PREANALITIČKA I ANALITIČKA KONTROLA KVALITETA QPCR

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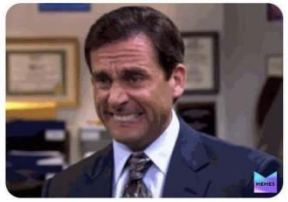
Time traveler: What year is it?

Me: 2020

Time traveler:

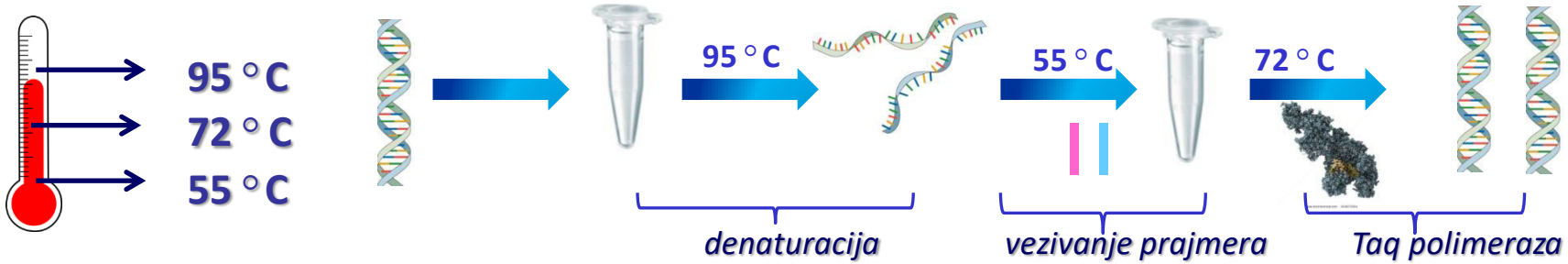


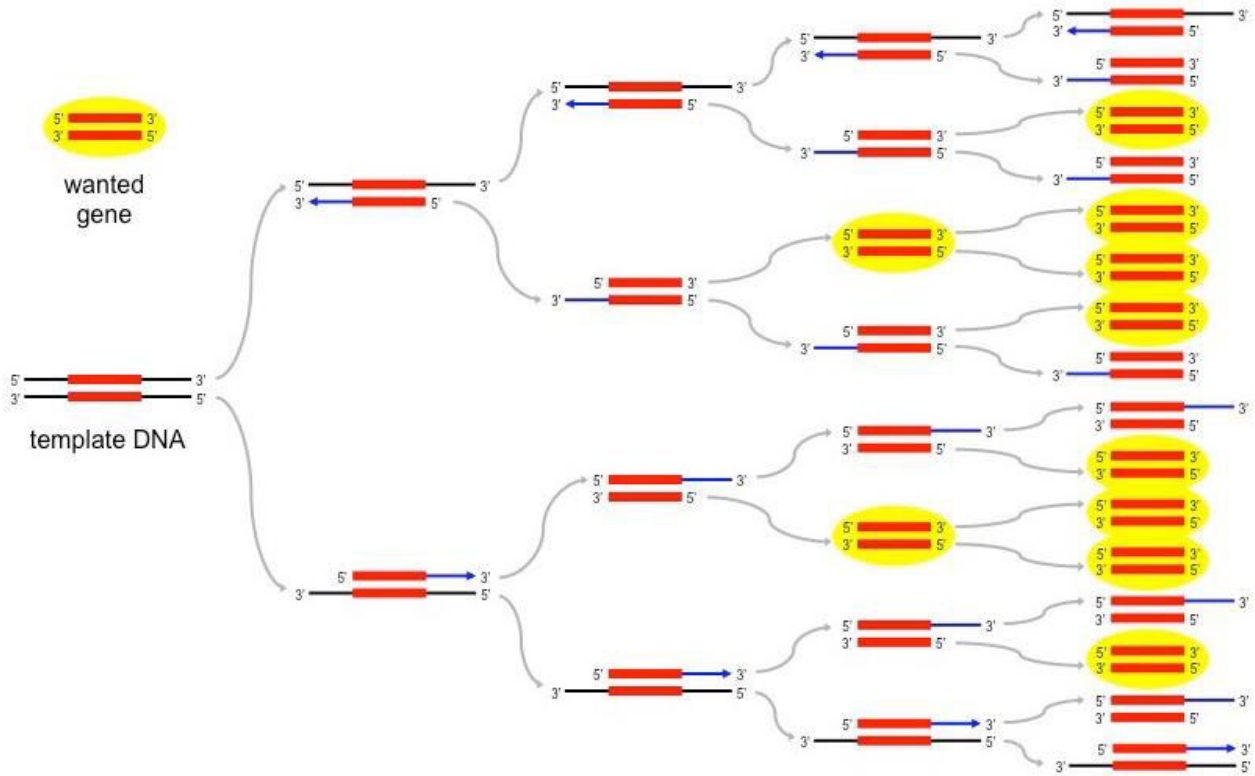
Finnaly, 2020 is over.
2021:

An Ancient Egyptian COVID-1900 B.C
nasal swab test (PCR test)

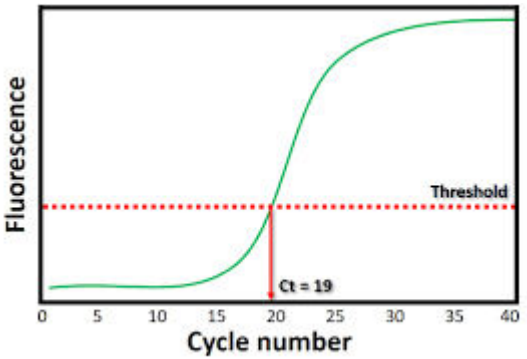
Any PCR test with
a cycle threshold above
35 is too sensitive and
cannot represent a 'positive'

PCR – *polimerase chain reaction*

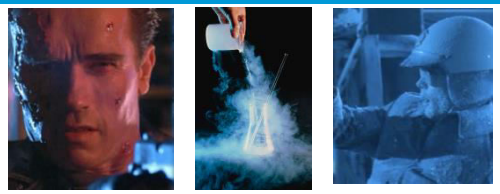


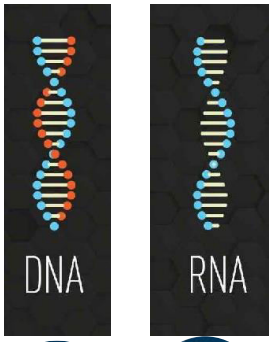


OSETLJIVOST



Preanalitička faza – uzorkovanje i čuvanje





4°C 4°C

-20°C -20°C

-80°C -80°C

CSH Cold Spring Harbor Laboratory

bioRχiv
THE PREPRINT SERVER FOR BIOLOGY

New Results

Performance of RNA purification kits and blood collection tubes in the Extracellular RNA Quality Control (exRNAQC) study

exRNAQC Consortium, Jasper Anckaert, Francisco Avila Cobos, Anneleen Decock, Jill Deleu, Olivier De Wever, Jilke De Wilde, Bert Dhondt, Thibaut D'huyvetter, Celine Everaert, Carolina Fierro, Hetty Hilde Helsmoortel, An Hendrix, Eva Hulstaert, Scott Kuersten, Pieter Mestdagh, Annelien Morlion, Nele Nijs, Justine Nuytens, Annouck Philippron, Thomas Piofczyk, Kathleen Schoofs, Gary P. Schroth, Eveline Vanden Eynde, Jo Vandesompele, Tom Van Maerken, Ruben Van Paemel, Kimberly Verniers, Nurten Yigit

doi: http://sci-hub.tw/10.1101/2021.05.11.442610

transcriptomes) using 189 synthetic spike-in RNAs as processing controls. When comparing blood tubes, so-called blood preservation tubes do not stabilize RNA very well, as is reflected by increasing RNA concentration and number of detected genes over time, and by compromised reproducibility. We also document large differences in

Preanalitička faza - izolacija

VARIKINA:
- 1 % rastvor
- tamne posude
- dnevna priprema

ETANOL:
- 70 % rastvor
- tamne posude
- dnevna priprema

INHIBITORI RNAza:
Komercijalno dostupni rastvori koji inhibiraju RNAze

RUKAVICE:
- nitrilne
- bez talka
- često menjati

UV STERILIZACIJA:
- 30 min

NASTAVCI:
- sterilni
- PCR nivo čistoće
- sa filterima

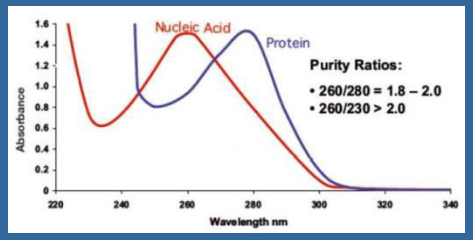
REAGENSI:
- PCR nivo čistoće

Nucleic Acid	DNA	RNA
Storage medium	1. TE Buffer-(Tris-EDTA Buffer) (10 mmol/L Tris, 1mmol/L EDTA, pH 7.4-8.0) 2. Sterile nuclease free water	1. Store RNA as precipitate in ethanol 2. TE Buffer 3. Sterile nuclease free water
Storage method	Divide DNA into small aliquots and store in sterile, nuclease free tubes.	Divide RNA into small aliquots and store in sterile, nuclease free tubes or diethylpyrocarbonate (DEPC) treated tubes. RNA can be reverse transcribed to cDNA and stored for better long term stability.
Storage Temperature	Recommended -70ºC Store at -200C if -70ºC not available Avoid repeated freeze-thaw of samples.	

Preanalitička faza – provera kvaliteta

UV analiza uzoraka omogućava kvantifikaciju NK i procenu kontaminacije organskim rastvaračima i proteinima:

- ✓ 230 nm
- ✓ 260 nm
- ✓ 280 nm

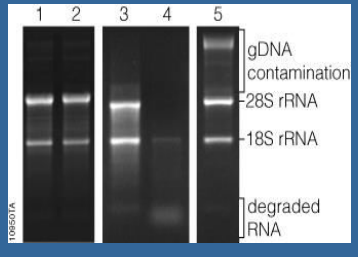


Fluorimetrija – posebno dizajnirane boje za detekciju (*Qubit 4™*):

- ✓dsDNA
- ✓ssDNA
- ✓RNA
- ✓microRNA
- ✓Proteina

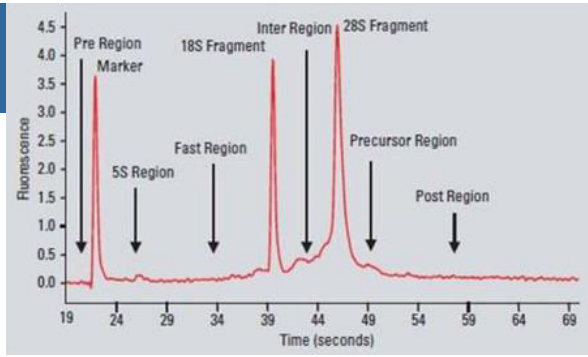
Elektroforeza na agaraznom gelu omogućava procenu očuvanosti:

- ✓ 28S rRNK
- ✓ 18S rRNK
- ✓ gDNK

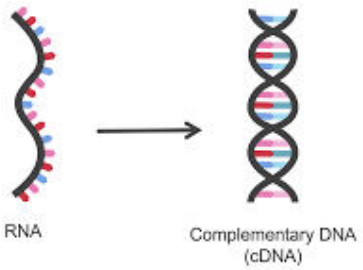


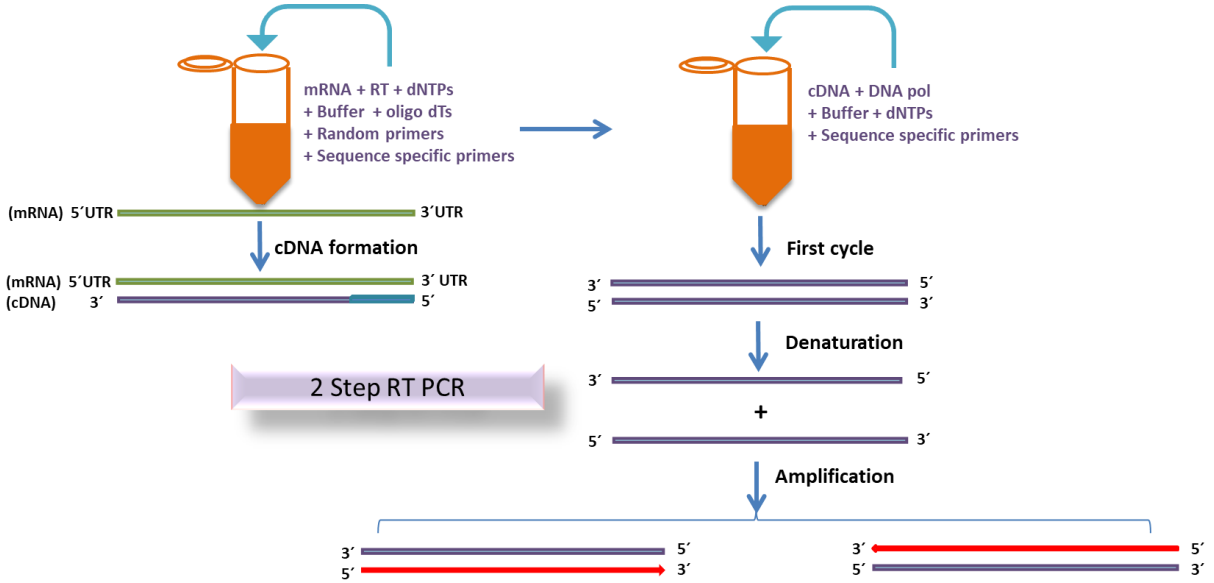
Kapilarna elektroforeza za procenu očuvanosti (*Bioanalyzer 2100™*):

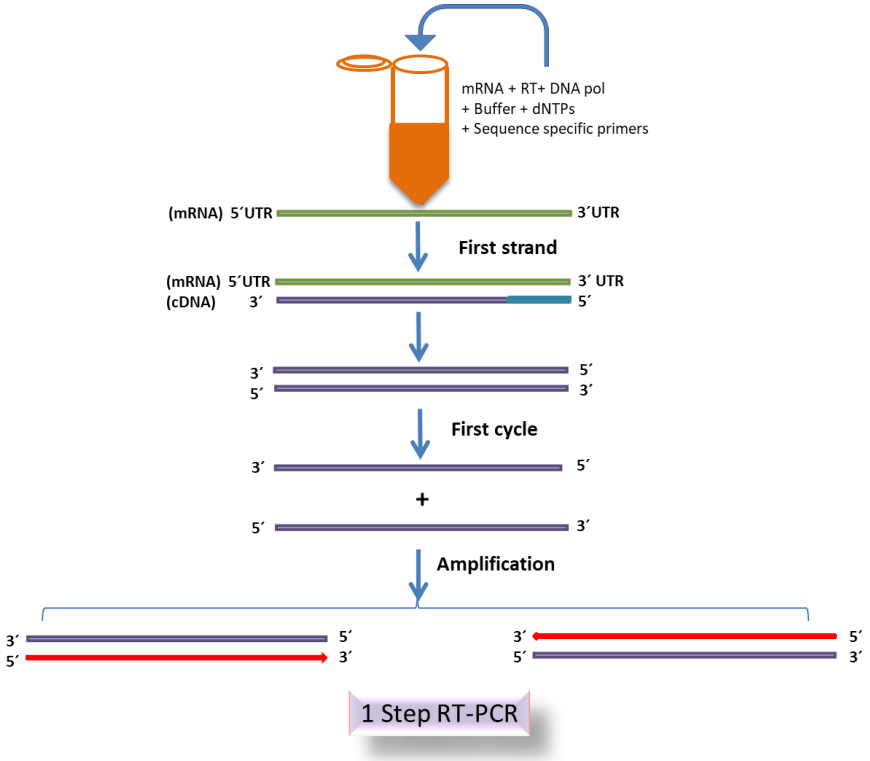
RNA integriti number (RIN™)



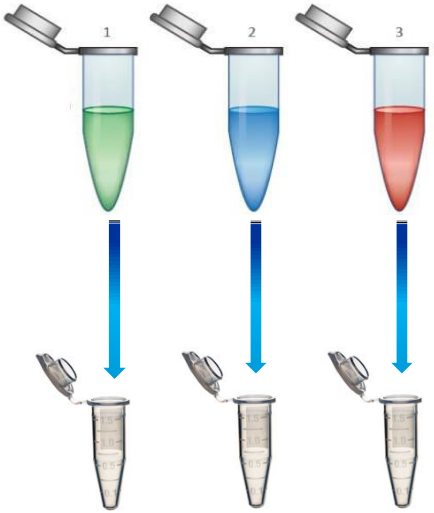
Analitička faza – Reverzna transkripcija





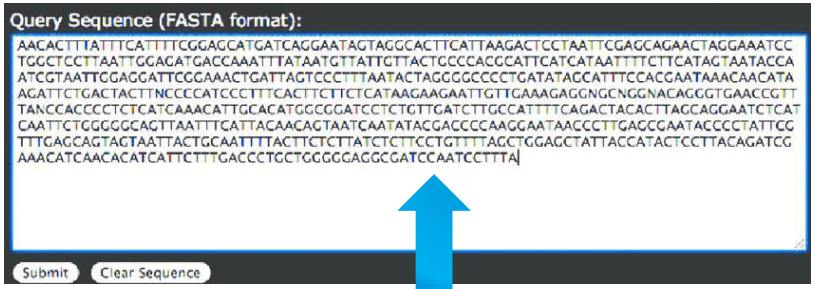


Efikasnost RT je zavisna od koncentracije RNK!



Analitička faza – odabir amplikona

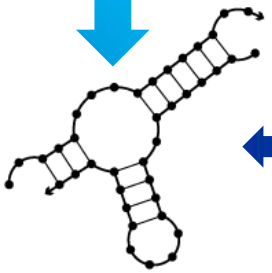
Dužina amplikona: 75-150 b



Ispitivanje target sekvence

5′ amplikon 3′

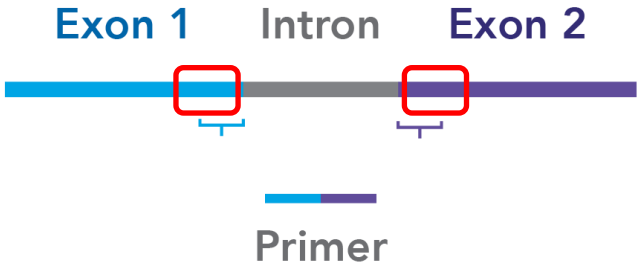
3′ 5′



Sekundarne strukture

A
EVE
RADAR
REVIVER
ROTATOR
LEPERS REPEL
MADAM I'M ADAM
STEP NOT ON PETS
DO GEESE SEE GOD
PULL UP IF I PULL UP
NO LEMONS, NO MELON
DENNIS AND EDNA SINNED
ABLE WAS I ERE I SAW ELBA
A MAN, A PLAN, A CANAL, PANAMA
A SANTA LIVED AS A DEVIL AT NASA
SUMS ARE NOT SET AS A TEST ON ERASMUS
ON A CLOVER, IF ALIVE, ERUPTS A VAST, PURE EVIL; A FIRE VOLCANO

Palindromske sekvence

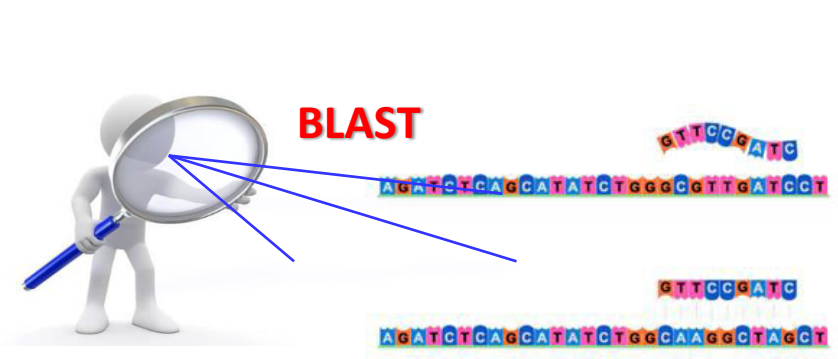


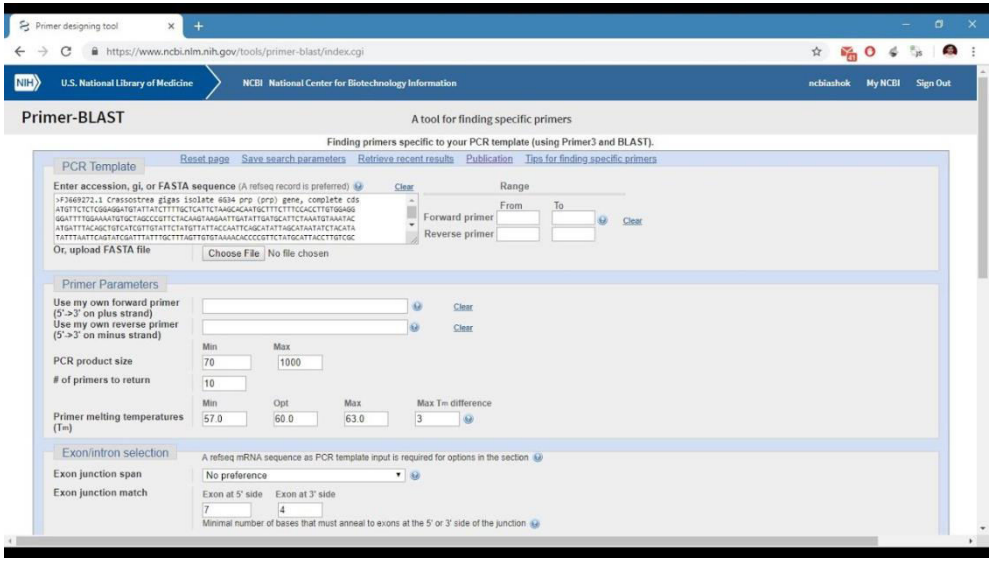
Amplikon „preko introna“

Analitička faza – in silico dizajn prajmera

- ☑ Dužine 20-24 baze
- ☑ Izbegavati homologiju unutar i između prajmera kako bi se smanjila verovatnoća stvaranja dimera
- ☑ Sekevenca prajmera ne bi trebalo da sadrži više od 4 uzastopna G ili C baze
- ☑ Sadržaj GC parova 40-60 %
- ☑ Kako bi se olakšalo vezivanje prajmera za matricu jedan od pet nukleotida do 3' kraja prajmera bi trebalo da bude G ili C
- ☑ Temperature topljenja dva prajmera bi trebalo da budu što bliže (57-61°C)

- ☒ Sekundarne strukture (Gibsova slobodna energija < -2 kcal/mol)
- ☒ Dimeri u okviru jednog prajmera (3' kraj →Gibsova slobodna energija < -5 kcal/mol; unutrašnji deo prajmera → Gibsova slobodna energija < -6 kcal/mol)
- ☒ Ukršteni dimeri (3' kraj →Gibsova slobodna energija < -5 kcal/mol; unutrašnji deo prajmera → Gibsova slobodna energija < -6 kcal/mol)





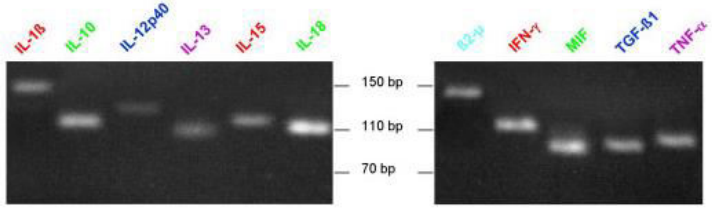
Analitička faza – optimizacija i validacija prajmera

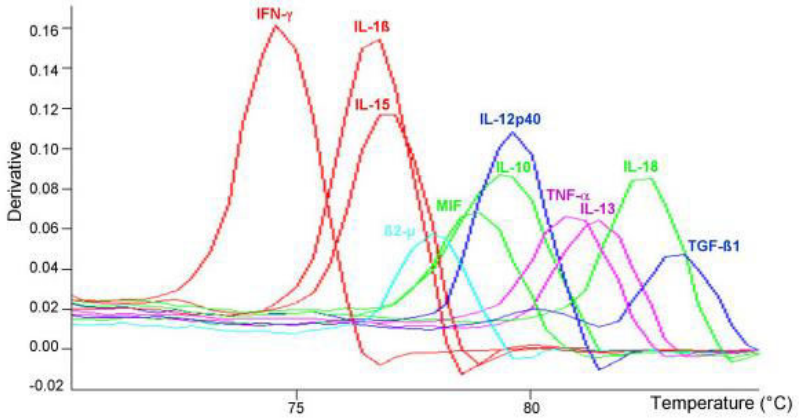
Optimizacija uslova reakcije:

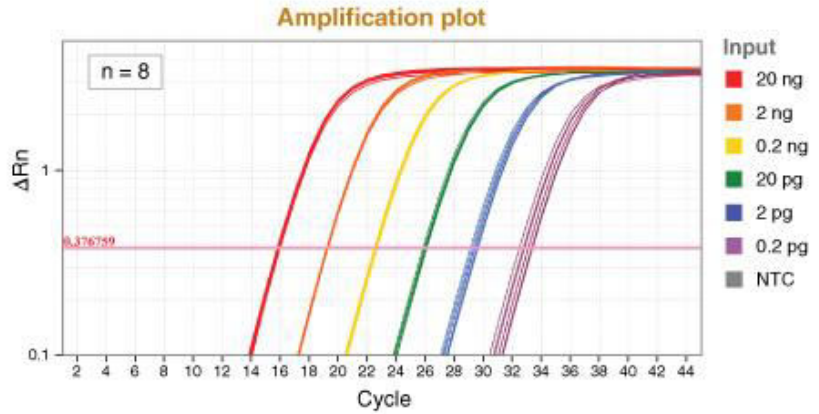
- ✓ *Koncentracija prajmera*
- ✓ *Temperatura vezivanja prajmera*
- ✓ *Koncentracije obeleženih proba*
- ✓ *Koncentracija uzorka*
- ✓ *Temperatura reakcije*
- ✓ *Elektroforeza i „melting curve“ analiza*

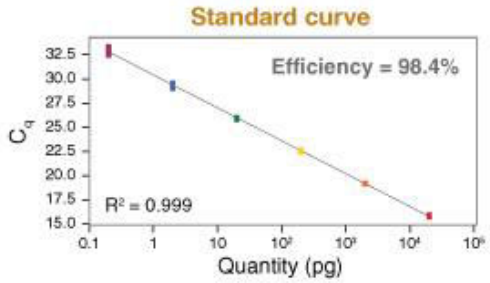
Efikasnost reakcije:

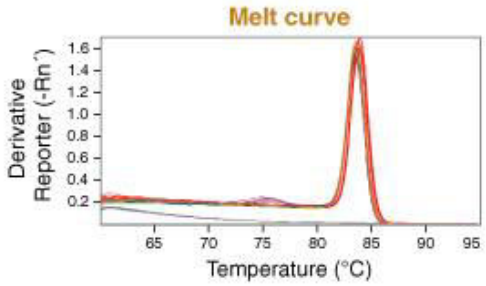
- ✓ 2^n
- ✓ *Standardna kriva*
- ✓ $E=10^{-1/nagib}$
- ✓ *E= 100% → Nagib = -3,32*
- ✓ *E → 90-110 %*



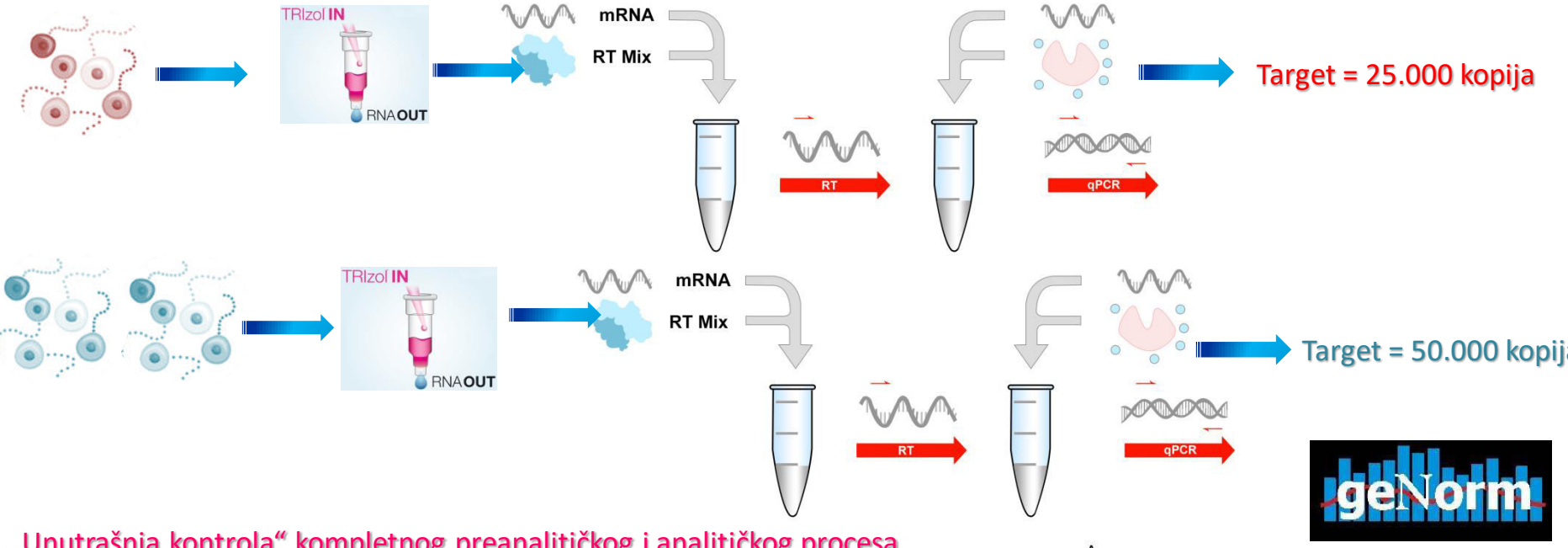




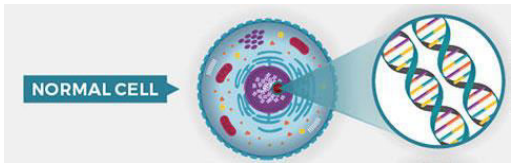


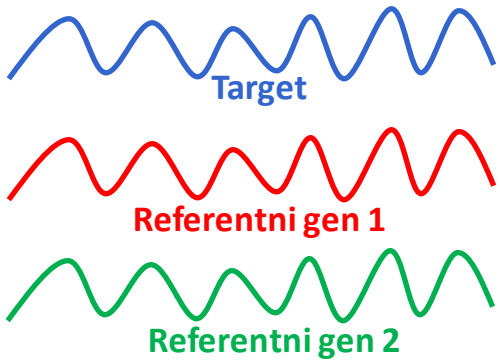


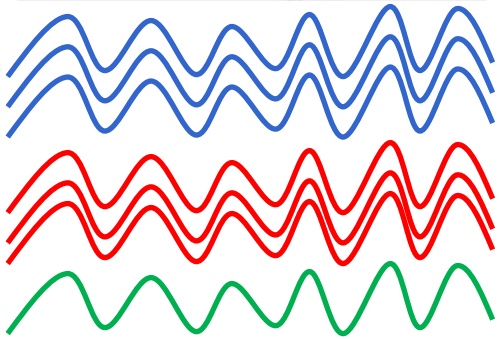
Analitička faza – odabir referentnog gena

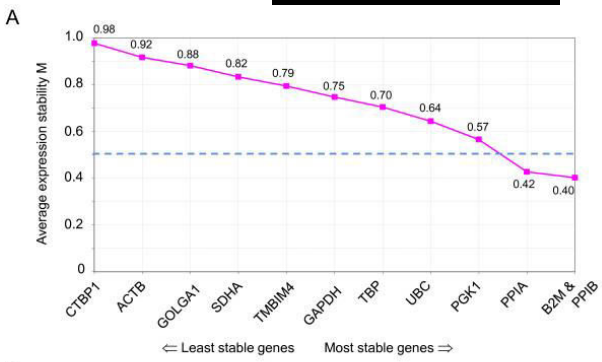


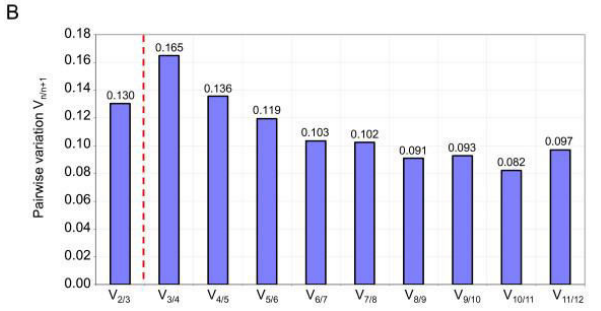
„Unutrašnja kontrola“ kompletnog preanalitičkog i analitičkog procesa
→ Referentni gen



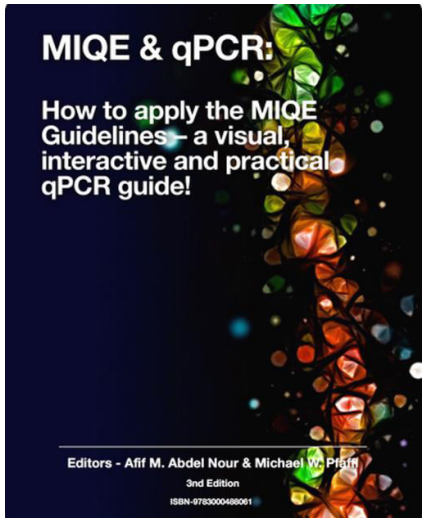








Standardi kvaliteta



ISO 20395:2019(en)
Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR

- *Endogena kontrola*
- *„Cell free RNA“*
- *„Spike in“*
- *Tačnost*
- *Preciznost*
- *LOD*
- *LOQ*