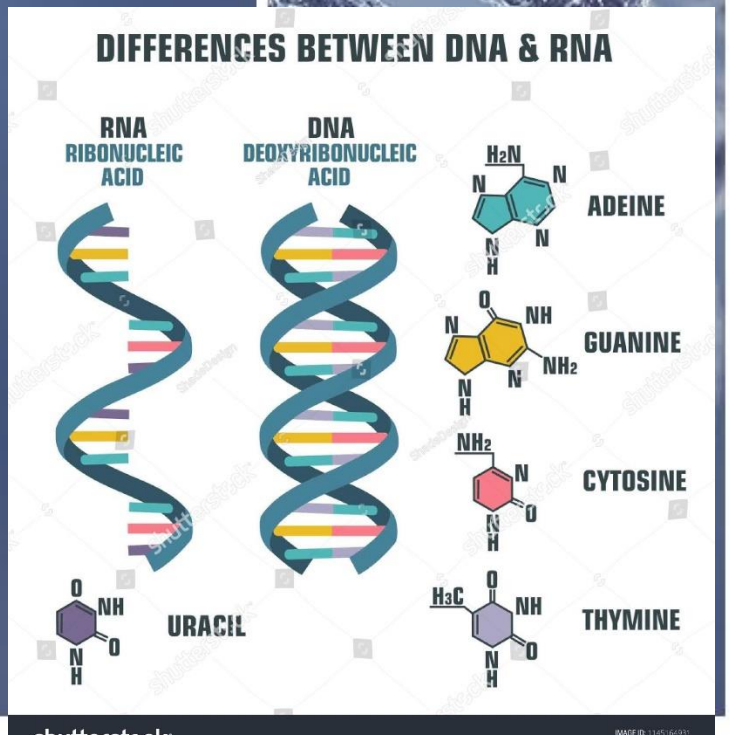
Tehnika reverzne transkripcije. Vizuelizacija produkata dobijenih upotrebom Real-time PCR.

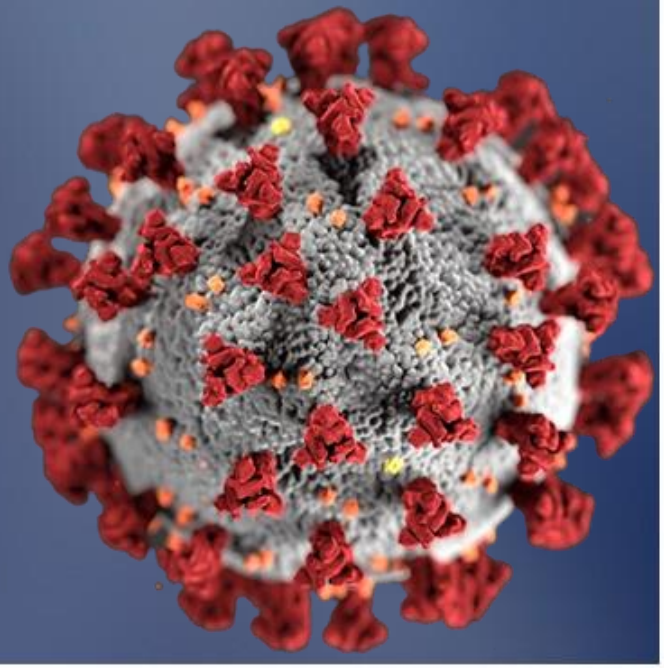
- **Polymerase chain reaction** (**PCR**) je metoda za brzo umnožavanje miliona/milijardi kopija specifične sekvence DNK u *in vitro* uslovima.

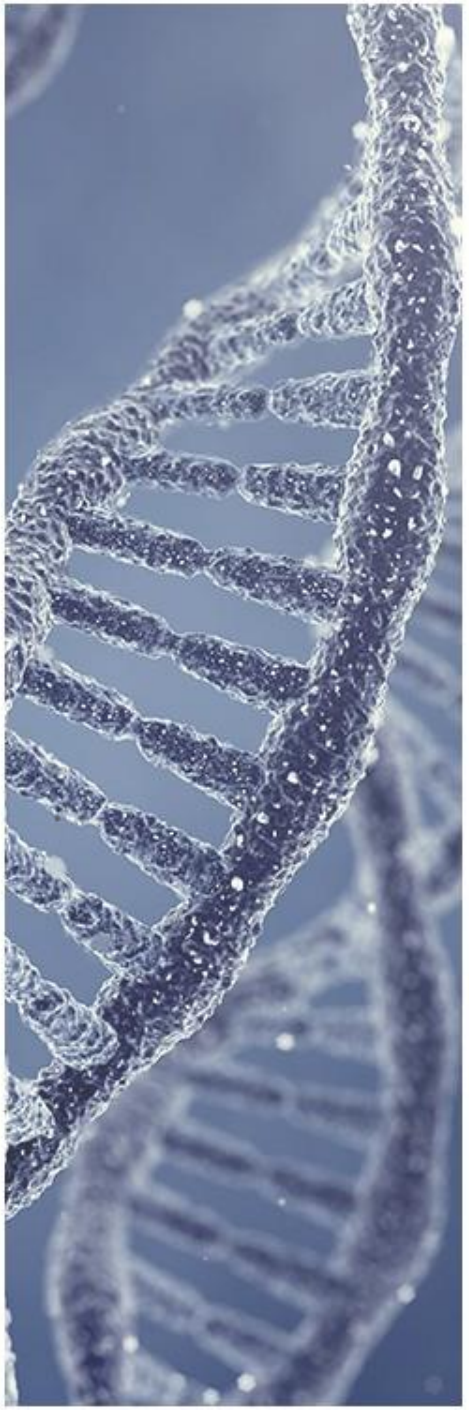
- *In vitro* amplifikacija RNK?



RNK

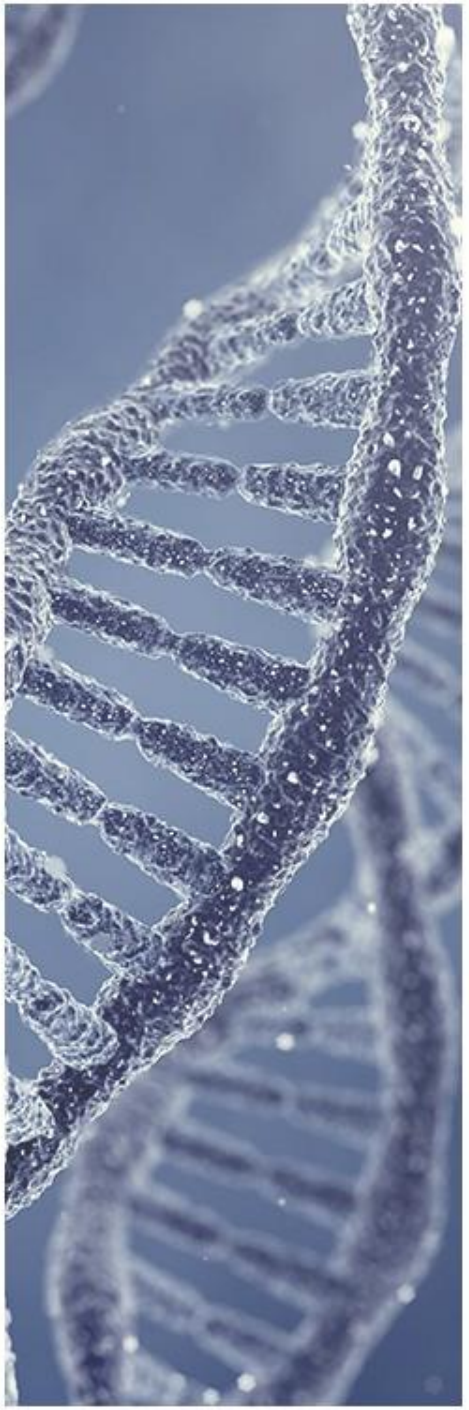
- Analizi eukariotske informacione RNK (iRNK) engl. messenger RNA (mRNA) – analizu genske ekspresije
- Amplifikaciju virusne RNK.





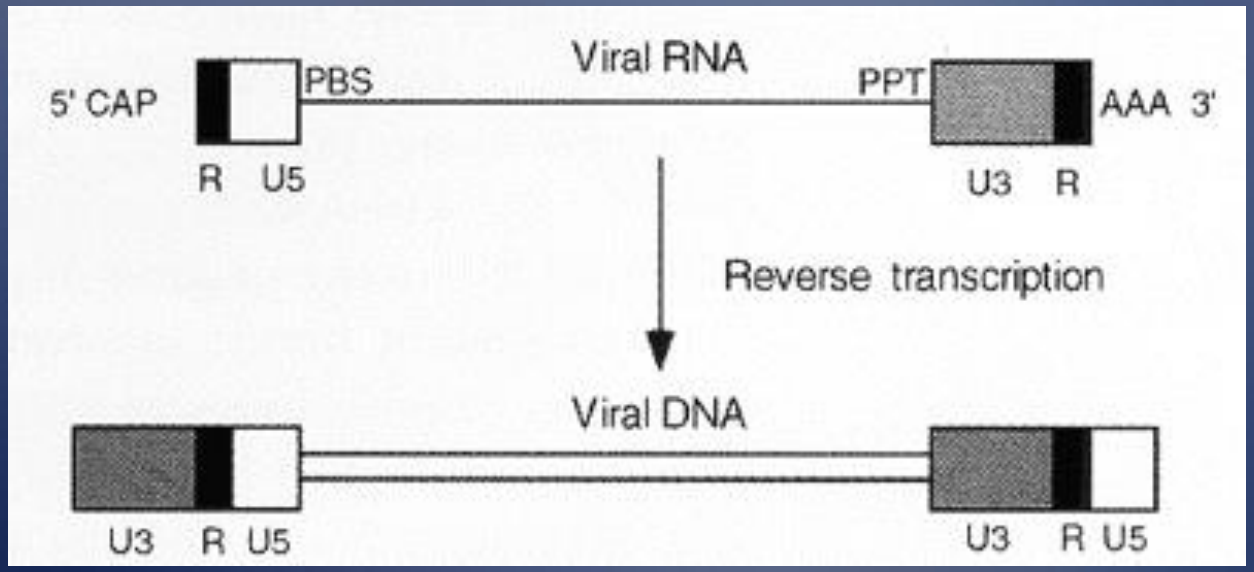
PCR amplifikacija

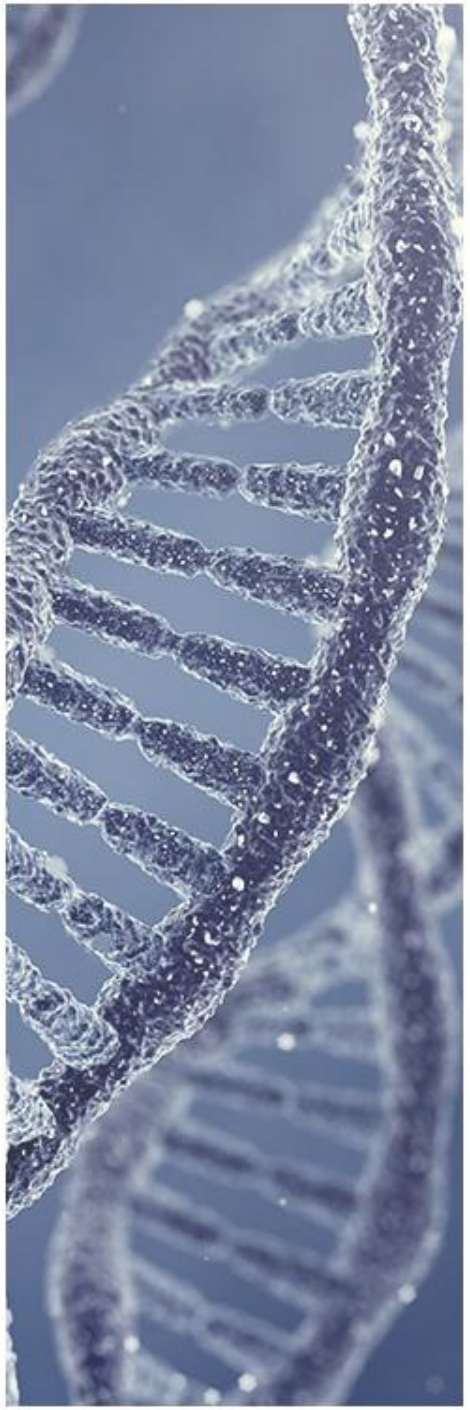
- DNK uzorak
- Polimeraza
- dNTP mix
- Prajmeri specifični za DNK sekvencu
- Puferi



Reverzna transkripcija

- Enzim reverzna transkriptaza – retrovirusi



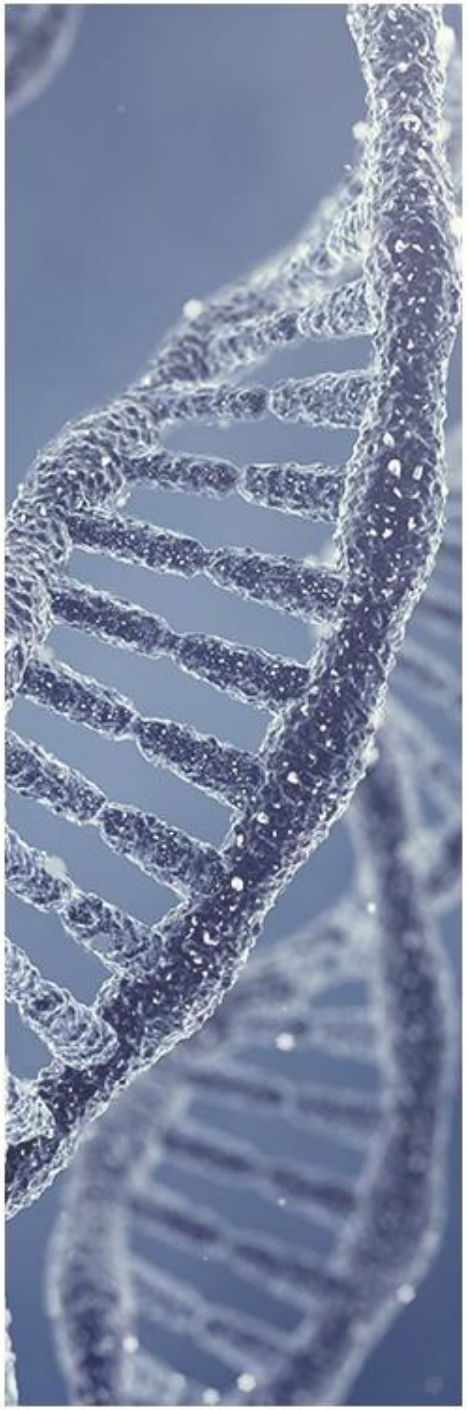


Koraci reverse transcription (RT)-PCR

- Prvi komplementarni DNK lanac
- Dvolančani hibrid RNK/DNK.
- Enzim RNK-aza razgrađuje RNK
- Sintetiše drugi DNK –

dvolančana DNK – cDNA (kDNK)

- PCR

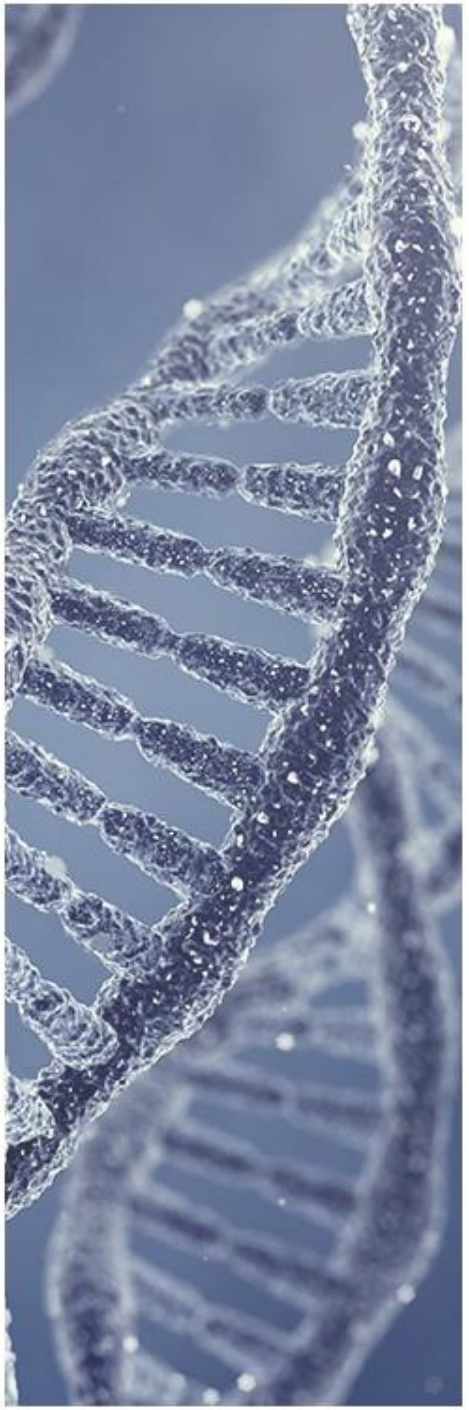


Prajmeri za RT-PCR

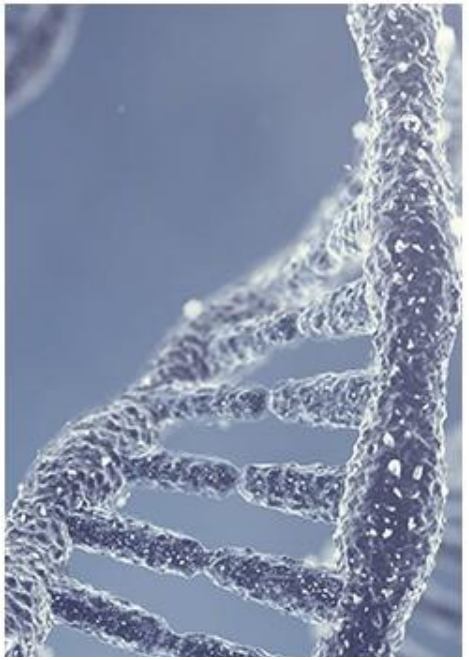
- **Oligo (dT) prajmeri** nekoliko uzastopnih deoksitimina (dT) koji se po sistemu komplementarnosi lepe za 3’ poli A repu (EUKARIOTI)

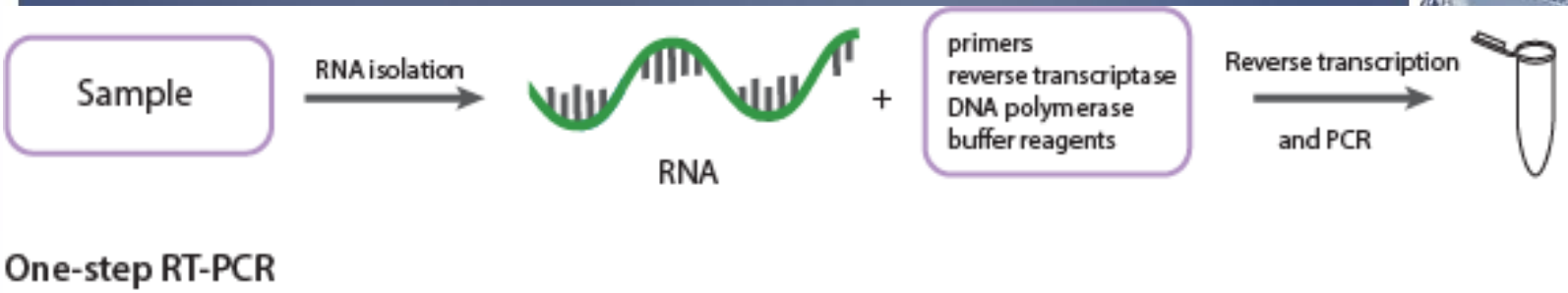
- **Random prajmeri** sekvence sa nasumičnim redosledom nukleotida – random heksameri

- **Specifični prajmeri**

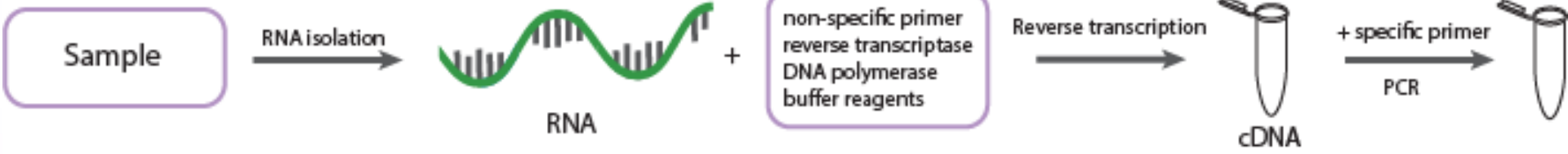


- **RT-PCR** - reverse transcription polymerase chain reaction
  - OneStep RT-PCR
  - TwoStep RT-PCR
    1. Reverzna transkripcija
    2. Amplifikacija





One-step RT-PCR

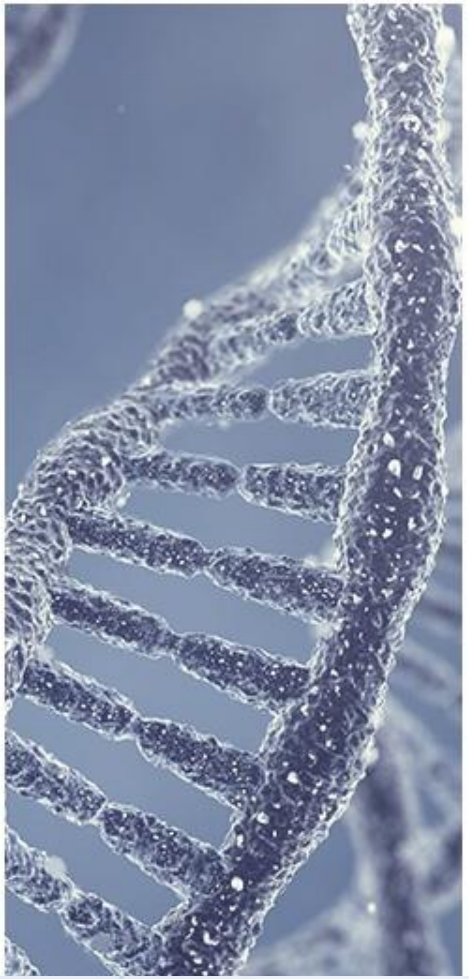


Two-step RT-PCR

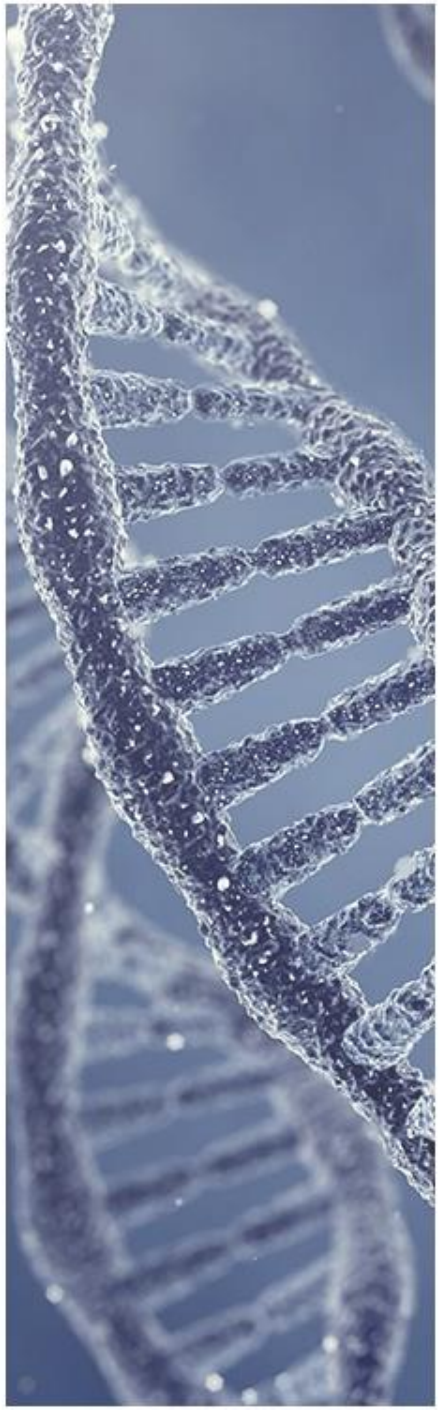
Real-time PCR (qPCR)

Real-time PCR

- Real-time PCR je modifkovni konvencionalni PCR koji omogućava praćenje amplifikacije (akumulacije amplifikata) u „real time“, odnosno tokom svakog ciklusa i amplifikacije.
- Real-time PCR pruža kvalitativne rezultate (prisustvo ili odsustvo sekvence), kao i kvantitativne rezultate (broj kopija DNK).
- Visoka specifičnost i osetljivost (1oo puta osetljivija od end point-PCR)



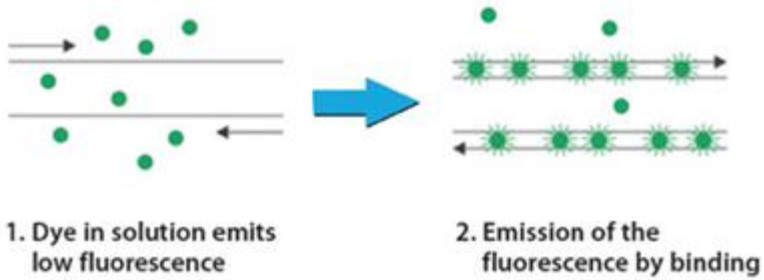
- **Reakciona smeša za real-time PCR reakciju sadrži :**
  - **DNK polimerazu**
  - **dNTP**
  - **par prajmera (forward & reverse)**
  - **Mg2+, pufer**
  - **PROBU ili**
  - **SYBER GREEN DYE**



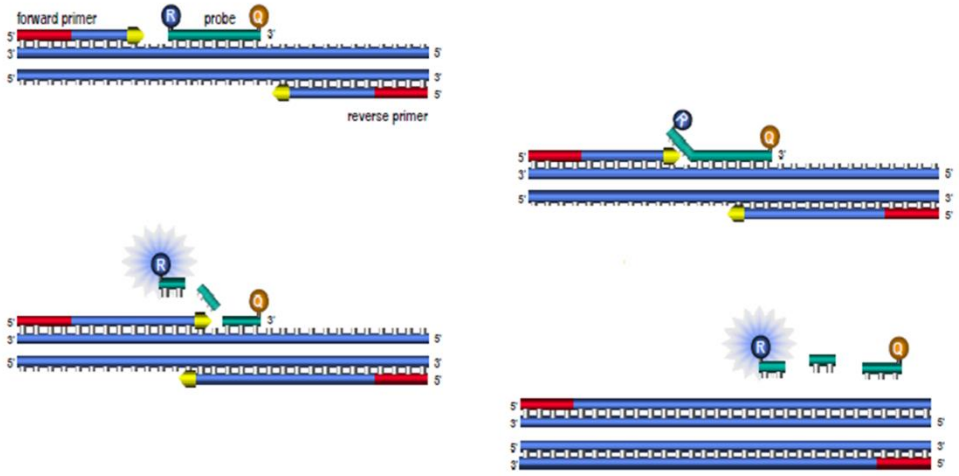
Kako real-time PCR radi?

- Razvijene su dve različite real-time PCR tehnologije:

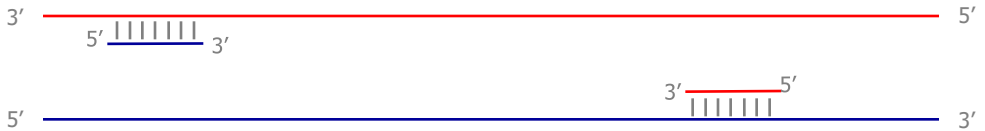
1. ***SYBR-Green® tehnologija***



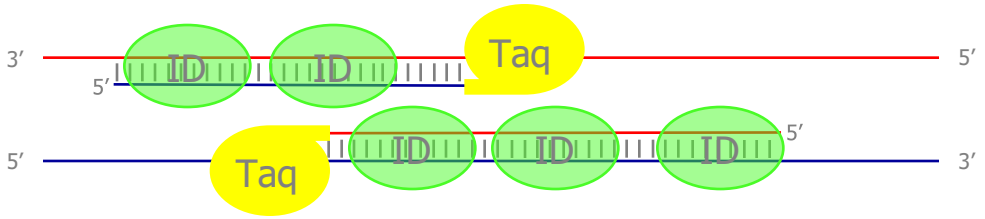
2. ***TaqMan® probe* tehnologija**



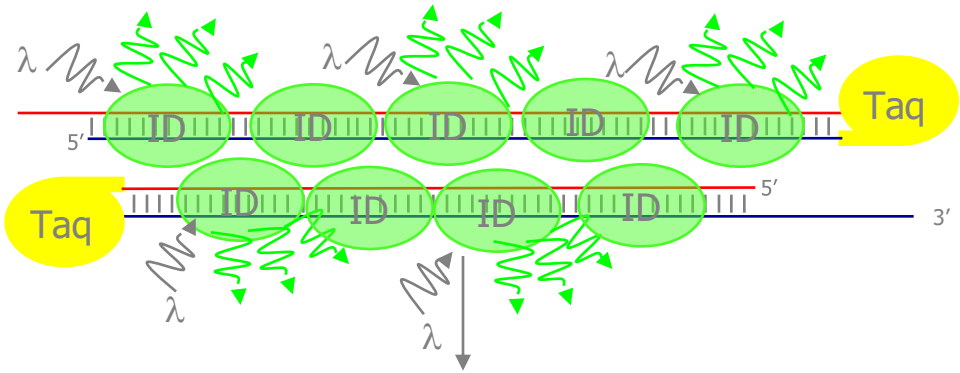
1. SYBR-Green® tehnologija



Ekstenzija

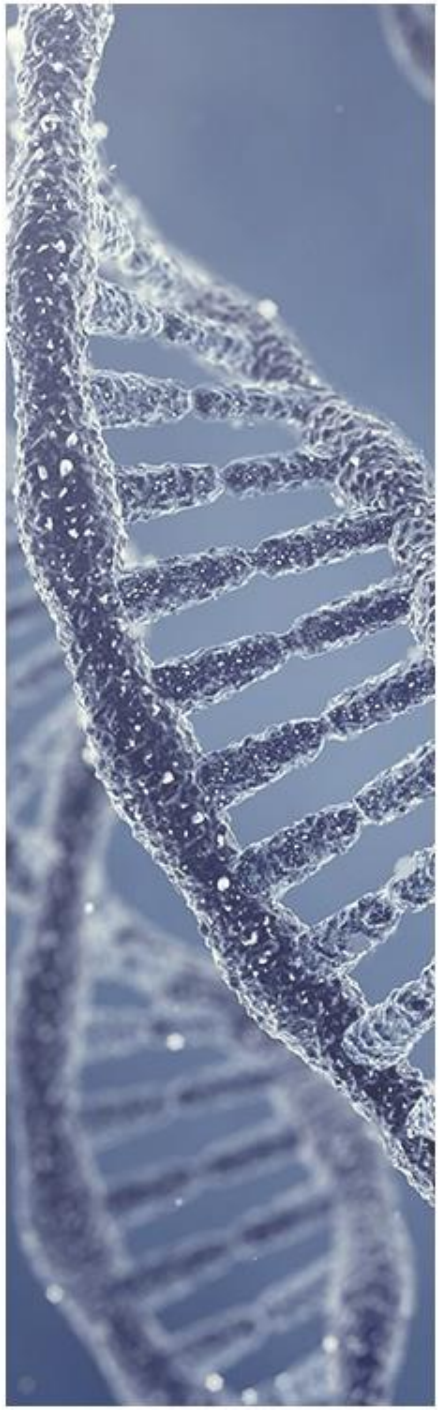


Vezivanje Syber green boje za dvolančanu strukturu

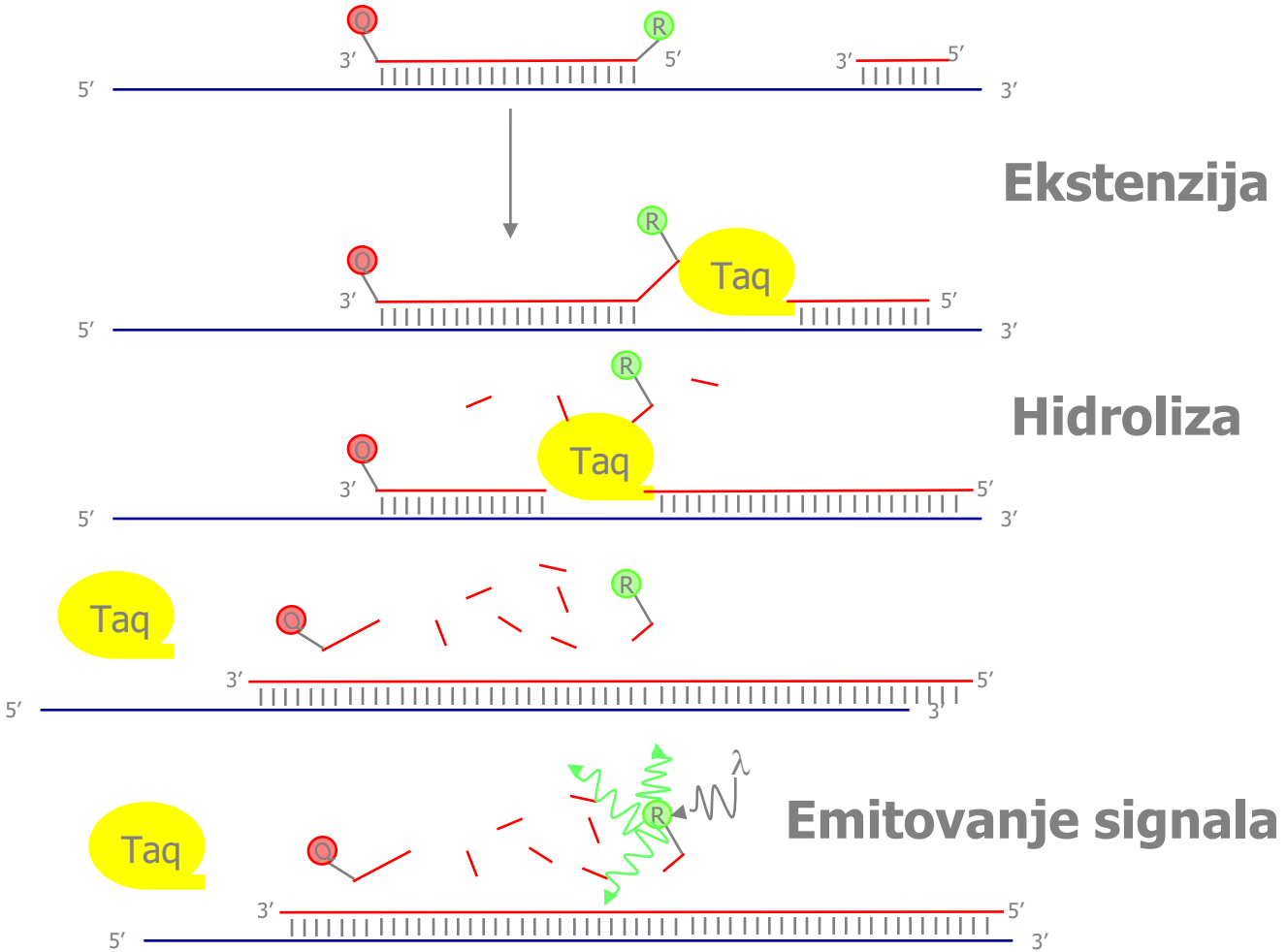


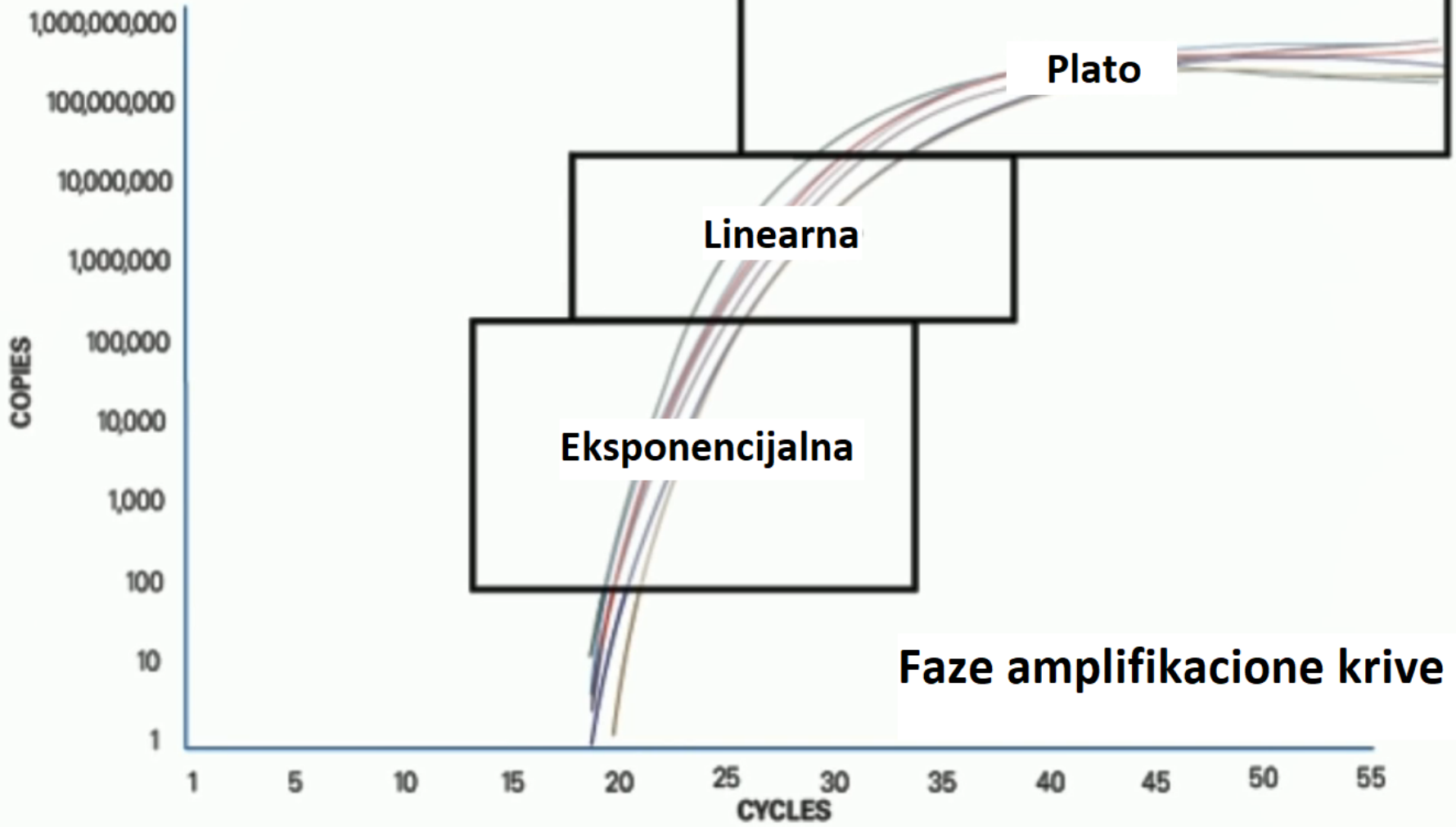
Emitovanje fluorescentnog signala

Kada se SYBER+DNK kompleks osvetljava sa svetlošću 490nm, on fluorescira na 520nm



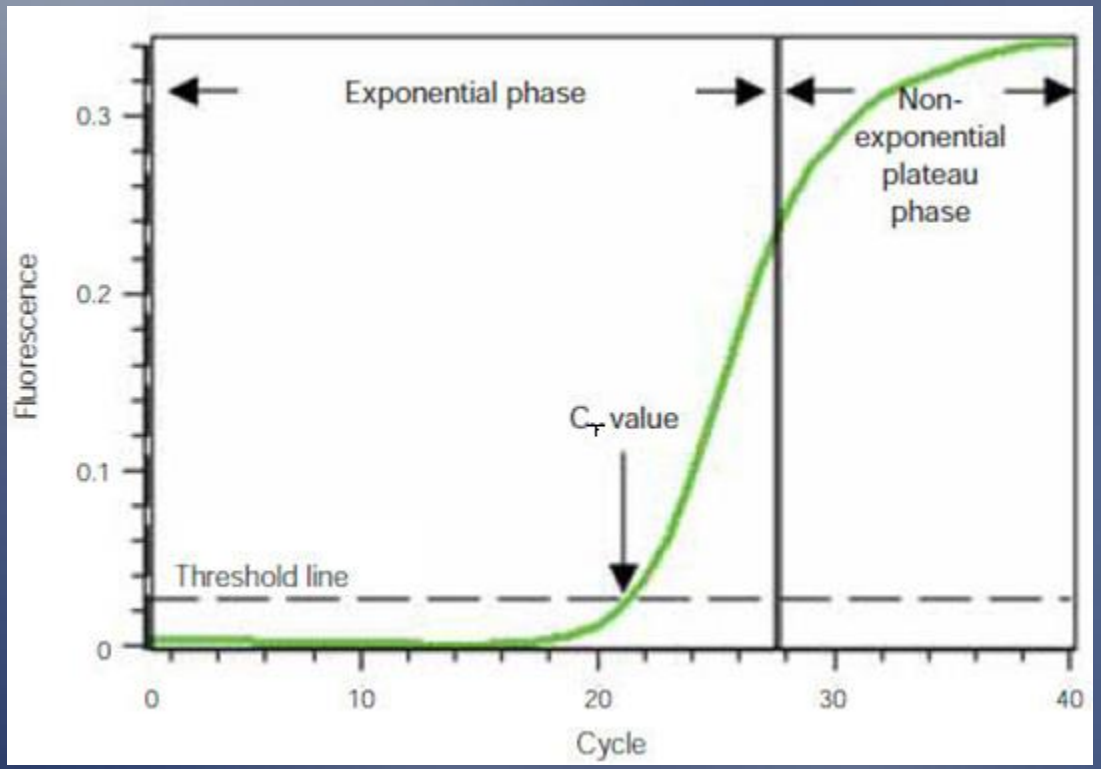
2. *TaqMan® probe* tehnologija

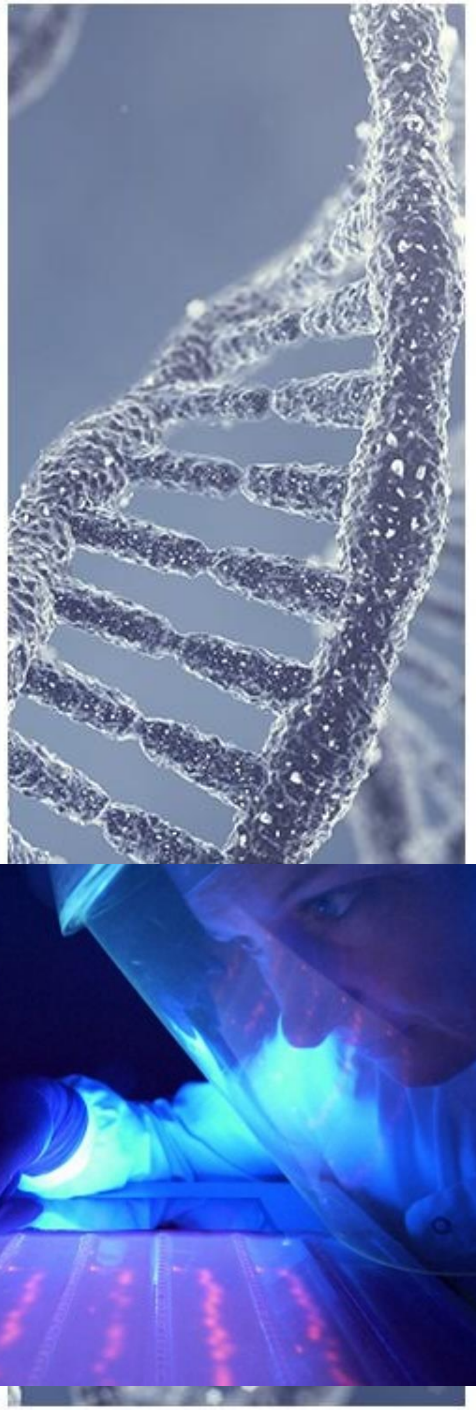




Faze amplifikacione krive

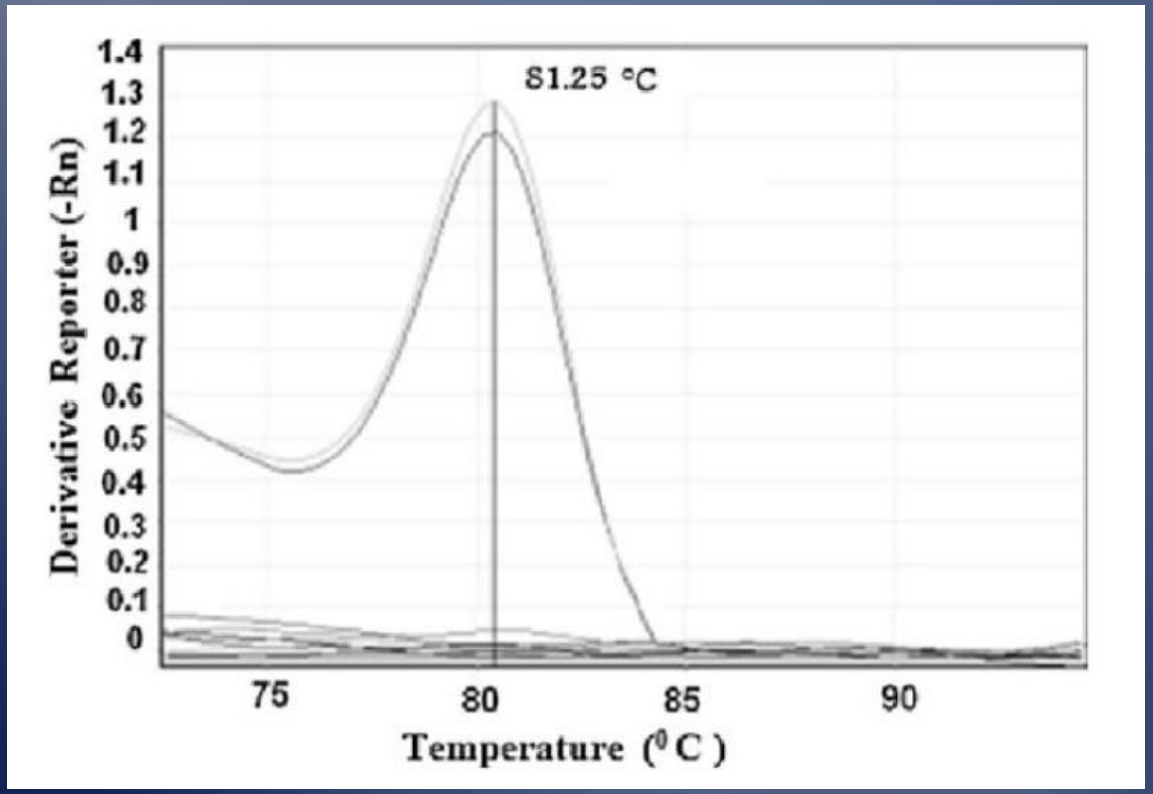
Amplifikaciona kriva

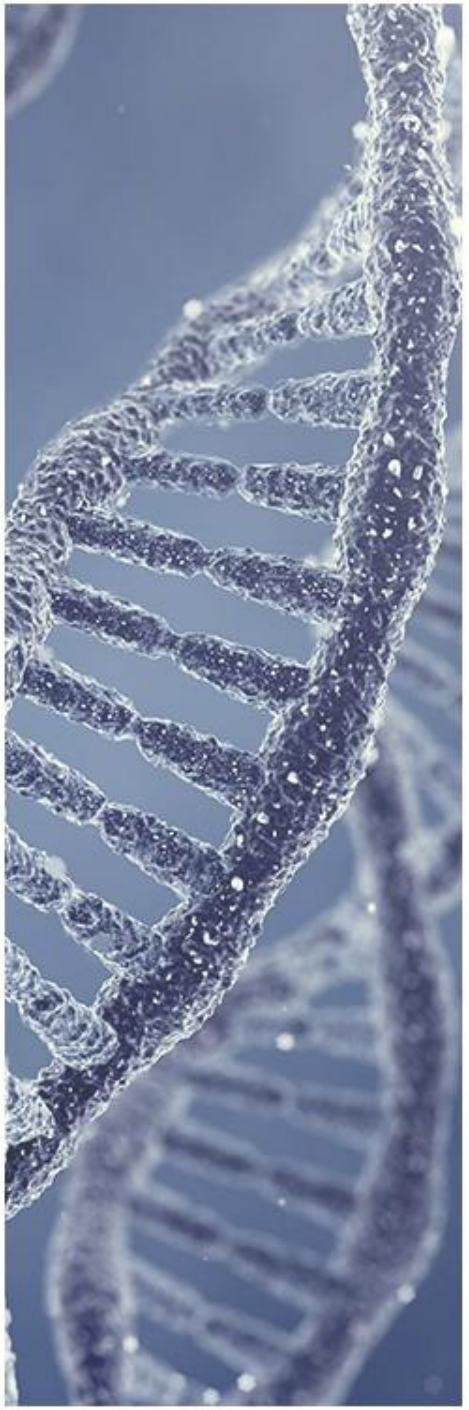


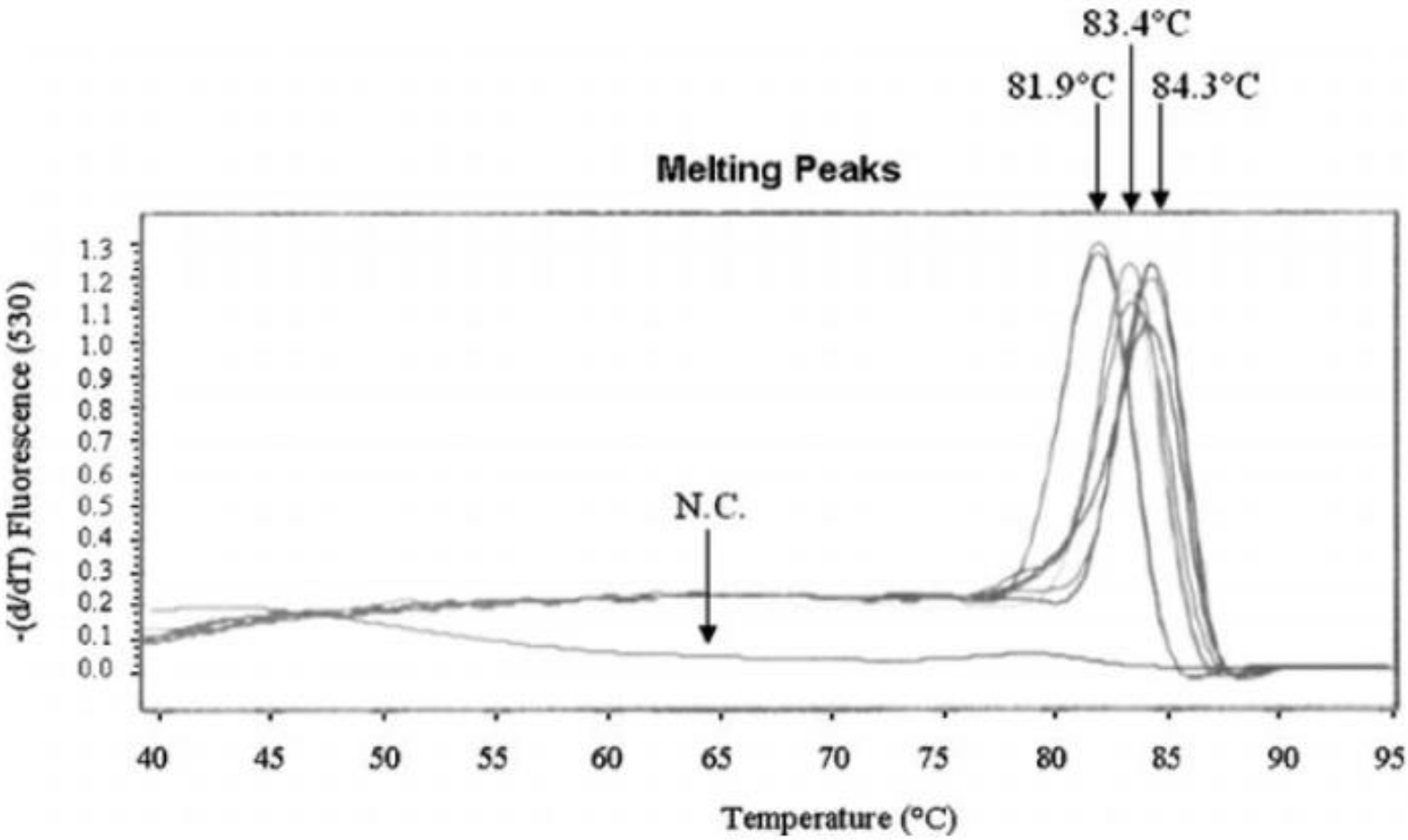


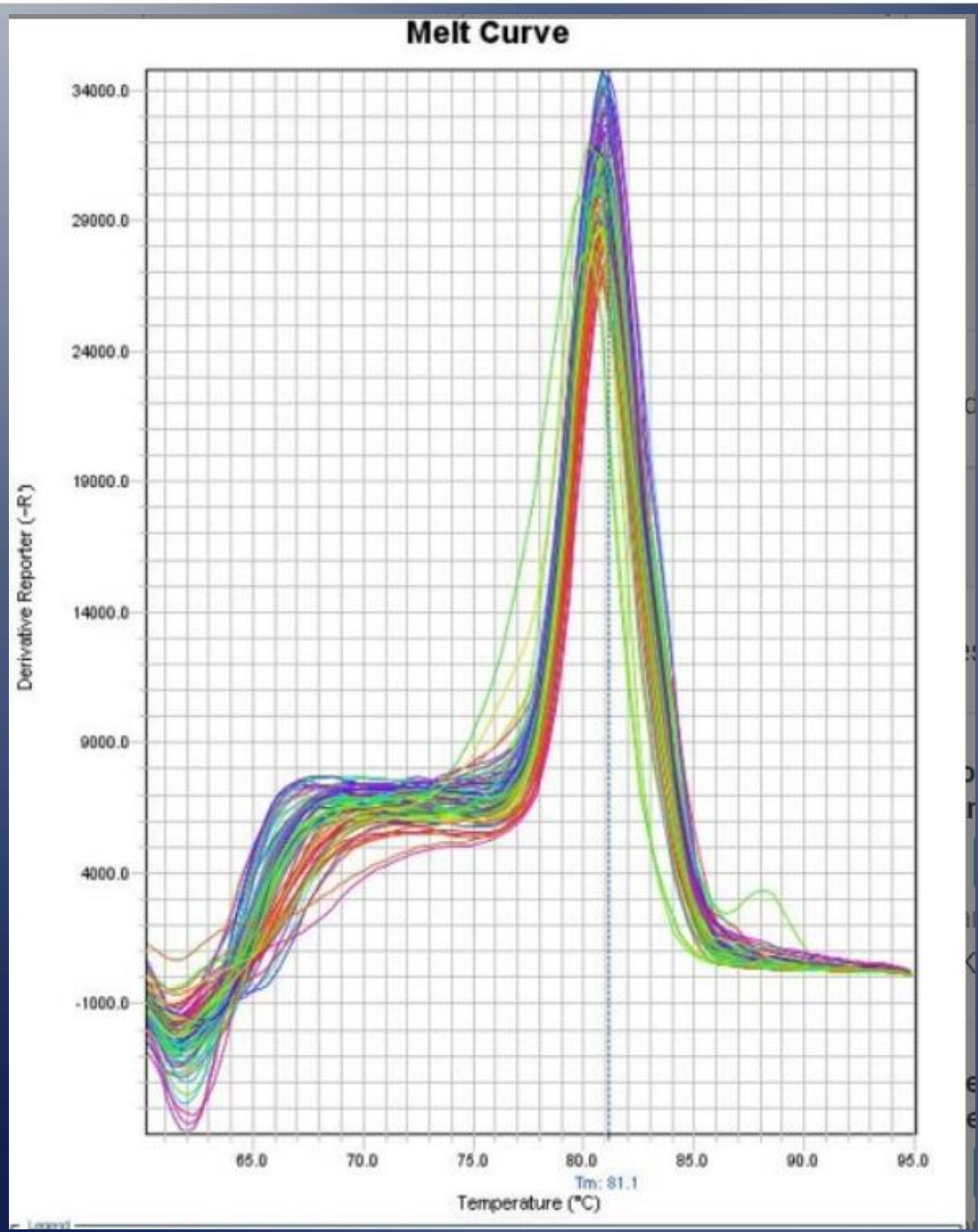
Ct vrednost definiše broj ciklusa kada amplifikaciona kriva preseče threshold.

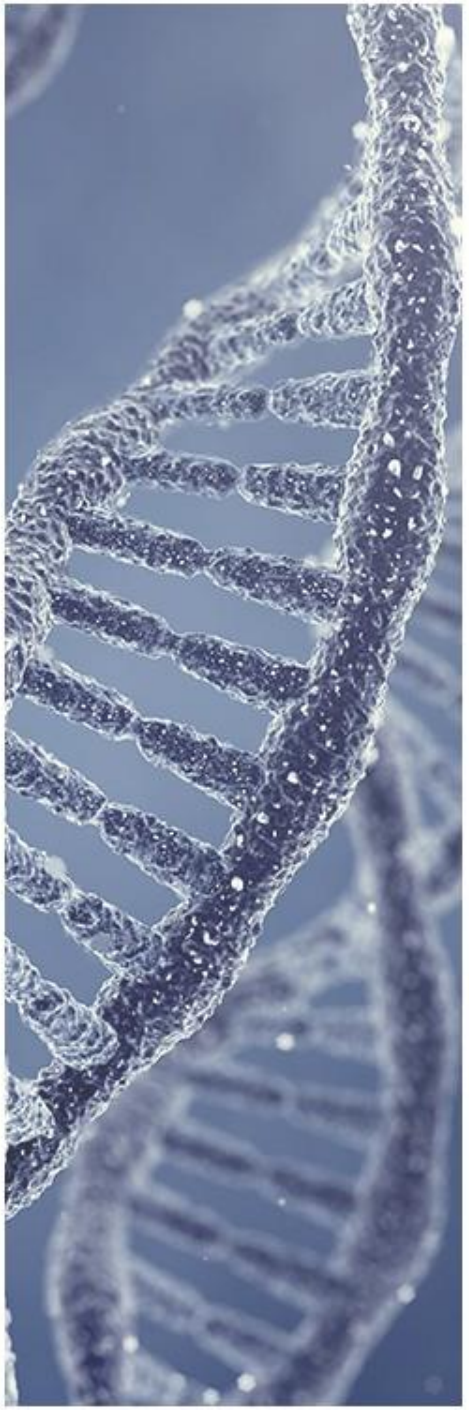
Melting curve

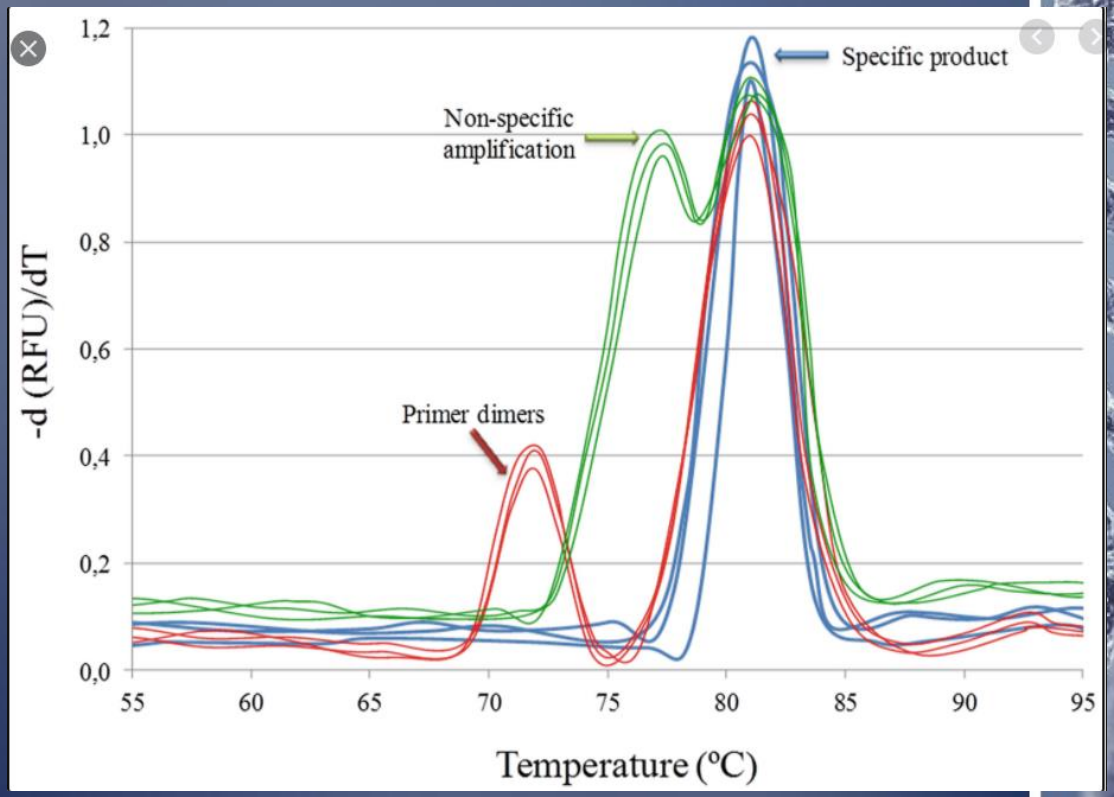




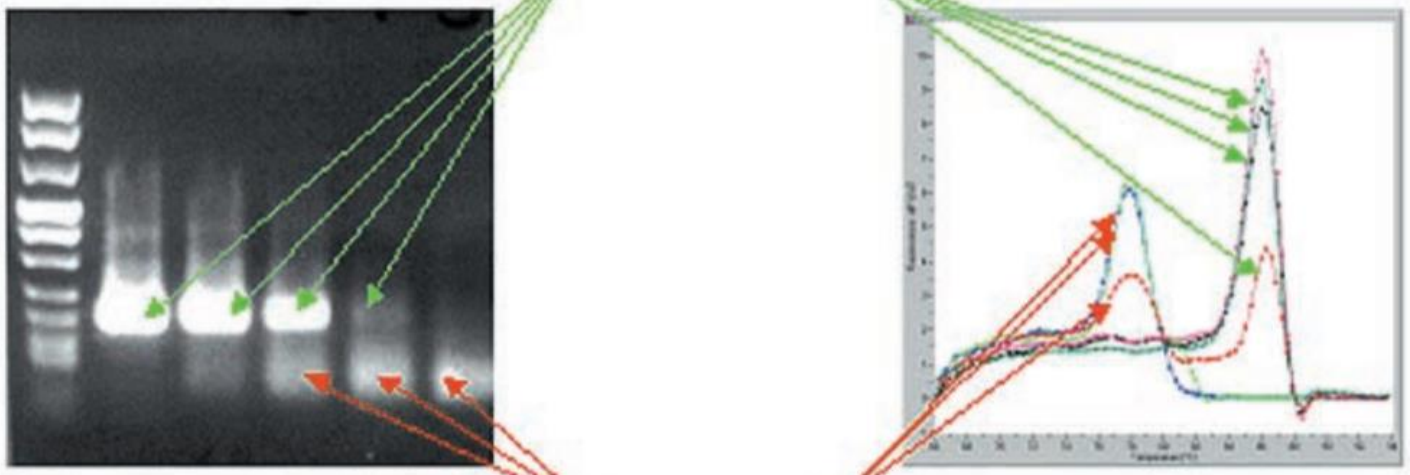

83.4°C
81.9°C
84.3°C
Melting Peaks
N.C.
-(d/dT) Fluorescence (530)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
40
45
50
55
60
65
70
75
80
85
90
95
Temperature (°C)


Melt Curve
34000.0
29000.0
24000.0
19000.0
14000.0
9000.0
4000.0
-1000.0
Derivative Reporter (-R')
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Tm: 81.1
Temperature (°C)



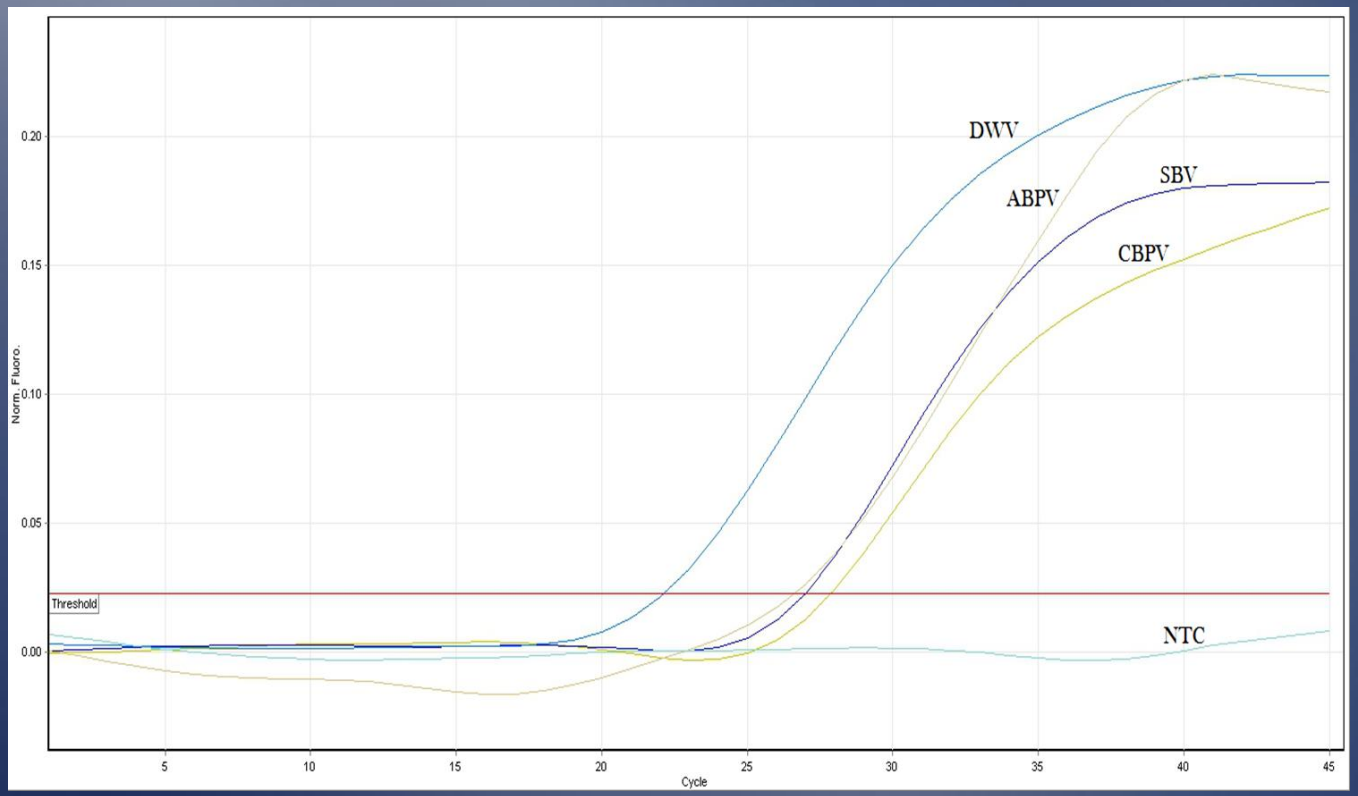

1,2
1,0
0,8
0,6
0,4
0,2
0,0
-d (RFU)/dT
55
60
65
70
75
80
85
90
95
Temperature (ºC)
Specific product
Non-specific amplification
Primer dimers

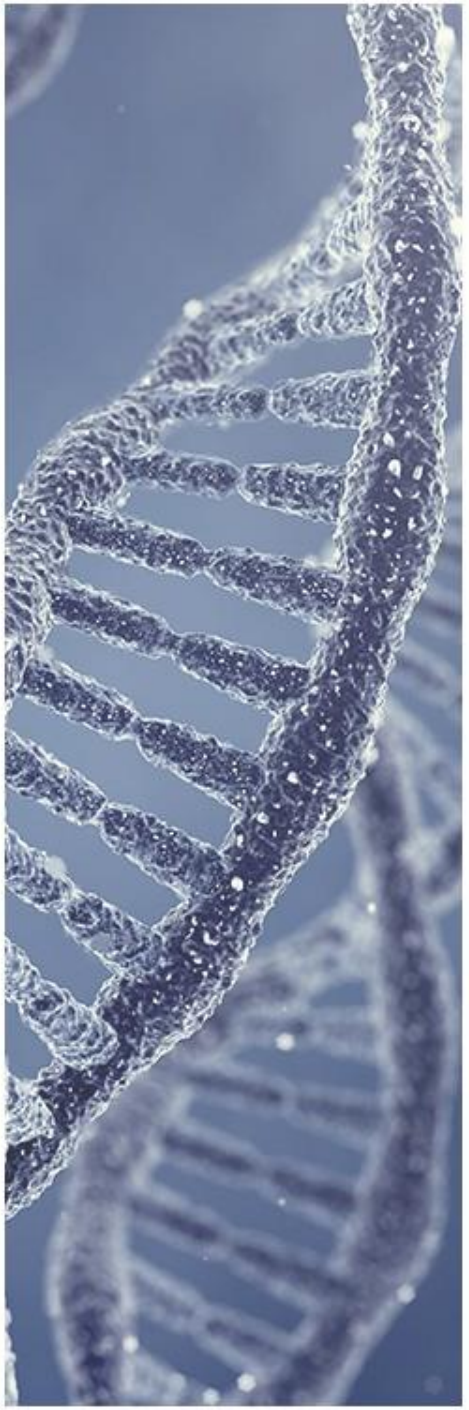
Amplification Product



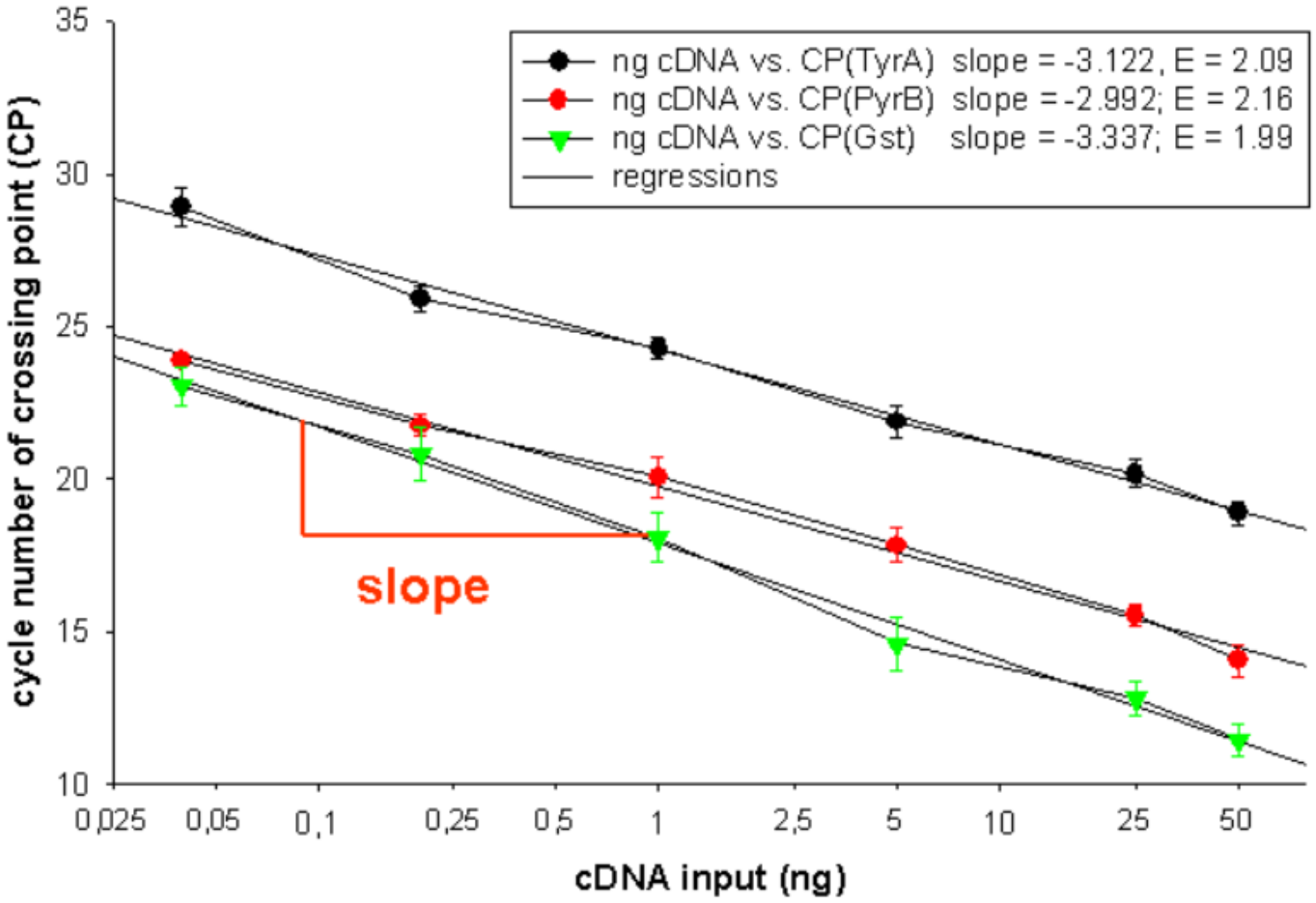
Primer Dimer

Naši rezultati





Calculation of real-time PCR efficiency

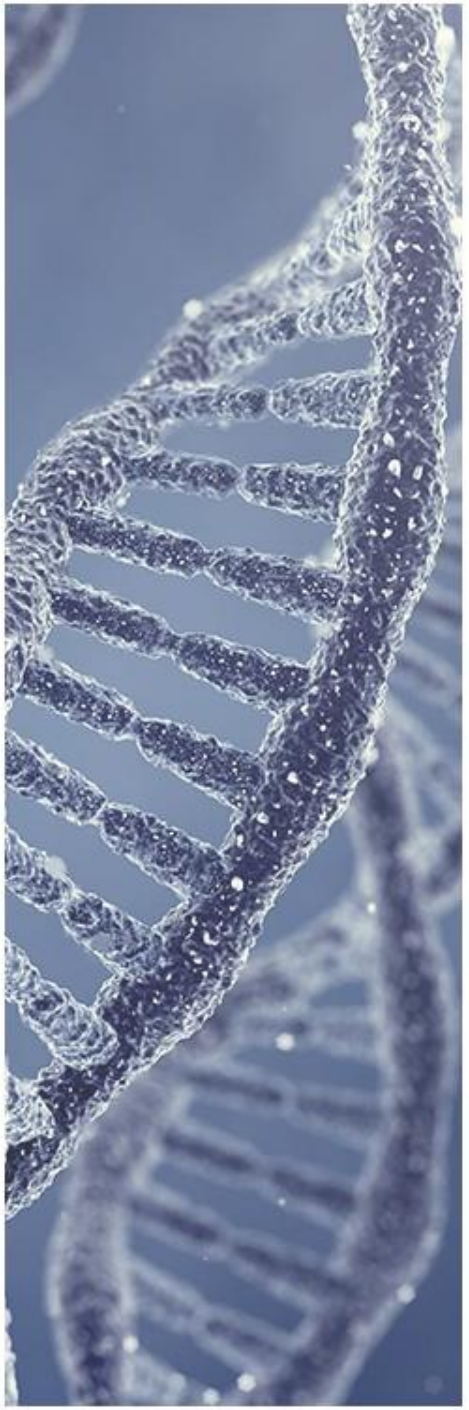


Kvantifikacija

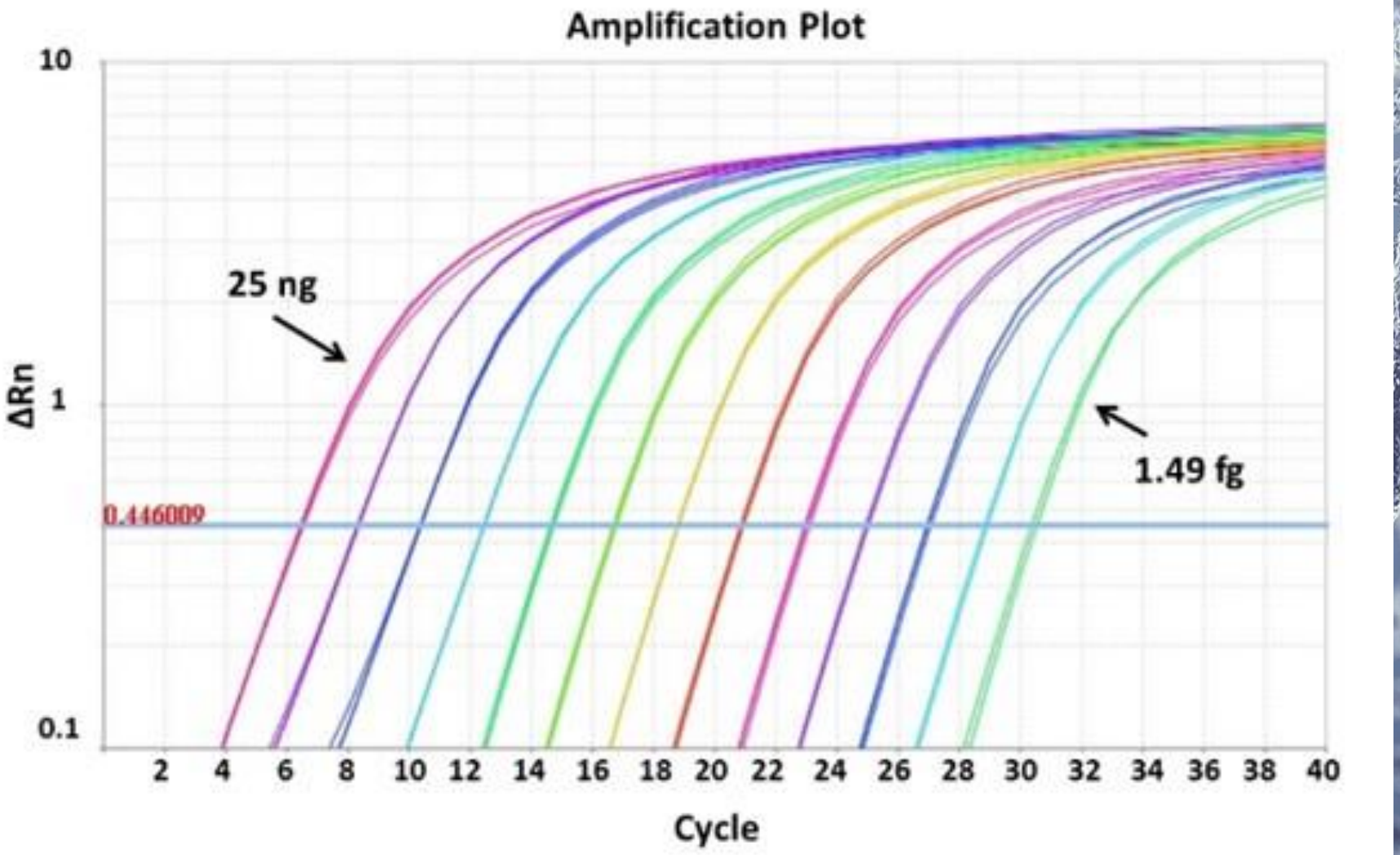
Kvantifikacija pomoću real-time PCR

Apsolutna kvantifikacija

Relativna kvantifikacija

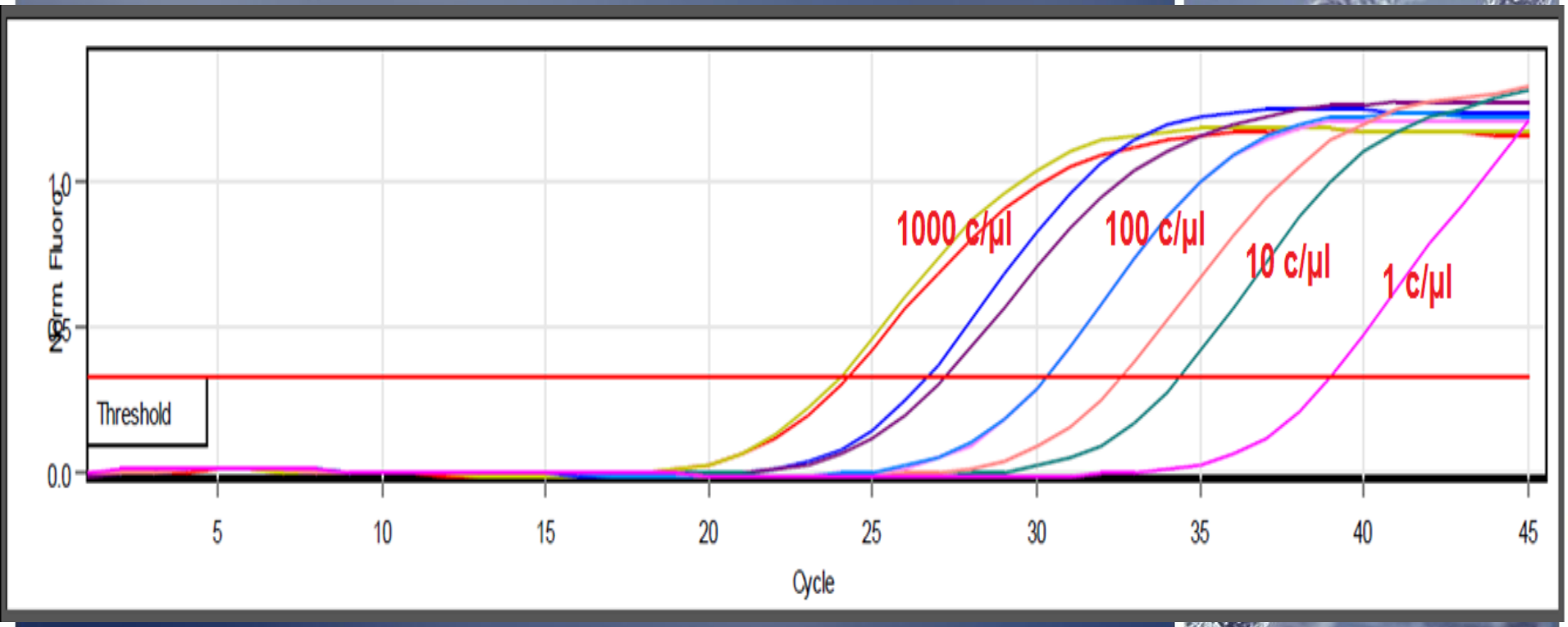


APSOLUTNA KVANTIFIKACIJA

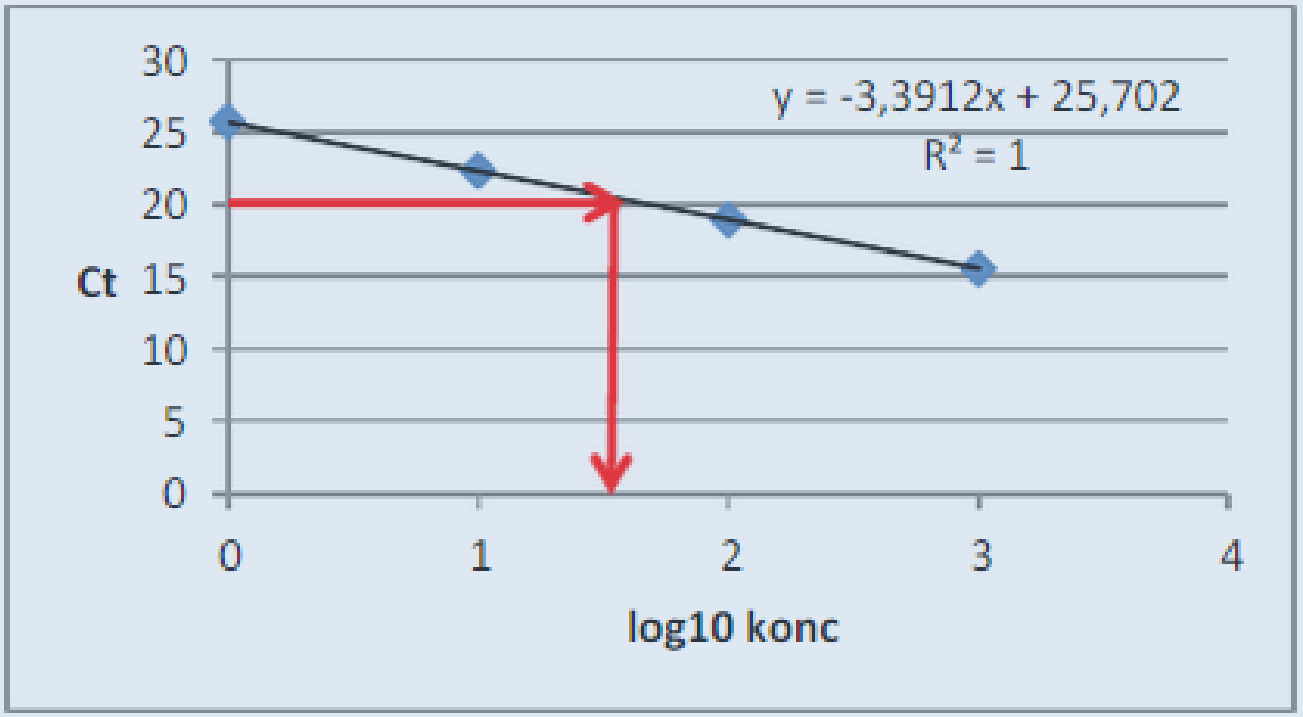


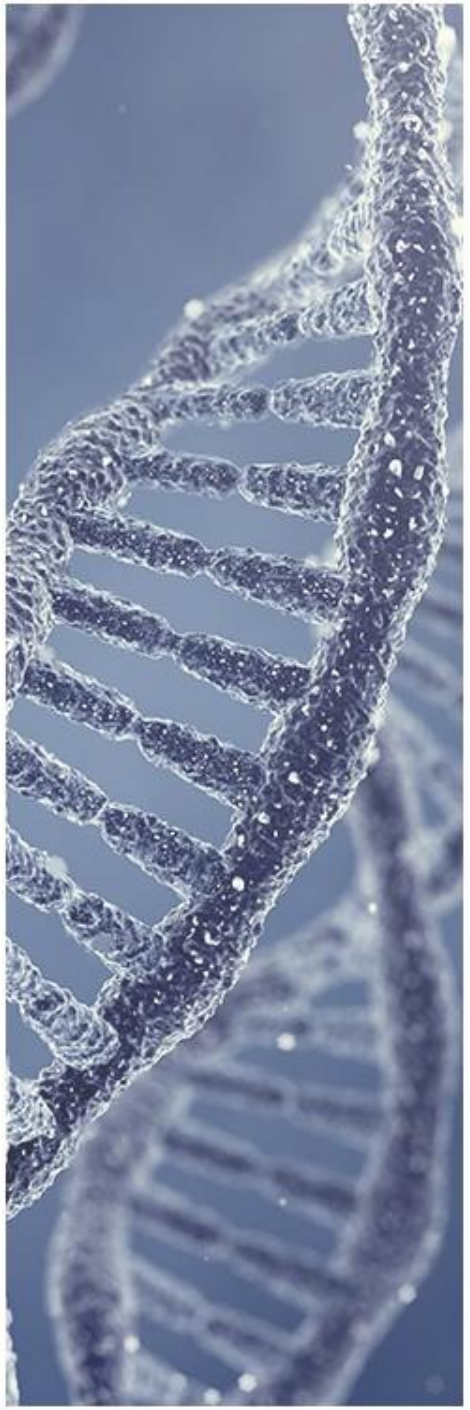
Razblazenja od 25 nanograma do 1,49 femtograma (10^{-15} gram).

APSOLUTNA KVANTIFIKACIJA



APSOLUTNA KVANTIFIKACIJA





Taric, E., Glavinic, U., Stevanovic, J., Vejnovic, B., Aleksic, N., Dimitrijevic, V. and Stanimirovic, Z., 2019. Occurrence of honey bee (Apis mellifera L.) pathogens in commercial and traditional hives. *Journal of Apicultural Research*, *58*(3), pp.433-443.

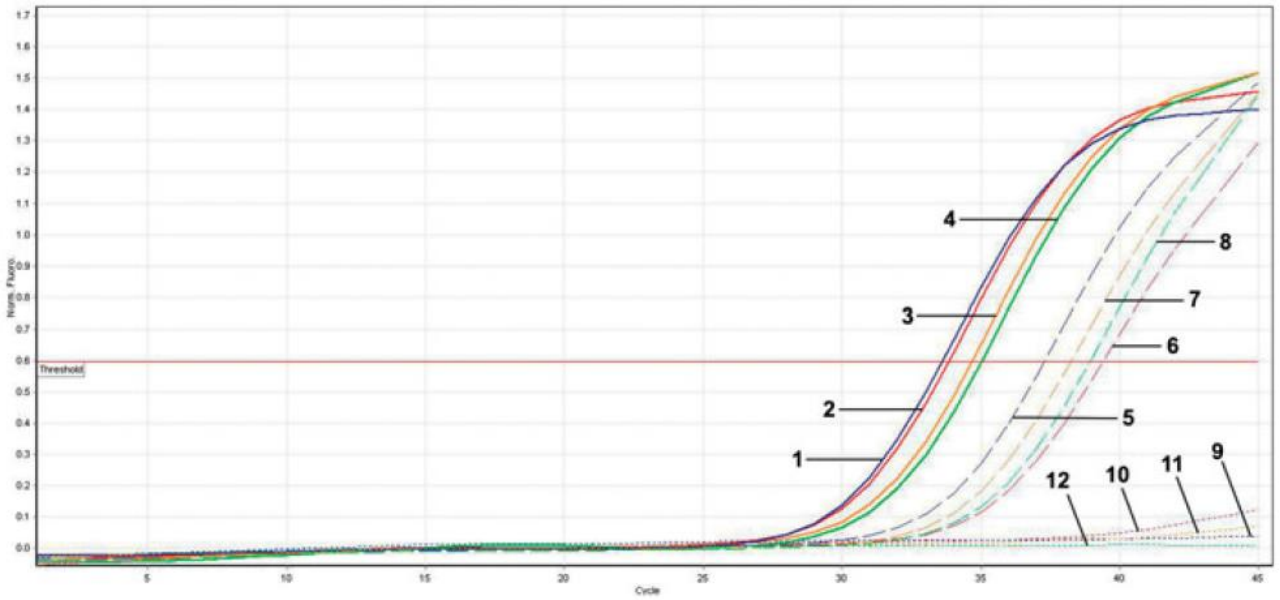
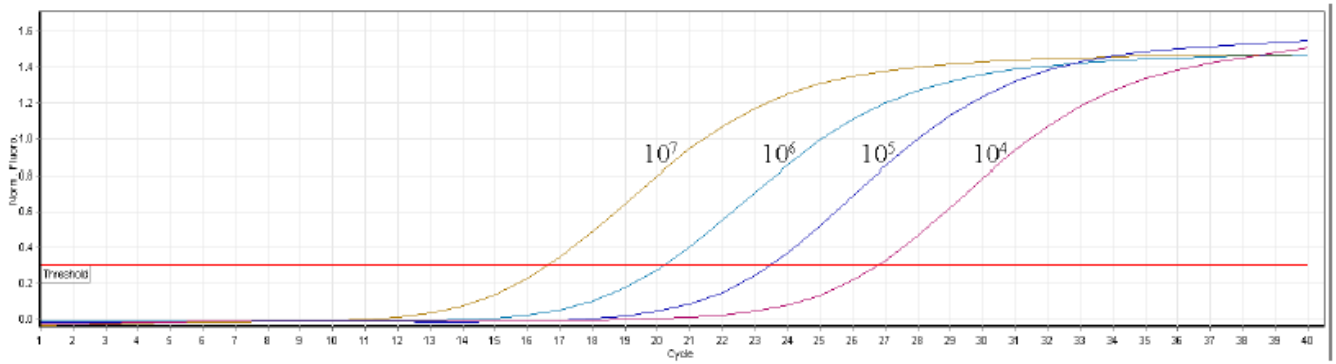
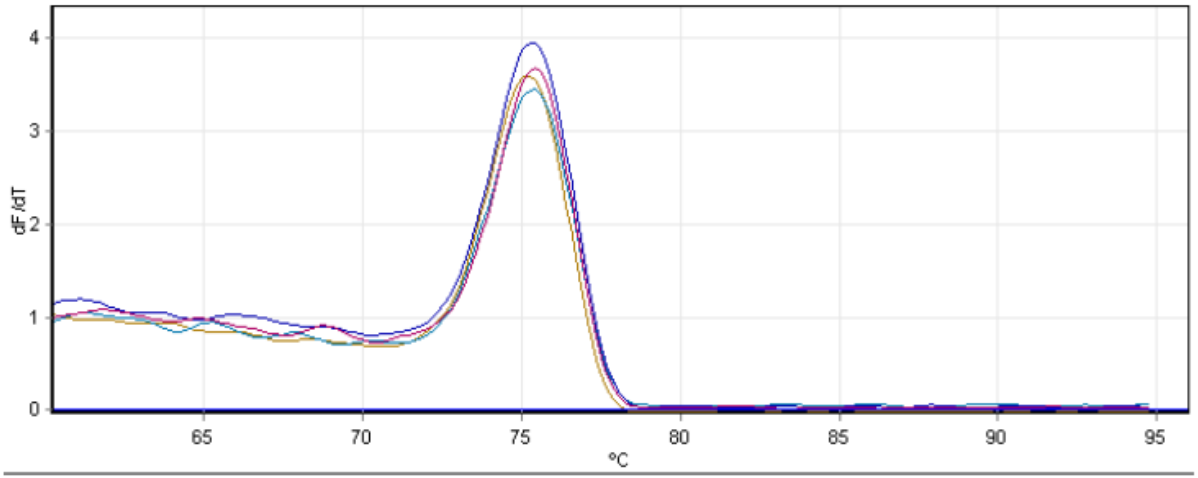


Figure 3. Amplification plots following real-time PCR demonstrating the detection of viruses in adult bees. Lines 1–4 – positive controls: (1) Blue line – for ABPV; (2) Red line – for CBPV; (3) Orange line – for DWV; (4) Green line – for SBV; Dashed lines (5–8) – samples, correspondingly. Dotted lines (9–12) – negative controls, correspondingly. Single replicates are shown for clarity.



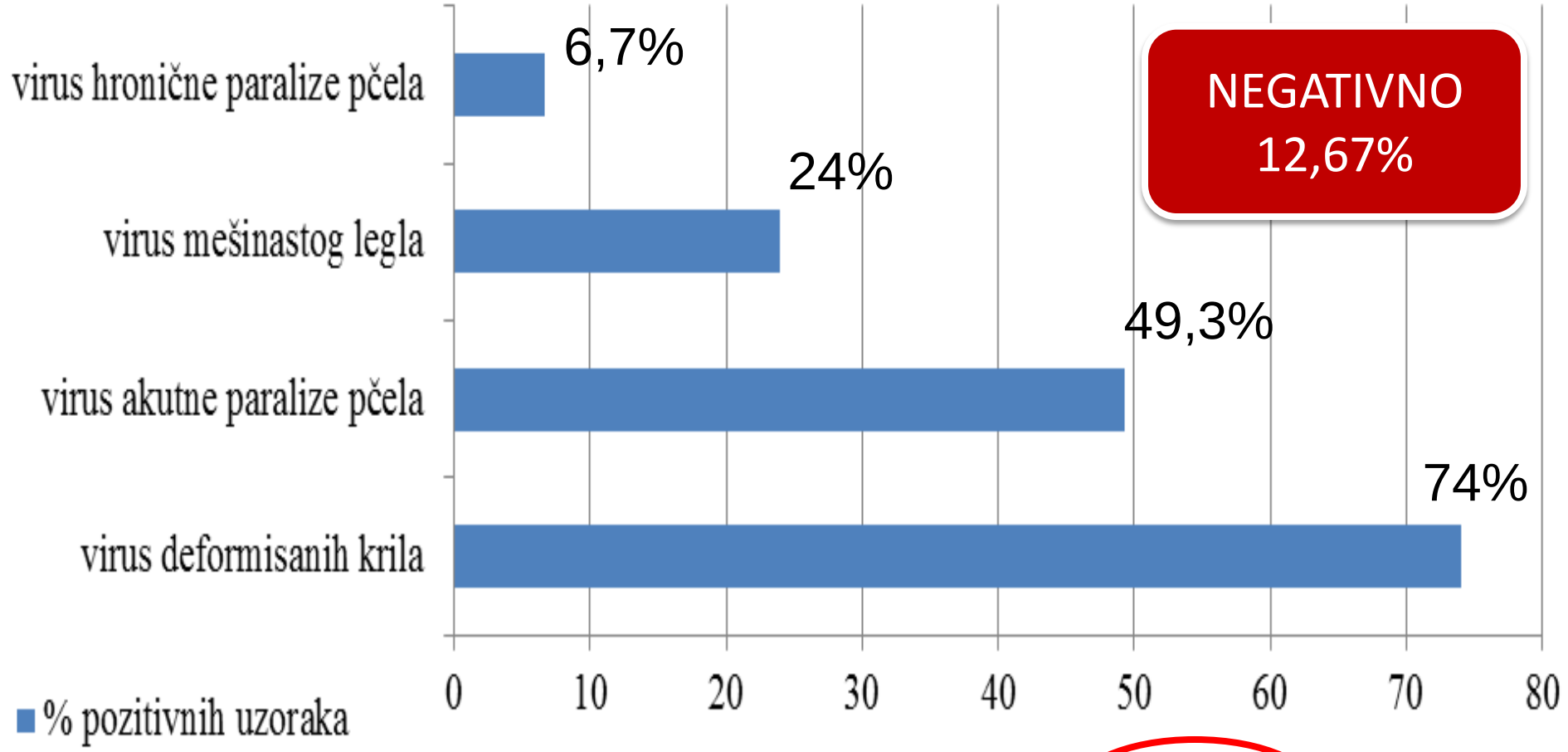
Slika 7. *Real-time* PCR fluorescentnih kriva standarda za *L. passim*. Standardi su dodati u serijskim razblaženjima od 1×10^7 do 1×10^4 plazmidne DNK, što rezultuje u 1×10^7 do 1×10^4 kopija po µl.



Slika 8. Topljenje (melting) produkata sa temperaturom u rasponu od 60 do 95°C, sa brzinom grejanja od 0,5°C po sekundi i konstantnim beleženjem nivoa fluorescencije

Naši primeri upotrebe real time PCR za apsolutnu kvantifikaciju

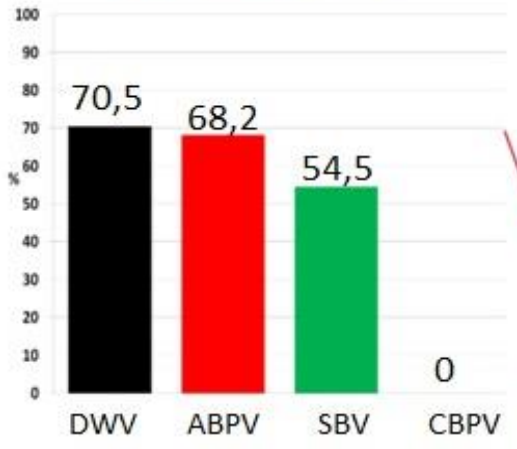
Naziv projekta:
MOLEKULARNO-GENETIČKA DETEKCIJA I IDENTIFIKACIJA UZROČNIKA VIRUSNIH I MIKROSPORIDIJALNIH INFEKCIJA ZASTUPLJENIH KOD PČELINJIH DRUŠTAVA NA TERITORIJI SRBIJE



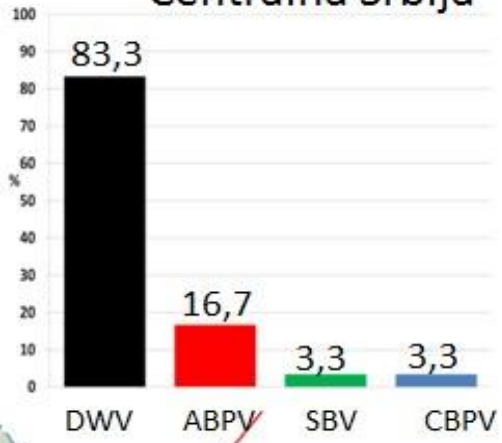
NEGATIVNO
12,67%

131 pozitivan uzorak → 87,33%

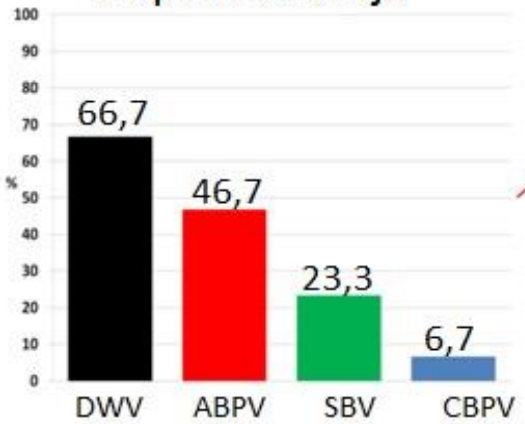
Severna Srbija

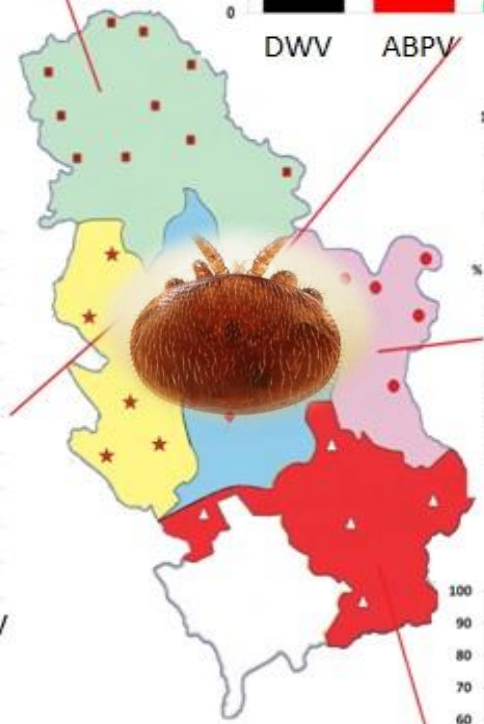


Centralna Srbija

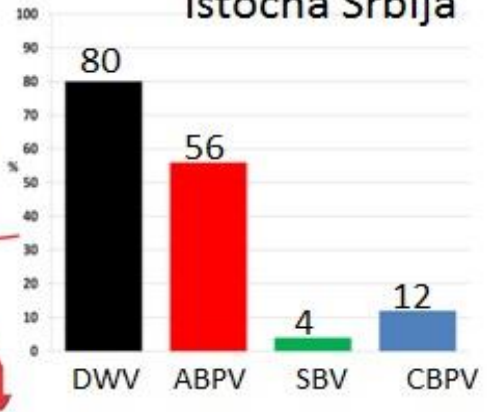


Zapadna Srbija

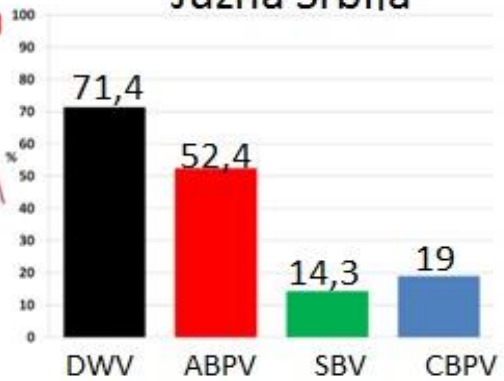


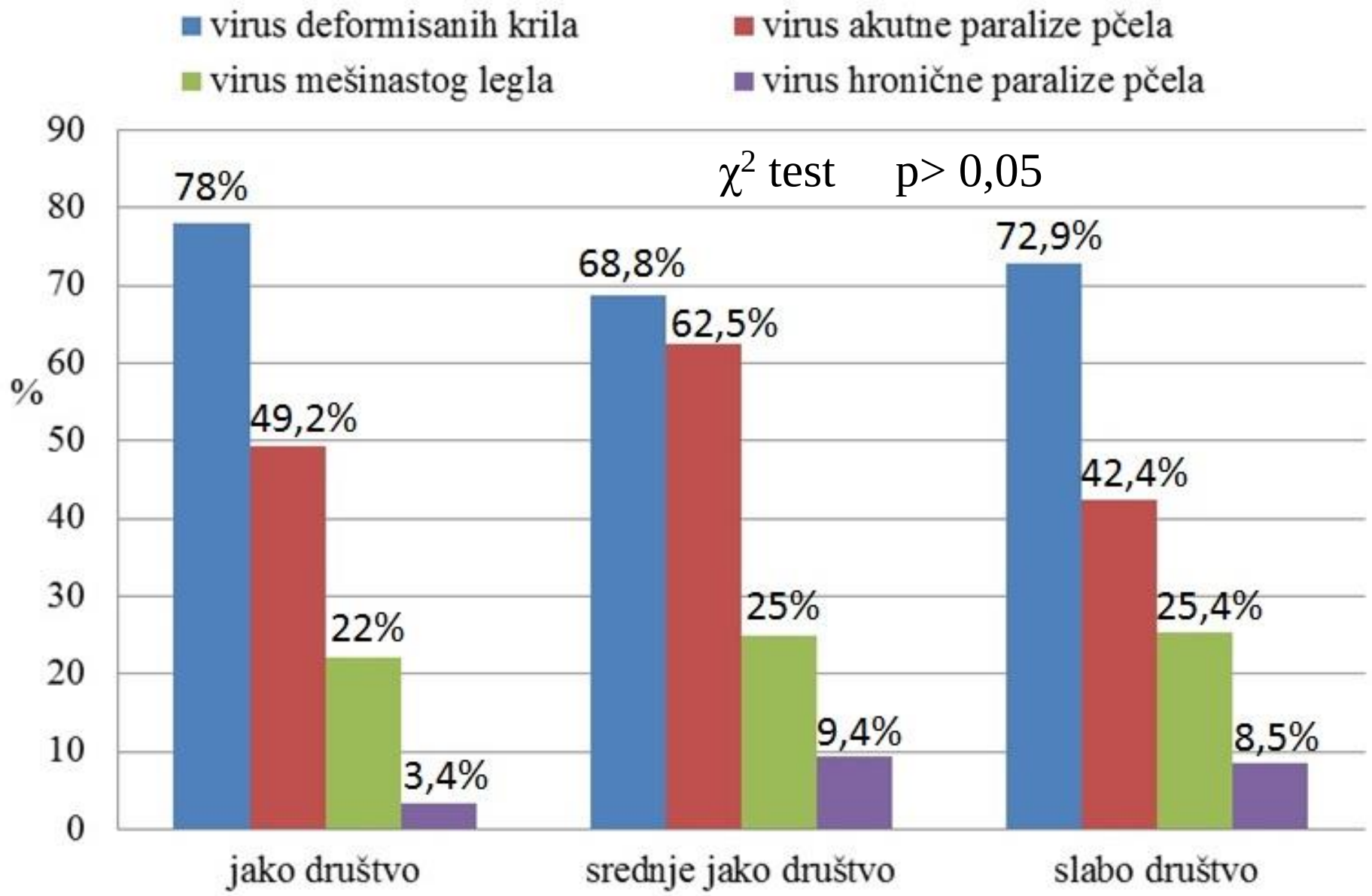


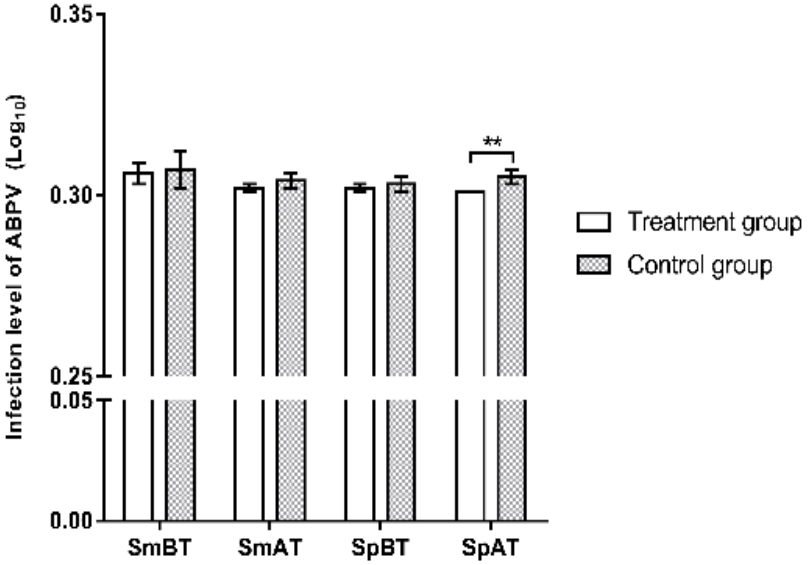
Istočna Srbija

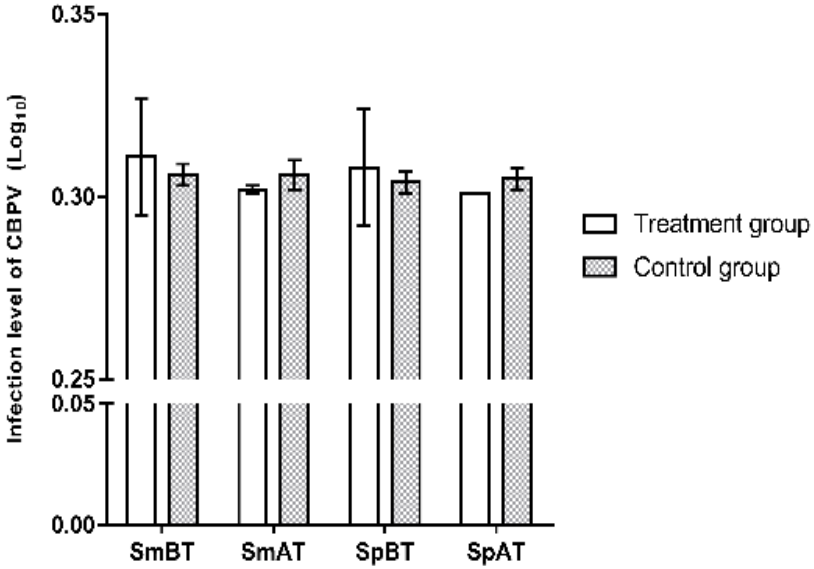


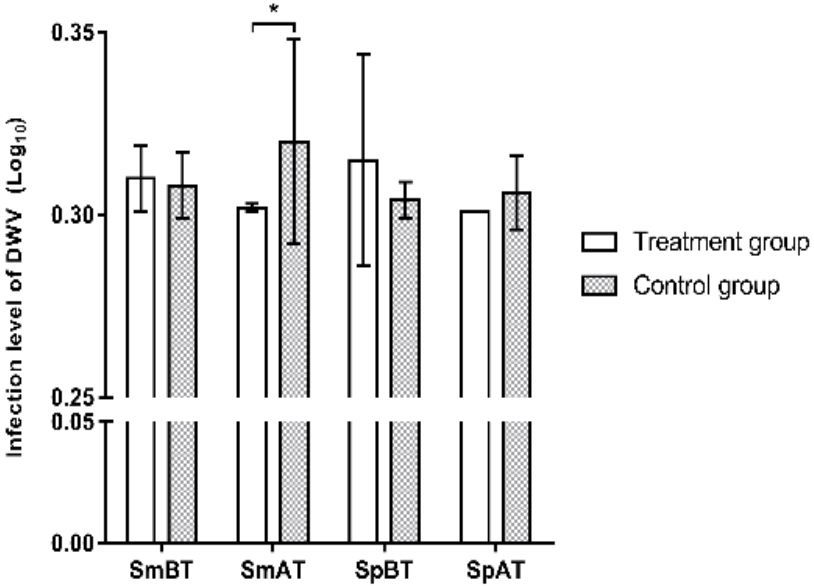
Južna Srbija

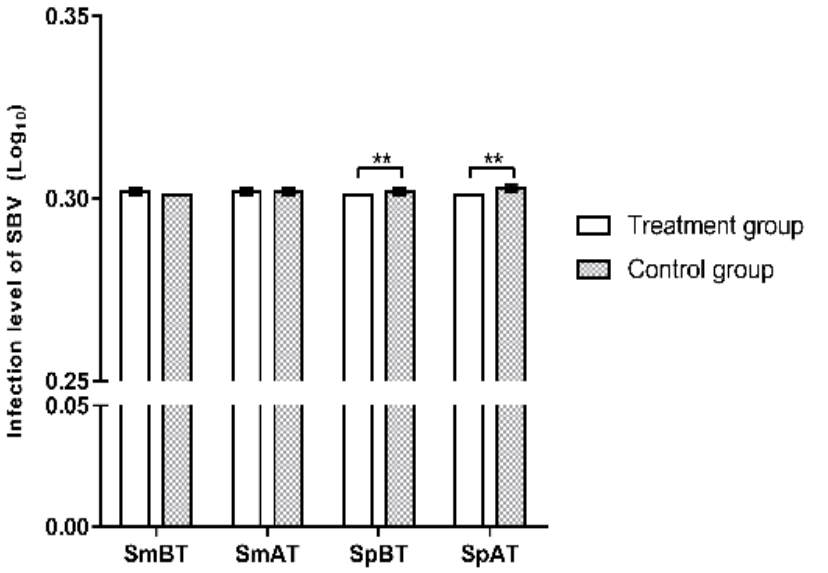



virus deformisanih krila
virus akutne paralize pčela
virus mešinastog legla
virus hronične paralize pčela
χ^2 test p> 0,05
%
90
80
70
60
50
40
30
20
10
0
78%
49,2%
22%
3,4%
68,8%
62,5%
25%
9,4%
72,9%
42,4%
25,4%
8,5%
jako društvo
srednje jako društvo
slabo društvo


Infection level of ABPV (Log10)
0.35
0.30
0.25
0.05
0.00
**
SmBT
SmAT
SpBT
SpAT
Treatment group
Control group


Infection level of CBPV (Log10)
0.35
0.30
0.25
0.05
0.00
SmBT
SmAT
SpBT
SpAT
Treatment group
Control group


Infection level of DWV (Log10)
0.35
0.30
0.25
0.05
0.00
*
SmBT
SmAT
SpBT
SpAT
Treatment group
Control group


Infection level of SBV (Log10)
0.35
0.30
0.25
0.05
0.00
**
**
SmBT
SmAT
SpBT
SpAT
Treatment group
Control group

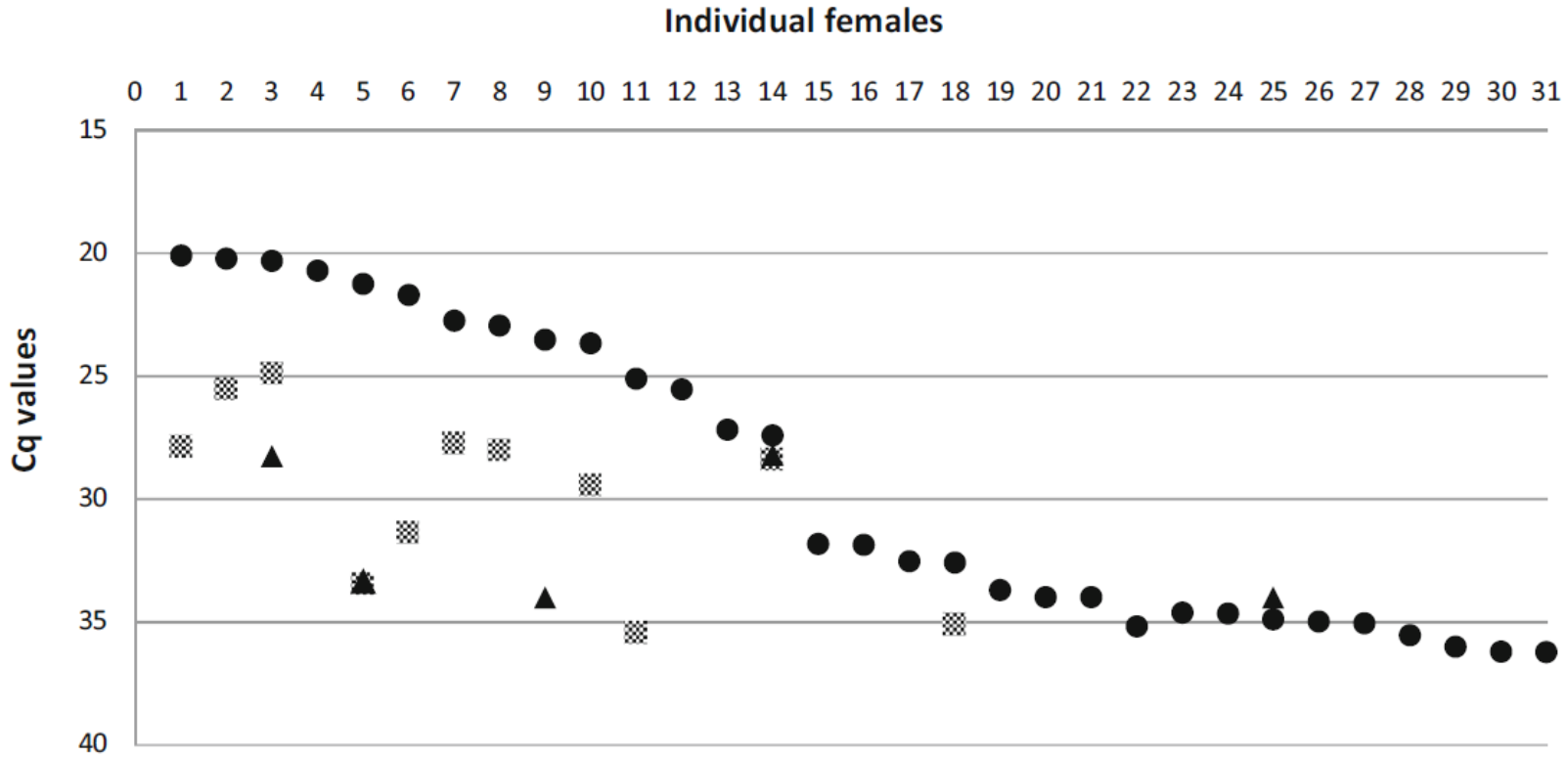


Fig. Viral RNA loads (Cq) of Individual females females orally fed with WNV NY99. Data are given for infection (data from bdomens, filled circles), dissemination (legs and wings, gray squares), and transmission (saliva, filled triangles). Females' progression number is reported as from the highest to the lowest viral RNA load in abdomens

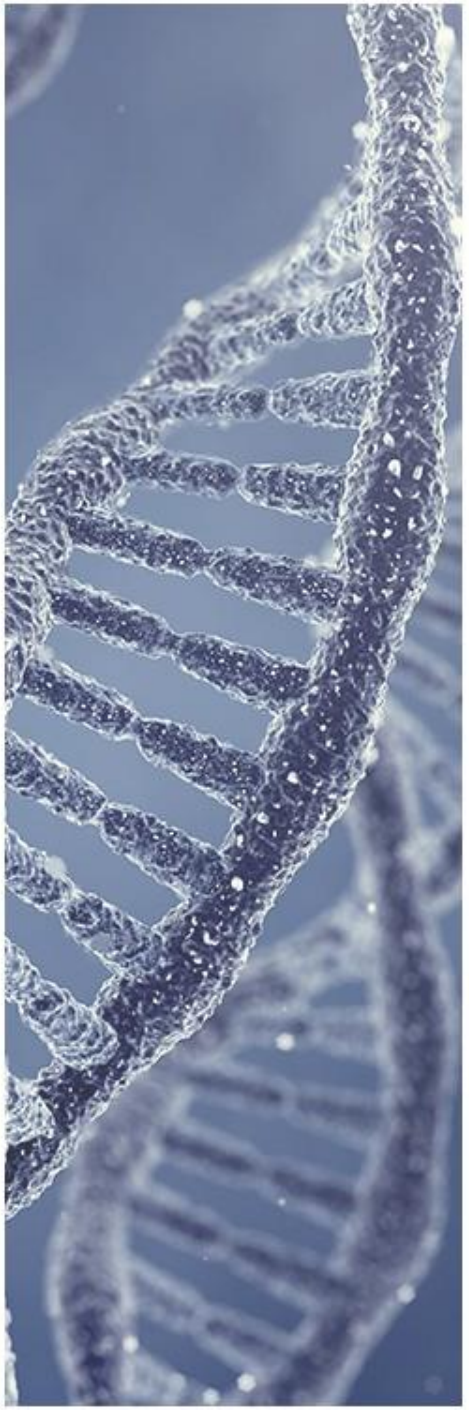
Relativna kvantifikacija

- poređenje dva uzorka jedan u odnosu na drugi: npr - pre i posle tretmana lekovima ili tretirana lekvima i kontrola (koja nije tretirana)

$$\Delta Ct = Ct_{(GI)} - Ct_{(HK)}$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{(calibrator)}$$

$$2^{-\Delta\Delta Ct}$$



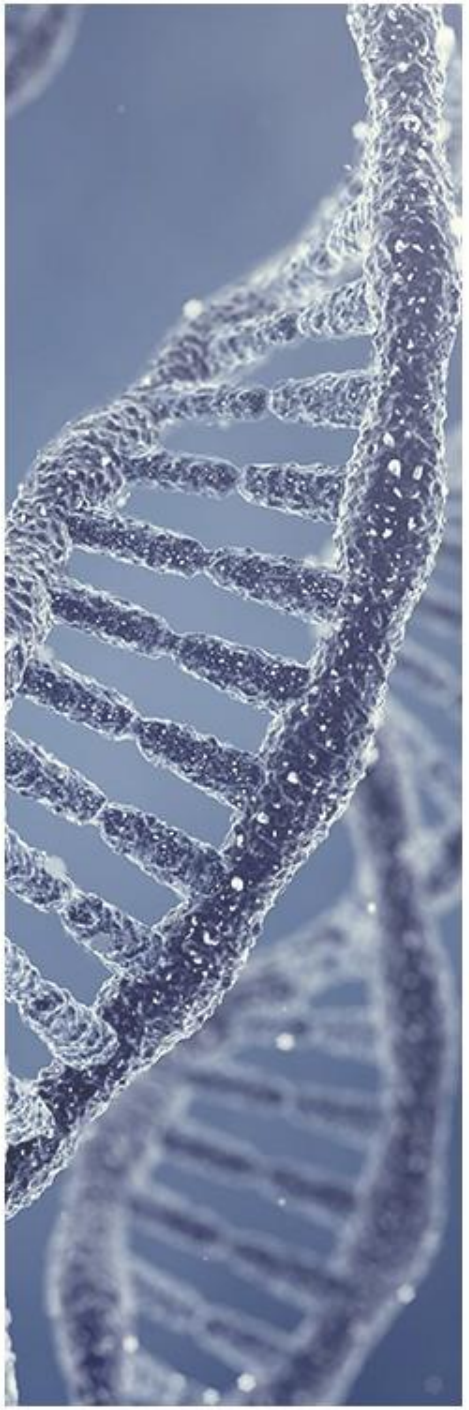
Relativna kvantifikacija

- poređenje dva uzorka jedan u odnosu na drugi: npr - pre i posle tretmana lekovima ili tretirana lekvima i kontrola (koja nije tretirana)

$$\Delta Ct = Ct_{(GI)} - Ct_{(HK)}$$

$$\Delta\Delta Ct = \Delta Ct - \Delta Ct_{(calibrator)}$$

$$2^{-\Delta\Delta Ct}$$

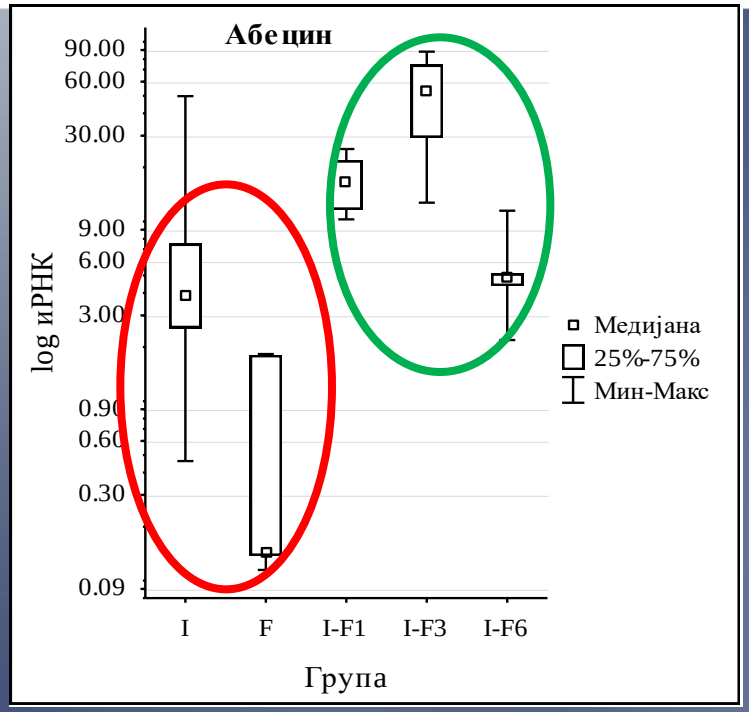


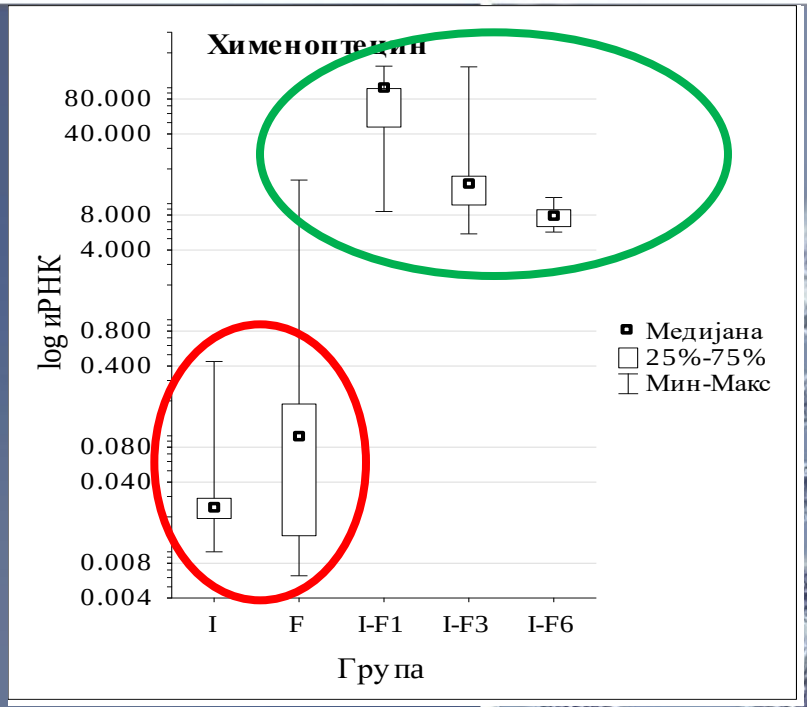
Naši primeri upotrebe real time PCR za relativnu kvantifikaciju

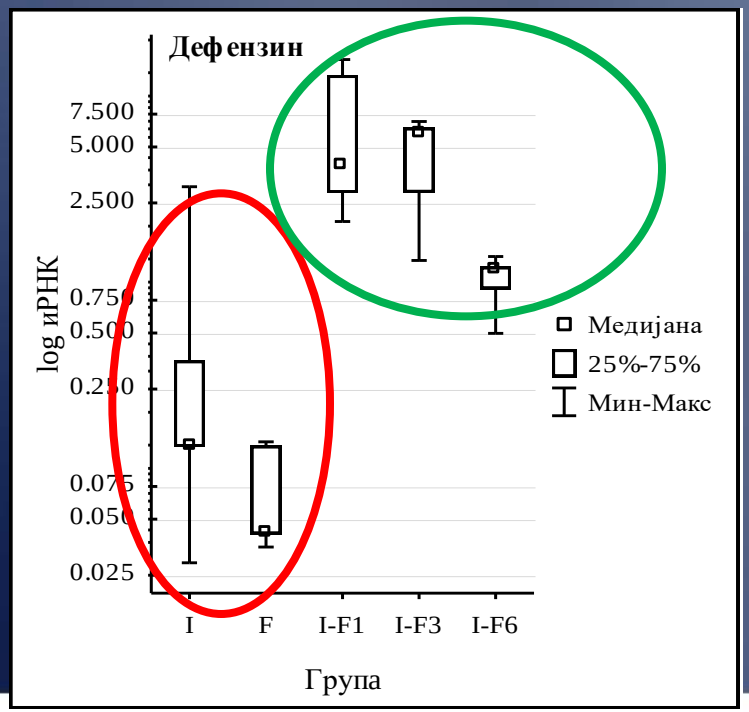
Naziv projekta:
Ispitivanje uticaja različitih preparata na ekspresiju gena koji kodiraju imune peptide pčela (*Apis mellifera*) inficiranih mikrosporidijom *Nosema ceranae*

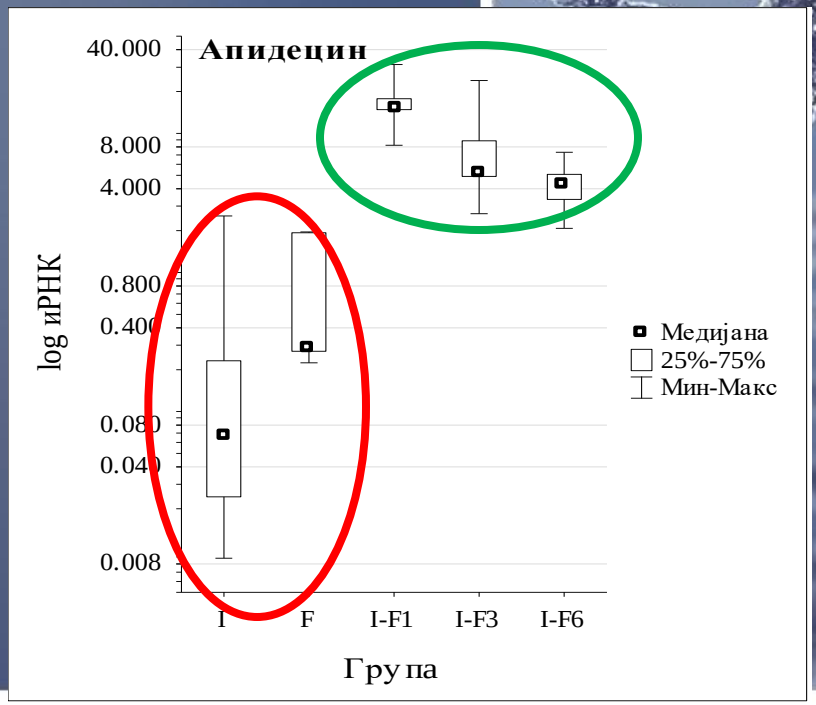
Експериментални дизајн

ГРУПА	Дан почетка третирања фумагилином (дан након излегања)	Заражавање спорама *N. ceranae* (дан након излегања)	Дан узоркована (дан након излегања)		
NI	-	-	6.	9.	15.
I	-	3.	6.	9.	15.
F	1.	-	6.	9.	15.
I-F1	1.	3.	6.	9.	15.
I-F3	3.	3.	6.	9.	15.
I-F6	6.	3.	-	9.	15.

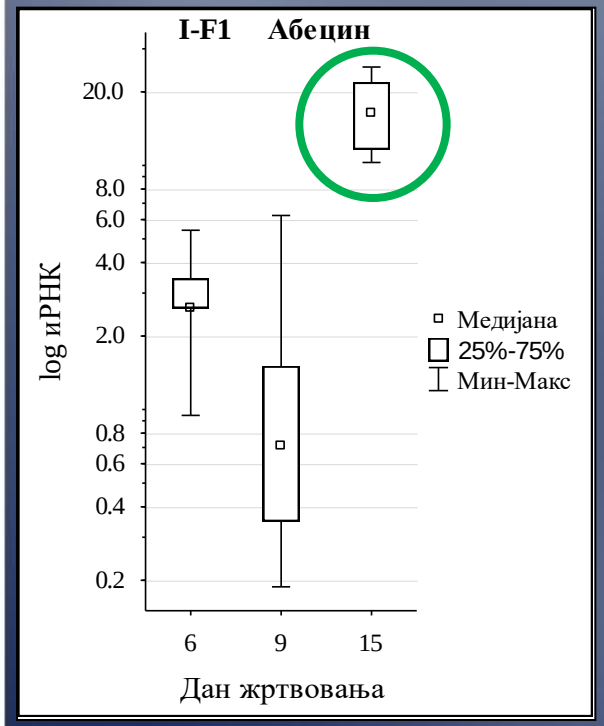

Абецин
90.00
60.00
30.00
9.00
6.00
3.00
0.90
0.60
0.30
0.09
log иРНК
I
F
I-F1
I-F3
I-F6
Група
Медијана
25%-75%
Мин-Макс

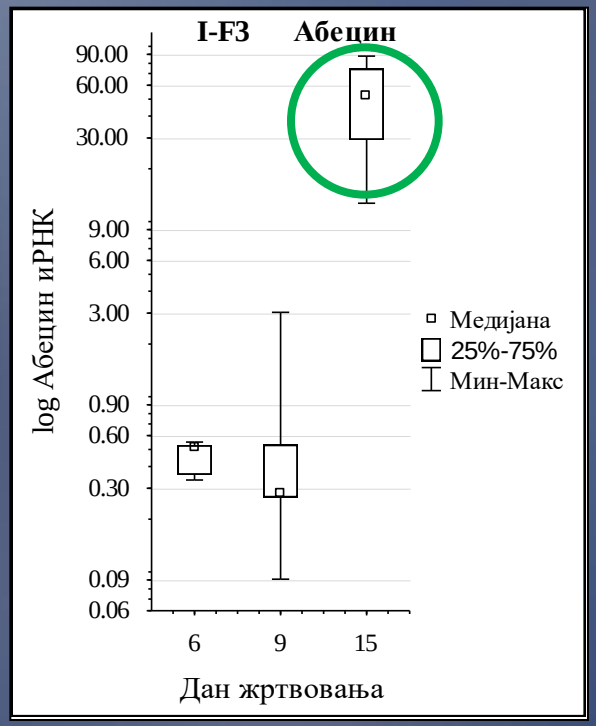

Хименоптецин
80.000
40.000
8.000
4.000
0.800
0.400
0.080
0.040
0.008
0.004
log иРНК
I
F
I-F1
I-F3
I-F6
Група
Медијана
25%-75%
Мин-Макс

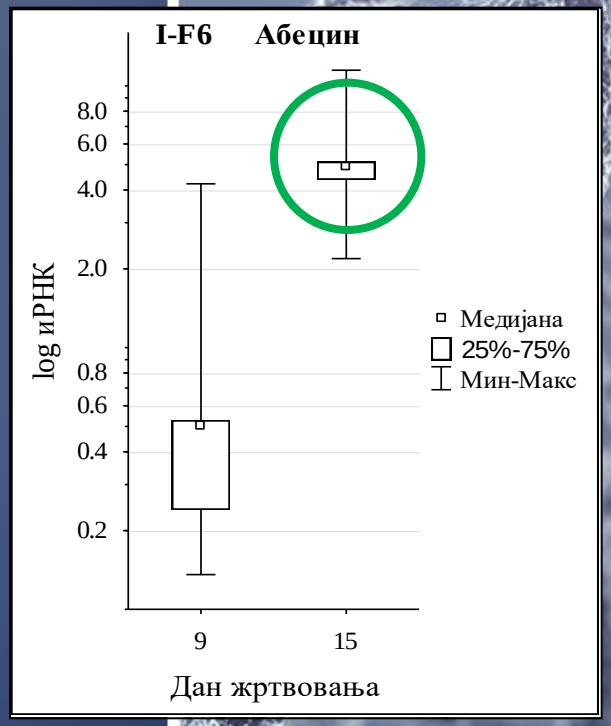

Дефензин
7.500
5.000
2.500
0.750
0.500
0.250
0.075
0.050
0.025
log иРНК
I
F
I-F1
I-F3
I-F6
Група
Медијана
25%-75%
Мин-Макс


Апидецин
40.000
8.000
4.000
0.800
0.400
0.080
0.040
0.008
log иРНК
I
F
I-F1
I-F3
I-F6
Група
Медијана
25%-75%
Мин-Макс

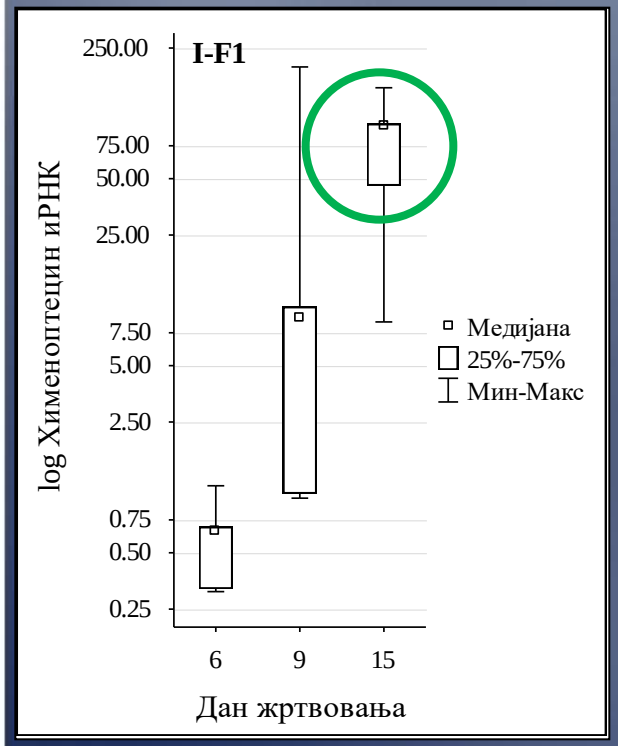
Нивои експресије гена за абецин током времена

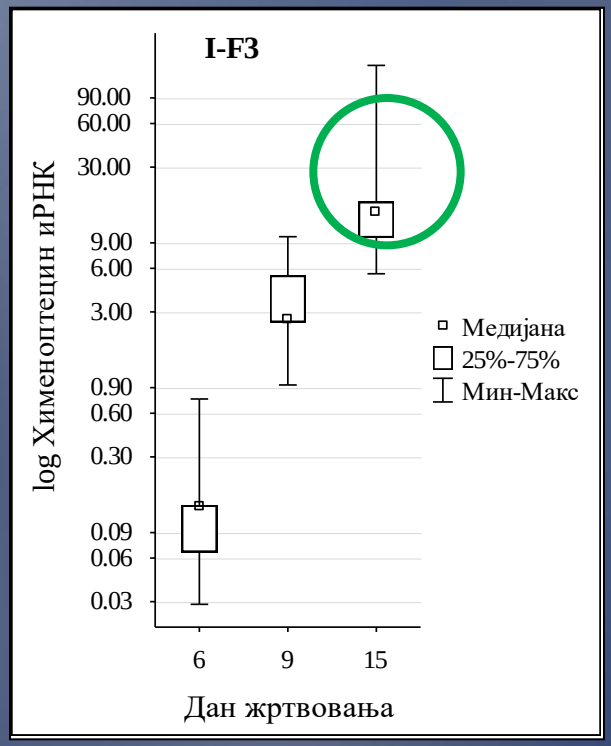


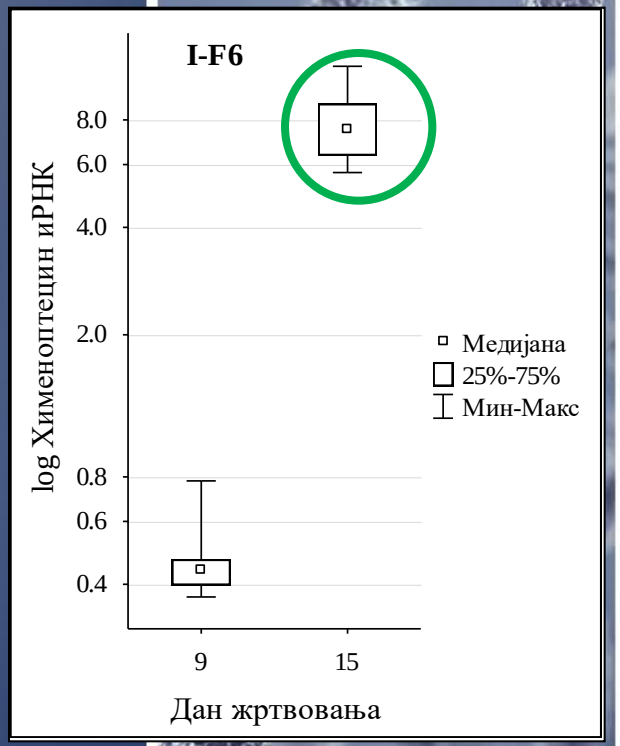




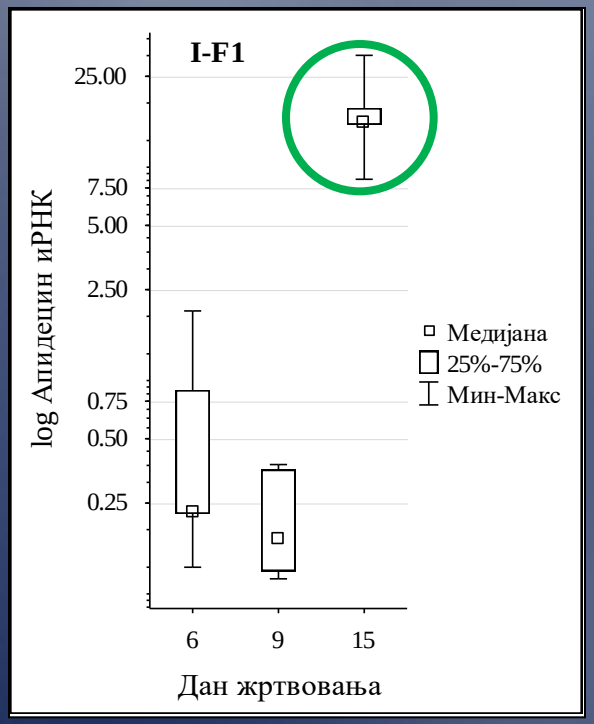
Нивои експресије гена за хименоптецин током времена

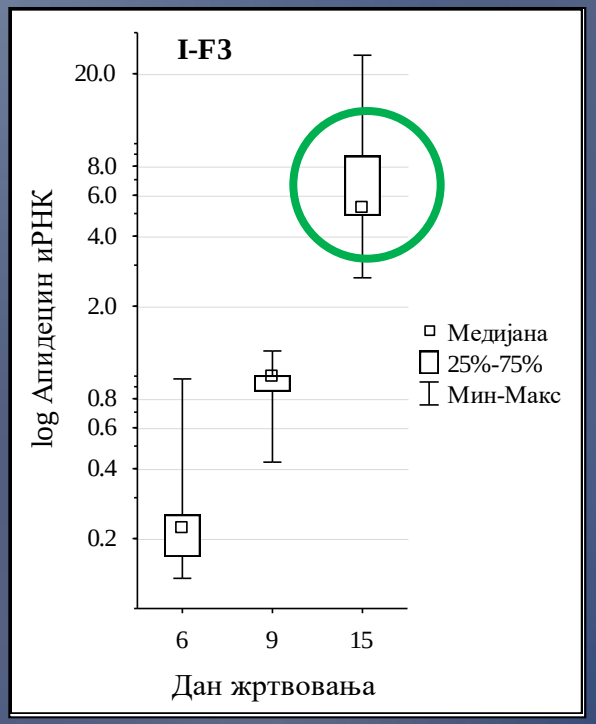


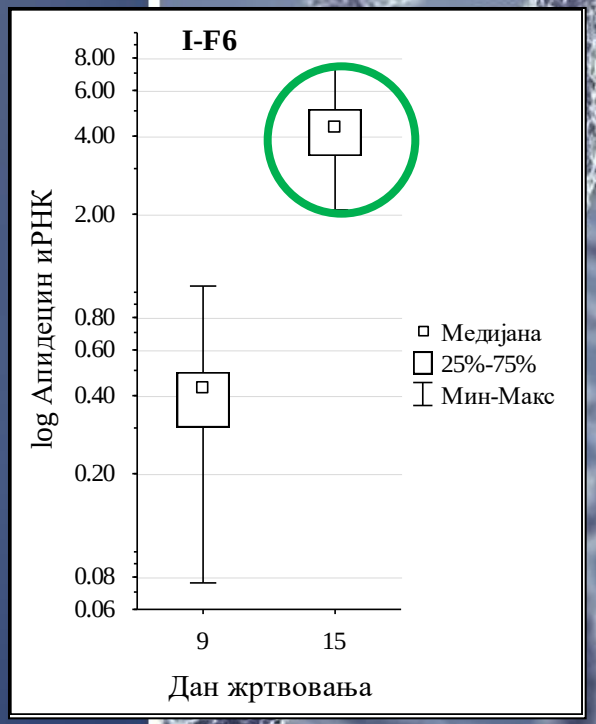




Нивои експресије гена за апидецин током времена







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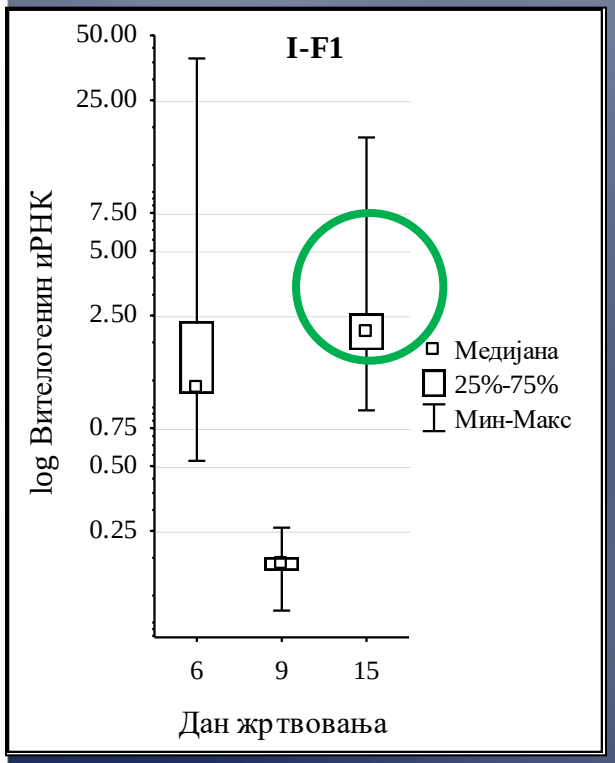
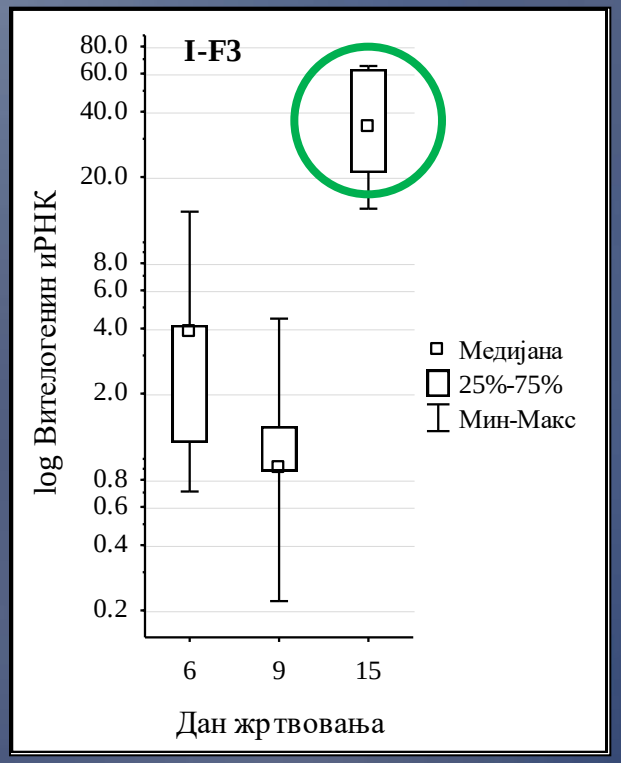
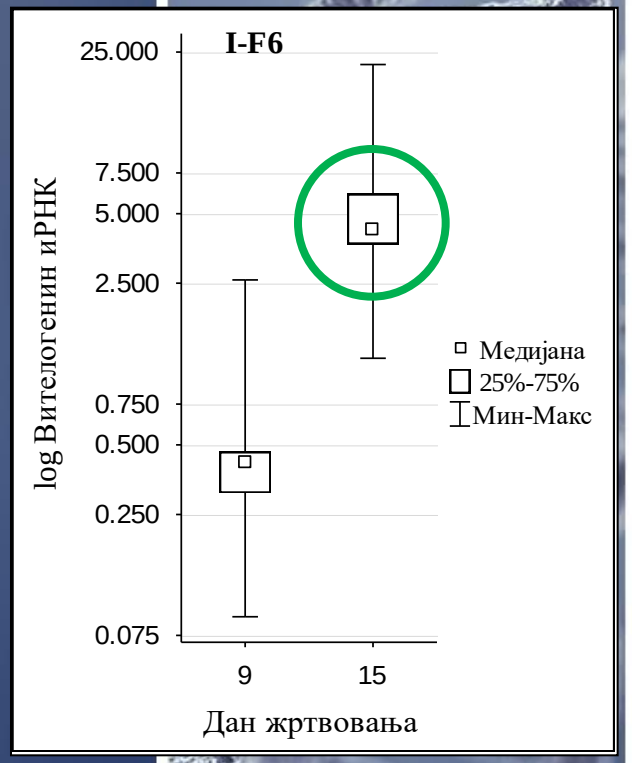
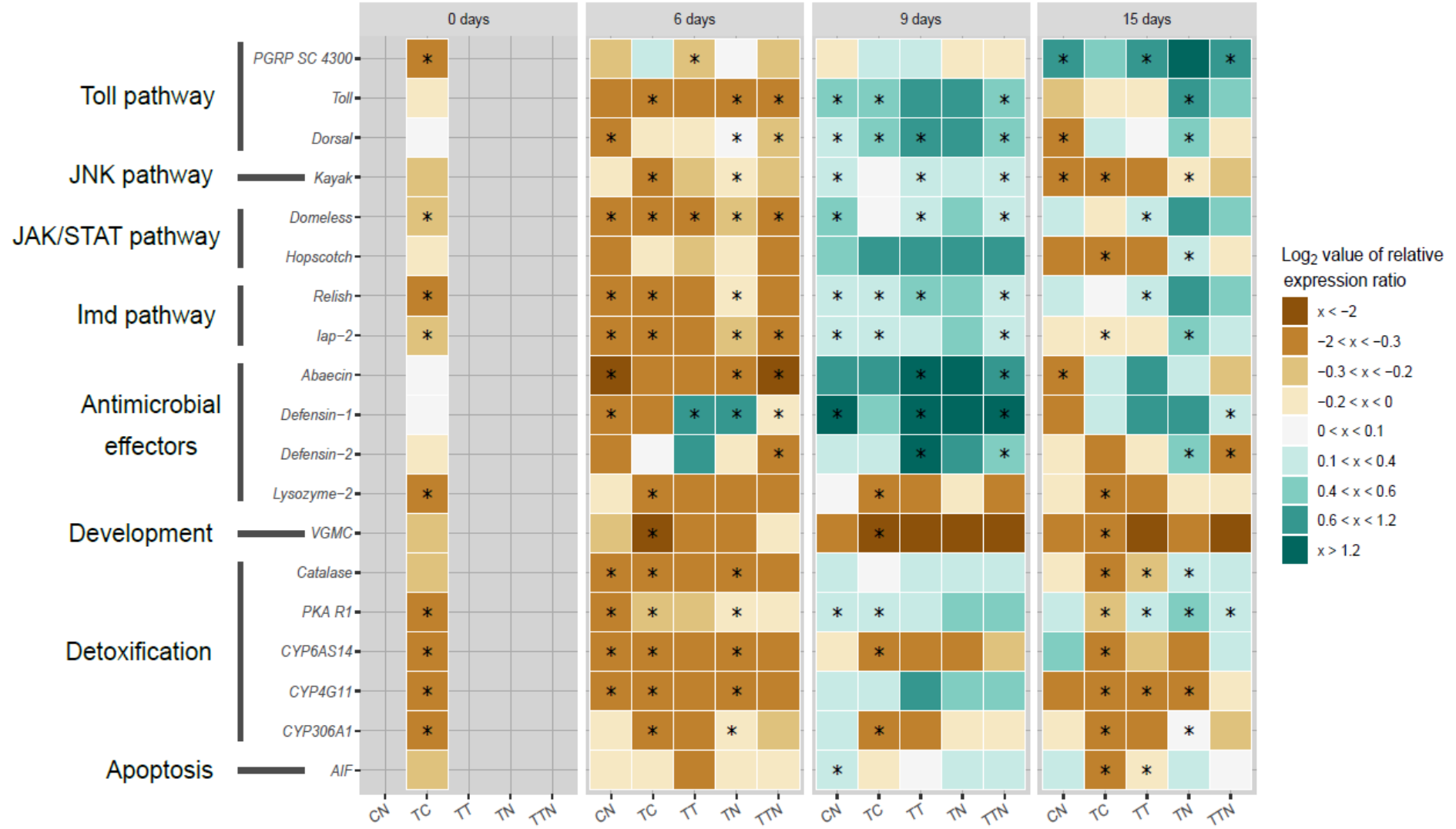
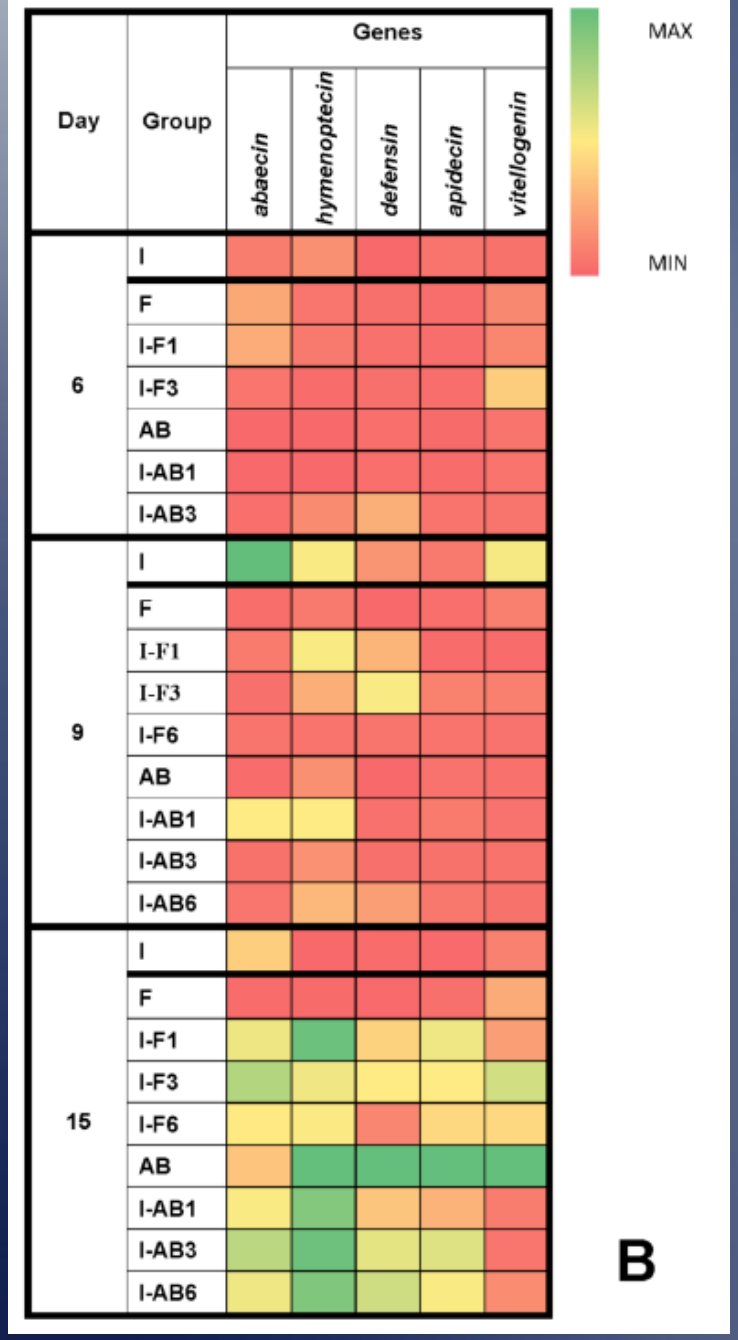


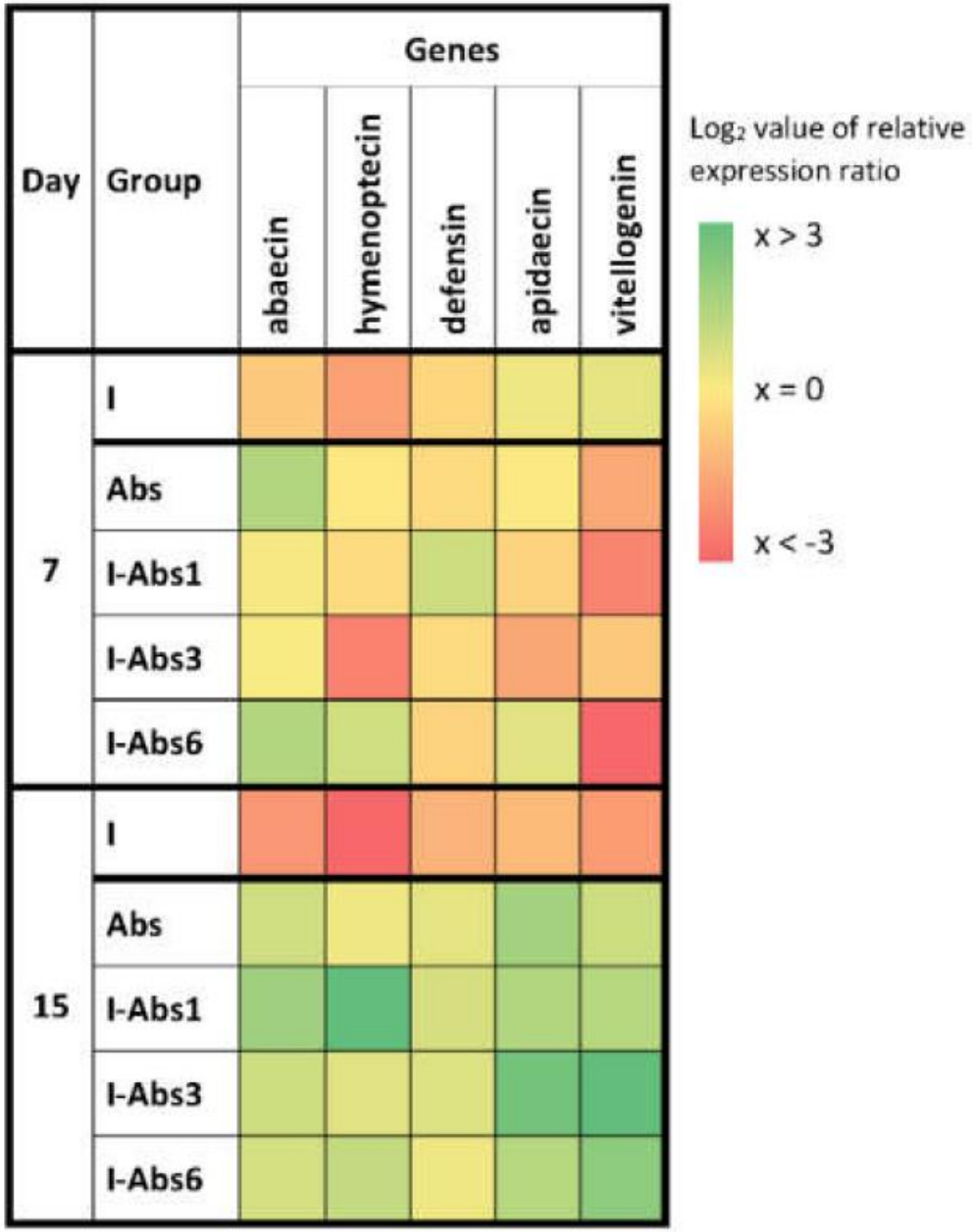




	Toll pathway					JNK pathway		JAK/S
	PGRPSC 4300	Spaetze	Toll	Dorsal-1	Cactus	BASKET	Kayak	Domel
PC_0	3,16	-0,47	1,96	1,21	1,43	0,07	0,82	4,48
CN_6	0,10	-0,59	0,34	0,20	-0,12	-0,65	-2,06	0,17
TN_6	-1,37	1,07	-1,80	-1,51	-1,49	0,18	3,18	-2,64
CN_9	0,14	0,89	-0,50	-0,16	0,73	0,29	-1,65	-0,60
TN_9	-0,81	-0,06	-0,91	-0,50	0,05	-0,17	-3,31	-0,43
CN_15	-0,28	-0,44	0,70	0,03	0,37	0,77	0,16	0,75
TN_15	0,40	-0,57	-0,08	0,47	-0,30	-0,28	*NA*	-0,00

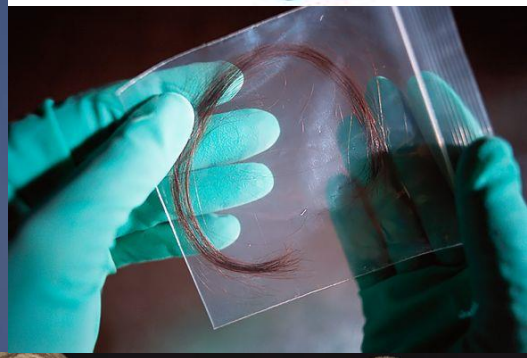

0 days
6 days
9 days
15 days
Toll pathway
JNK pathway
JAK/STAT pathway
Imd pathway
Antimicrobial effectors
Development
Detoxification
Apoptosis
PGRP SC 4300
Toll
Dorsal
Kayak
Domeless
Hopscotch
Relish
Iap−2
Abaecin
Defensin−1
Defensin−2
Lysozyme−2
VGMC
Catalase
PKA R1
CYP6AS14
CYP4G11
CYP306A1
AIF
CN
TC
TT
TN
TTN
Log2 value of relative expression ratio
x < −2
−2 < x < −0.3
−0.3 < x < −0.2
−0.2 < x < 0
0 < x < 0.1
0.1 < x < 0.4
0.4 < x < 0.6
0.6 < x < 1.2
x > 1.2

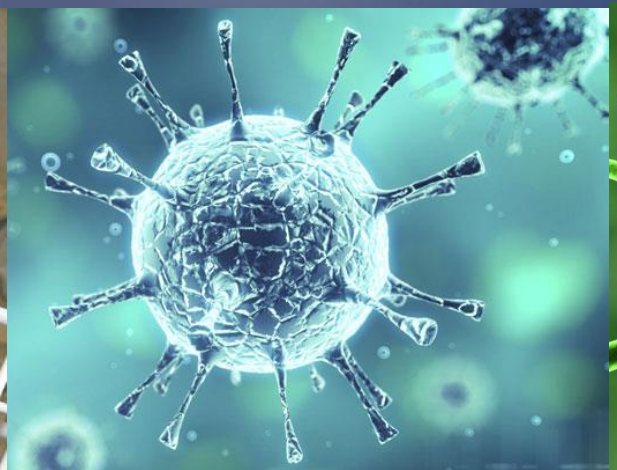

Day
Group
Genes
abaecin
hymenoptecin
defensin
apidecin
vitellogenin
6
I
F
I-F1
I-F3
AB
I-AB1
I-AB3
9
I
F
I-F1
I-F3
I-F6
AB
I-AB1
I-AB3
I-AB6
15
I
F
I-F1
I-F3
I-F6
AB
I-AB1
I-AB3
I-AB6
MAX
MIN
B


Day
Group
Genes
abaecin
hymenoptecin
defensin
apidaecin
vitellogenin
7
I
Abs
I-Abs1
I-Abs3
I-Abs6
15
I
Abs
I-Abs1
I-Abs3
I-Abs6
Log2 value of relative expression ratio
x > 3
x = 0
x < -3

Primena

- medicina
  - genetičko testiranje (nasledna oboljenja) (prisustvo mutacija koja dovode do oboljenja)
  - preimplantaciona i prenatalna dijagnostika
  - efiksnost lekova, suplemenata, aditiva...
- forenzika
  - identifikacija osoba pomoću DNK profila (“*genetic fingerprinting*”)
  - određivanje srodstva (očinstvo, majčinstvo,)
- infektivne bolesti, mikrobiologija, virusologija
- naučna istraživanja





Real-time PCR vs PCR

- **Real-time PCR**

- Senzitivniji, brži, nema manipulacije sa amplifikatom nakon reakcije, manja mogućnosti za kontaminaciju...

- **PCR**

- End point...

Nije automatizovan, etidijum bromid, rezultati su kvalitativni, ne kvantitativni...

Hvala na pažnji !!!