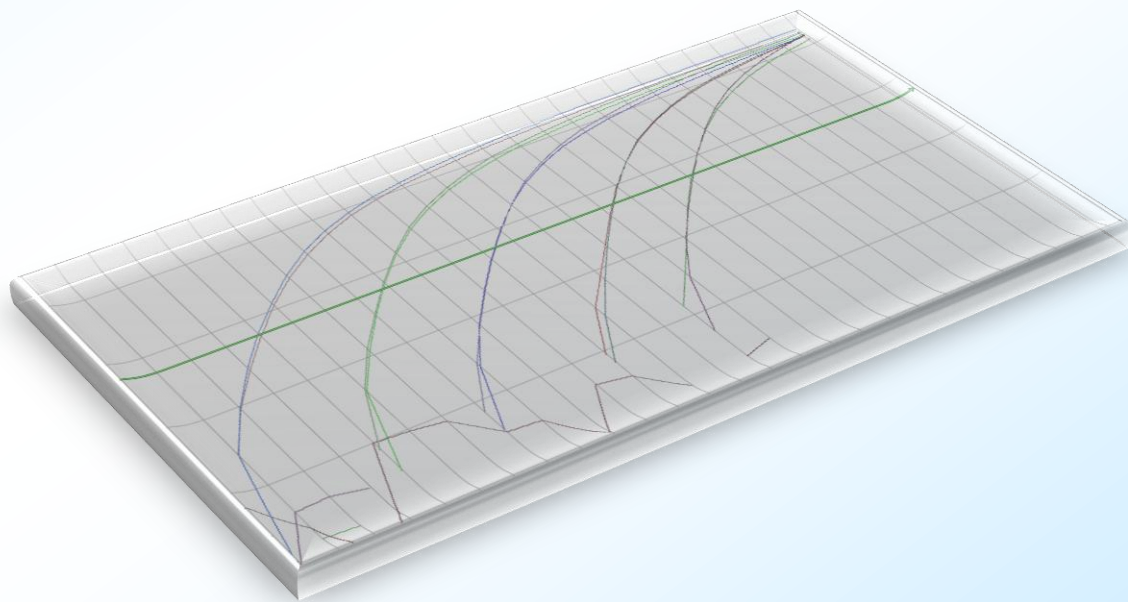


ÚVOD DO KVANTITATIVNÍ REAL-TIME PCR



Tipy na začátek

- Negativní kontrola, pozitivní kontrola
- Příprava rozložení vzorků na destičce – no template control tak, aby nedošlo ke kontaminaci – co nejdále od vzorků s vysokou koncentrací
- Všechno rozmrazit na ledu, v temnu
- Destičku připravit v jiné místnosti než kde probíhala izolace DNA
- Reakční směs zhruba o 10% víc než je potřeba
- Napipetovanou destičku lze uchovat až 10 hod v temnu a chladu
- Správně a úplně zalepit destičku fólií u na okrajích
- Aspoň duplikáty
- Po napipetování spin na centrifuze

Kontrolly

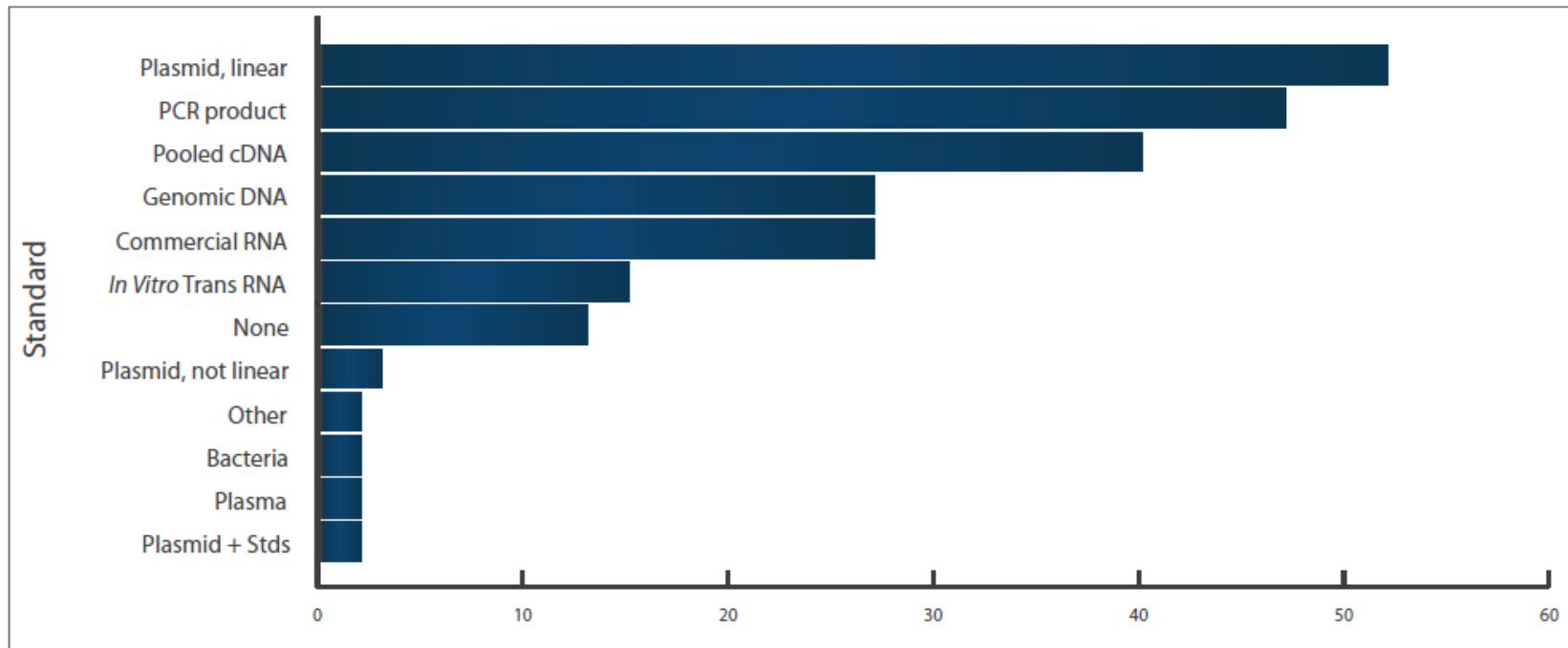


Figure 21: Most frequently used quantification standards. From Nucleic Acid Research Group, (NARG) survey 2007, <http://www.abrf.org/NARG/>

Frekvence kontrol

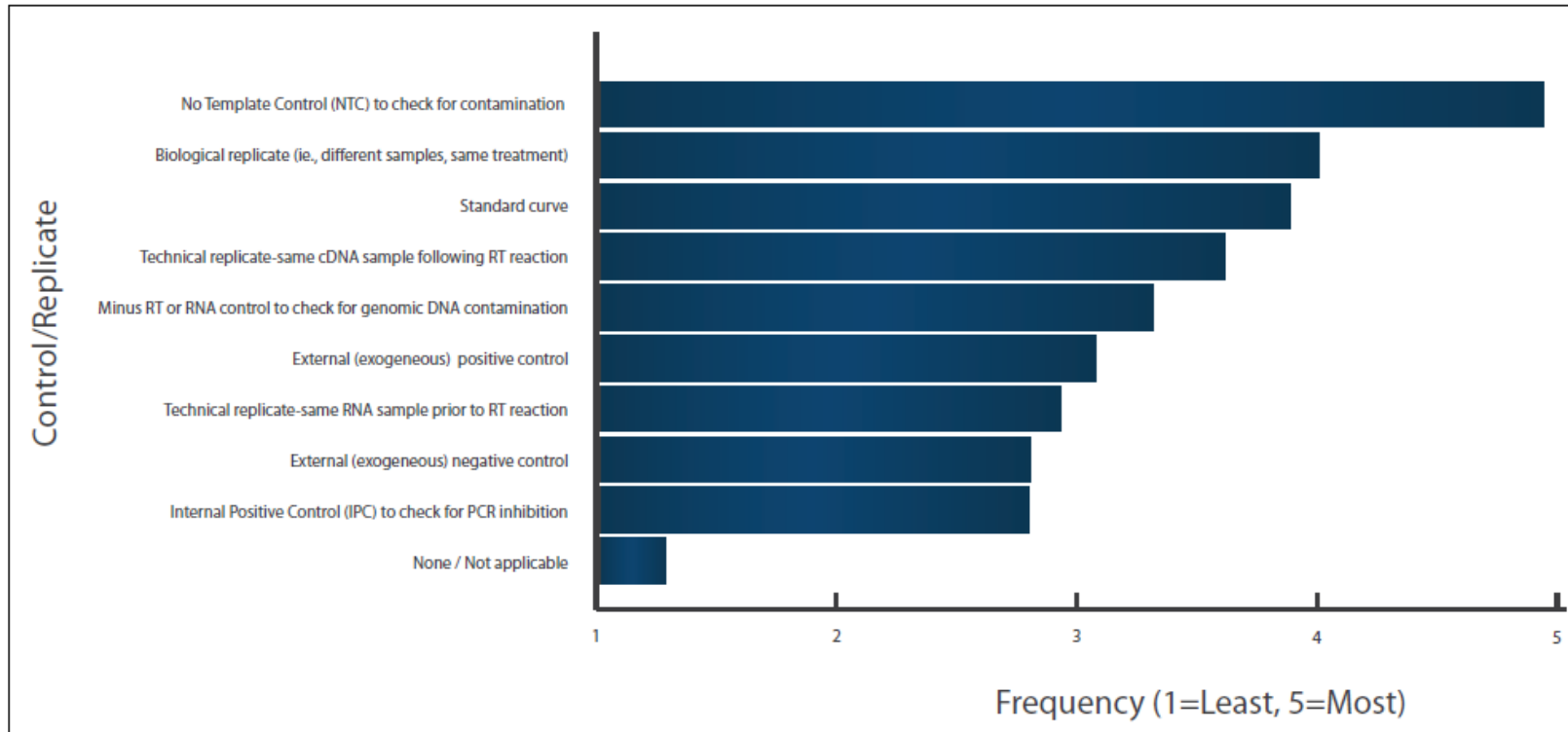


Figure 22: Frequency of replicates controls. From Nucleic Acid Research Group, NARG survey 2007, <http://www.abrf.org/NARG/>

Negativní kontroly

- NTC- voda a reakční směs – žádný signál nebo alespoň o 8 cyklů později, 2 krát
- NAC- no amplification control, bez polymerázy
- No RT- pokud ukáže amplifikaci- kontaminace genomovou DNA

Pozitivní kontroly

- Detekce kvality reagensií, přítomnost inhibitorů, poškozený vzorek
- Endogenní- druhý target kromě detekovaného genu, v multiplexu nebo zvlášť, standardizace množství DNA a RNA
- Exogenní- je známa exprese daného genu (předchozí reakce, plazmid.....)
- Spike control

Kontroly

qPCR controls



NEGATIVE

- No Template Control
- No Amplification Control
- No RT Control

AIM

- Detection of primers dimers and contamination
- Detection of probe's degradation
- Detection of genomic DNA contamination



POSITIVE

- Endogenous Control
(same sample, different target)
- Exogenous Control
(same target, different sample)
- Spiking Control
(additional DNA spiked into the sample,
different target)

AIM

- Check quality of reagents.
Also used for normalization.
- Check quality of reagents.
- Detect inhibitors presence
Reject false negative in diagnostic assays

Figure 23. Summary of the main existing controls in qPCR and their interest.

RT-qPCR

- Accuracy, sensitivity, fast results
- Monitoring of amplification in real-time

RT-PCR

- Experimental design
- RNA extraction
- RNA quality control
- Reverse transcription
- Primer and amplicon design
- qPCR validation
- Choice of reference genes
- Experimental reproducibility

Experimental design

- mRNA transcription sensitive to external stimuli- need to minimize
- Define
 - procedures
 - control groups
 - type and number of replicates
 - experimental conditions- minimize variability

RNA extraction

- From 'fresh' material if possible
- RNA stored at -80C or in RNA storage solution
- Minimize handling time- 10-20 samples
- DNase I treatment

RNA quality control

- High purity (no contamination)
- High integrity (not degraded)
- Impurities- PCR inhibition
- Purity- protein contamination
- $OD_{260/280}$ 1.8-2.0 no protein
- $OD_{260/230}$ 1.8-2.0 no organic contaminants
- $RIN > 7$
- Consistency in purity and integrity-reduction of variability of samples
- Immediately follow with procedures, store only cDNA

RT

- Immediately after isolation- no degradation of RNA from freeze/thaw
- Consistent and complete coverage of transcribed regions
- Always enter same amount of RNA and same reaction time for all samples
- Ctrl
 - no RT samples (contamination with genomic DNA)
 - no template control (contamination)
- RT Buffer
 - mix of random primers
 - RNase H
 - RT enzyme- broad dynamic range

Primer and amplicon design

- Essential for specific and efficient amplification
- Target sequences
 - Unique
 - 75-100 bp
 - GC content 50-60%
 - No secondary structures
- Primers
 - GC content 50-60%
 - Melting temperature 55-65C
 - No long stretches of G or C
 - G or C at the end of primer
 - Primer blast, MFOLD, experience

qPCR validation

- Assessed for optimal range of primer annealing temperatures, efficiency, specificity using a standard set of samples
- Reaction conditions, buffers, primers optimized
- cDNA samples not contaminated
- *www.bio-rad.com/genomics/pcrsupport*

qPCR validation

- Optimal annealing T for primers- temperature gradient
- Analysis of PCR product- melt curve analysis (single sharp peak)
- Samples run on gel
- NTC necessary (primer -dimer, DNA contamination)

PCR efficiency

- Measure of rate at which polymerase converts the reagents to amplicon
- Maximum increase per cycle is 2-fold - 100% efficiency
- Low efficiency
 - inhibitors of polymerase
 - high or suboptimal annealing temperature
 - old/inactive Taq
 - poorly designed primers
 - secondary structures

Standard curve

- 10-fold dilution - 8 points
- Broad dynamic range
- For each point- in triplicates, get Ct values
- Need tight technical replicates
- If OK- Ct values separated by 3.32 cycles
- Need 90-110% Efficiency
- R values- how well data fit on curve
- $R^2 > 0.985$ OK
- Will define dynamic range of reaction

qPCR

- Commercial qPCR kits
- Sample volumes- 10-50ul in 96 well plate format
- Software analysis
 - Flexibility in set up info
 - Group wells
 - Gene expression analysis
 - Ability to combine multiple plates

Choice of reference genes

- Perfect reference gene- no expression changes between samples from various experimental conditions, time points
- How to find:
 - Extract RNA from 1-2 samples from each condition or time point, confirm purity and quality
 - Normalize concentration, do RT from same volume
 - Do qPCR from same volume of cDNA
 - geNorm method to calculate stability
(med-gen.ugent.be/genorm/)
 - Need 3-5 genes

Experimental reproducibility

- 2 sources of variability:
 - Biological- differences of organisms, tissues, cell cultures
 - Technical- pipetting, samples quality...
- 3 biological and 2 technical replicates
- 3 biological replicates- separate and independent experiments

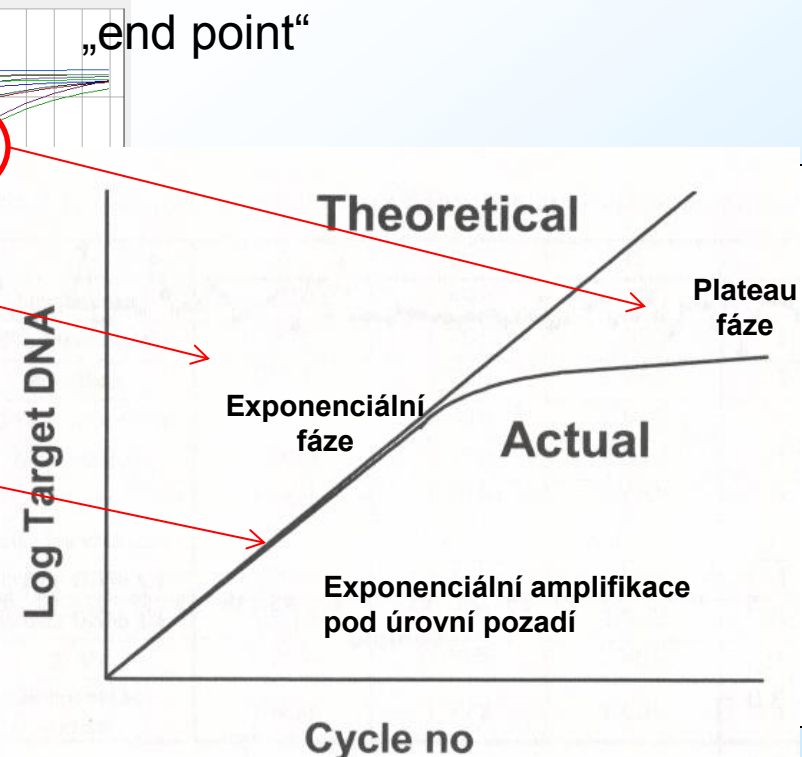
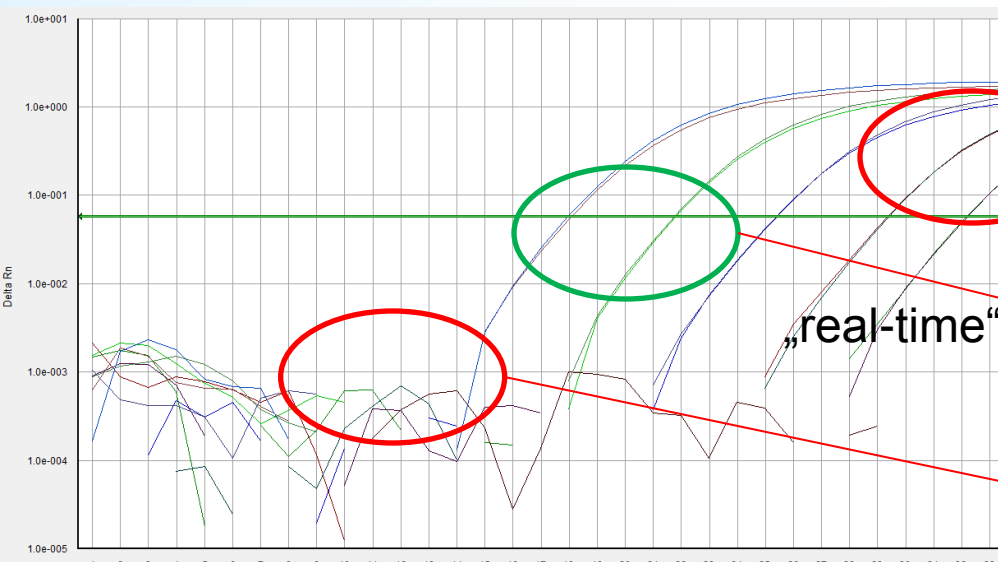
Key steps for qPCR

- Appropriate number of biological replicates and control samples
- Strict protocols for acquisition, processing and storage
- RNA purity and integrity
- RT
- Proper design of PCR

Kvantitativní vztah mezi

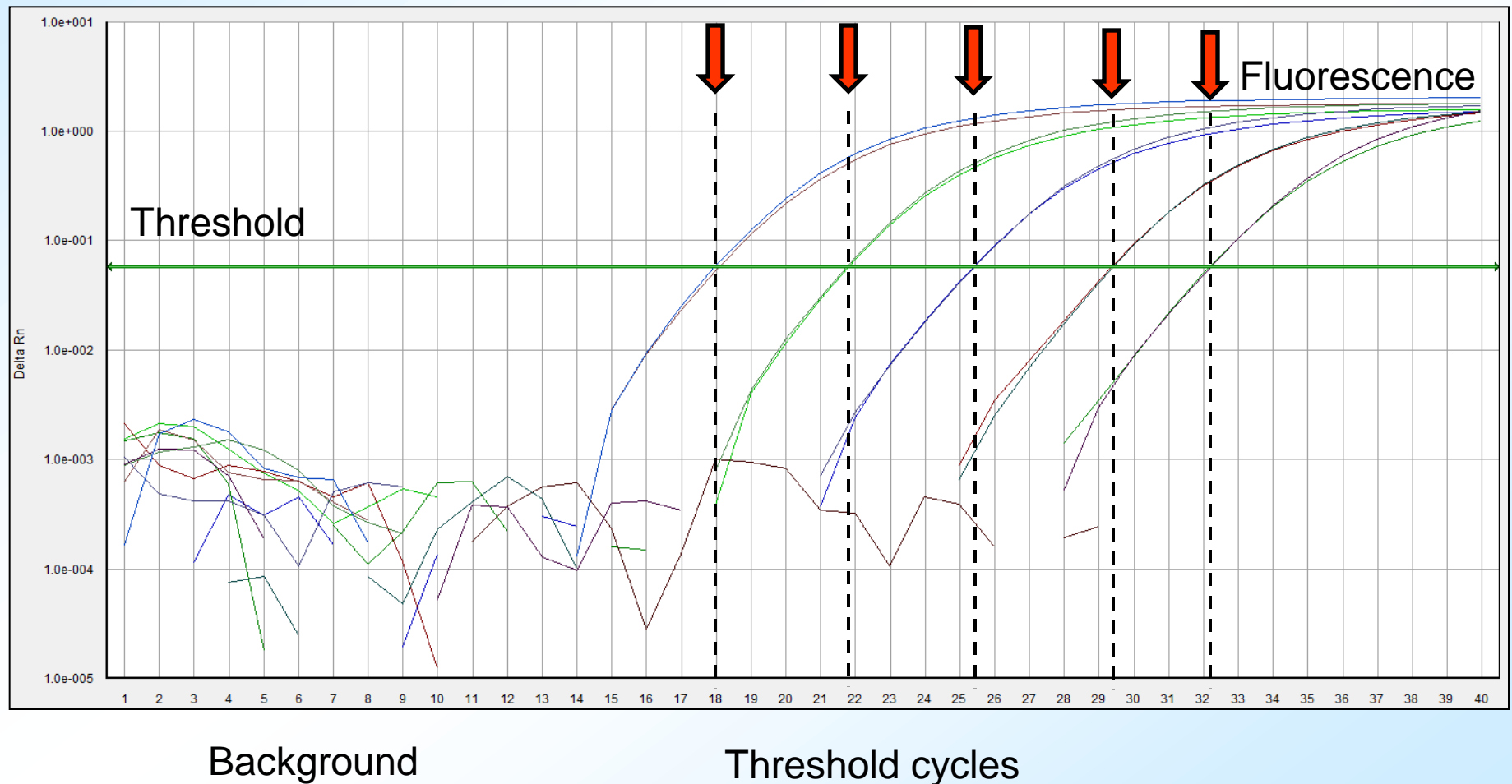
množstvím PCR produktu (amplikonu) a intenzitou fluorescence

- Amplifikační práh detekce (Ct)



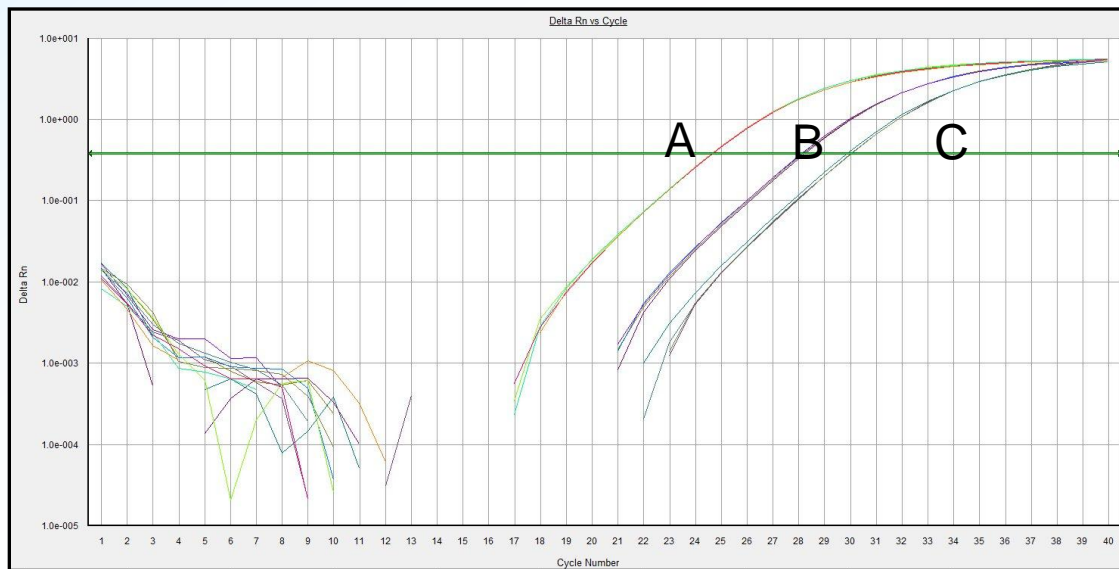
Threshold cycle „Ct“

- určený na základě hodnoty fluorescence pozadí (background) a aktuální fluorescence vzorku
- kvantitativní výstup pro každý vzorek



Threshold cycle „Ct“

- počáteční množství kopií templátu
- definovaný v exponenciální fázi PCR
- stejná účinnost PCR ve všech reakcích
- účinnost štěpení fluorogenní sondy nebo vazby fluoroforu na DNA
- citlivost detekce
- čím menší Ct - tím větší počet kopií templátu na začátku reakce



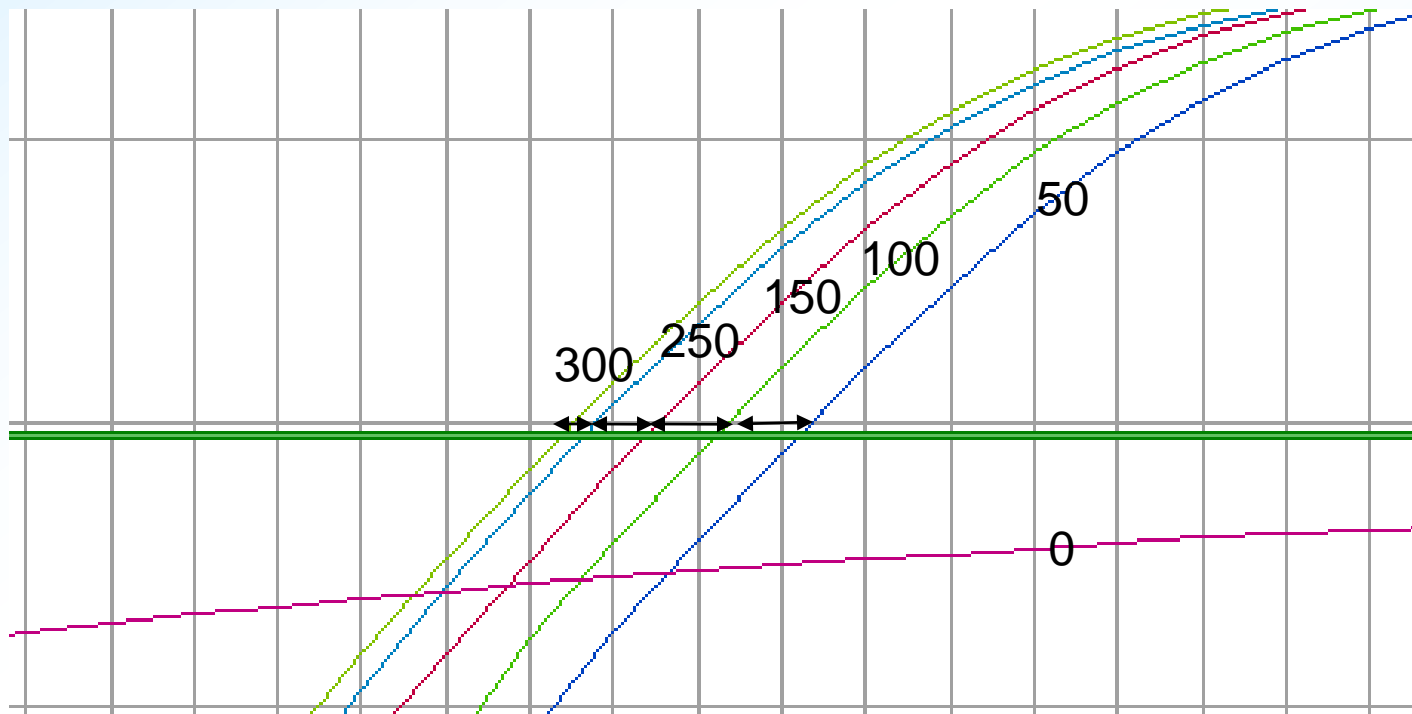
$A > B > C$

Threshold cycle „Ct“

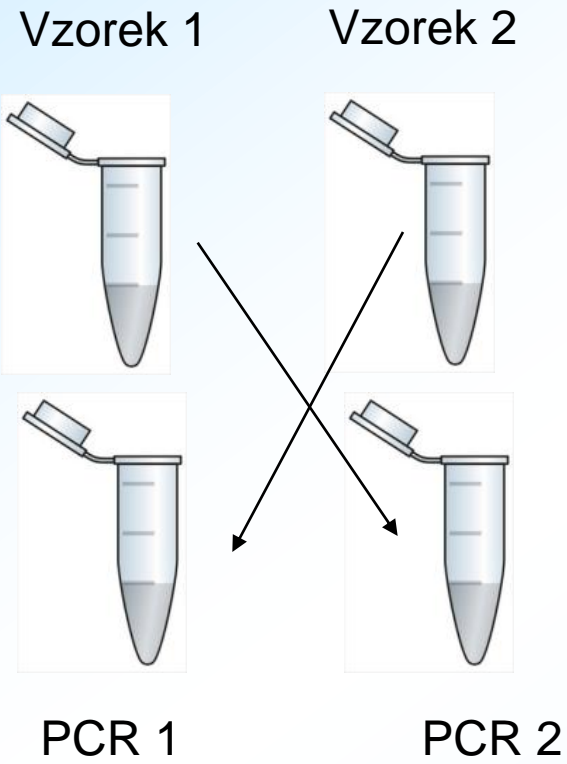
- rozdíl 1 Ct – dvojnásobné množství templátu $2^1 = 2$
- kolika cyklům odpovídá odpovídá 10ti násobný rozdíl v množství templátu?

(předpokládáme 100% účinnost PCR) $2^n = 10$

n=3,32



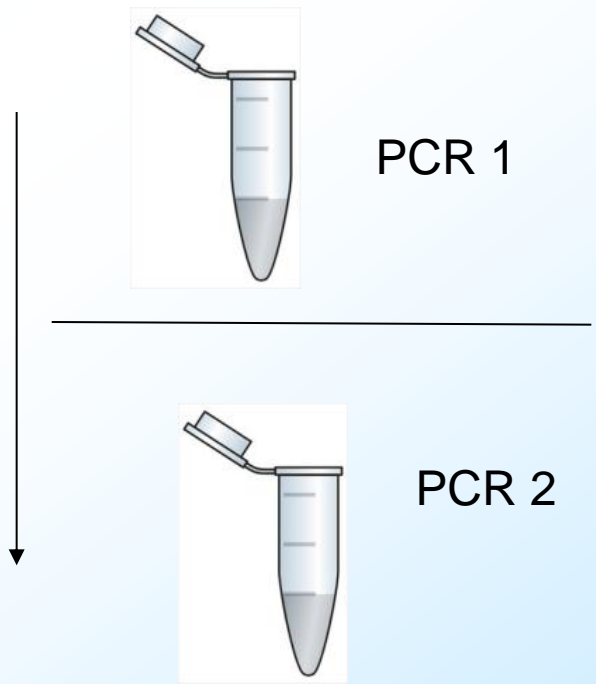
Cross contamination



Vzájemná kontaminace vzorků

Přenos amplikonu do dalších PCR

Carry-over contamination

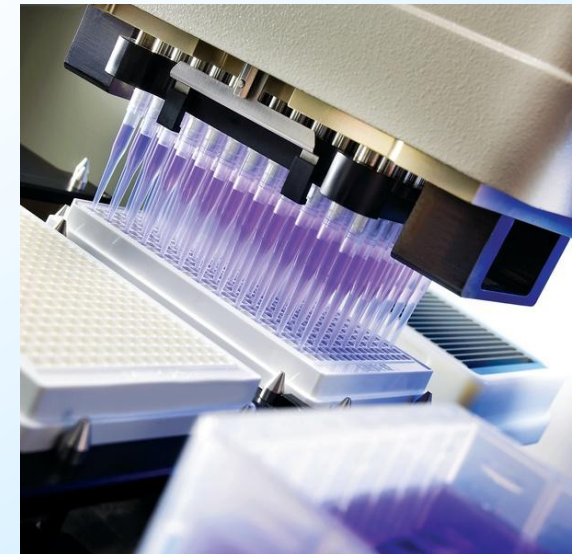
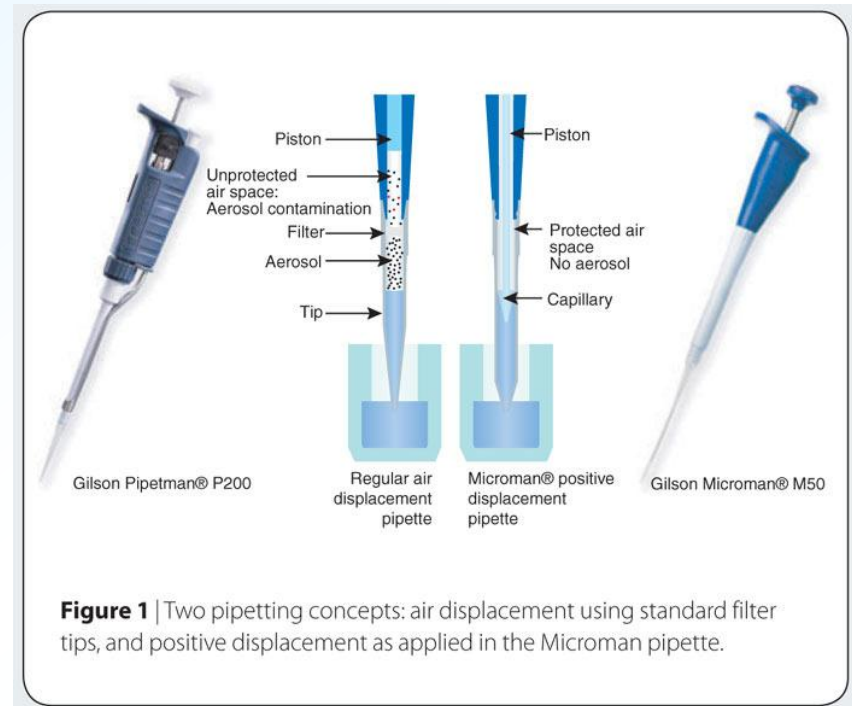
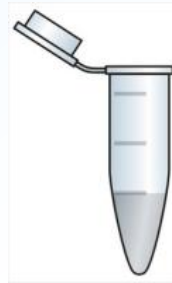


Jak předejít kontaminaci

- Správná laboratorní praxe
- Plastik v RNA kvalitě
- Automatizace



×



Prevence kontaminace

- Fyzické oddělení pre a postPCR procesů
- Pipety a jiné nástroje oddělit
- Rukavice měnit často
- Reakci připravit v laminárním boxu
- Filtrované špičky
- Reagencie – pracovat opatrně, zabránit kontaminaci
- UV světlo pro dekontaminaci místnosti, 10% savo