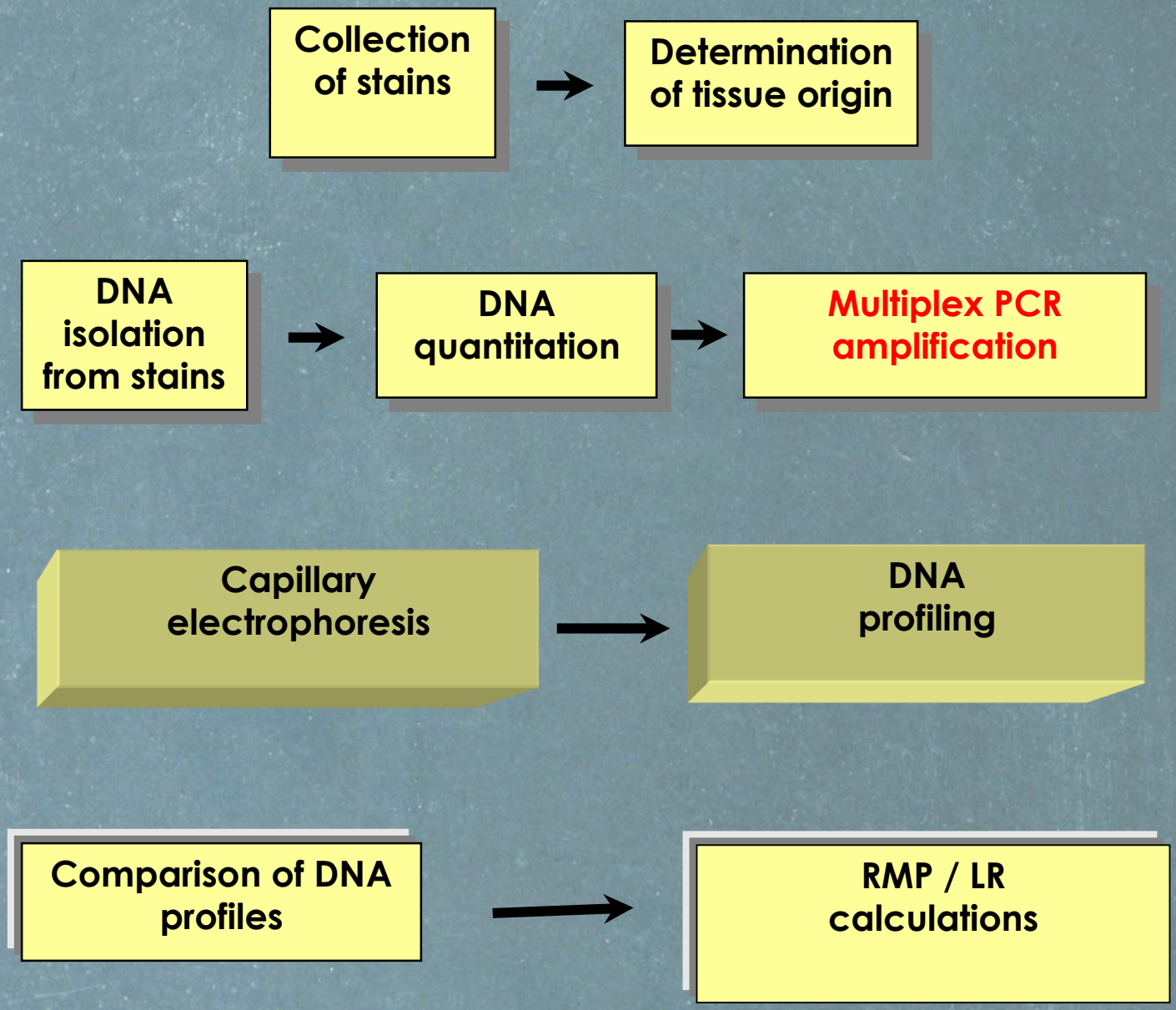
Forensic Genetics and Legal Medicine 2019-2020

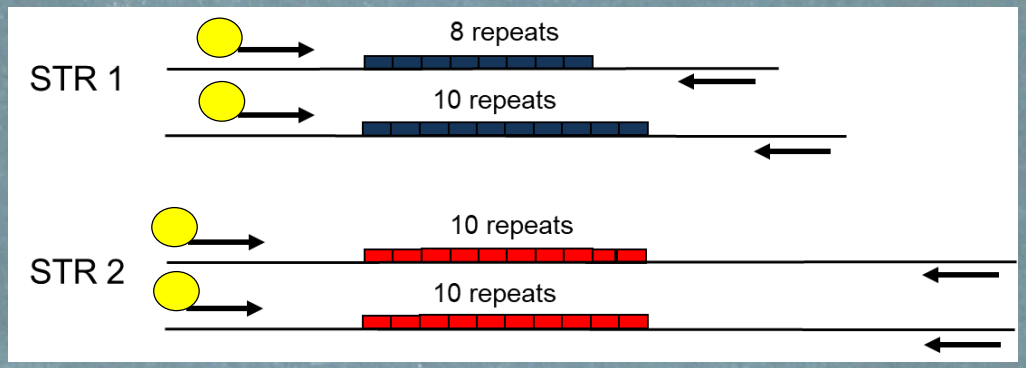
20th April 2020

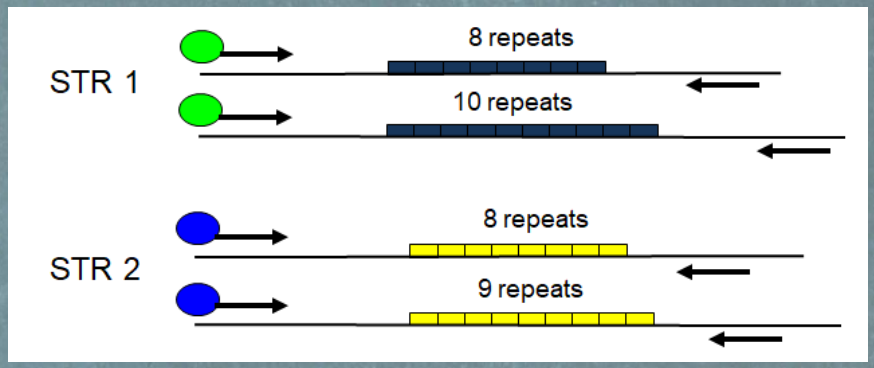
Standard typing techniques Part I


Collection of stains
Determination of tissue origin
DNA isolation from stains
DNA quantitation
Multiplex PCR amplification
Capillary electrophoresis
DNA profiling
Comparison of DNA profiles
RMP / LR calculations

✓ PCR primers targeting non polymorphic flanking regions can be used to amplify a specific STR in every individual

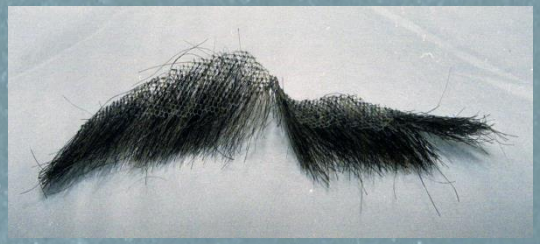
✓ since the amount of DNA available in forensic samples is often limited, in order to maximize informativity many STRs are amplified in the same test tube. For STRs with overlapping amplicon size range, different amplification products can be distinguished coupling each PCR primer pair with a fluorescent dye having a different color (**multiplex PCR amplification**)

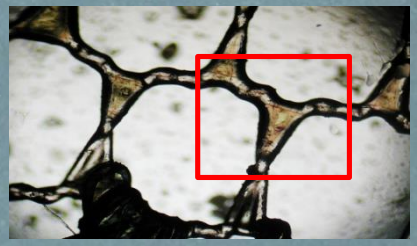


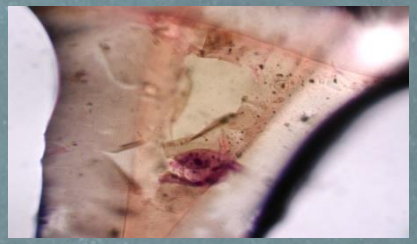


Sensitivity of PCR

✓ each nucleated cell contains 0.006 nanograms (ng) of genomic DNA
✓ about 0.1 ng of genomic DNA (the content of 16 cells) is enough to obtain PCR products for STRs from forensic stains leading to highly reliable DNA profiles







Type of sample	Amount of DNA
Liquid blood	20,000-40,000 ng/mL
stain	250-500 ng/cm²
Liquid semen	150,000-300,000 ng/mL
Postcoital vaginal swab	10-3,000 ng/swab
Hair (with root)	
Plucked	1-750 ng/root
Shed	0-1 ng/root
Liquid saliva	1,000-10,000 ng/mL
Oral swab	100-1500 ng/swab
Urine	1-20 ng/mL
Bone	3-10 ng/mg
Tissue	50-500 ng/mg

[a]Quantity of DNA recovered from evidentiary samples is significantly affected by environmental factors.

PCR

An in vitro reproduction of in vivo DNA replication

Ingredients

✓ template DNA: original DNA isolated from the stain (optimal amount 0.3-0.5 ng)

✓ at least one pair of PCR primers: short synthetic stretches of DNA that hybridize ("anneal") to template DNA, each on one side of the target STR region

- 18-30 bps
- Tm 55-72°C
- ΔTm between F and R primer ≤ 5°C
- % GC 40-60%
- No haipins
- No primer dimers
- No long runs of the same base

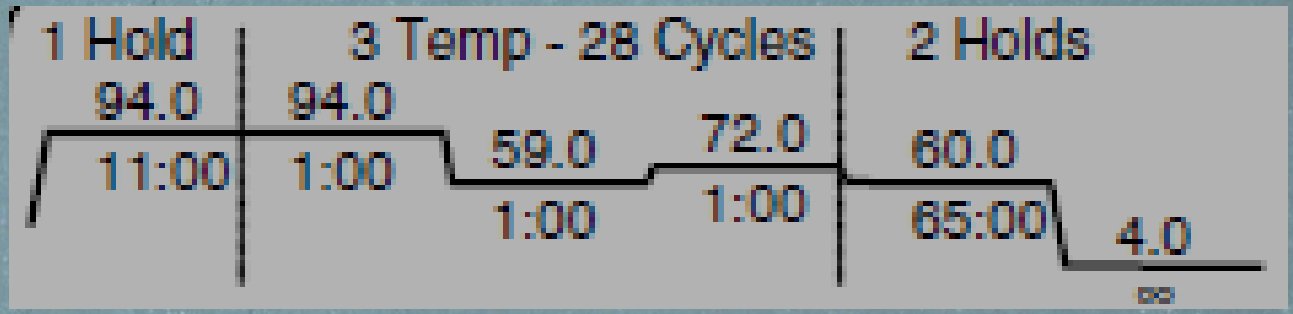
http://primer3.ut.ee/

✓ Taq DNA polymerase: a thermostable enzyme isolated from bacteria living in hot springs which can tolerate the high temperatures involved in PCR

✓ a saline buffer (TRIS-HCl pH 8.3) including:

• a mix of the 4 deoxynucleotide triphosphates (dNTPs), which are incorporated in the nascent DNA strand during replication

• a critical concentration of magnesium chloride ($MgCl_2$), which is necessary for optimal primer-template annealing and Taq polymerase functioning



Hot-start: Initial activation of chemically modified Taq (rendered inactive unitl heated)

Denaturation: DNA double helix opens up

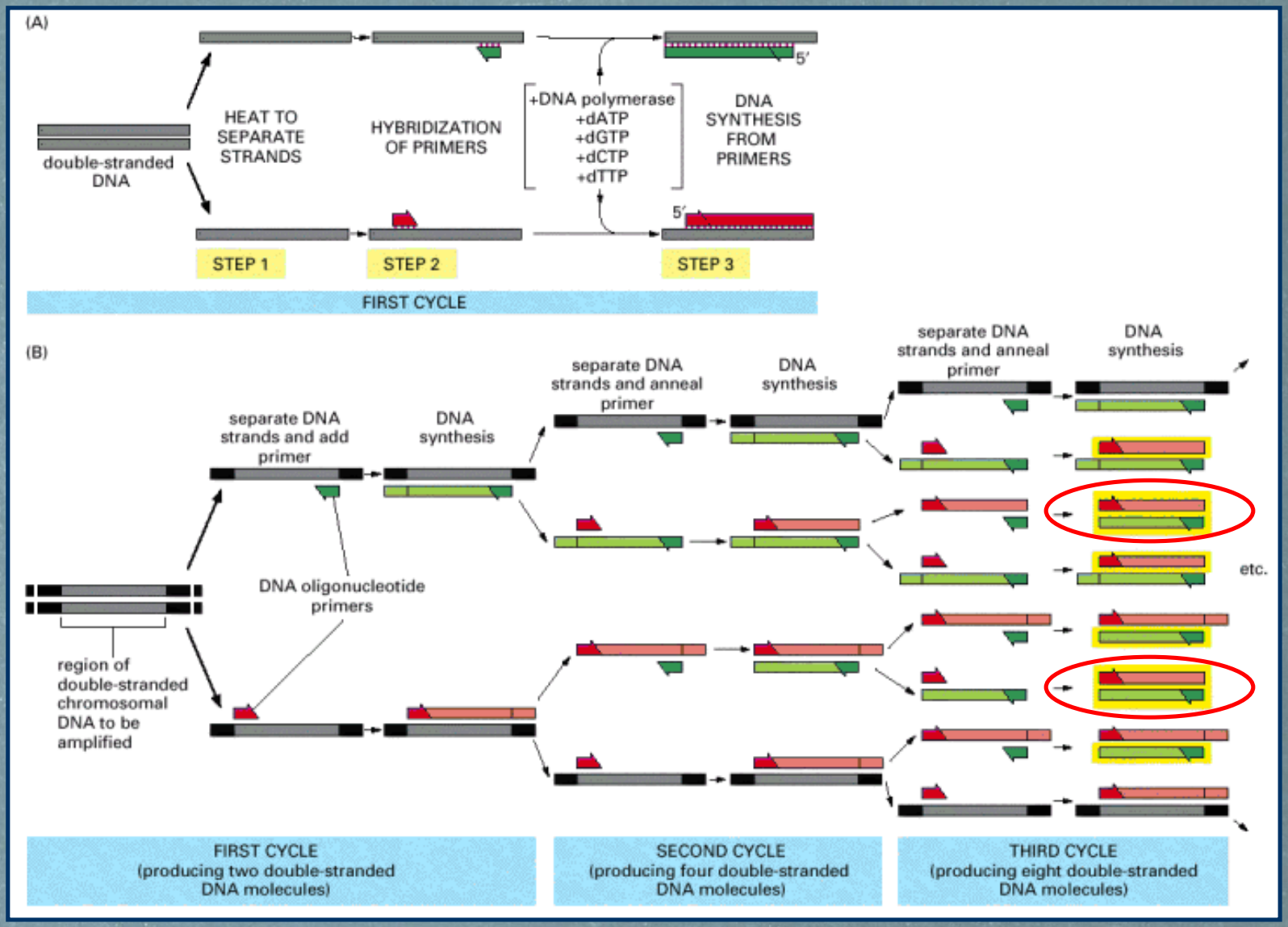
Annealing: PCR primers bind to complementary bases on template DNA strands

It depends on primers' melting temperature (Tm)

Extension: Taq polymerase adds dNTPs complementary to template DNA strand starting from 3' of primer

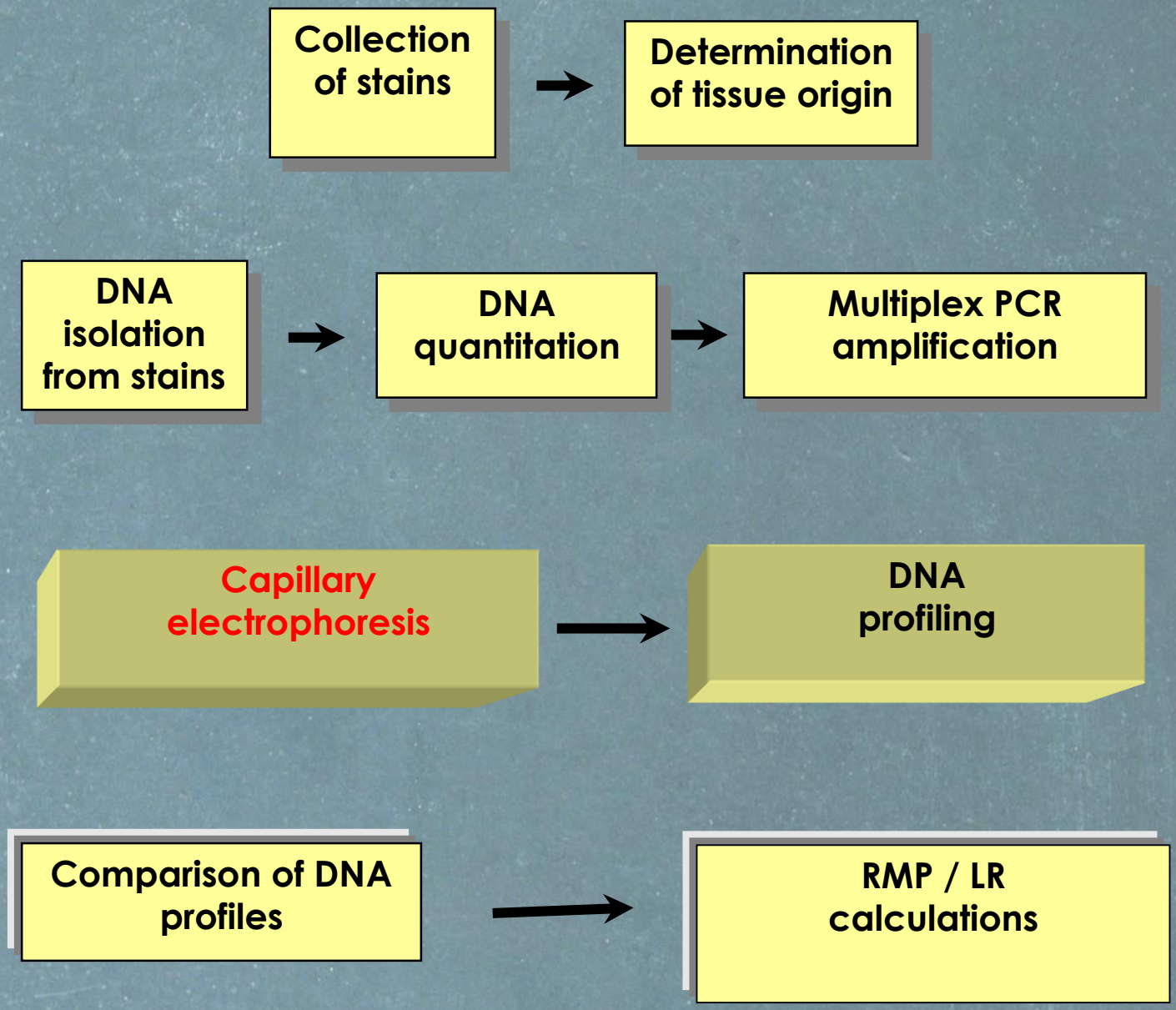
Final extension (to reduce non-template addition artifacts)

DNA can be kept refrigerated in the thermalcycler for several ours



✓ forensic PCR protocols normally include 28-32 cycles: $2^{26} > 6*10^6$; $2^{30} > 1*10^9$

✓ even though PCR efficiency is never 100%, million/billion of copies of the target STR region are produced at the end of the process, allowing much easier down-stream analysis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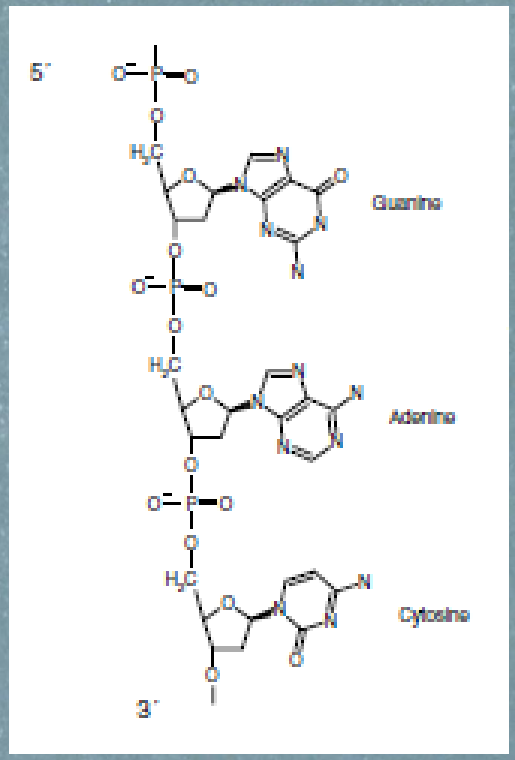
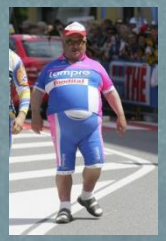
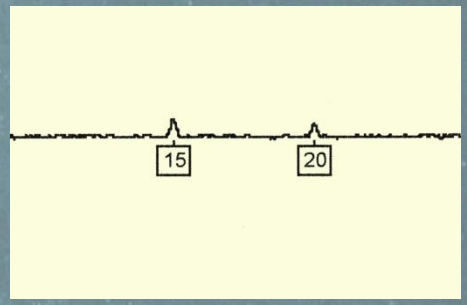
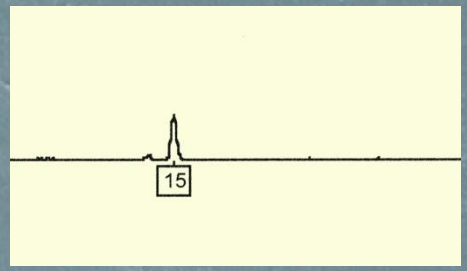
Electrophoresis









t

\- +

DNA is a negatively charged molecule in a neutral pH environment

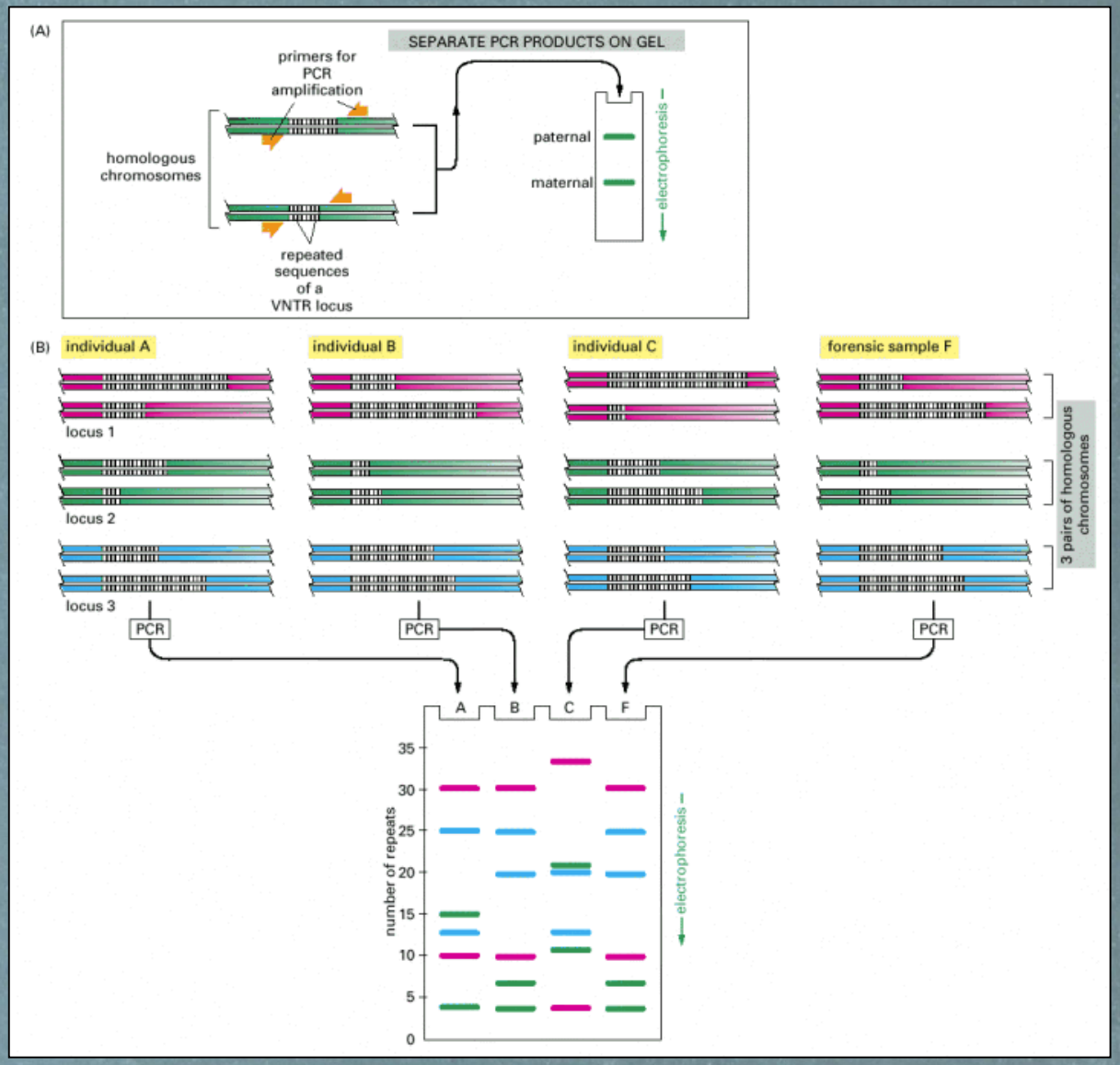
✓ If subjected to voltage, amplified DNA fragments will migrate towards the positive electrode

✓ shorter fragments will reach the positive electrode in less time (t) than larger fragments

✓ Alleles within an STR differ in lenght, and therefore in electrophoretic migration time
✓ Even for STRs having equal lenght of PCR products, simultaneous amplification and discrimination of genotypes is possible by means of the flurescent dyes coupled to primers

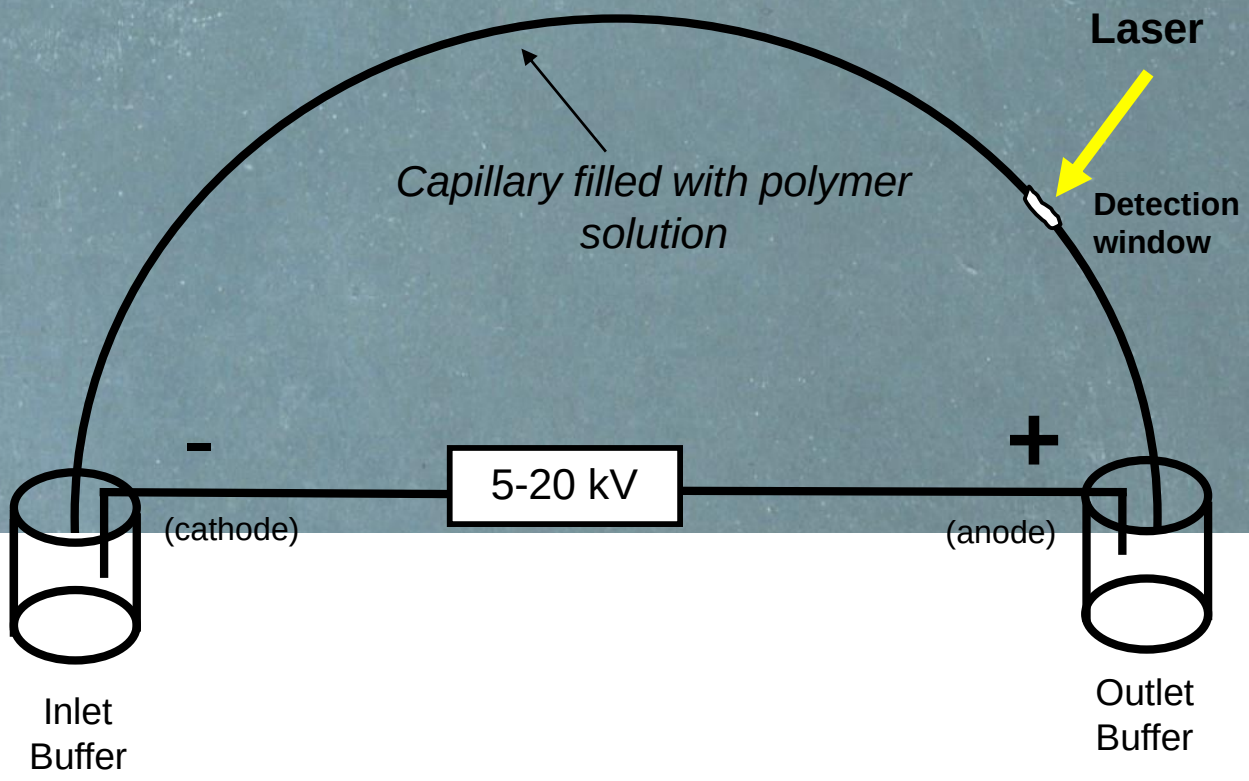
Current forensic systems allow the combination of 4 to 5 different dyes
In multiplex PCR reaction

Originally electrophoresis was performed seeding PCR products in vertical slab gels

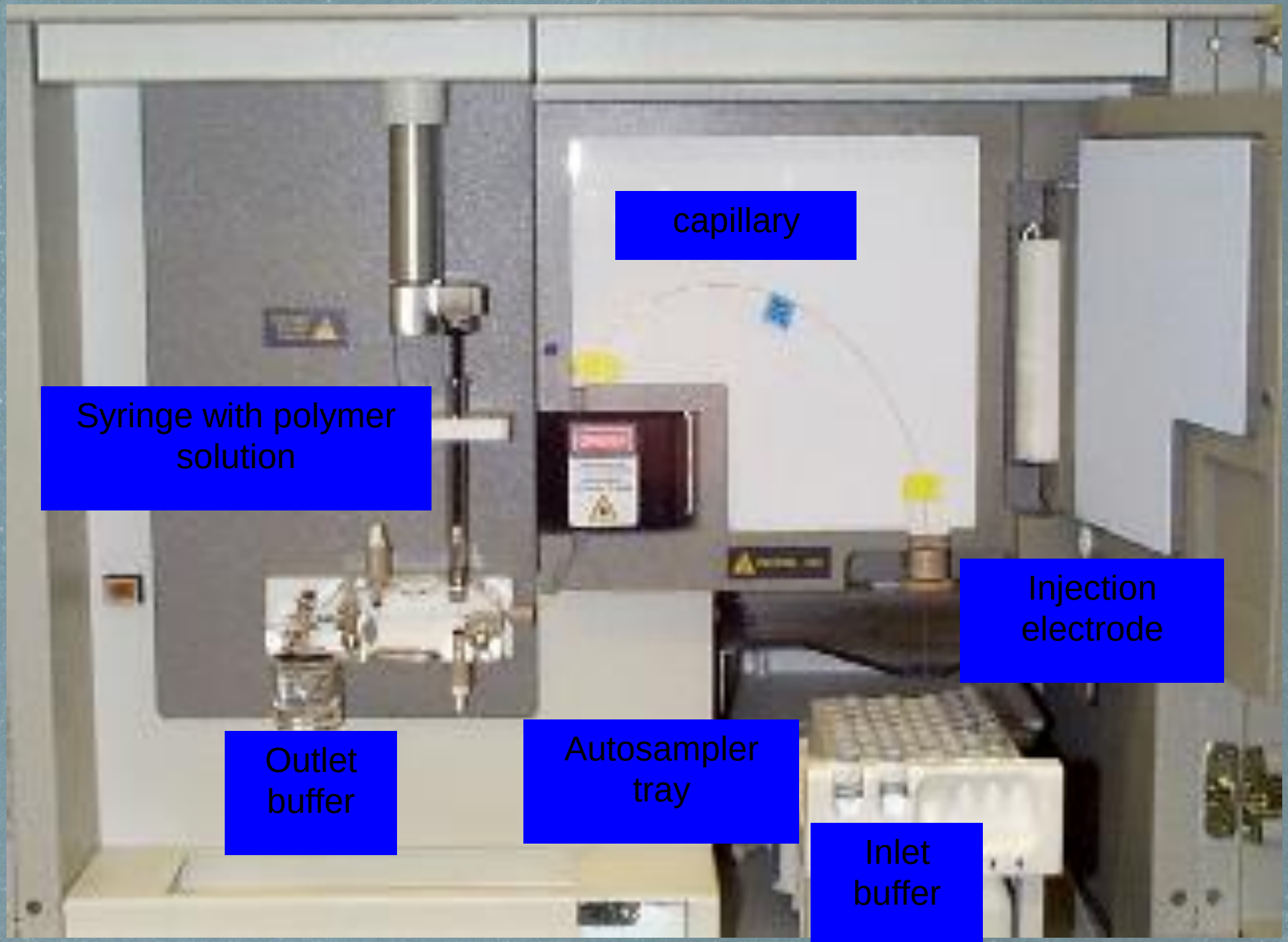


Capillary electrophoresis (CE)

Nowadays, electrophoresis is performed injecting PCR products inside tiny plastic capillaries filled with a gel matrix (polymer)



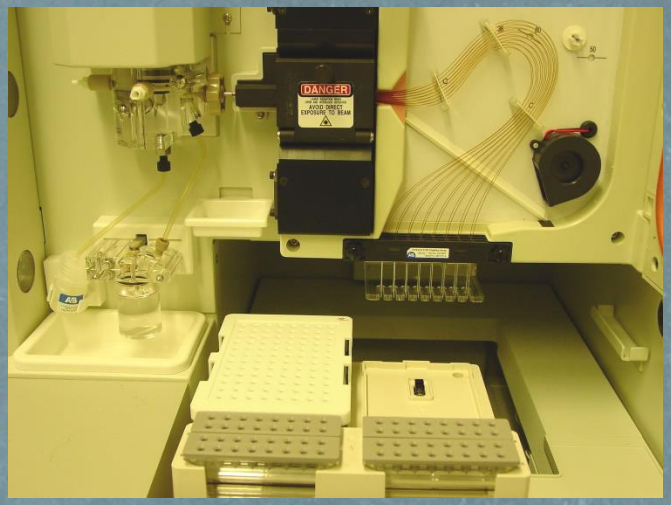
Single capillary system



High throughput multicapillary systems

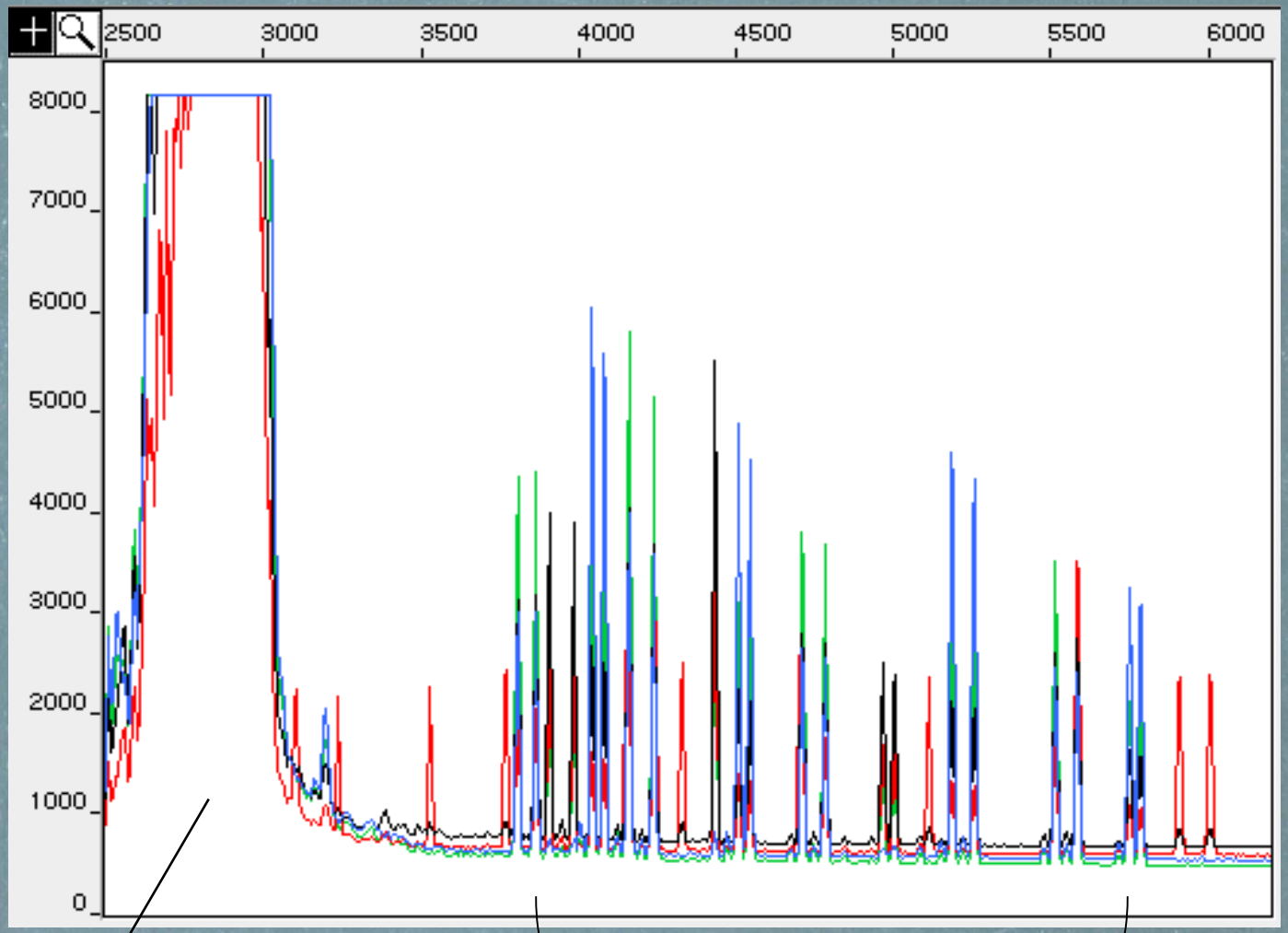
8 capillaries system

4 capillaries fully integrated system



16 capillaries system

✓ The laser hitting the detection window causes fluorescence emission, whenever a PCR fragment (coupled to its dye-labelled primer) reaches the window

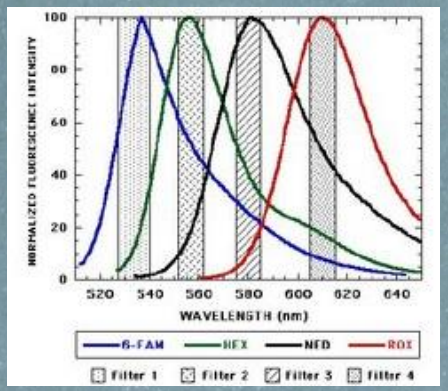


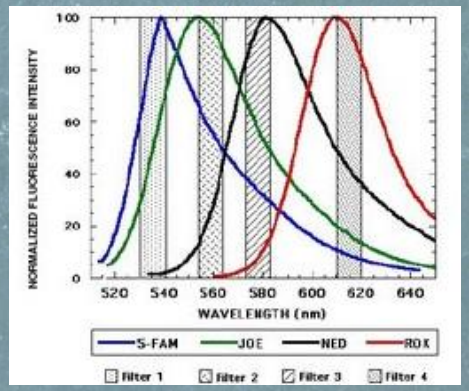
Electropherogram (epg)

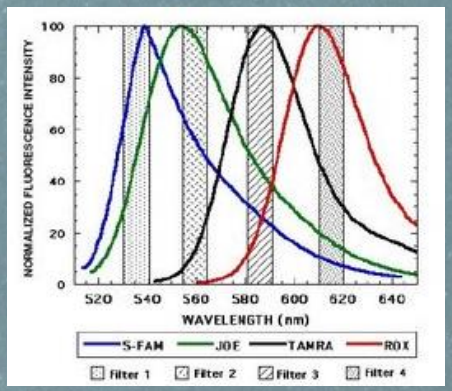
Primers not incorporated in PCR reaction

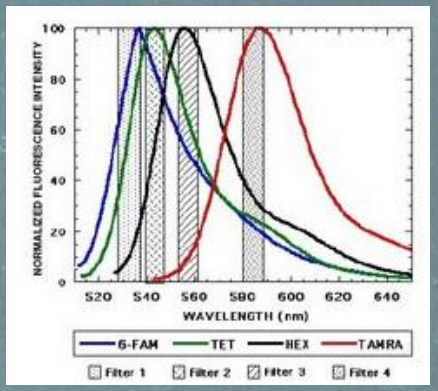
PCR fragments

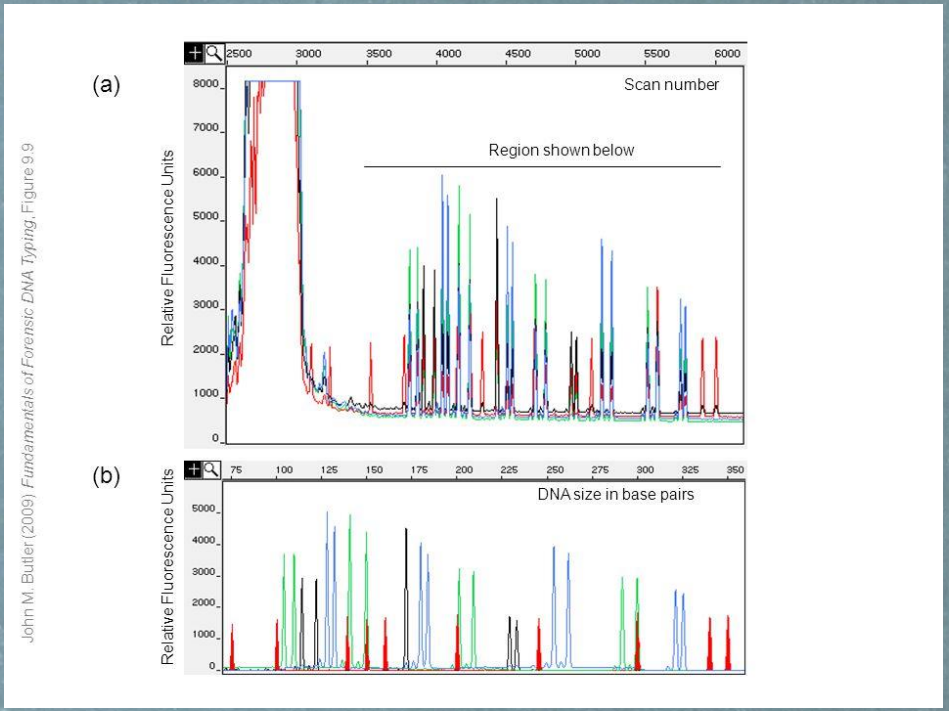
✓ Signal is cleaned by reading florescent emission only in specific wavelenght frames, chosen according to the combination of dyes used for PCR primers labelling (virtual filter) and minimizing overlap





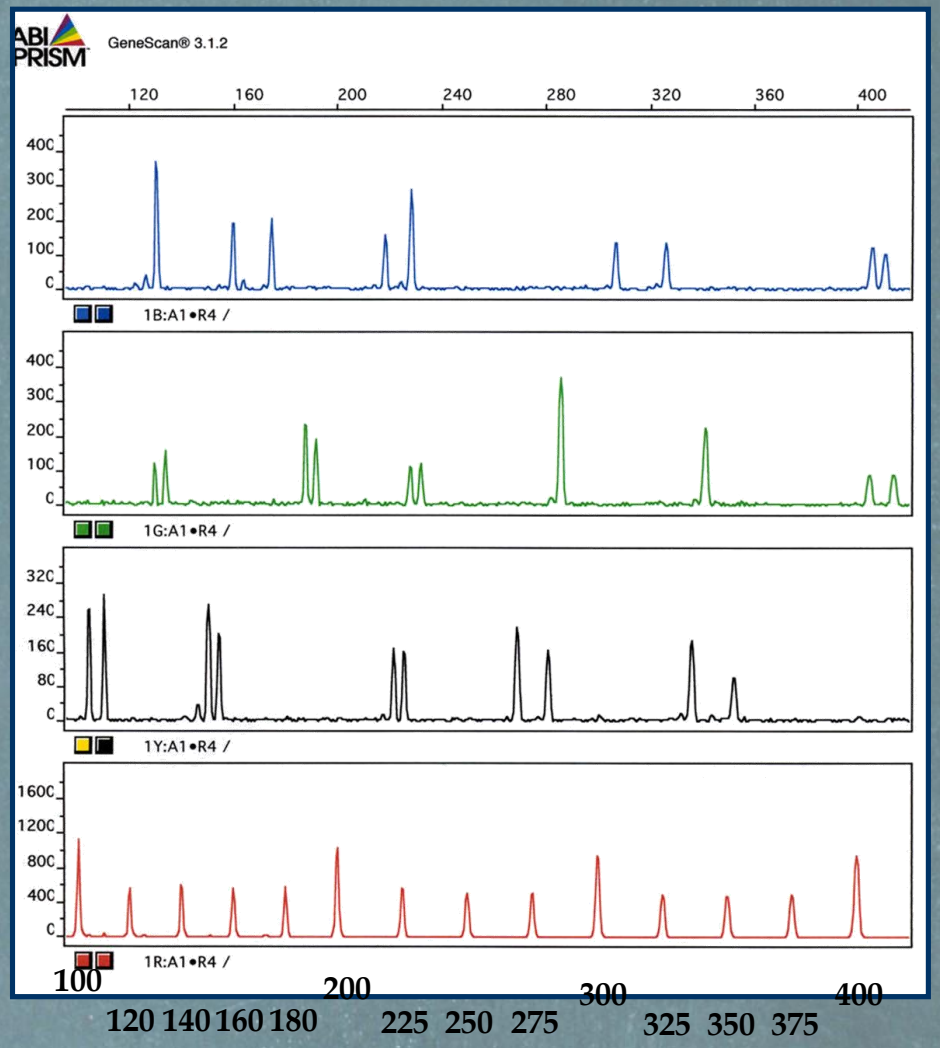




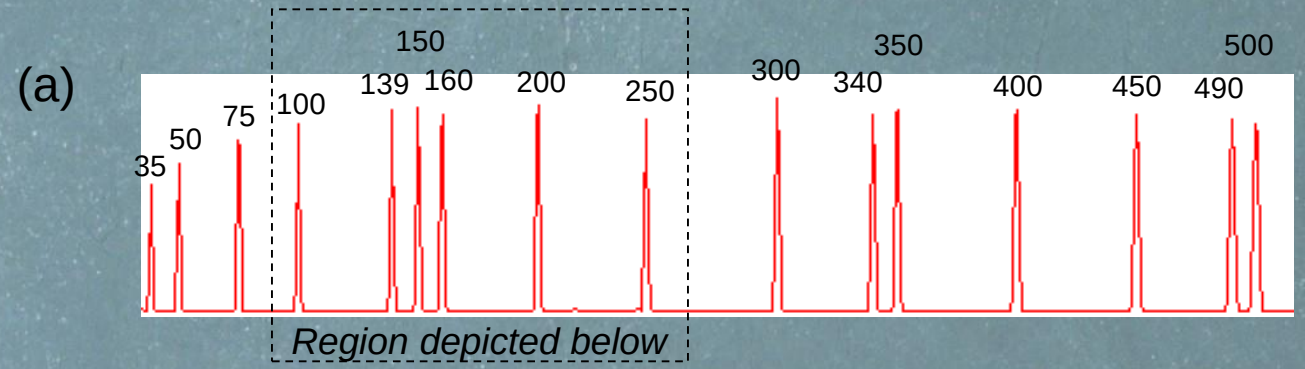


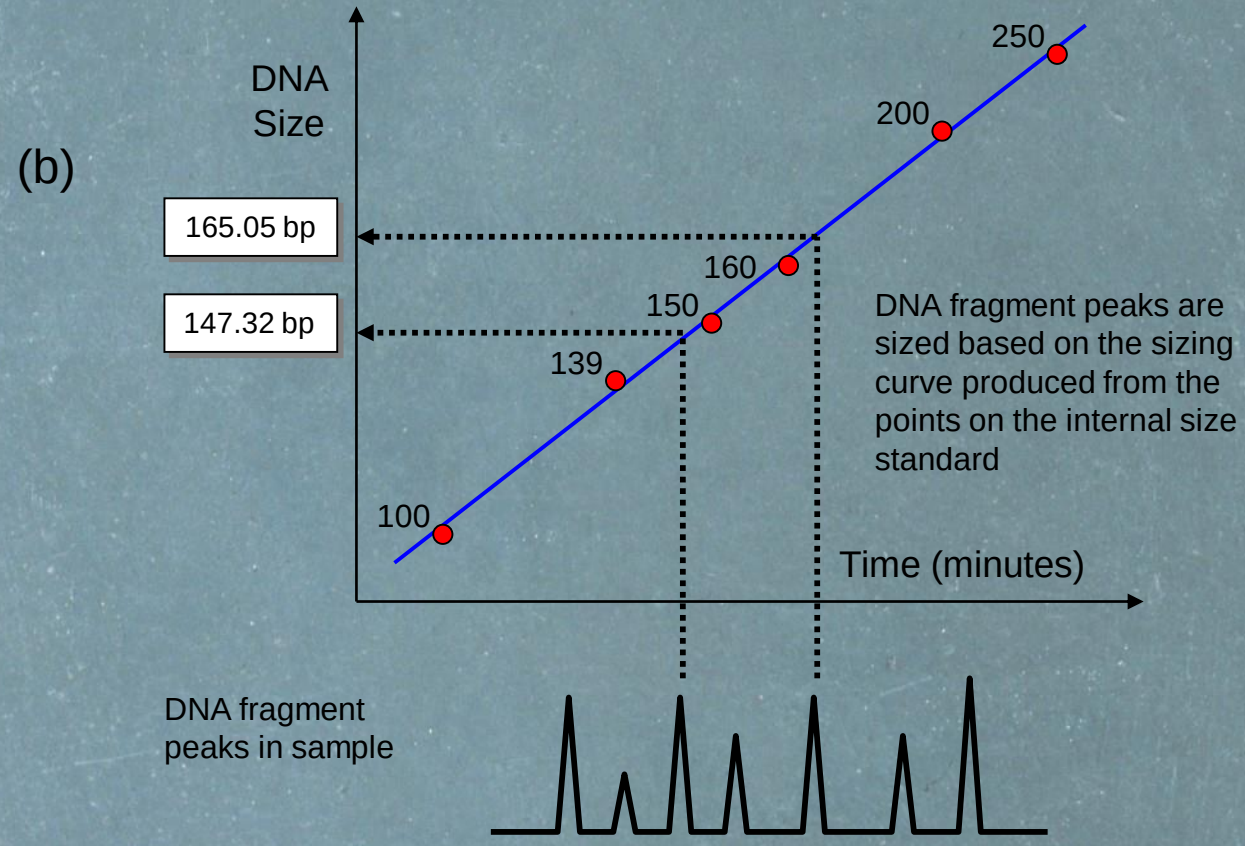
✓ cleaned signal can be graphically separated according to dye-label color

- ✓ Before being injected in the capillary, the PCR products obtained from each DNA sample are premixed with a combination of synthetic DNA fragments of known lenght (**internal size standard, ILS**)
- ✓ ILS is labelled with a fluorescent dye different from those included in multiplex PCR

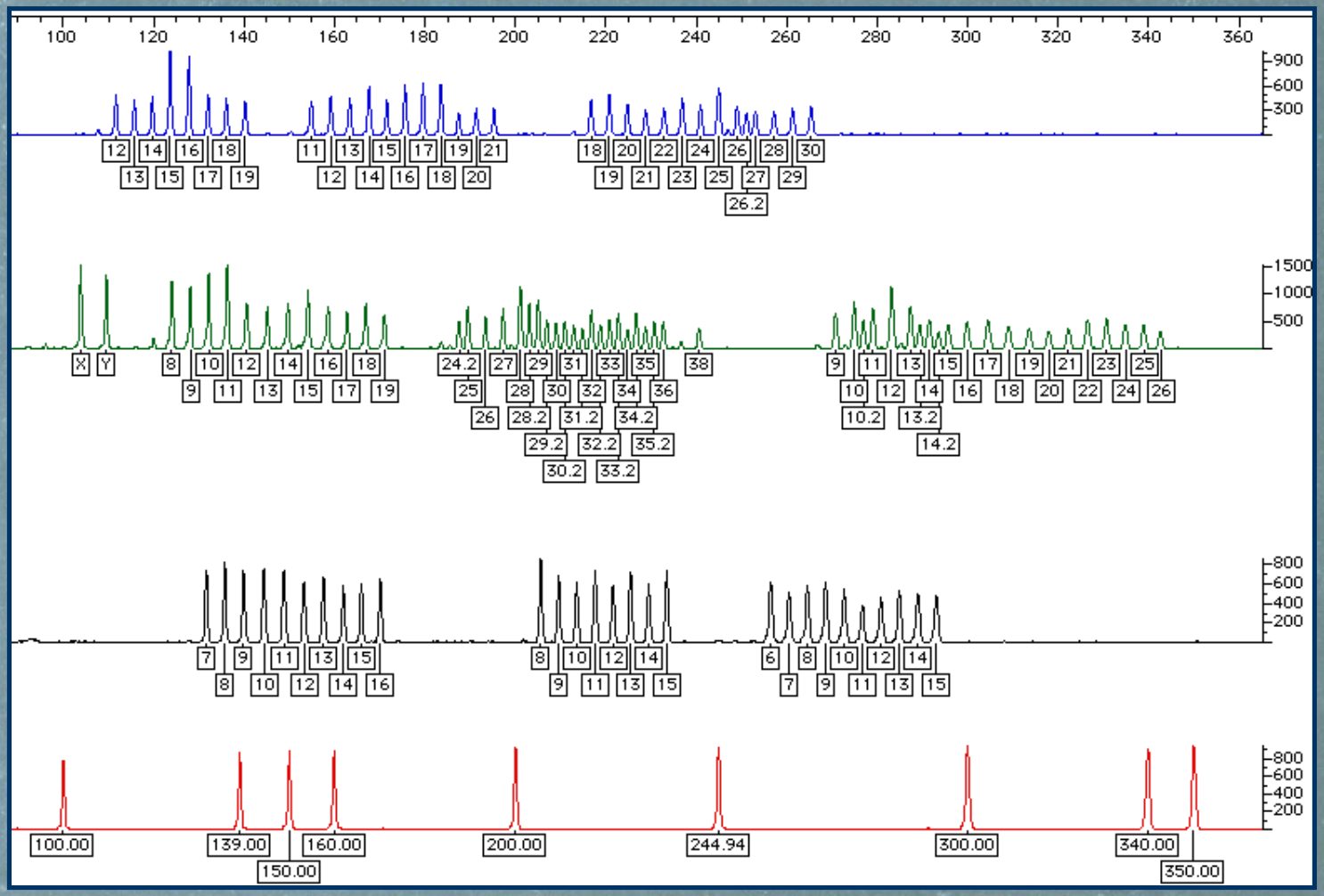


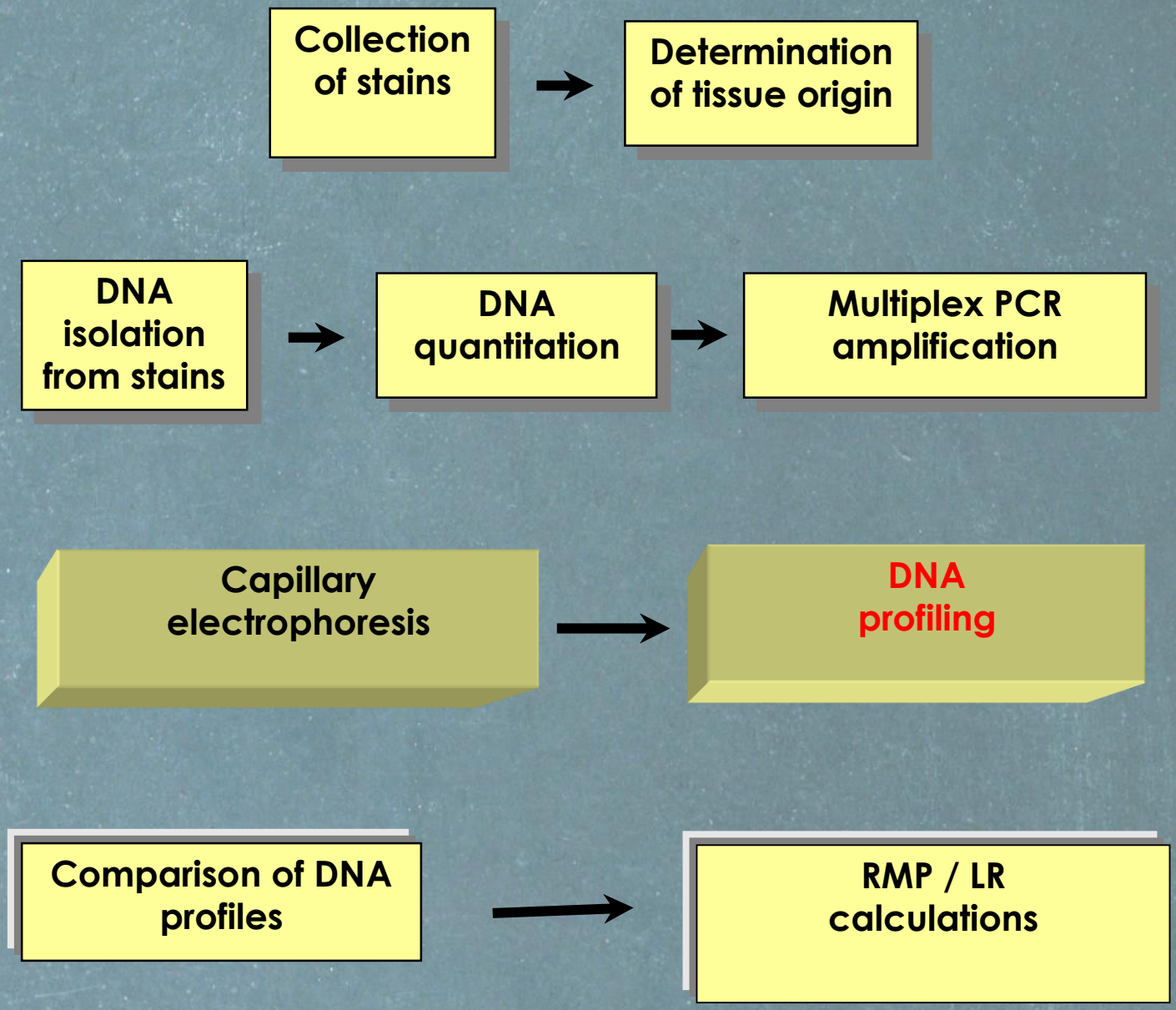
Peak Sizing with an Internal Size Standard





✓ final assignment of genotypes is made by comparison of peak bps in the sample and an **allelic ladder,** which includes all the most common alleles present in the population for the tested STRs, and which s injected in the capillary before, after or parallely (in multicapillary systems) to unknown DNA samples. Bps of allele peaks in the allelic ladder are determined through comparison with the same ILS used for unknown DNA samples.

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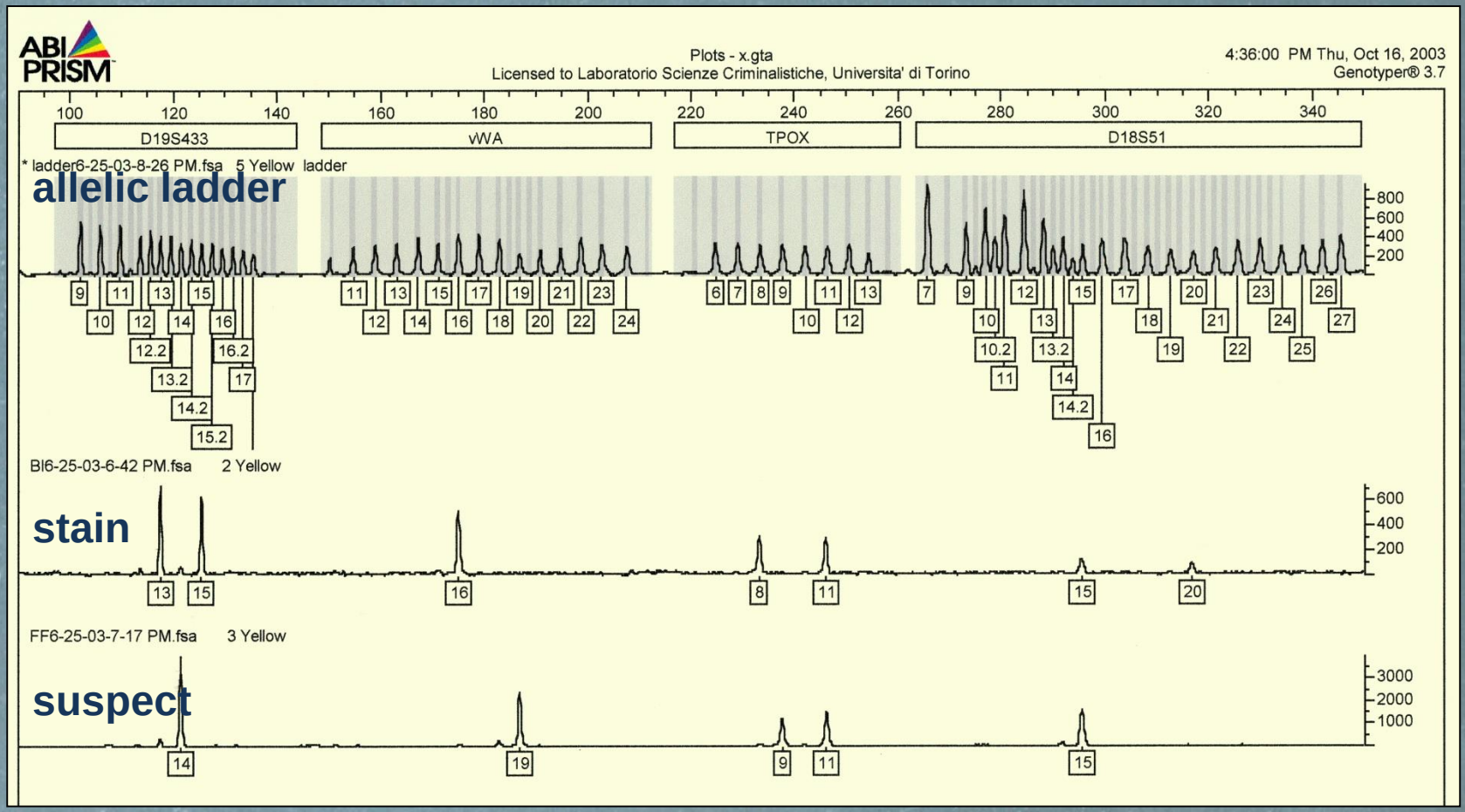
✓ the combination of genotypes of a stain (or of an individual) for a particular set of STRs is called a **DNA profile**



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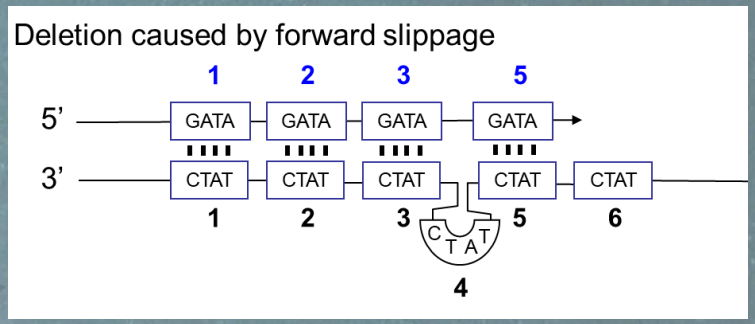
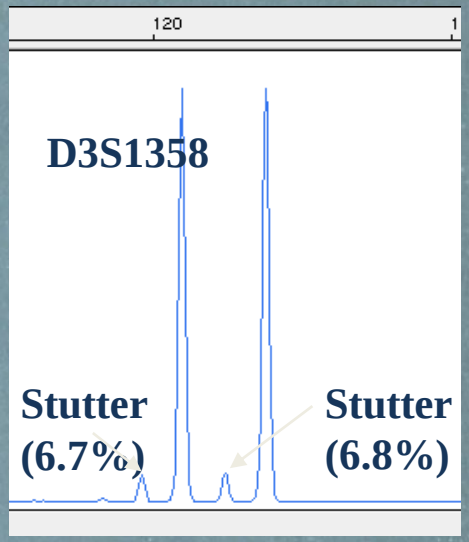
Standard typing techniques Part II

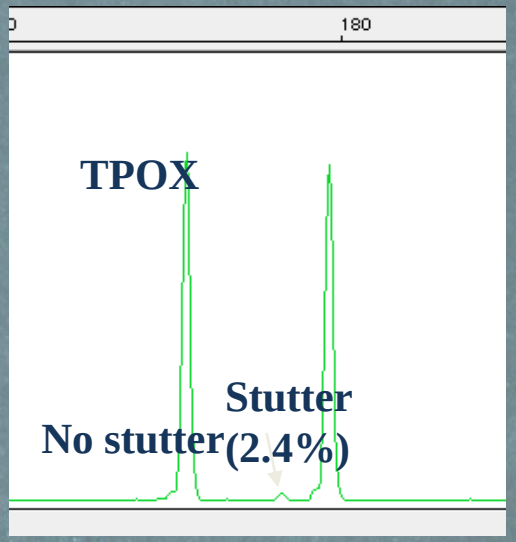
PCR artifacts affecting CE analysis of STRs

- ✓ **stutter**
- Peaks that show up primarily one repeat less than the true allele as a result of strand slippage during DNA synthesis
- Stutter is less pronounced with larger repeat unit sizes (dinucleotides > tri- > tetra- > penta-)
- Stutter rates are locus specific (but generally <15% in forensic STRs, usually higher for longer alleles)



Epgs analysis software include locus-specific settings to filter out stutters



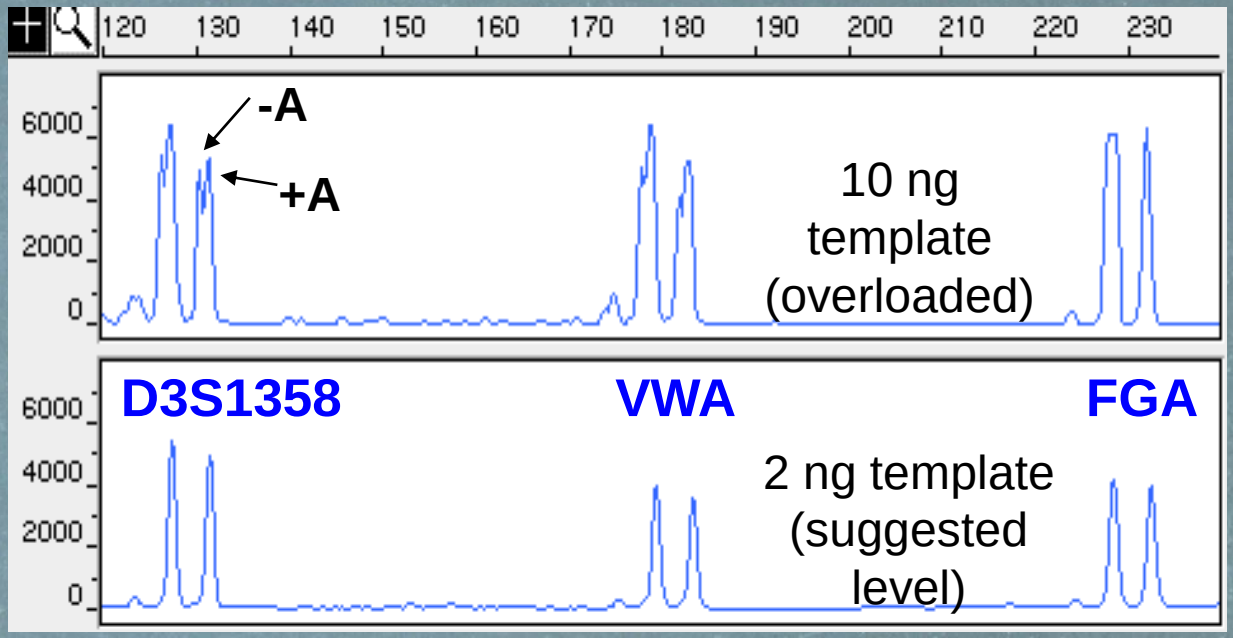


As we will later see, stutters can have an impact on the interpretation of DNA profiles obtained from degraded or mixed DNA

✓ PCR artifacts due to variation in template DNA quantity/quality

a) non-template addition

- Taq polymerase tends to adds an extra nucleotide to the 3'-end of a PCR product; most often an "A" (termed "adenylation")
- Excess amounts of DNA template in the PCR reaction can result in incomplete adenylation
- a mixture of "+/- A" amplicons at the end of PCR give rise to split peaks in CE
- Split peaks are typically caused by excess input DNA in PCR

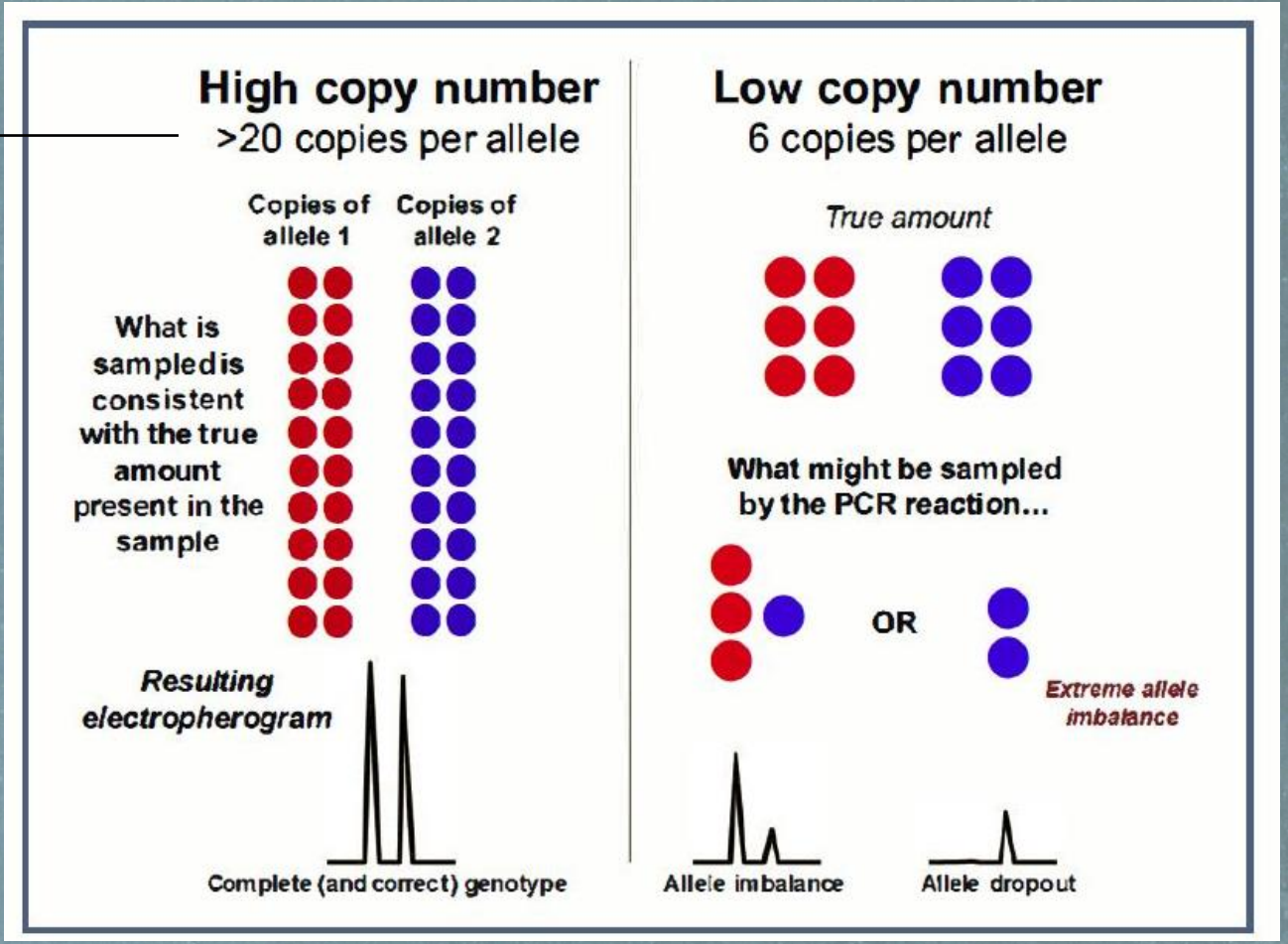


Split peaks can affect genotyping of loci including .1 or .3 allele variants

b) Allele imbalance, allele/locus drop-out

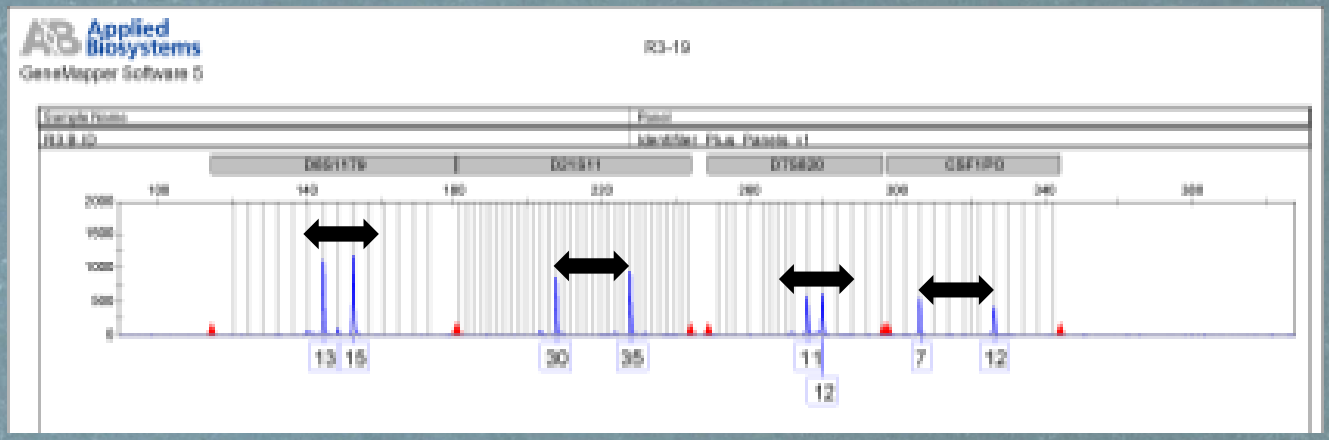
- Stochastic effect of PCR due to unequal sampling of alleles in low template (LT) DNA samples. it results in partial (allele imbalance) or complete (allele drop-out) failure to detect an allele. As a consequence heterozygous genotypes can appear as actually homozygous.
- Failure to amplify both alleles causes (locus drop-out)

> 0.1 ng DNA

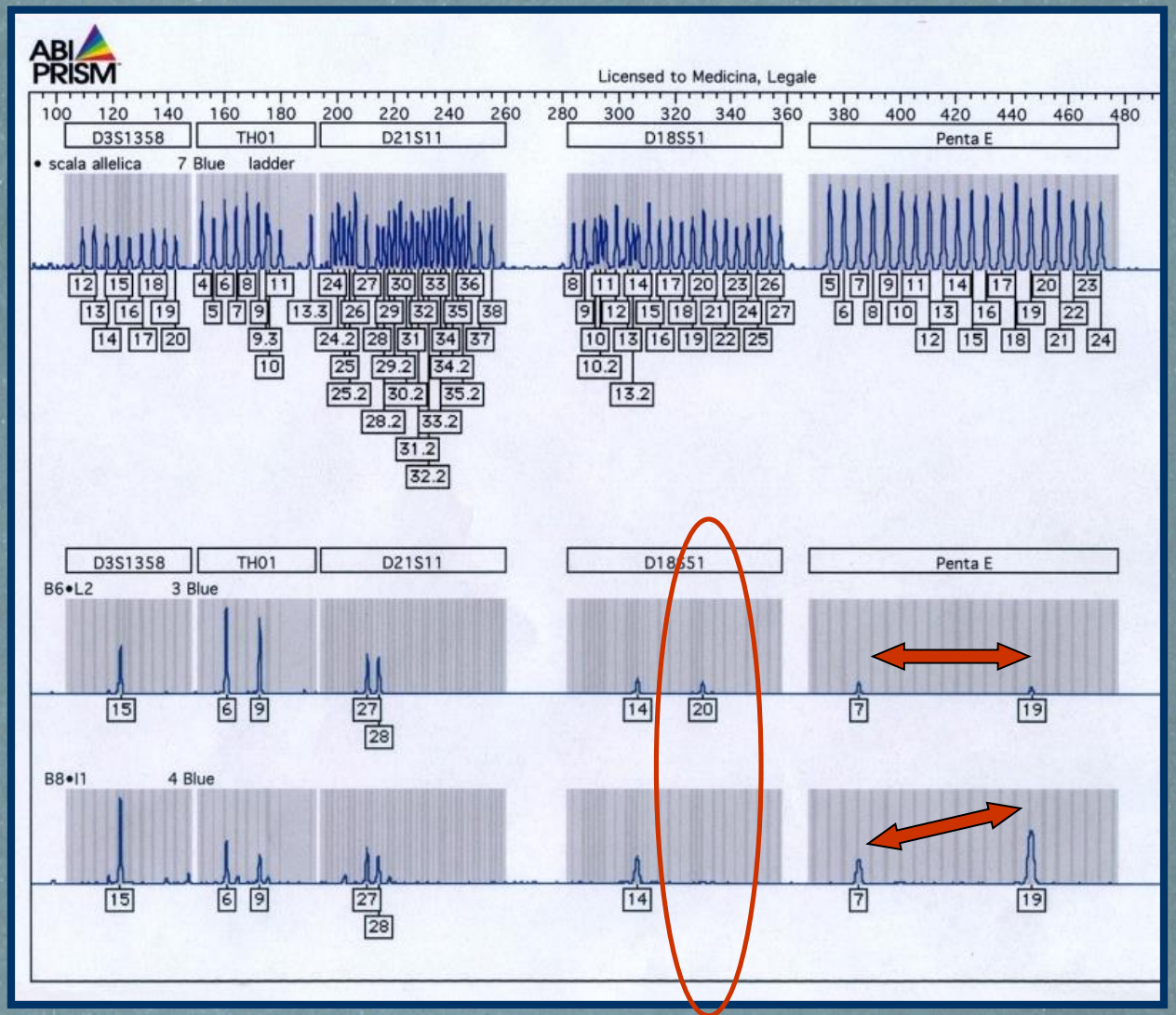


LT-DNA: can derive from a small amount of total DNA originally present in the source stain, but also from DNA degradation limiting the amount of preserved DNA fragments long enough to act as template in PCR amplification of STR loci

Allele imbalance: although short-amplicon loci are amplified with greater efficiency compared to long-amplicon, within each locus multiplex PCR is quantitative and reflects the starting amount of DNA present in the amplification reaction. Normally, in good quality samples (e.g. reference samples) peak height ratio (PHR)* between alleles of a heterozygous genotypes is never below 60%



* Peak height of smaller peak in heterozygous genotype / peak height of higher peak in heterozygous genotype



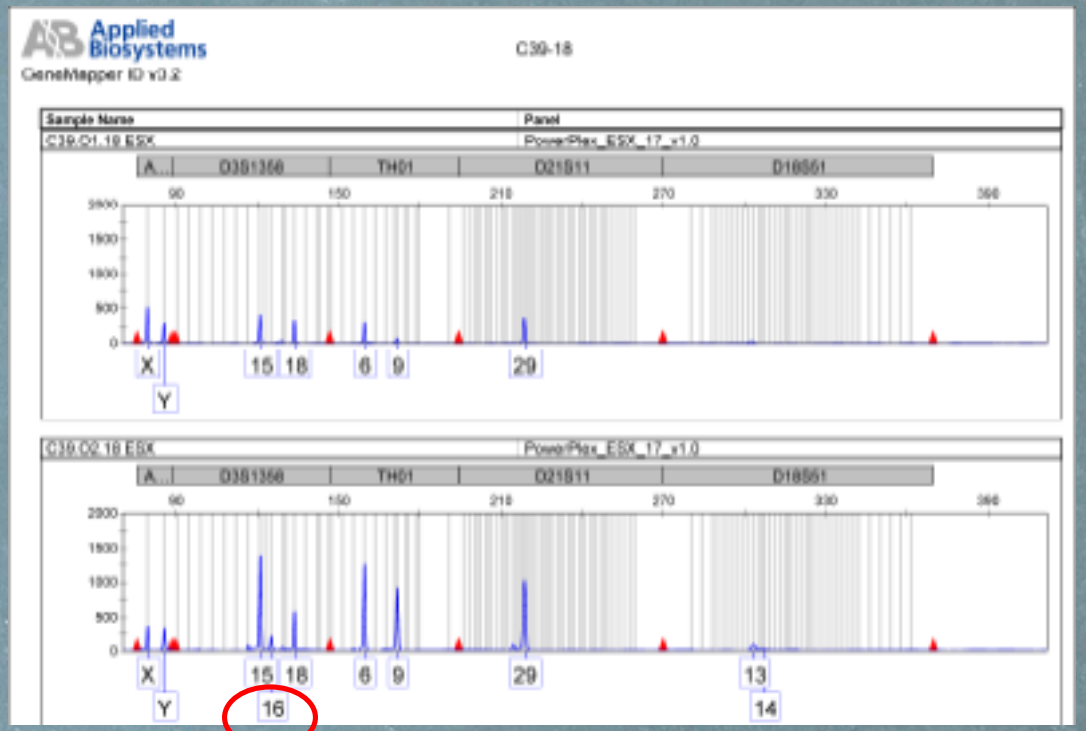
Same DNA,
different input in PCR

Allele dropout

Allele imbalance (PHR < 60%)

c) Allele drop-in

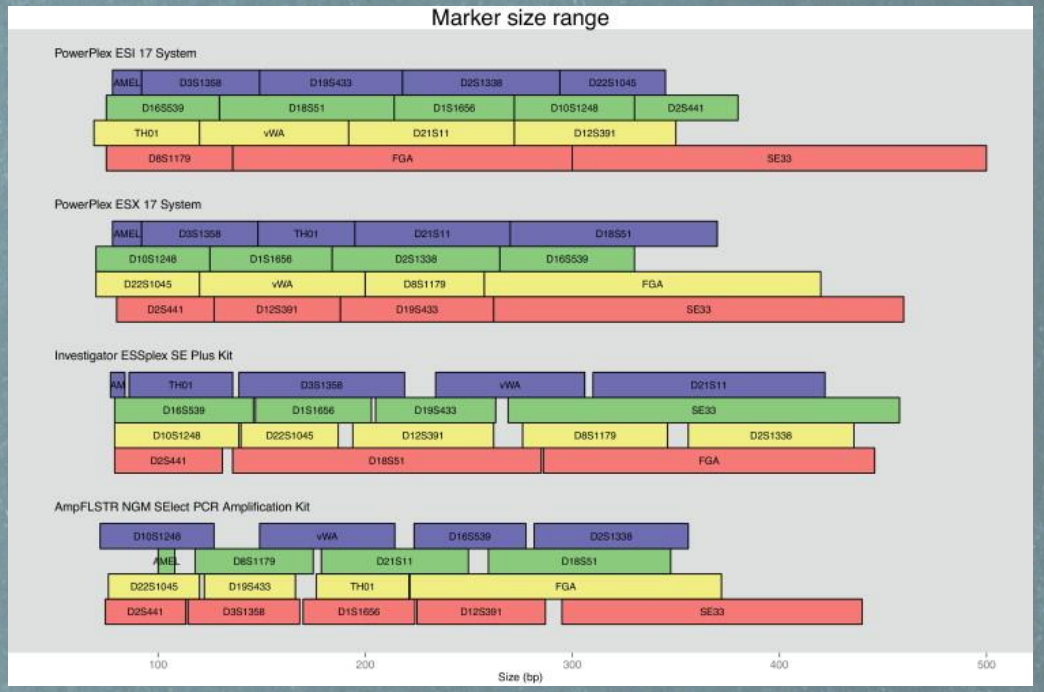
- Stochastic effect of PCR consisting in the observation of spurious alleles due to sporadic microcontamination. It is typically observed in LT-DNA, in which the limited amount of template DNA cannot mask microcontaminations.
- Although forensic multiplex PCR panels for amplification of STR loci are highly efficient, when different DNAs are present in the template, only the DNA of the major contributor is observed when present in ~20-fold concentration compared to minor contributor
- Allele drop-in is a stochastic (random) event and should not be confused with proper contamination in which the same unexpected allele/s appear in PCR replicates

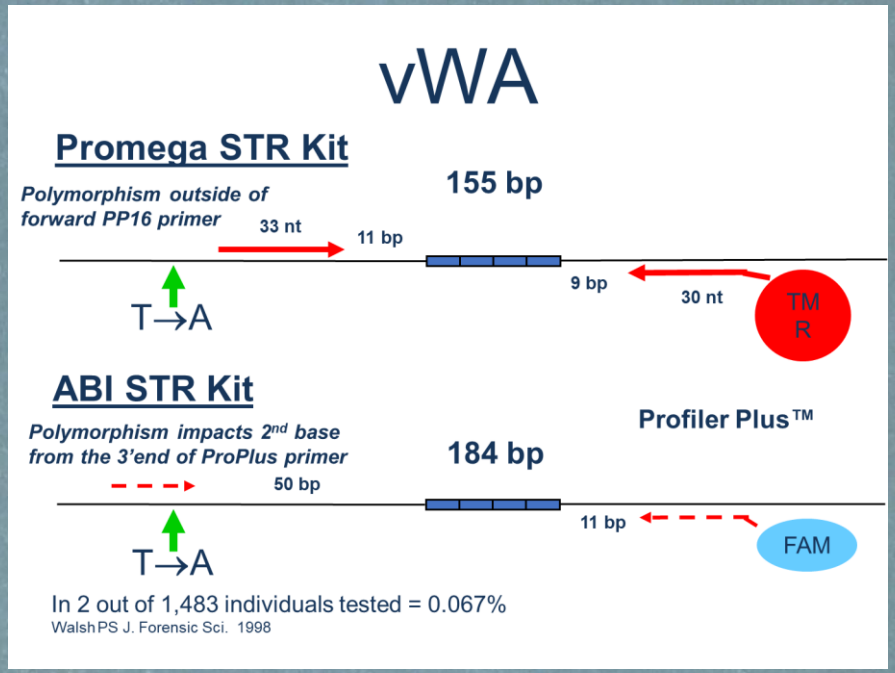


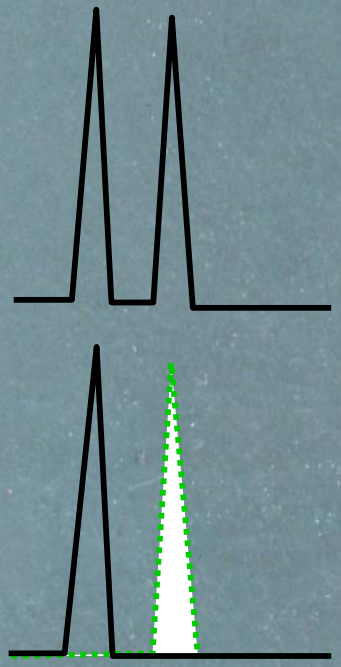
→PCR replicate 1

→PCR replicate 2

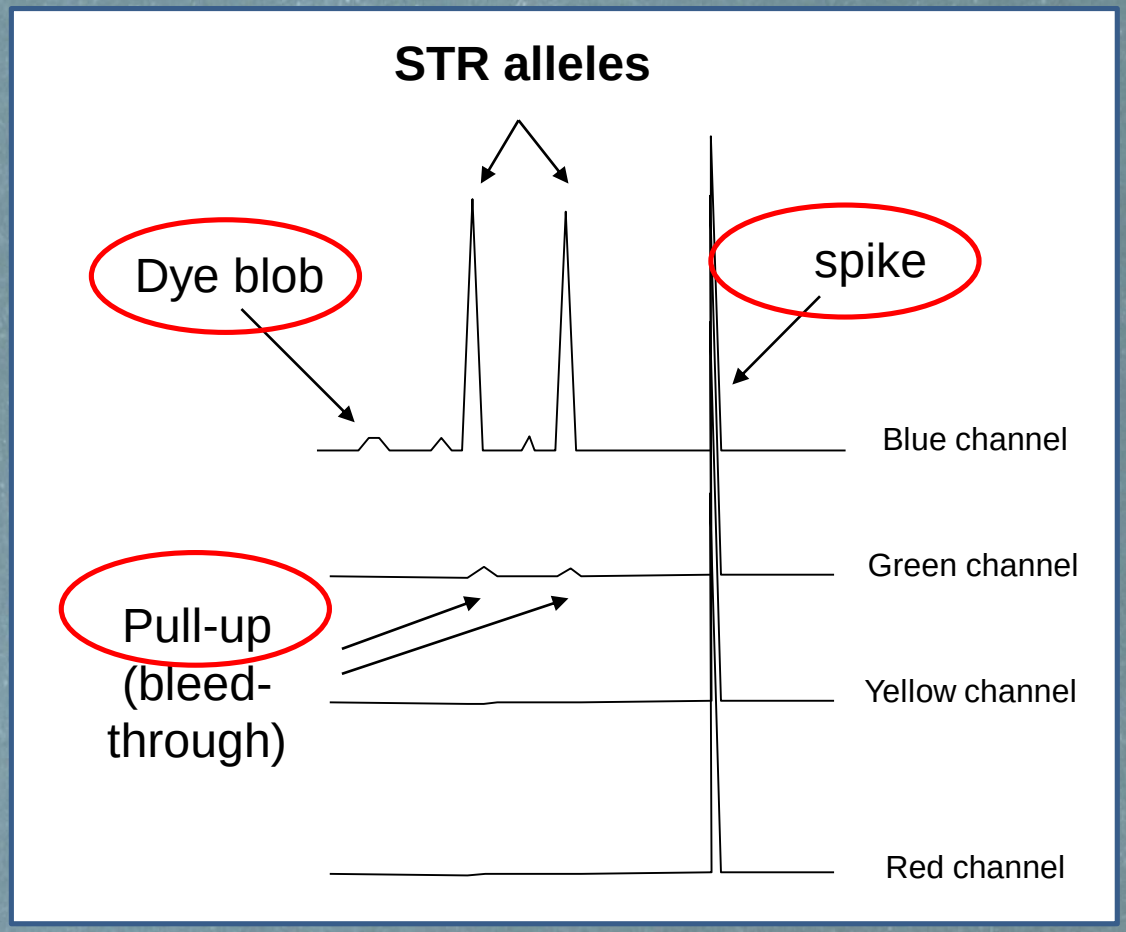
- ✓ **Primer binding site mutation**
- Different commercial kit are available for the amplification of overlapping sets of forensic STRs
- Different kits use different primer sets
- Point mutations in primer binding sites can differentially affect different kits giving rise to inconsistencies in genotyping (null allele)







CE artifacts affecting analysis of STRs

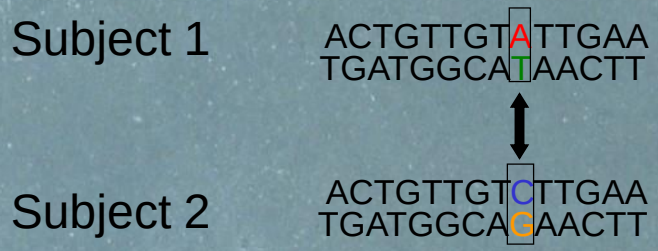


- Dye blob: free dye not coupled to PCR primer (affect single color channel)
- Pull up: overamplification of DNA hampers virtual filtering, causing the apperance of spurious peaks in other color channels (affect color channels with most overlapping emission wavelenght)
- Spike: crystals in polymer or buffer (affect all color channels)

Non-STR forensic DNA polymorphisms

✓ **SNPs**

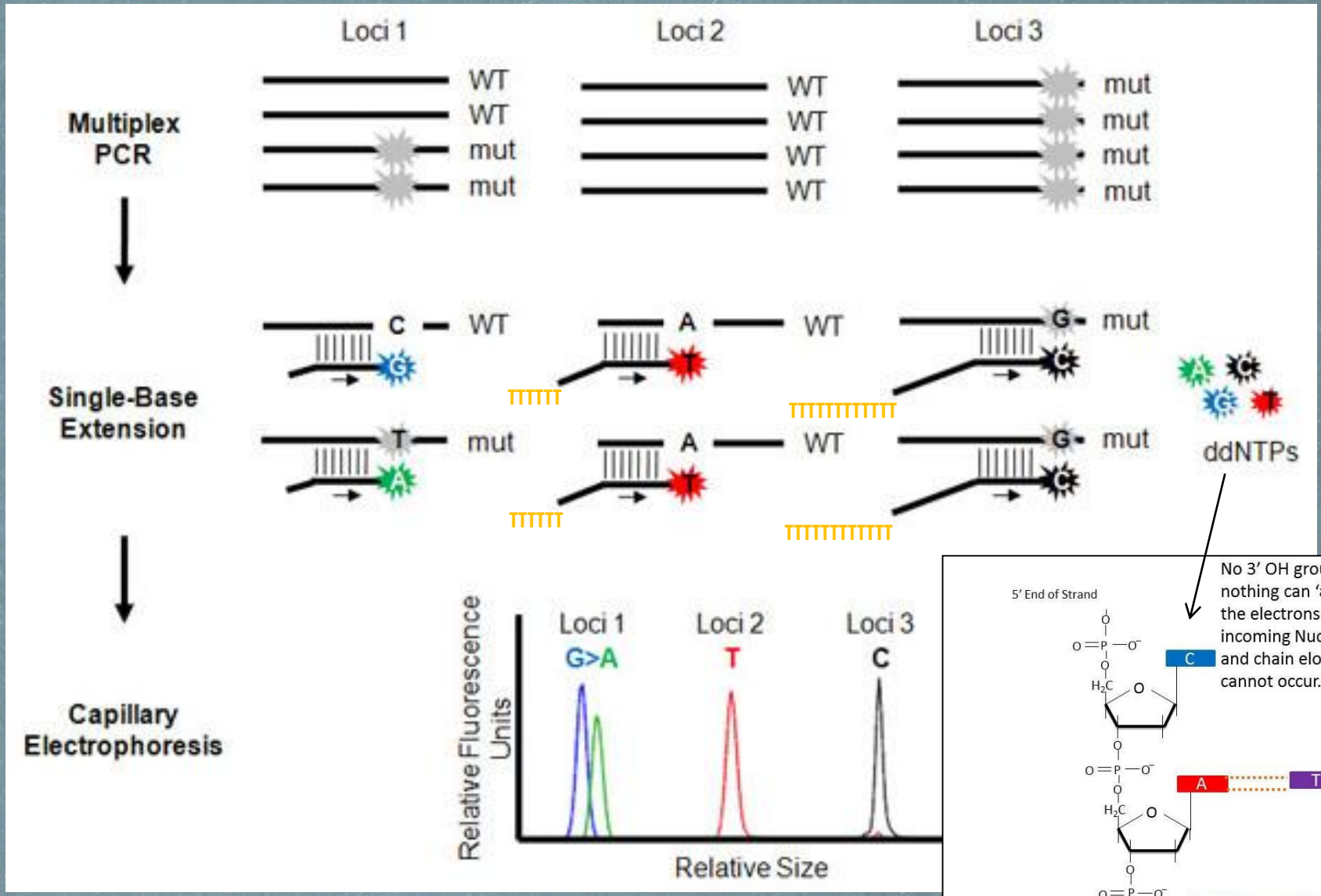
- interindividual difference does not consist in variable number of repertitions, but in the substitution of single bases in the DNA sequence



- analysis of SNPs through capillary electrophoresis is possible via Single Base Extension (SBE) technique consisting of:

a) multiplex PCR with unlabelled primers of SNP regions

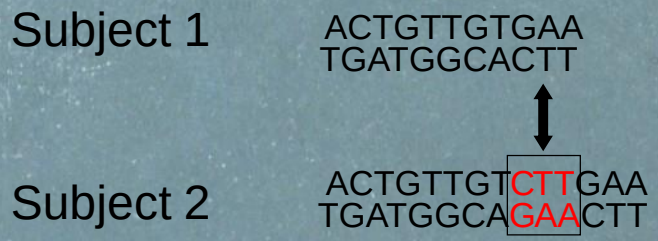
b) sequencing with primers whose 3'-end anneals to the base directly preceeding the SNP position (which can be differentiated through modifying tails of different lenght) and terminator ddNTP labelled with base-specific flourescent dyes



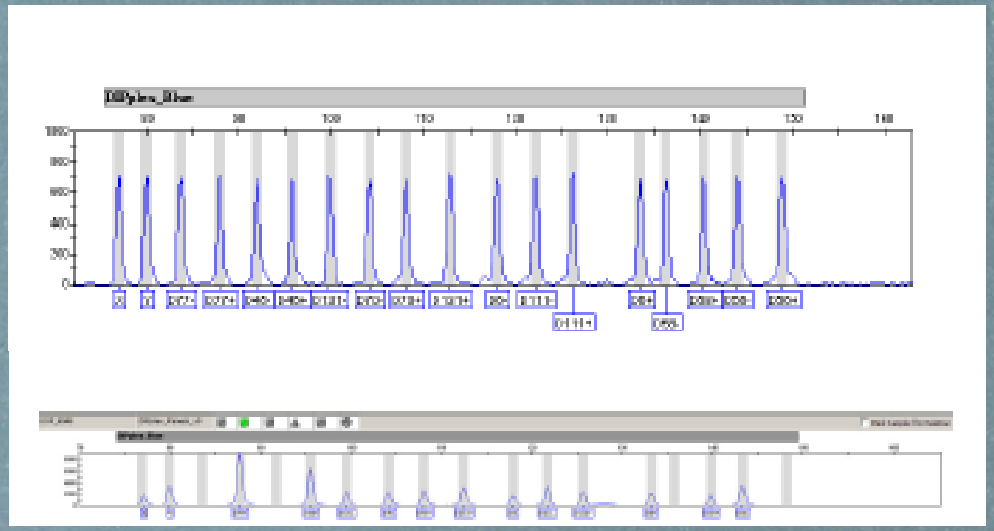
T_n = modifying tails

✓ **Indels**

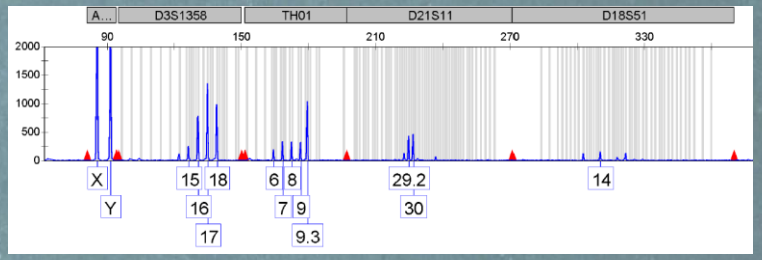
- interindividual difference consist in presence/absence of sequence stretch of variable lenght



- Like STRs, indels are amplified by multiplex PCR with dye labelled primers. Insertion/deletion variants can then be separated by CE in the presence of an ILS and genotyped in comparison to allelic ladders

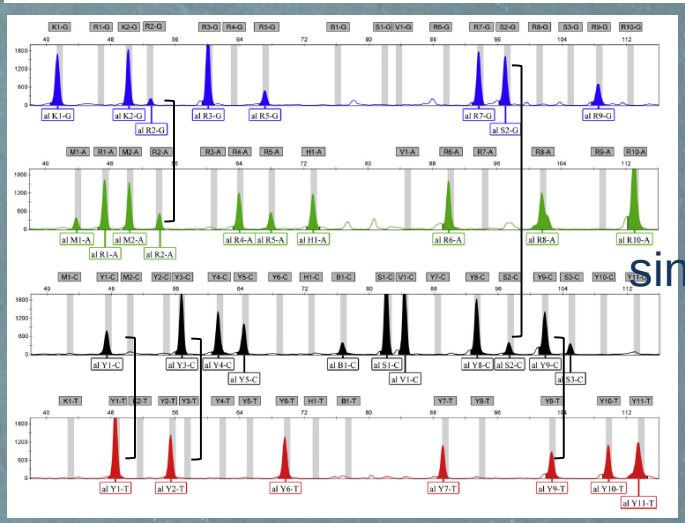


- Being multiallelic STRs easily detected mixed stains (> alleles at multiple loci)



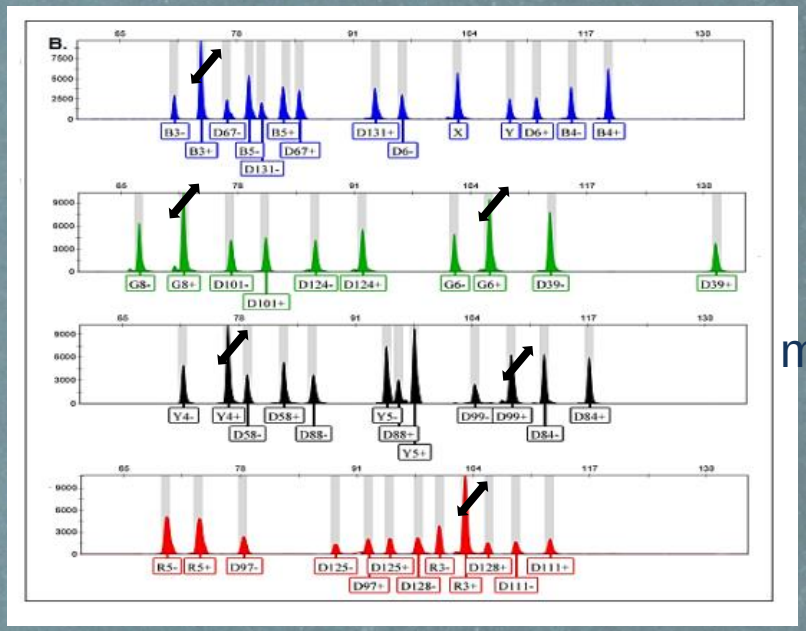
mixed

- Base incorporation in SBE is not sensitive to original DNA quantity, so SNPs cannot easily identify mixed stains (unless triallelic SNPs are included in the assay)



single source

- Although Indels are biallelic, typing is fully based on PCR and therefore quantitative within single loci (as in the case of STRs): imbalance in PHR at multiple Indel loci detects mixed samples



mixed

✓mtDNA haplotypes have been routinely identified by Sanger sequencing

• the whole CR can be amplified in a single PCR reaction, while trace samples usually require amplification of overlapping short regions of CR in different PCR experiments

• both strands of the PCR products are then sequenced through amplification with a single primer and a buffer including both dNTPs and fluorescent dye-labelled ddNTPs, which –once incorporated- interrupt elongation by the polymerase