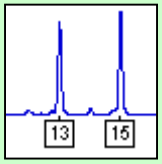
Evidence

	D3	vWA	FGA
S1	14,15	17,18	23,24
S2	15,18	17,19	23.2,24
E	15,18	17,19	23.2,24

Information is tied together with multiplex PCR and data analysis

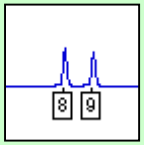


D8S1179

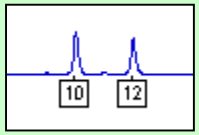


D21S11

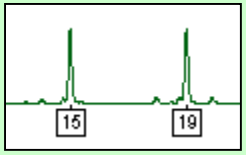
D7S820



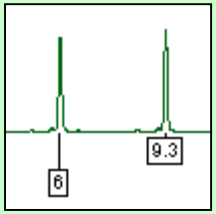
CSF1PO



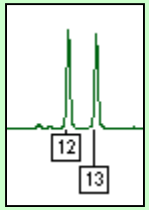
D3S1358



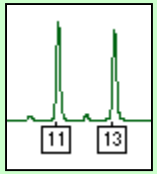
TH01



D13S317

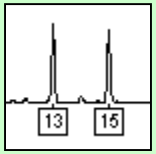


D16S539



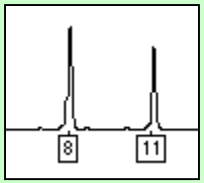
D2S1338

D19S433

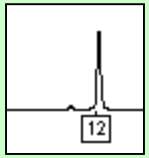


VWA

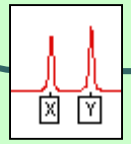
TPOX



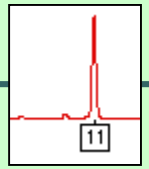
D18S51



AMEL



D5S818

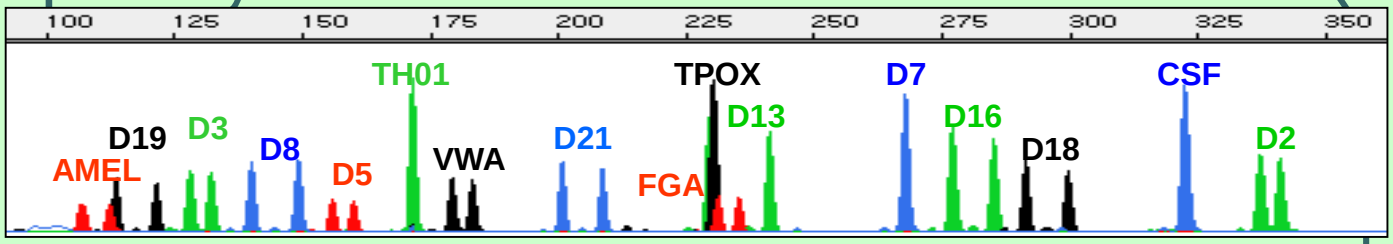


FGA

1 integrated analysis vs. 16 separate runs

DNA Profile Frequency

AmpFlSTR® 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^{15})**

CODIS DNA Database

Combined DNA Index System

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

- 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 × B2 (1 in 25 × 1 in 100) = 1 in 2500
C	3	1 in 320	A1 × B2 × C3 (1 in 25 × 1 in 100 × 1 in 320) = 1 in 800,000
D	4	1 in 75	A1 × B2 × C3 × D4 (1 in 25 × 1 in 100 × 1 in 320 × 1 in 75) = 1 in 60 million