MUNI
MED

Metody detekce nukleotidových polymorfismů

Andrea Knight, PhD
ÚPF Lékařská fakulta MU

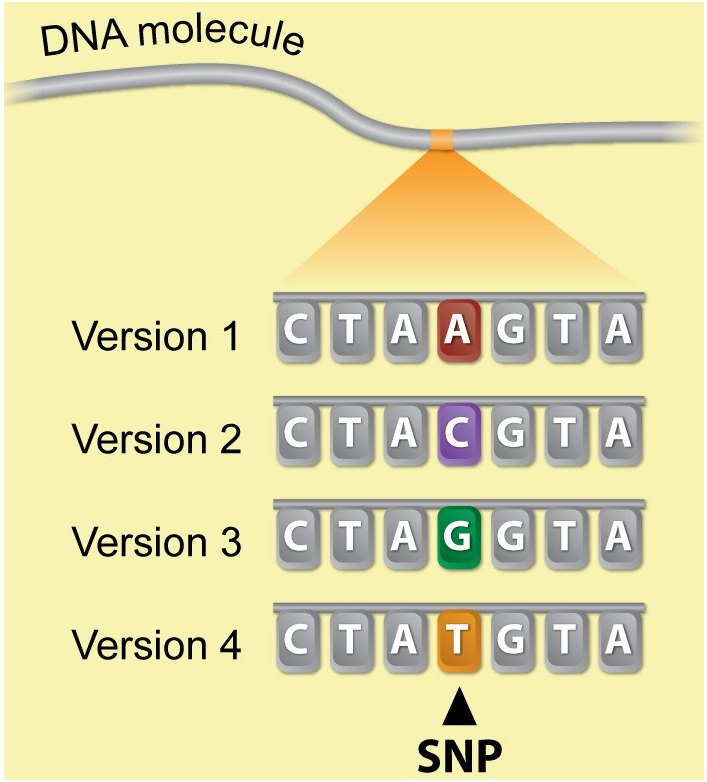
Point mutation vs polymorphism

- Both are single nucleotide differences in DNA sequence
- Point mutation:
- Polymorphism:
  - a DNA sequence at a certain locus that varies among individuals (is present as two or more alleles)
  - any sequence variant present at a frequency of > 1% in a population

Types of polymorphisms

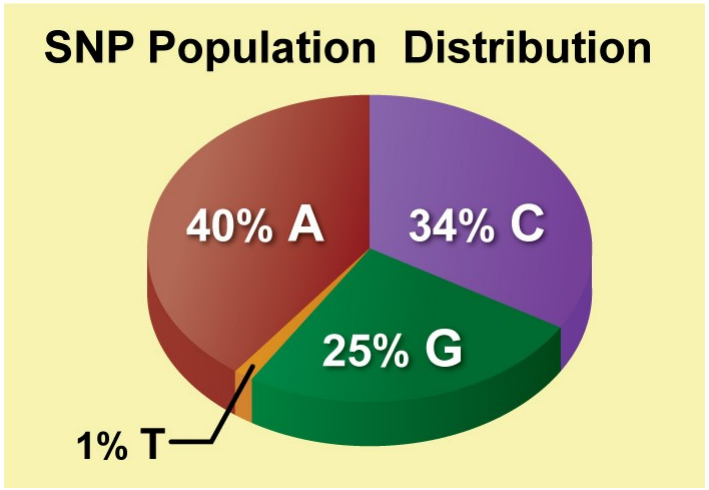
- single nucleotide polymorphism (SNP)
- short tandem repeats (STR), microsatellites
  - Repeat length 2 -5 bps
- variable number of tandem repeats (VNTR) or minisatellites
  - Repeat length 10 - 200 bps
- restriction fragment length polymorphisms (RFLPs)
- insertion/deletion

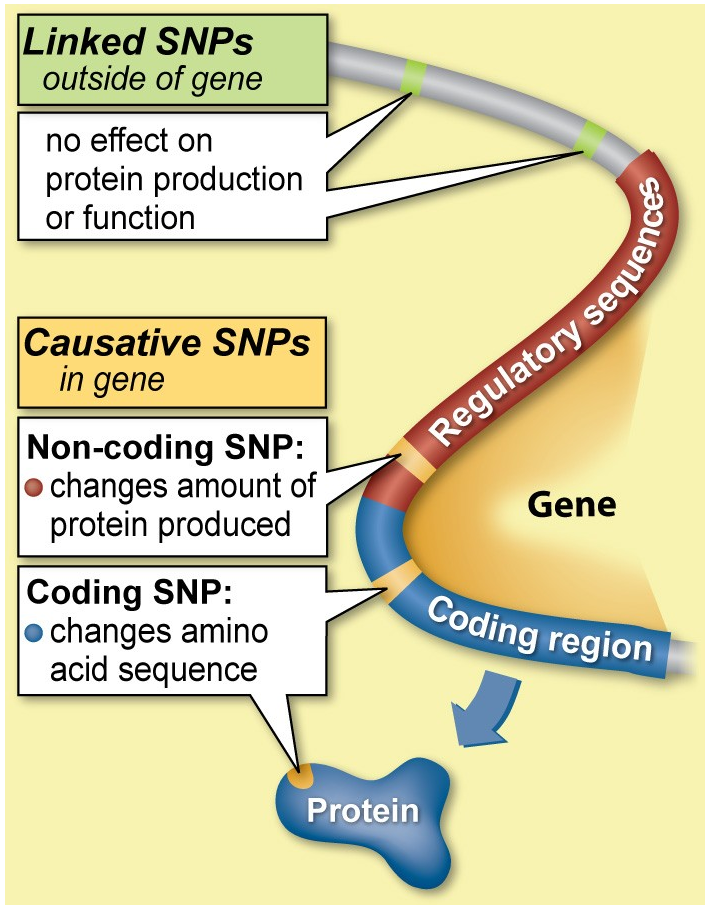
To be classified as a SNP:
Two or more versions of a sequence must each be present in at least 1% of the general population.



Single nucleotide polymorphisms

- Nucleotide: A, T, C, G
- SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T)
- occurs once in every 300 nucleotides
- there are ~ 10 million SNPs in the human genome
  - *Human genome completed & published: 1st draft April 2003, May 2006 (incl. chr.1)*
- most commonly found in DNA between genes
- when SNPs occur within a gene or in a regulatory region near a gene, they plays a direct role in disease by affecting the gene's function





Linked SNPs:

-do not reside within genes

-do not affect protein function

-do correspond to a particular drug response or to the risk for getting a certain disease

Causative SNPs:

-affect the way a protein functions

-correlating with a disease or influencing a person's response to medication

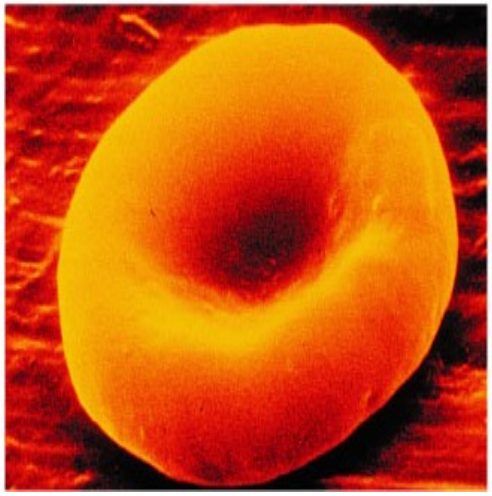
Polymorphisms

- are harmless/non-pathogenic sequence variants
- are useful for:
    - forensic analysis (DNA profiling)
    - mapping disease genes
    - inheritance of disease genes in the family
    - as predictive markers of effectiveness to drugs
- are now intensively studied https://www.ncbi.nlm.nih.gov/snp/

- **Databases of SNPs** contains human single nucleotide variations, microsatellites, insertions and deletions, population frequency, molecular consequence, genomic and RefSeq mapping information for both common variations and clinical mutations.

SNP example - Sickle cell anaemia

- Impaired production of red blood cells (RBC)
- Inheritance of two abnormal Beta-globin gene (chr 11)
- Estimated that 7% of world's population (~420 million) are carriers
- The gene defect is a SNP, where *GAG* codon changes to *GTG* and results in glutamic acid being substituted by valine (E6V)



Polymorphism of regulatory gene

- IRF5; interferon regulatory factor 5
- =transcription factor
- Associated with increased risk of SLE
- In type-I diabetes
- In melanona
- Correlates with acute rejection of liver transplant

Metody detekce SNPs

- PCR
- Real-time PCR
- RFLP
  - ELFO na agaróze
- PAGE
  - SSCP, heteroduplexní analýza
- Kapilární elektroforéza
  - v podstatě varianta PAGE

Strategie volby metody

- Flexibilita
- Cena
- Dostupnost
- Rychlost

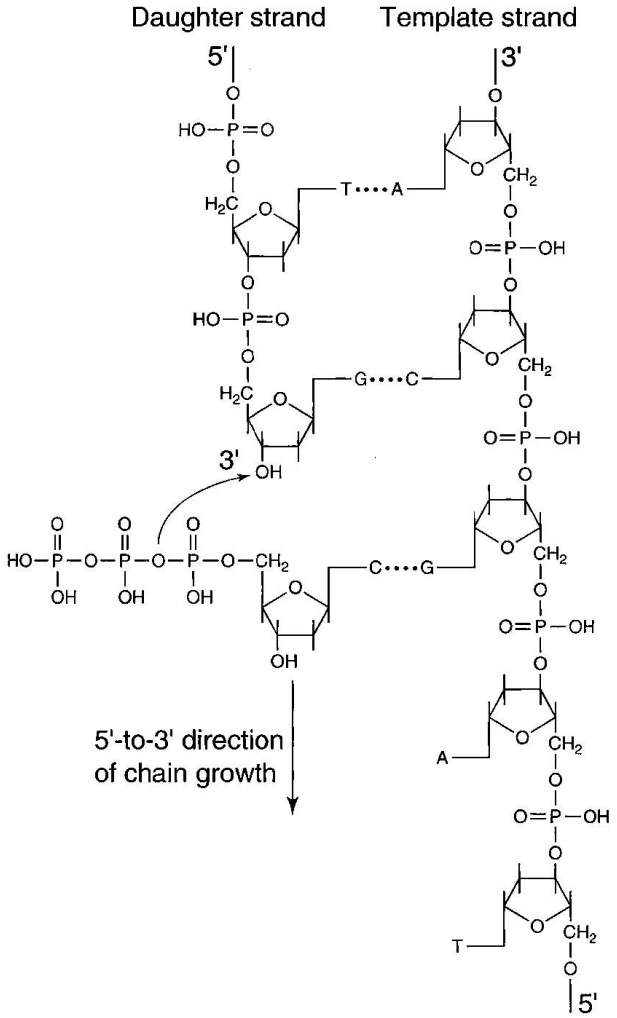
Polymerase Chain Reaction

1983 Kary Mullis

1993 Nobel Prize

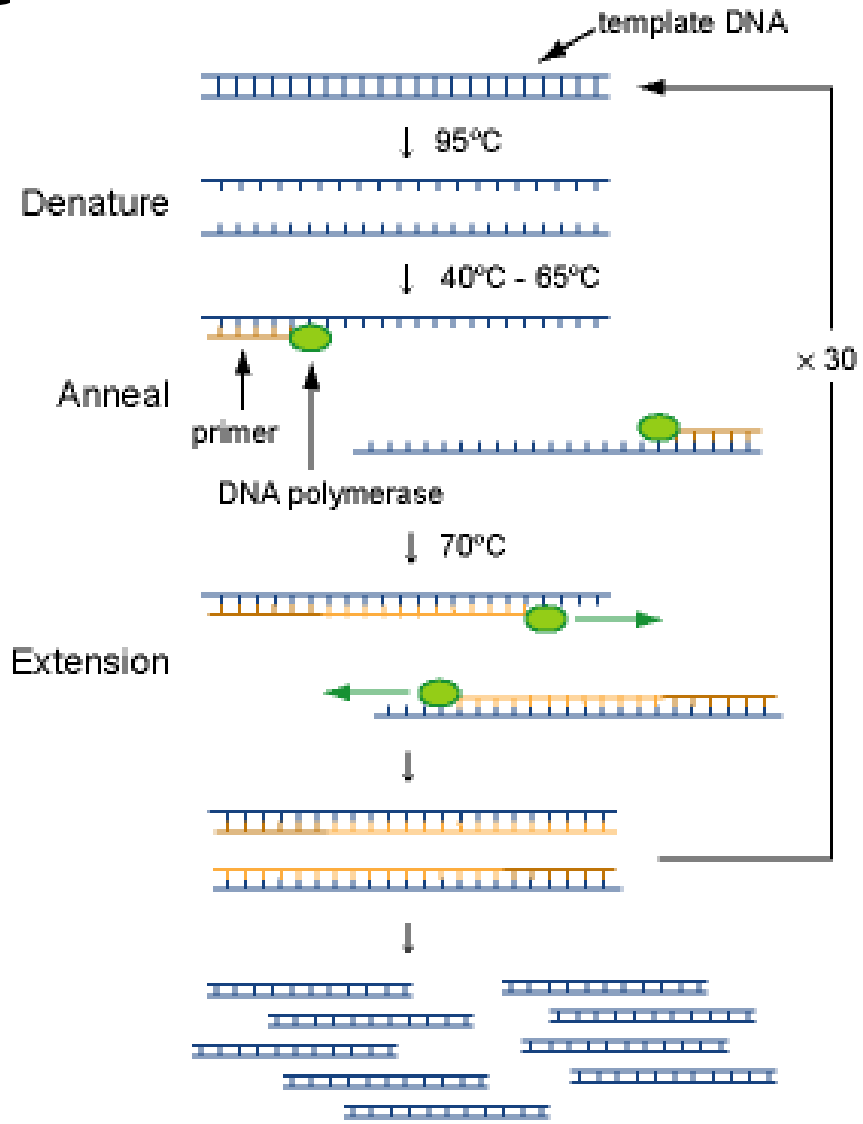
PCR is a DNA synthesis

- DNA primers – complementary to the sequences that flank the region to be amplified
- Deoxynucleotides (dNTPs)
  - dATP+dTTP+dCTP+dGTP
- Mg^{2+} co-factor for enzyme
- Buffer
- DNA polymerase – heat resistance Taq polymerase
  - Thermophilus Aquaticus



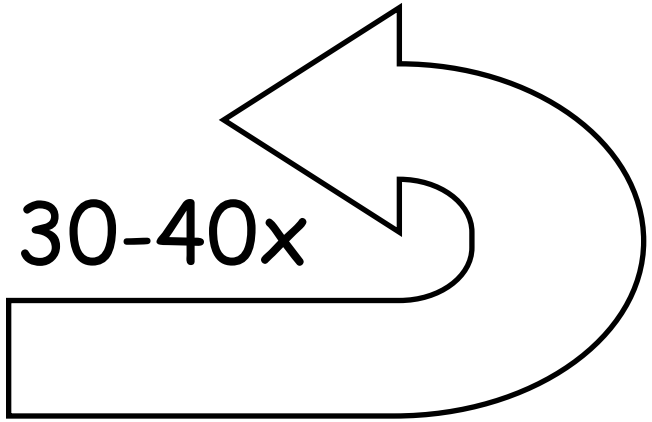
PCR

- PCR is DNA synthesis
- First strands are separated (denaturation)
- Heat to 95ºC
- DNA primer instead of RNA primer to provide free 3’ OH
- DNA primers designed to be complementary to sequences that flank the region to be amplified
- Primers hybridize to DNA (anneal)
- DNA is synthesised (extension)
- Use heat resistant DNA polymerase – *Taq polymerase*



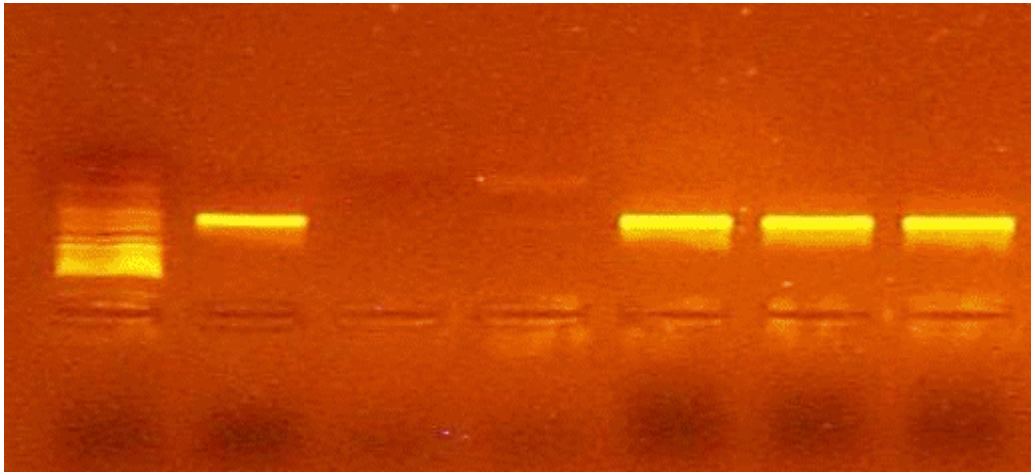
Reakce

- 96°C Iniciální denaturace
- 96°C denaturace
- 40-72°C annealing
- 72°C elongace
- 72°C závěrečná elongace
- 4°C zchlazení



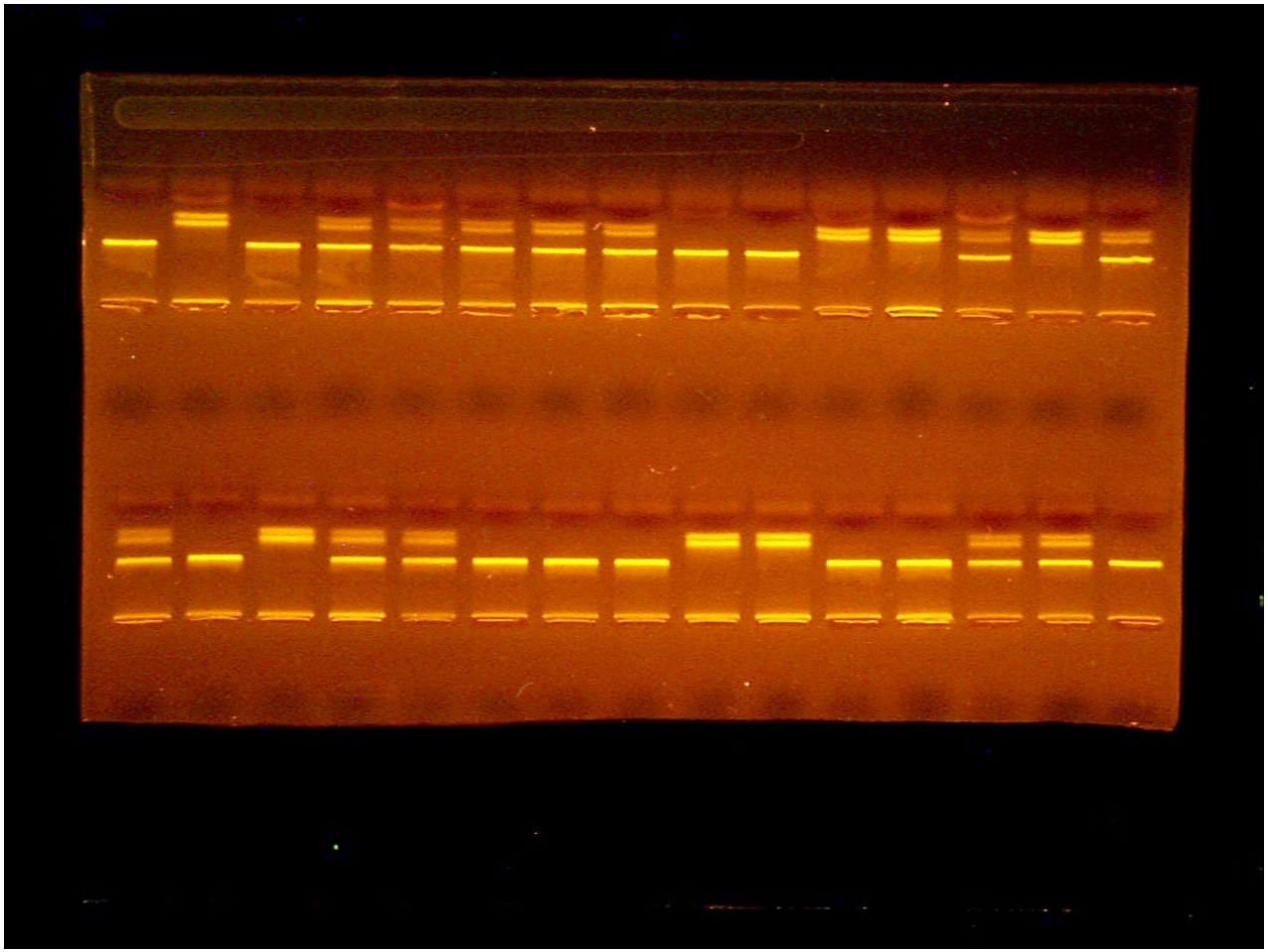
Agarose gel electrophoresis

+



-

Agarose gel electrophoresis: discuss



PCR s následnou RFLP

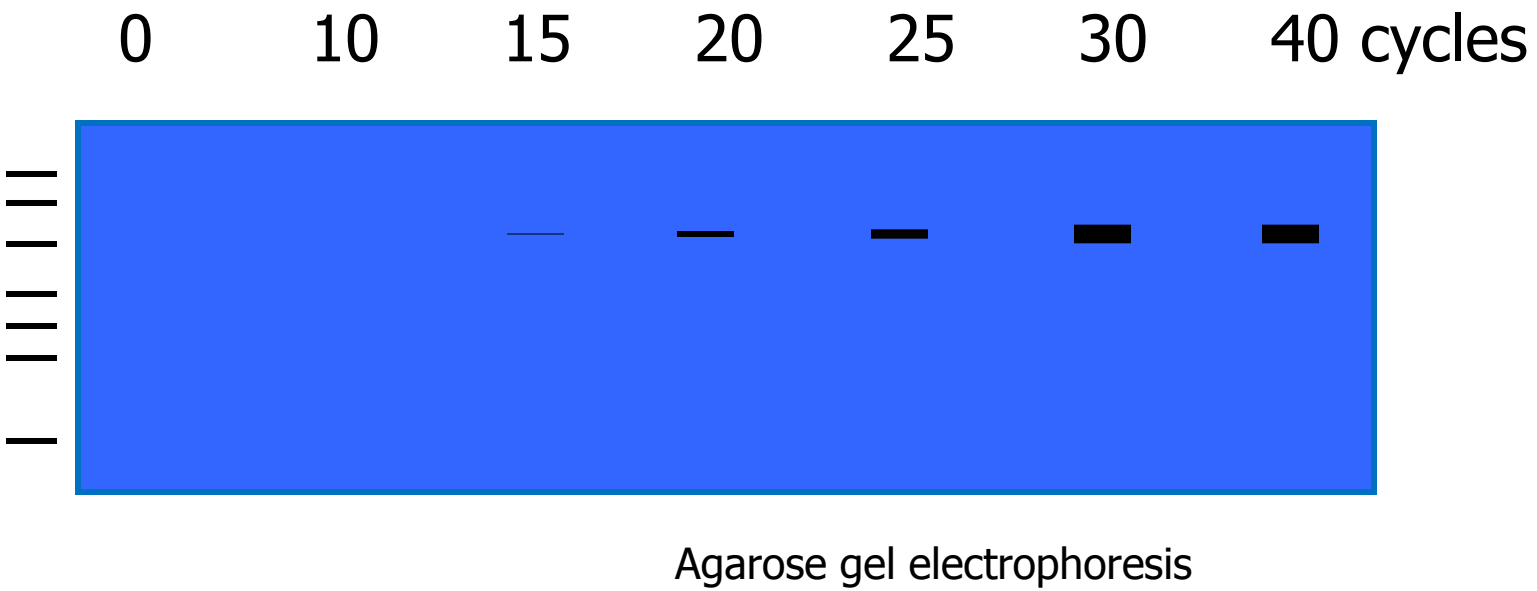
- Pokud polymorfismus způsobuje vznik restrikčního místa:
  - podrobíme produkt PCR působení daného enzymu a na agarové elektroforéze pozorujeme výsledek

- Mismatch primery – umožňují vnést restrikční místo do blízkosti SNP a umožní jeho detekci pomocí endonukleáz

PCR s analýzou délky produktů

- Pokud je polymorfismus způsoben insercí/delecí většího počtu bází:
  - produkt PCR analyzujeme přímo na agarové elektroforéze
- V případě analýzy dinukleotidových repetic používáme kapilární elektroforézu
- Alelově specifické primery – poskytnou produkt jen za přítomnosti dané alely

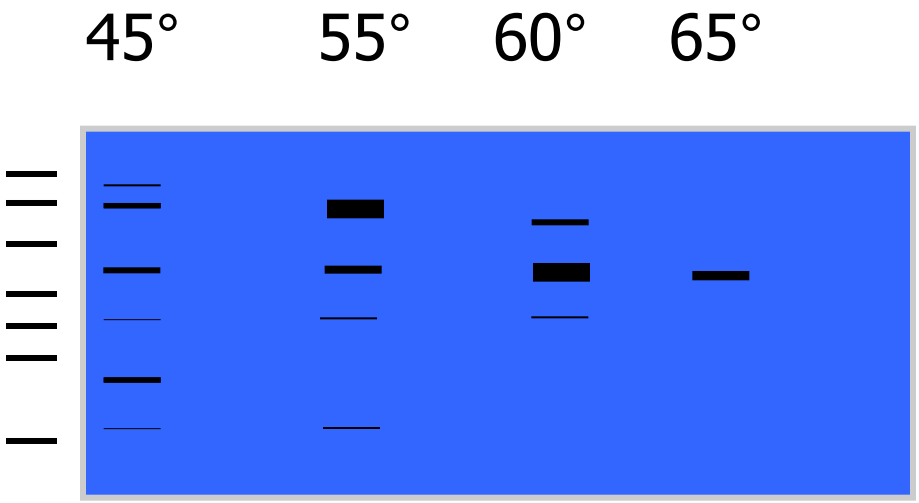
PCR Question #1



Agarose gel electrophoresis

Explain the intensity of the bands as the number of PCR cycles increases

PCR Question #2



Agarose gel electrophoresis

Explain the pattern of the bands as annealing temperature is changed

Real-time PCR

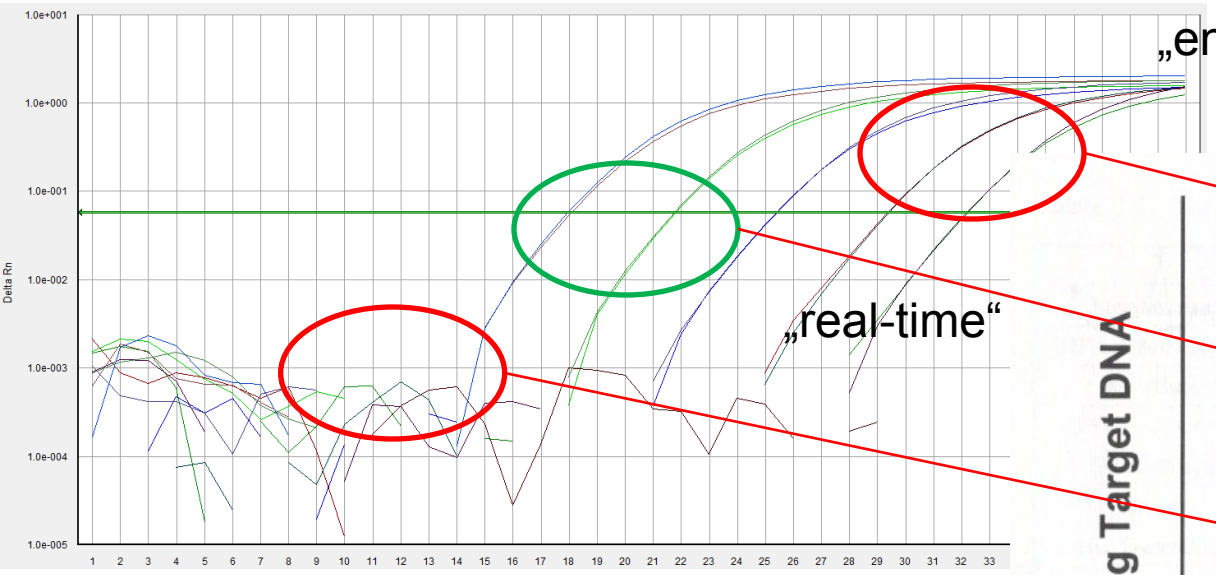
- Různé systémy sond (TaqMan, FRET)
- Melting curve analysis

Real – time detekce amplifikace

Kvantitativní vztah mezi :

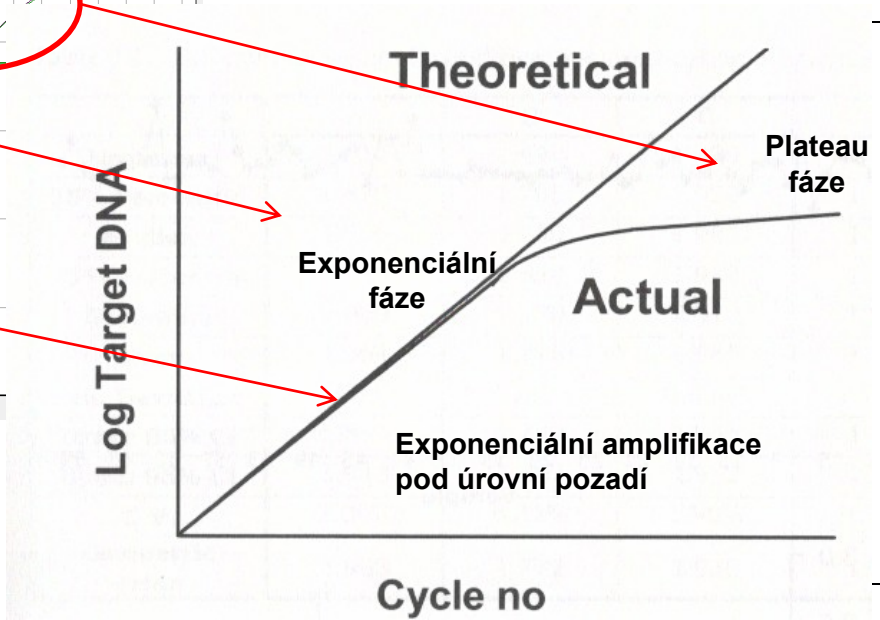
množstvím PCR produktu (amplikonu) a intenzitou fluorescence

- Amplifikační práh detekce (Ct)



„end point“

„real-time“



Threshold cycle „Ct“

– určený na základě hodnoty fluorescence pozadí (backround) a aktuální fluorescence vzorku

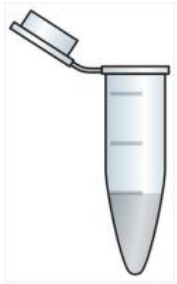
– kvantitativní výstup pro každý vzorek

Background

Threshold cycles

Jak předejít kontaminaci

- Správná laboratorní praxe
- Plastik v RNA kvalitě
- Automatizace



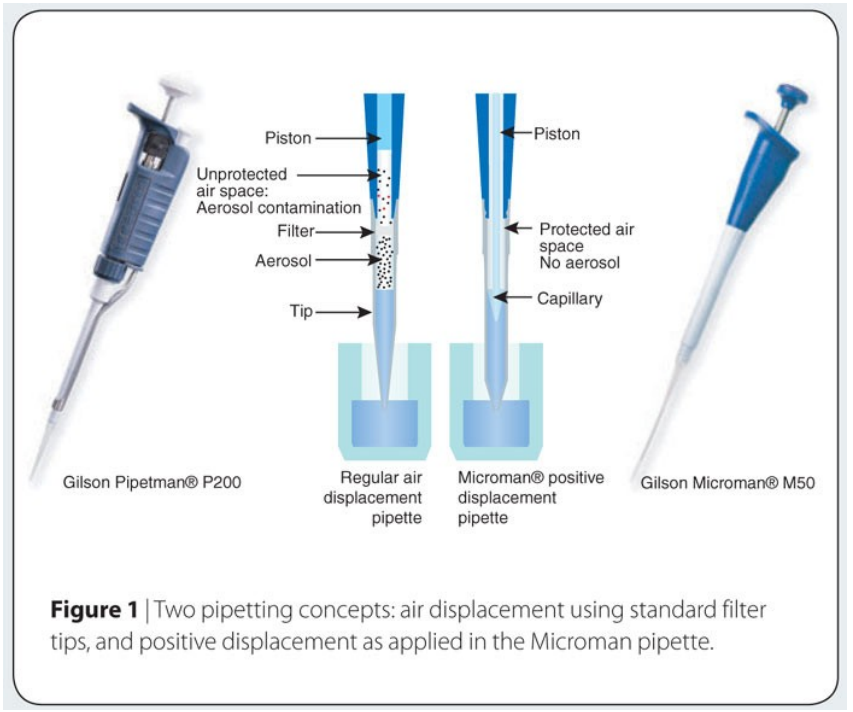
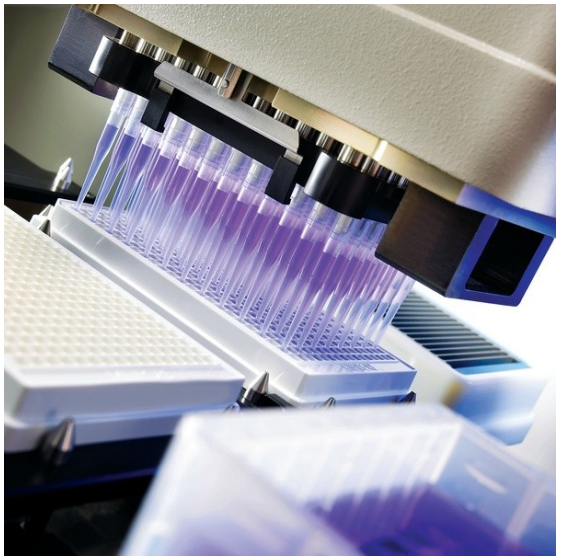


Figure 1 | Two pipetting concepts: air displacement using standard filter tips, and positive displacement as applied in the Microman pipette.



www.millipore.com; www.appliedbiosystems.com

Application note: Contamination-pipetting: relative efficiency of filter tips compared to Microman® positive displacement pipette. *Nature Methods* 3 June 2006

Shrnutí

- Co jsou SNPs
- Metody detekce
- Praktikum