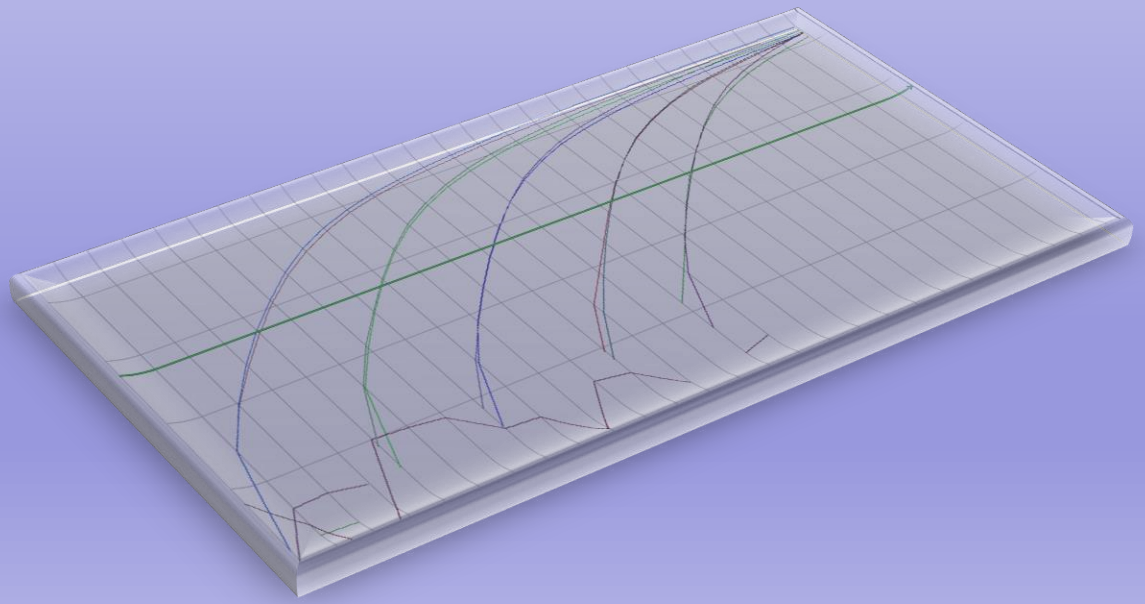
ÚVOD DO KVANTITATIVNÍ REAL-TIME PCR



Tipy na začátek

- Experimentální design
  - Co dělám?
  - Proč to dělám?
  - Jak to dělám?

- Kontrola všech chemikálií
  - Voda – alikvoty
  - Kit na RT – jen pro 1 studenta
  - Master mixy – omezené množství lidí

Tipy na začátek

- Oddělení místností pre a post PCR
  - Izolace DNA, RNA
  - RT
  - PCR a post-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
- Reakční směs zhruba o 10% víc než je potřeba
- Destičku lze uchovat až 10 h v temnu a chladu
- Správně a úplně zalepit destičku fólií i na okrajích
- Alespoň duplikáty
- Po napipetování spin na centrifuze

Kontroly

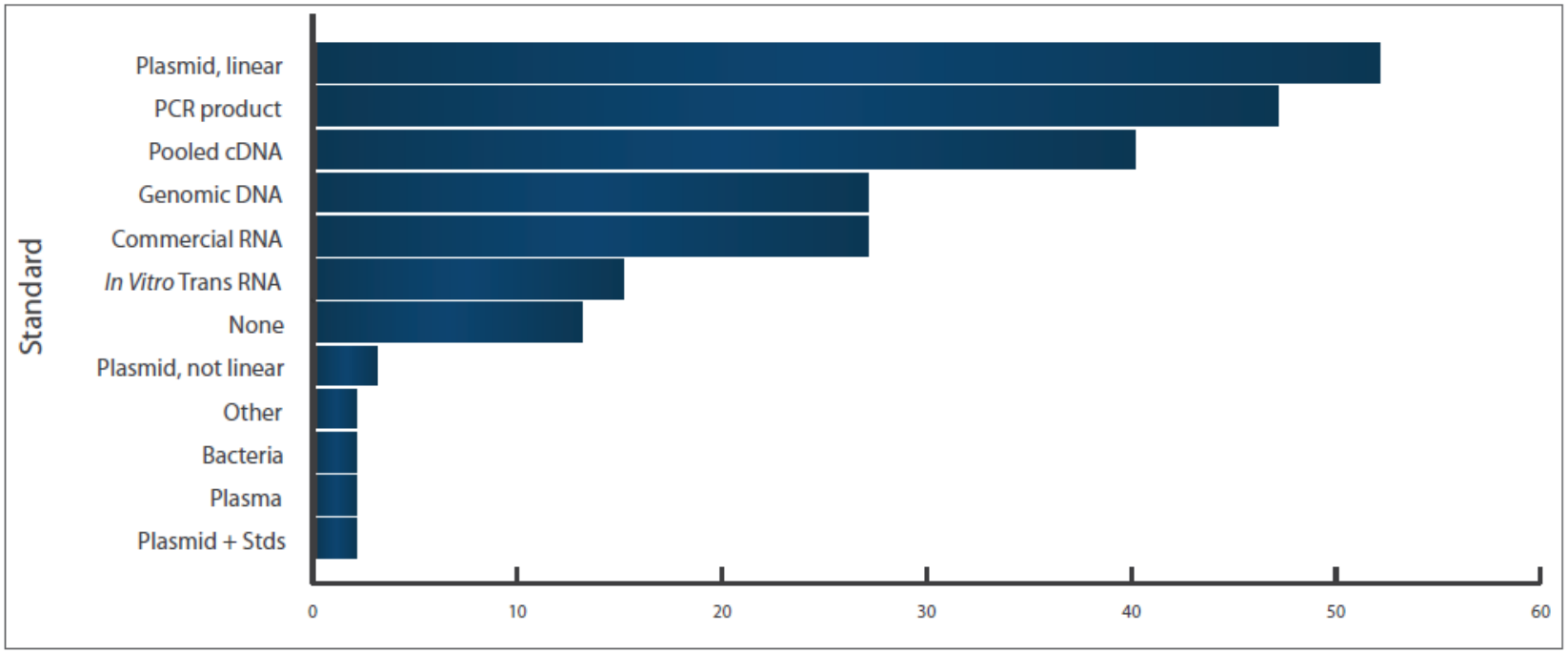


Figure 21: Most frequently used quantification standards. From Nucleic Acid Research Group, (NARG) survey 2007, http://www.abrf.org/NARG/

Frekvence kontrol

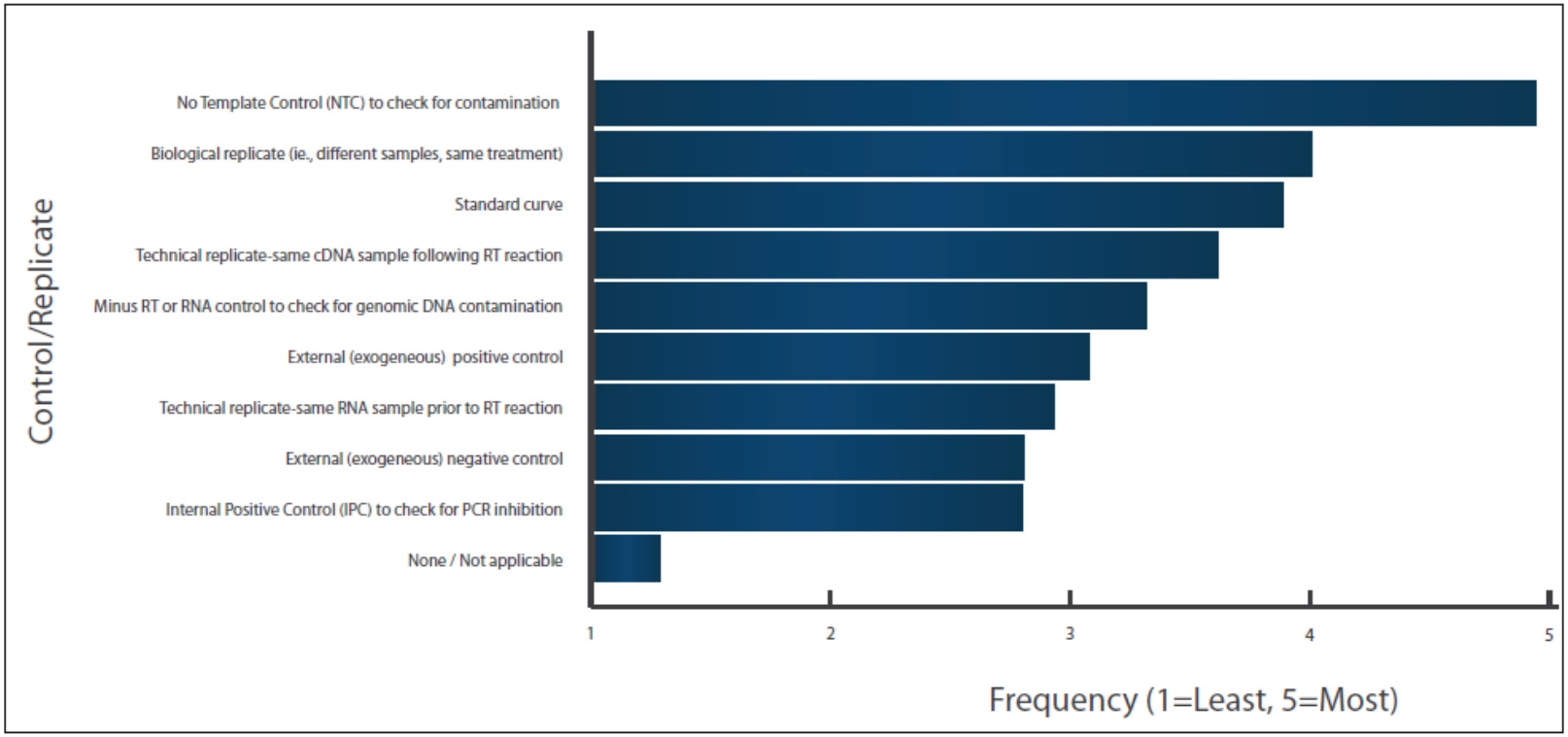


Figure 22: Frequency of replicates controls. From Nucleic Acid Research Group, NARG survey 2007, http://www.abrf.org/NARG/

Negativní kontroly

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

Pozitivní kontroly

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

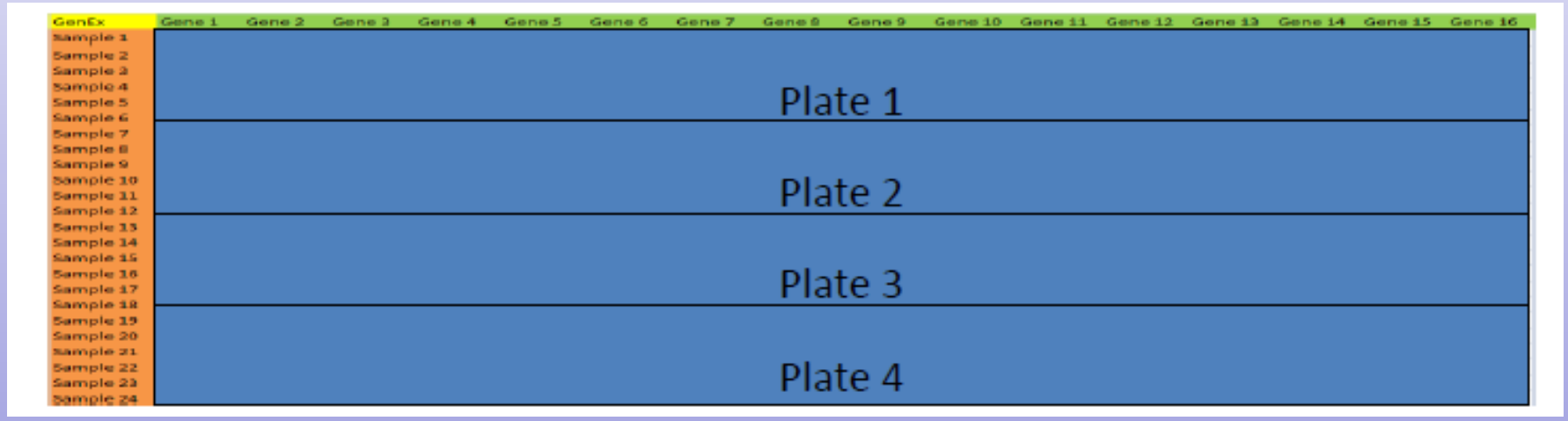
Interplate control

- Interplate calibrators are used to compensate for variations between runs due to instrument settings (base-line correction and threshold settings). These variations are independent of assay, but depend on instrument channel used.
- The Cq of an interplate calibrator must be determined with very high accuracy, else interplate calibration may add more variance to the data than the systematic variation it removes.
- It is highly discouraged to perform independent interplate calibrations per assay!

Interplate control

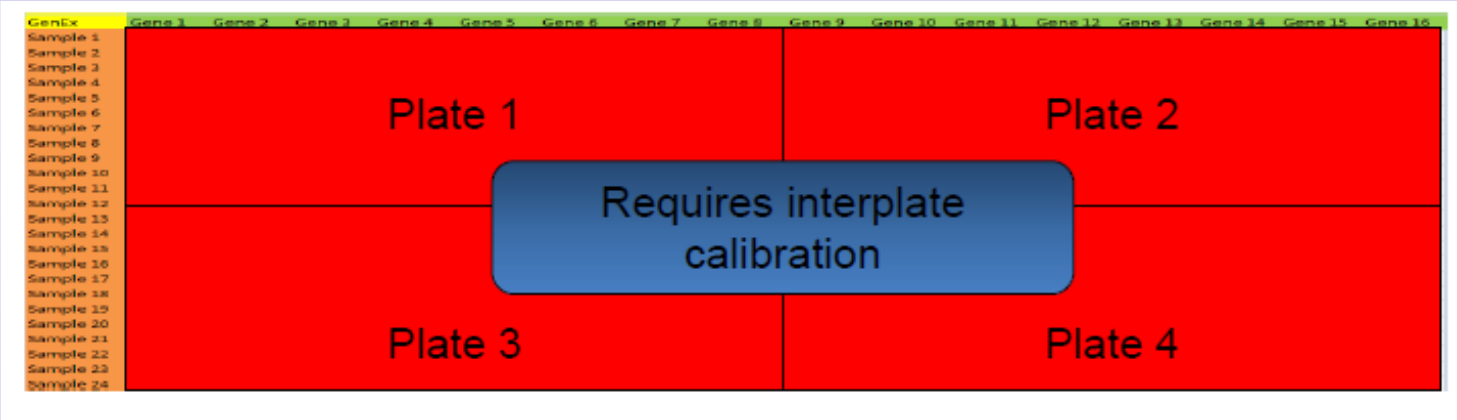
- When expression of genes and samples is compared, multiple runs can be merged for common analysis without correction if either:
  - All genes analyzed for each sample are assayed in the same plate (“All genes”)
  - All samples analyzed for each gene are assayed in the same plate (“All samples”)
  - MIXED analysis: Requires interplate calibration
    - very robust assay
    - SD of triplicate < 0.1 Cq
    - uncomplicated and stabilized template at fairly high concentration (15 < Cq< 20)
    - provided in 50 aliquots ready to use (-20°C storage)
    - IPC should be run in replicates (minimum triplicates)

Kdy není potřeba IPC – ALL GENES



Kdy není potřeba IPC – ALL SAMPLES

Kdy je potřeba IPC - MIXED



Kontroly

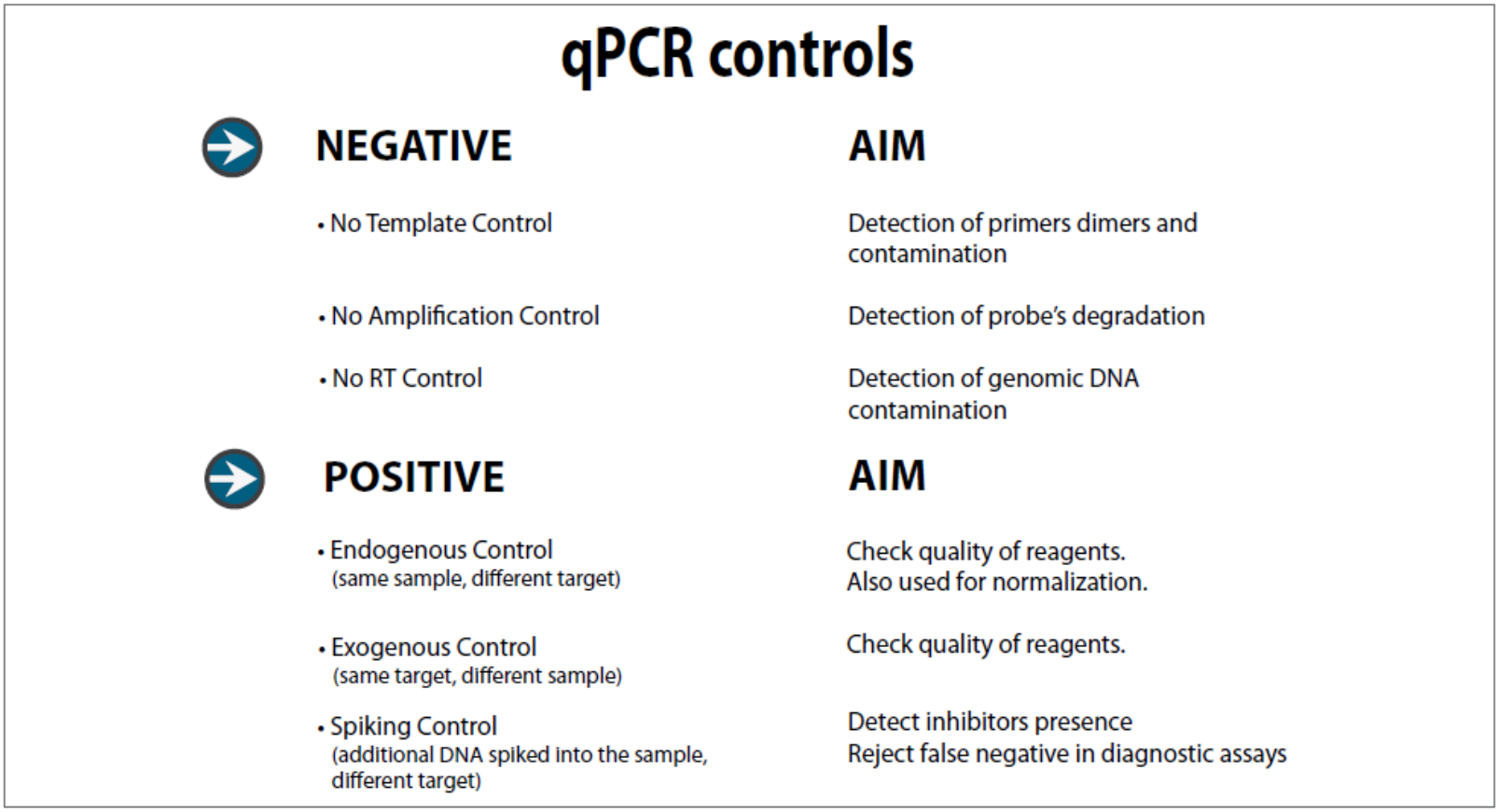


Figure 23. Summary of the main existing controls in qPCR and their interest.

RT-qPCR

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

Bustin, 2010

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

Reverse Transcription

- 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

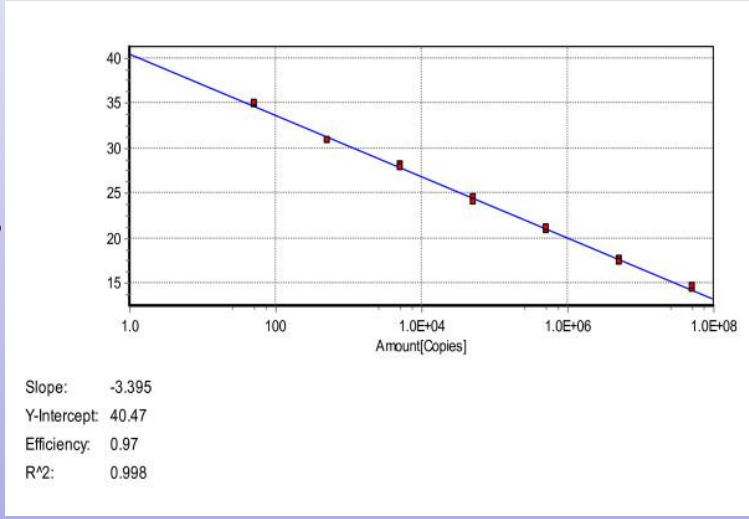
- Optimal annealing T for primers- temperature gradient
- Analysis of PCR product- melt curve analysis (single sharp peak)
- Samples run on gel
- NTC necessary (primer-dimer, DNA contamination)

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

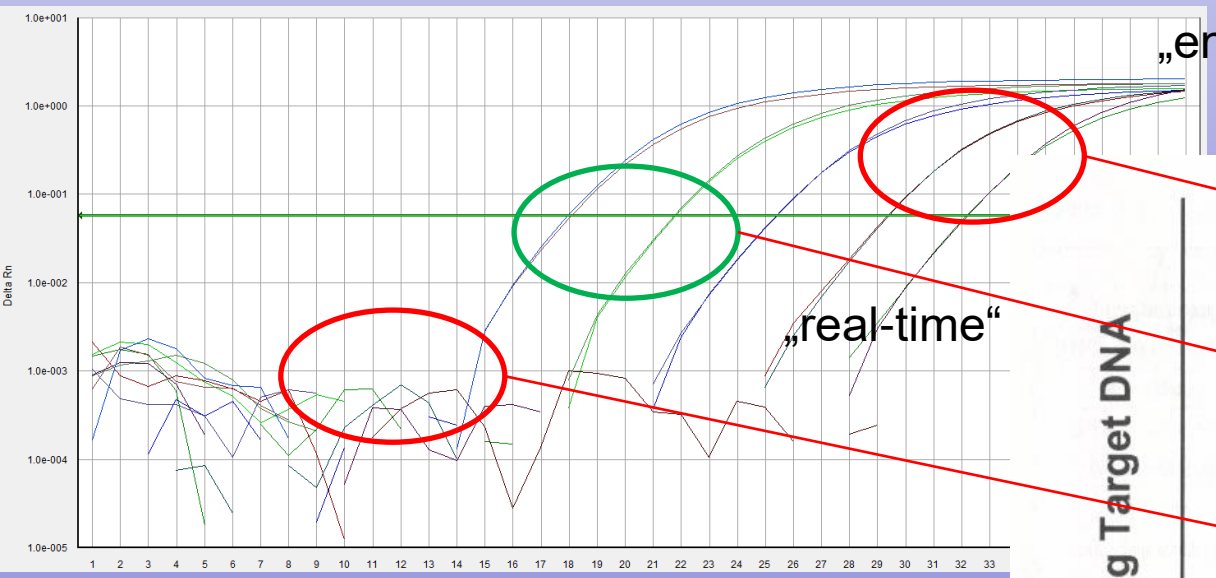
- Appropriate number of biological replicates and control samples
- Strict protocols for acquisition, processing and storage
- RNA purity and integrity
- Reverse Transcription
- Proper design of PCR

Real – time detekce amplifikace

Kvantitativní vztah mezi

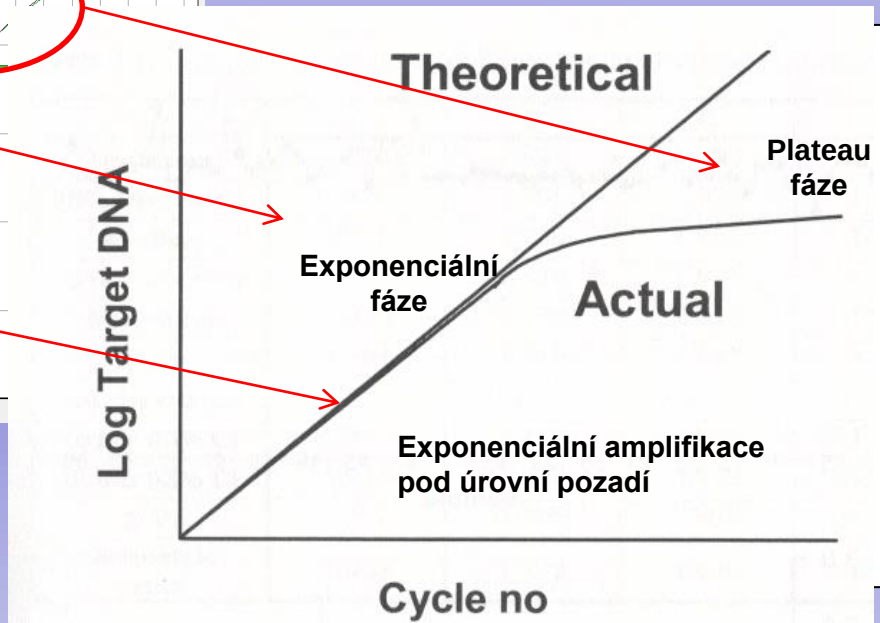
množstvím PCR produktu (amplikonu) a intenzitou fluorescence

- Amplifikační práh detekce (Ct)



„end point“

„real-time“



Threshold cycle „Ct“

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

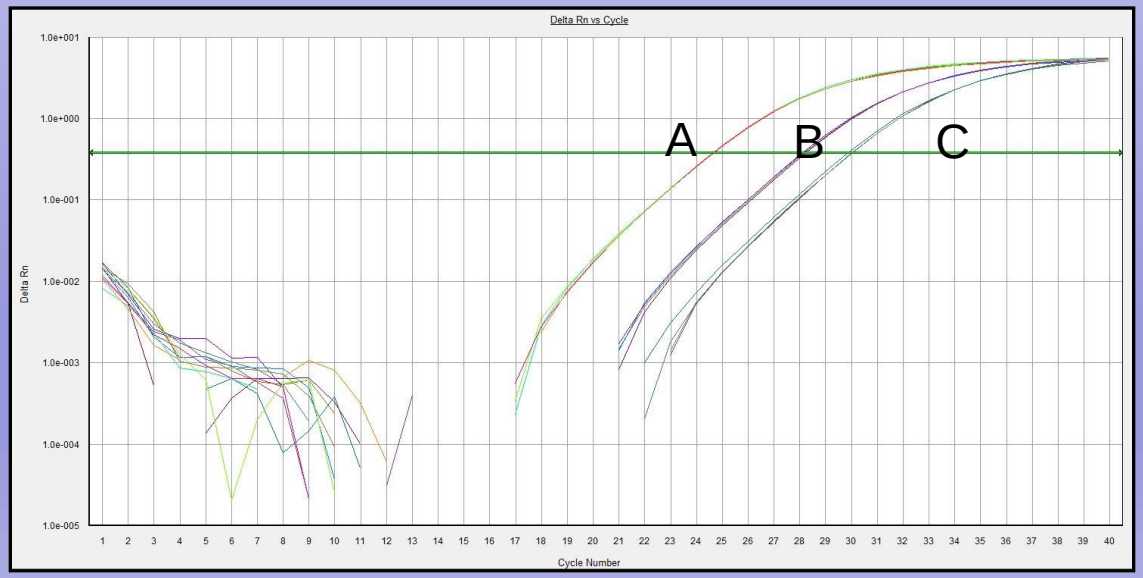
– kvantitativní výstup pro každý vzorek

Background

Threshold cycles

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

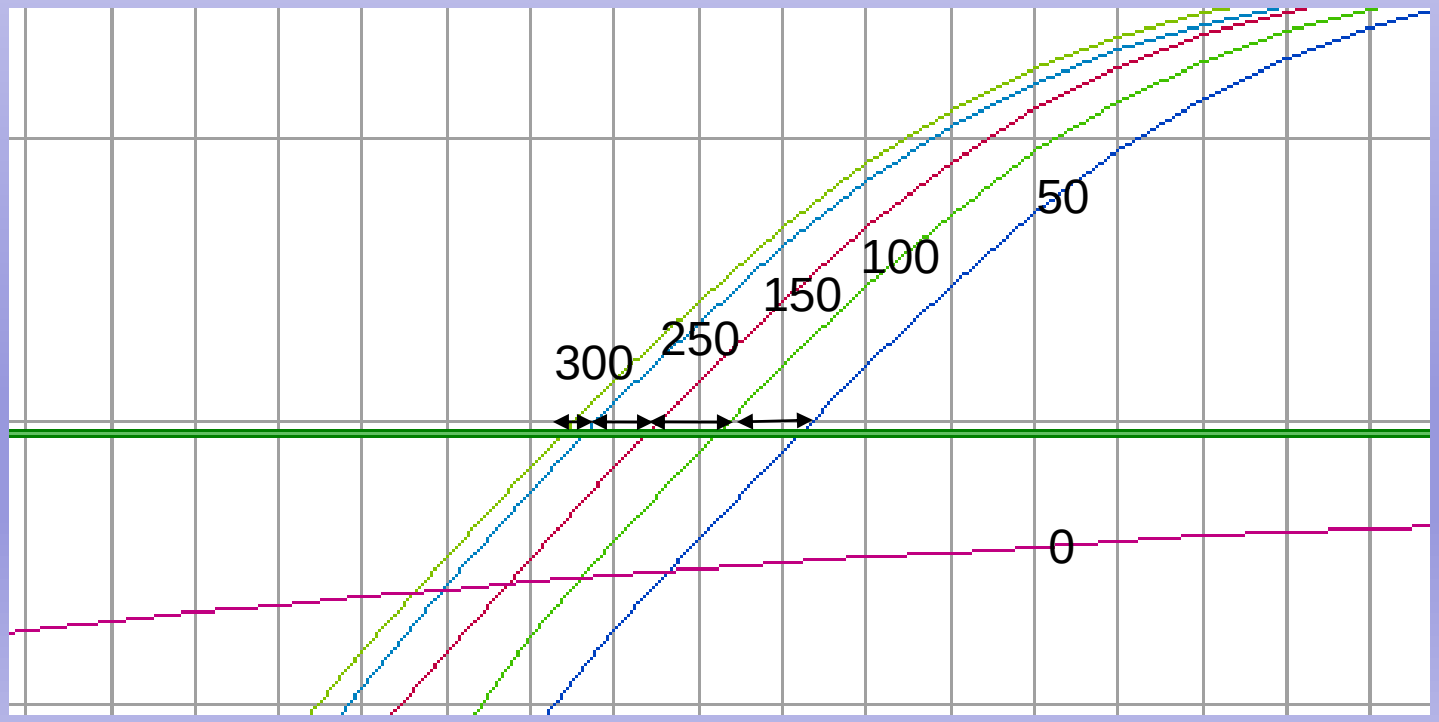
Threshold cycle „Ct“

- 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



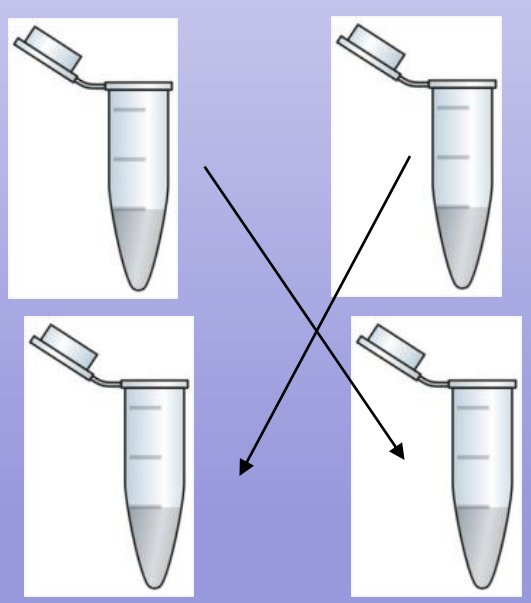
c [ng/µl]	Ct
50	28,16
100	27,16
150	26,66
250	26,06
300	25,66

Kontaminace PCR

Cross contamination

Vzorek 1

Vzorek 2



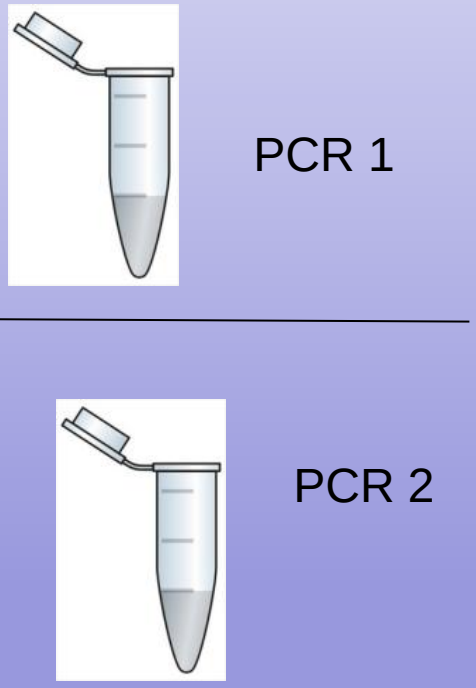
PCR 1

PCR 2

Vzájemná kontaminace vzorků

Carry-over contamination

Přenos amplikonu do dalších PCR



PCR 1

PCR 2

Jak předejít kontaminaci

- Správná laboratorní praxe
- Plastik v RNA kvalitě
- Automatizace

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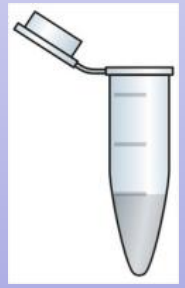


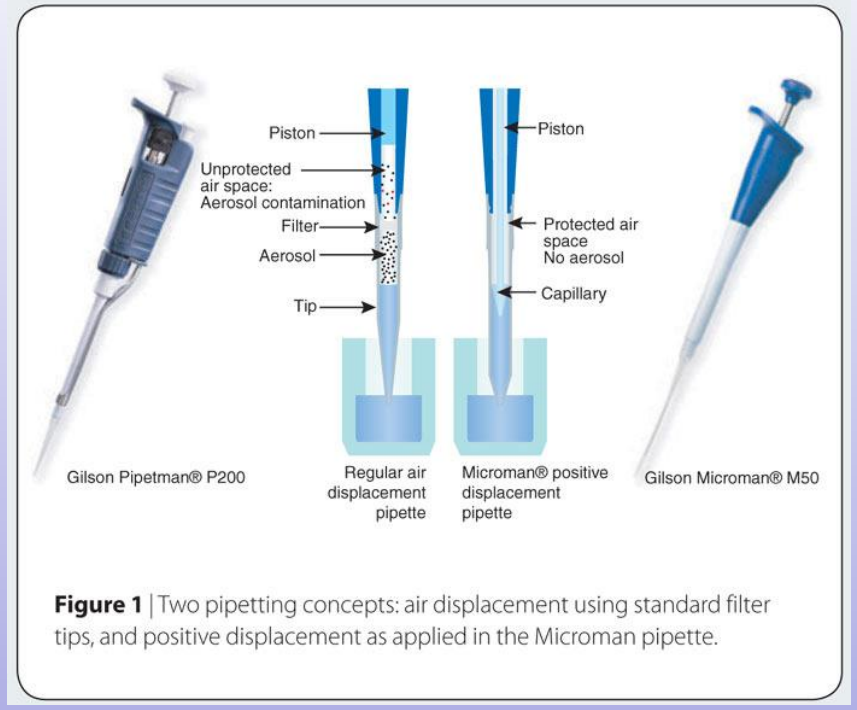
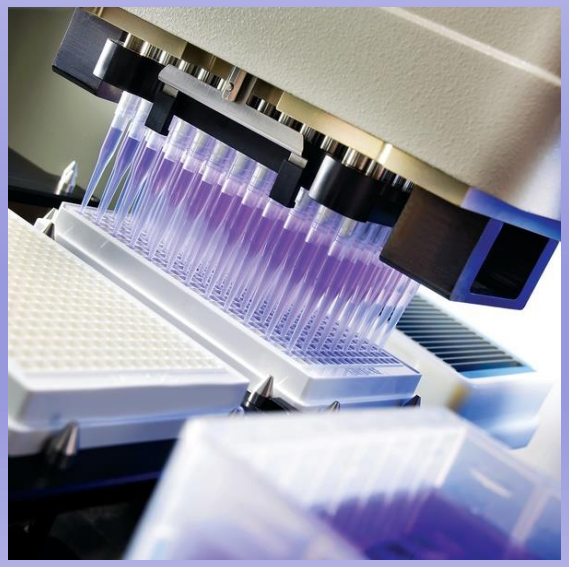


Figure 1 | Two pipetting concepts: air displacement using standard filter tips, and positive displacement as applied in the Microman pipette.



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

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

Nejčastější důvody špatné PCR

- Špatný design primerů a sond
- Špatná kvalita RNA
- Nepoužívání master mixů
- Cross-contamination
- Nepoužití no RT control (NAC ctrl)
- Špatná normalizační kontrola
- Sybr green – melting curve
- Ct cycle – v exponenciální oblasti
- Špatná efektivita reakce
- Špatně udělaná standardní křivky

Co je sonda

- qPCR založeno na detekci fluorescenčního signálu
  - Taqman probes (FRET)
  - Molecular beacons (FRET)
  - Scorpions (FRET)
  - Sybr green
- Detekce PCR produktů vyrobením fluorescenčního signálu

Co jsou primery

- Sekvenčně specifické úseky DNA