OPTIMIZATIONS OF DETECTION AND QUANTIFICATION OF GMOS

Mojca Milavec

National Institute of Biology
Department of Biotechnology and Systems Biology
Večna pot 111, SI-1000 Ljubljana, Slovenia

E-mail: mojca.milavec@nib.si

NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

Introduction

National reference laboratories for genetically modified organisms (GMOs) and plant pathogens

Partner in two **European Union Reference Laboratories** (for viruses and phytoplasmas, and for bacteria)

ISO 17025 since 2003, flexible scope since 2006

Development and validation or verification of methods

Routine analysis (plant pathogens, GMOs: food, feed, seed, plants)

Designated as national metrological institute - Holder of **National Standard** in the Field of Amount of Substance/Bioanalysis of Nucleic Acids/GMOs and Microorganisms

Genetically Modified Organisms

…are by the EU legal definition[1]: organisms, with the exception of humans, in which the genetic material has been altered through the use of biotechnological methods, in a way that does not occur naturally by mating and/or natural recombination.

NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

[1] Directive 2001/18/EC of the European Parliament and the Council of 12 March 2001 on the deliberate release in to the environment of genetically modified organisms and repealing Council Directive 90/220/EC

GMOs in the European Union

Regulation No. 1829/2003

Labelling "...shall not apply to foods/feed containing material which contains, consists of or is produced from GMOs in a proportion **no higher than 0.9 per cent** of the **food ingredients** considered individually or food consisting of a single ingredient..."

Regulation No. 619/2011 (so called LLP regulation)

"...it is appropriate to set as a Minimum Required Performance Limit (MRPL) the lowest level of GM material which is considered by the EU-RL for the validation of quantitative methods. This level corresponds to **0.1% related to mass fraction** of GM material in feed and is the lowest level where results are satisfactorily reproducible between official laboratories ..."

Three partite PCR-based testing scheme

screening

identification

quantification

More general information

More specific information

Three partite PCR-based testing scheme - screening

screening

More general information

More specific information

Three partite PCR-based testing scheme - identification

screening

identification

More general information

More specific information

Three partite PCR-based testing scheme - quantification

screening

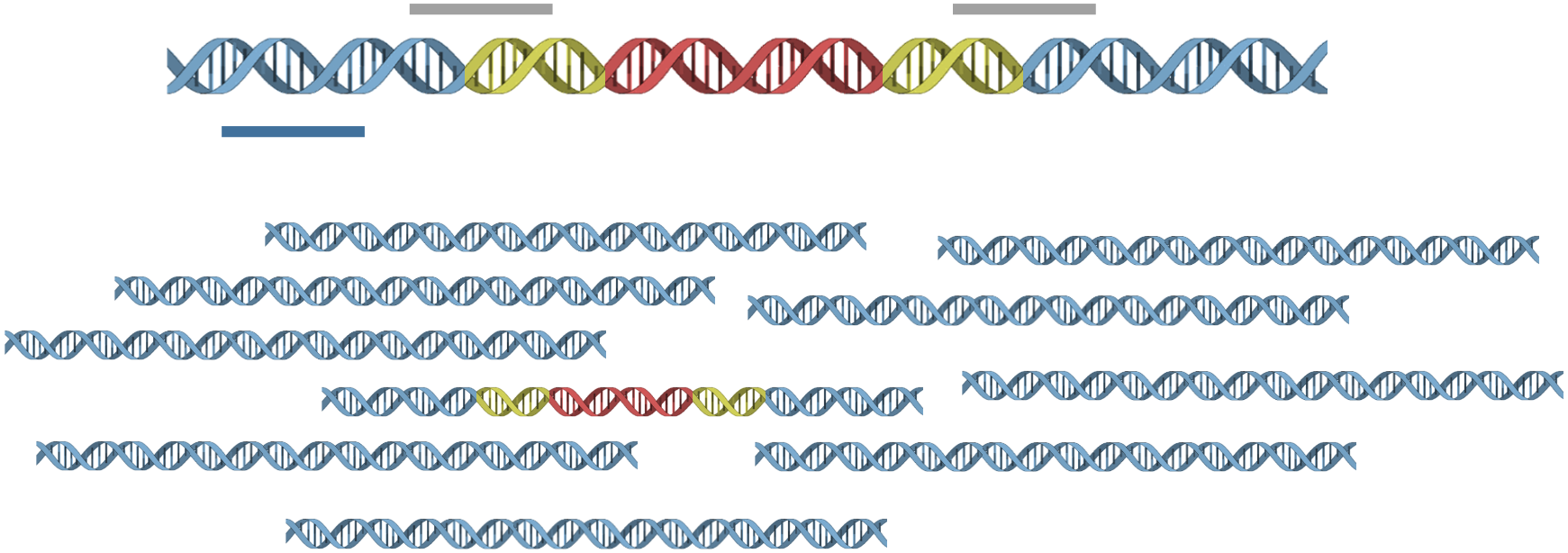
identification

quantification

More general information

More specific information

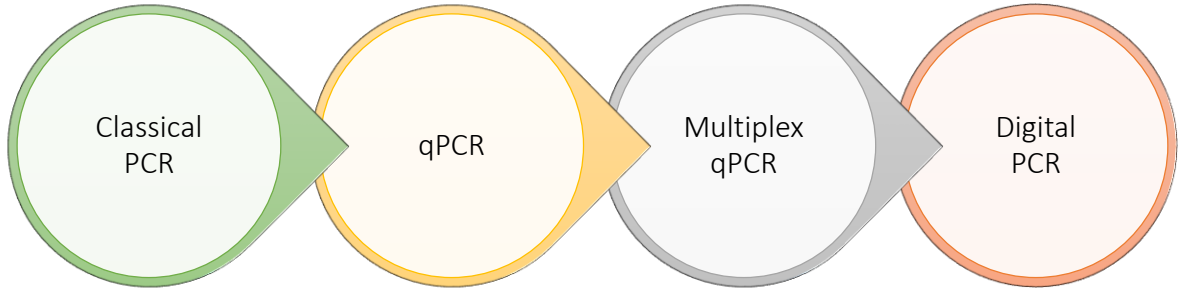
Three partite PCR-based testing scheme - quantification



1 GM + 9 non GM = 10% GMO

Challenges in GMO testing

- Unknown GMOs
- Unauthorized GMOs
- **Increasing number of GMOs**
- **GMOs without common elements**
- **GMOs in challenging matrices**



SAFEFOODERA
European excellence in food safety research programming

Increasing number of GMOs

GMOseek

GMOval project
Real-time PCR screening methods

Food Standards Agency

Federal Office of Consumer Protection and Food Safety

Screening for common elements and decision support after the screening

5 common elements in pentaplex:	
P35S	promoter region of the cauliflower mosaic virus
Tnos	nopalin synthase gene terminator from *Agrobacterium tumefaciens*
bar	basta resistance gene from *Streptomyces hygroscopicus*
pat	phosphinothricine acetyltransferase gene from *Streptomyces viridochromogenes*
ctp-cp4-epsps	construct containing a 6-enolpyruvylshikimate-3-phosphate-synthase gene from *Agrobacterium tumefaciens* strain cp4 with an upstream sequence of the ctp2 chloroplast transit peptide from *Arabidopsis thaliana*

Results of testing feed (containing soybean and maize) with pentaplex:

P-35S - present
Tnos - present
bar – not present
pat - not present
ctp-cp4-epsps - not present

In the next step presence of 8 soybean lines and 9 maize lines should be tested (marked in red)

NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

Increasing number of GMOs

Screening for common elements and decision support after the screening

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development and Validation of Duplex, Triplex, and Pentaplex Real-Time PCR Screening Assays for the Detection of Genetically Modified Organisms in Food and Feed

Ingrid Huber,*[,†] Annette Block,[†] Daniela Sebah,[†] Frédéric Debode,[‡] Dany Morisset,[§] Lutz Grohmann,[⊥] Gilbert Berben,[‡] Dejan Štebih,[§] Mojca Milavec,[§] Jana Žel,[§] and Ulrich Busch[†]

[†]Bavarian Health and Food Safety Authority (LGL), Veterinärstrasse 2, D-85764 Oberschleissheim, Germany

[‡]Walloon Agricultural Research Centre (CRA-W), Chaussée de Namur 24, B-5030 Gembloux, Belgium

[§]Department of Biotechnology and Systems Biology, National Institute of Biology (NIB), Vecna pot 111, SI-1000 Ljubljana, Slovenia

[⊥]Federal Office of Consumer Protection and Food Safety (BVL), Mauerstrasse 39-42, 10117 Berlin, Germany

GMOs without common elements

With screening for selected common elements it is not possible to detect all in EU authorized GMOs

Event (Unique identifier)	Species	CTP2-CP4-EPSPS	Tnos	P35S	bar	pat
A2704-12 (ACS-GMØØ5-3)	soybean	0	0	1	0	1
A5547-127 (ACS-GMØØ6-4)	soybean	0	0	1	0	1
MON40-3-2 (MON-Ø4Ø32-6)	soybean	0	1	1	0	0
MON89788 (MON-89788-1)	soybean	1	0	0	0	0
DP356043-5 (DP-356Ø43-5)	soybean	0	0	1	0	0
DP305423-1 (DP-3Ø5423-1)	soybean	0	0	0	0	0
CV127 (BPS-CV-127-9)	soybean	0	0	0	0	0
MON87701 (MON-877Ø1-2)	soybean	0	0	0	0	0
MON87769 (MON-87769-7)	soybean	0	0	0	0	0
68416 (DAS-68416-4)	soybean	0	0	0	0	1
MON87708 (MON-87708-9)	soybean	0	0	0	0	0
MON87705 (MON-877Ø5-6)	soybean	1	0	0	0	0
FG72 (MST-FGØ72-2)	soybean	0	1	0	0	0
DAS44406 (DAS-44406-6)	soybean	0	0	0	0	1
DAS81419 (DAS-81419-2)	soybean	0	0	0	0	1
SYHT0H2 (SYN-ØØØH2-5)	soybean	0	1	1	0	1
MON87751 (MON-87751-7)	soybean	0	0	0	0	0
GMB151 (BCS-GM151-6)	soybean	0	0	1	0	0

Event (Unique identifier)	Species	CTP2-CP4-EPSPS	Tnos	P35S	bar	pat
A2704-12 (ACS-GMØØ5-3)	soybean	0	0	1	0	1
A5547-127 (ACS-GMØØ6-4)	soybean	0	0	1	0	1
MON40-3-2 (MON-Ø4Ø32-6)	soybean	0	1	1	0	0
MON89788 (MON-89788-1)	soybean	1	0	0	0	0
DP356043-5 (DP-356Ø43-5)	soybean	0	0	1	0	0
DP305423-1 (DP-3Ø5423-1)	soybean	0	0	0	0	0
CV127 (BPS-CV-127-9)	soybean	0	0	0	0	0
MON87701 (MON-877Ø1-2)	soybean	0	0	0	0	0
MON87769 (MON-87769-7)	soybean	0	0	0	0	0
68416 (DAS-68416-4)	soybean	0	0	0	0	1
MON87708 (MON-87708-9)	soybean	0	0	0	0	0
MON87705 (MON-877Ø5-6)	soybean	1	0	0	0	0
FG72 (MST-FGØ72-2)	soybean	0	1	0	0	0
DAS44406 (DAS-44406-6)	soybean	0	0	0	0	1
DAS81419 (DAS-81419-2)	soybean	0	0	0	0	1
SYHT0H2 (SYN-ØØØH2-5)	soybean	0	1	1	0	1
MON87751 (MON-87751-7)	soybean	0	0	0	0	0
GMB151 (BCS-GM151-6)	soybean	0	0	1	0	0

Event (Unique identifier)	Species	CTP2-CP4-EPSPS	Tnos	P35S	bar	pat
A2704-12 (ACS-GMØØ5-3)	soybean	0	0	1	0	1
A5547-127 (ACS-GMØØ6-4)	soybean	0	0	1	0	1
MON40-3-2 (MON-Ø4Ø32-6)	soybean	0	1	1	0	0
MON89788 (MON-89788-1)	soybean	1	0	0	0	0
DP356043-5 (DP-356Ø43-5)	soybean	0	0	1	0	0
DP305423-1 (DP-3Ø5423-1)	soybean	0	0	0	0	0
CV127 (BPS-CV-127-9)	soybean	0	0	0	0	0
MON87701 (MON-877Ø1-2)	soybean	0	0	0	0	0
MON87769 (MON-87769-7)	soybean	0	0	0	0	0
68416 (DAS-68416-4)	soybean	0	0	0	0	1
MON87708 (MON-87708-9)	soybean	0	0	0	0	0
MON87705 (MON-877Ø5-6)	soybean	1	0	0	0	0
FG72 (MST-FGØ72-2)	soybean	0	1	0	0	0
DAS44406 (DAS-44406-6)	soybean	0	0	0	0	1
DAS81419 (DAS-81419-2)	soybean	0	0	0	0	1
SYHT0H2 (SYN-ØØØH2-5)	soybean	0	1	1	0	1
MON87751 (MON-87751-7)	soybean	0	0	0	0	0
GMB151 (BCS-GM151-6)	soybean	0	0	1	0	0

Instead of screening for common elements, we are screening for presence of all in EU authorized lines:

The same set up of the qPCR for all runs

Plates (384 wells) for qPCR are partly prepared in advance – primers and probes are distributed on the plate and stored <-15°C

Samples and controls are mixed with the mastermix for qPCR and added to the plate

Event:		MON87701	DP-305423-1	CV127	MON87769	MON87708	MON40-3-2	DP356043	MON89788	MON87705	A2704-12	A5547-127	FG72	DAS68416	DAS44406	DAS81419
Sample:		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
NTC1	A	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1	NTC1
poz. control	B	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control
	C	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control	poz. control
G217/19 -1 5x	D	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x
	E	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x	G217/19 -1 5x
G217/19 -2 5x	F	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x
	G	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x	G217/19 -2 5x
G222/19 -1 1x	H	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x
	I	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x	G222/19 -1 1x
G222/19 -2 1x	J	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x
	K	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x	G222/19 -2 1x
G223/19 -1 1x	L	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x
	M	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x	G223/19 -1 1x
G223/19 -2 1x	N	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x
	O	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x	G223/19 -2 1x
NTC2	P	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2	NTC2

GMOs in challenging matrices

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Digital PCR as an effective tool for GMO quantification in complex matrices

Alexandra Bogožalec Košir*, Tina Demšar, Dejan Štebih, Jana Žel, Mojca Milavec

Department of Biotechnology and Systems Biology, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

GMOs in challenging matrices

vs.

vs.

Use of dPCR for quantification of GM soybean line in complex samples, containing PCR inhibitors.

GMOs in challenging matrices

Feed sample containing GM soybean – MON40-3-2 (Roundup Ready)

qPCR – sample exhibits great inhibition for both event specific sequence (MON40-3-2) and reference gene/endogene (Le1) up to 100 × dilution, sample cannot be quantified → pLOQ = 49 %

Methods (validated) were transfered to dPCR

GMOs in challenging matrices

Feed sample containing GM soybean – MON40-3-2 (Roundup Ready)

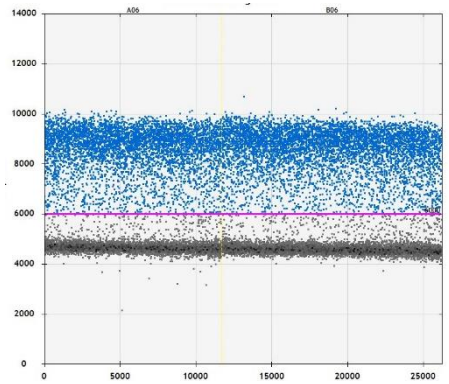
qPCR – sample exhibits great inhibition for both event specific sequence (MON40-3-2) and reference gene/endogene (Le1) up to 100 × dilution, sample cannot be quantified → pLOQ = 49 %

Methods (validated) were transfered to dPCR

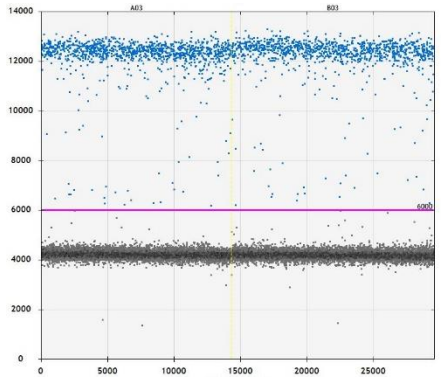
ddPCR	cdPCR	Bias
35.45	31.07	14.09 %

dPCR is not completely „immune“ to inhibitors

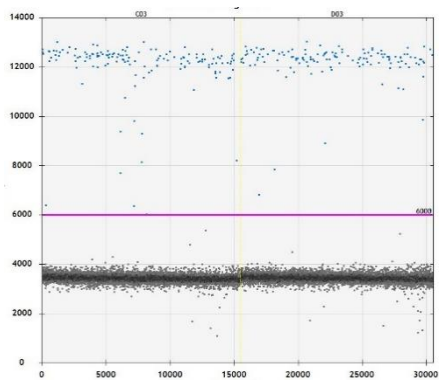
1× dilution – 14000 cp/µL



10× dilution – 2300 cp/µL

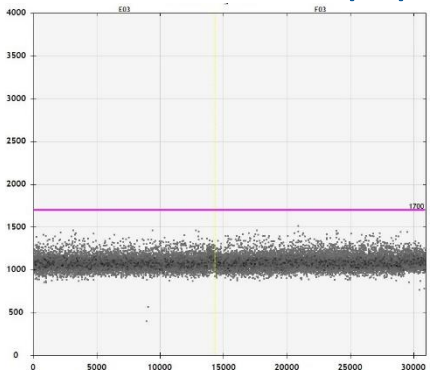


100× dilution – 260 cp/µL

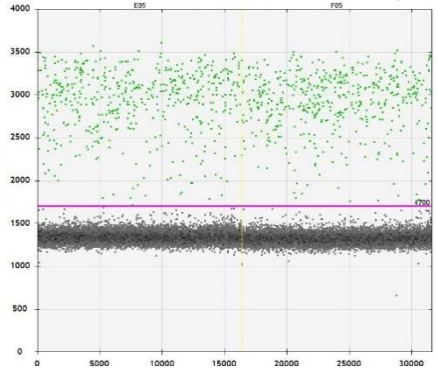


Reference gene

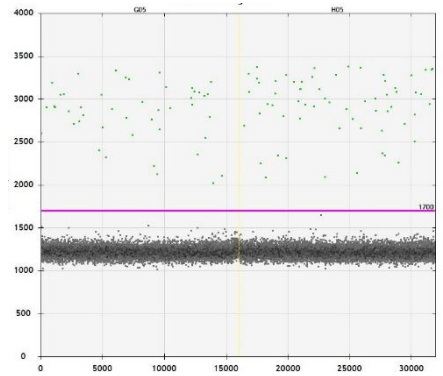
1× dilution – 0 cp/µL



10× dilution – 810 cp/µL



100× dilution – 90 cp/µL

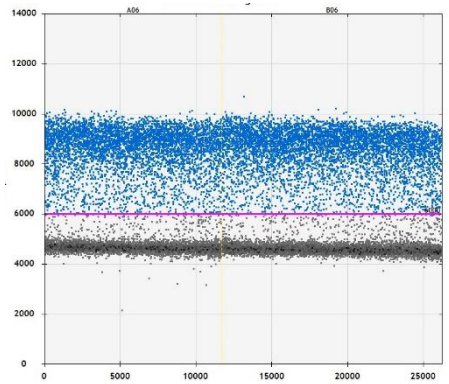


GMO

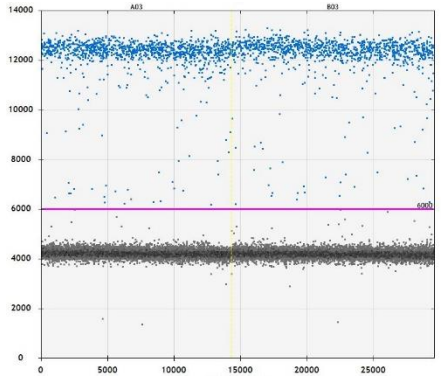
NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

dPCR is not completely „immune“ to inhibitors

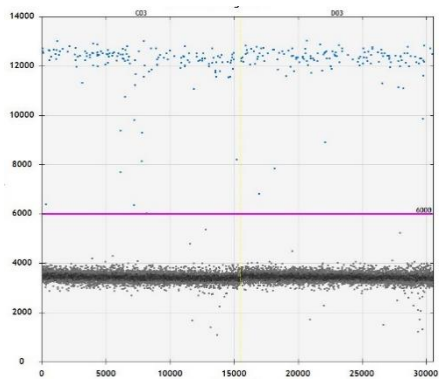
1× dilution – 14000 cp/µL



10× dilution – 2300 cp/µL

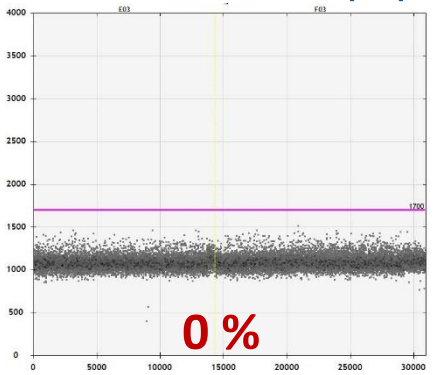


100× dilution – 260 cp/µL

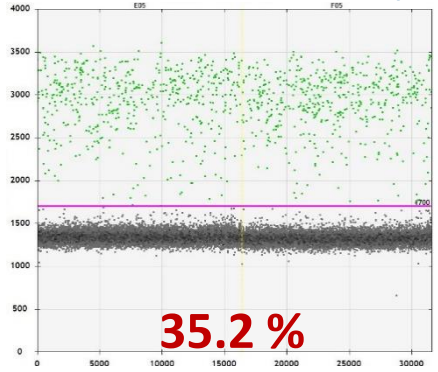


Reference gene

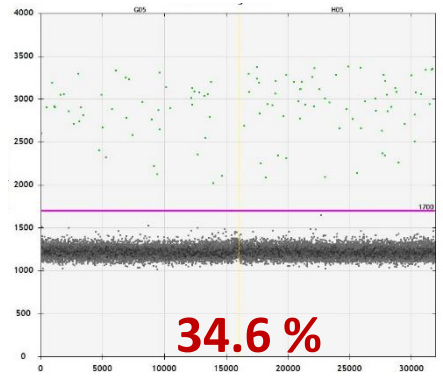
1× dilution – 0 cp/µL



10× dilution – 810 cp/µL



100× dilution – 90 cp/µL



GMO

NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

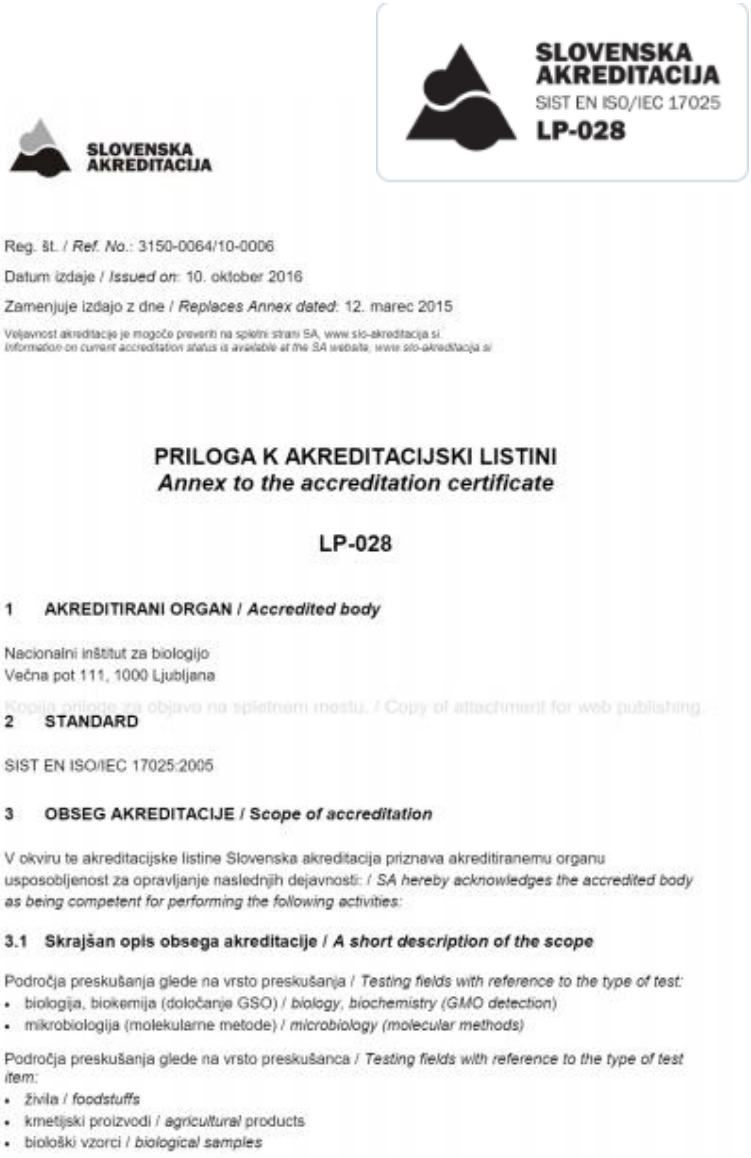
Accreditation

2 simplex assays

Soybean endogene *Le1*

MON40-3-2 or Roundup Ready soybean

A duplex assay for quantification of MON40-3-2



SLOVENSKA AKREDITACIJA

SLOVENSKA AKREDITACIJA
SIST EN ISO/IEC 17025
LP-028

Reg. št. / *Ref. No.*: 3150-0064/10-0006

Datum izdaje / *Issued on*: 10. oktober 2016

Zamenjuje izdajo z dne / *Replaces Annex dated*: 12. marec 2015

Veljavnost akreditacije je mogoče preveriti na spletni strani SA, www.slo-akreditacija.si
Information on current accreditation status is available at the SA website, www.slo-akreditacija.si

PRILOGA K AKREDITACIJSKI LISTINI
Annex to the accreditation certificate

LP-028

1 AKREDITIRANI ORGAN / *Accredited body*

Nacionalni inštitut za biologijo
Večna pot 111, 1000 Ljubljana

Kopija priloge za objavo na spletnem mestu. / *Copy of attachment for web publishing.*

2 STANDARD

SIST EN ISO/IEC 17025:2005

3 OBSEG AKREDITACIJE / *Scope of accreditation*

V okviru te akreditacijske listine Slovenska akreditacija priznava akreditiranemu organu usposobljenost za opravljanje naslednjih dejavnosti: / *SA hereby acknowledges the accredited body as being competent for performing the following activities:*

3.1 Skrajšan opis obsega akreditacije / *A short description of the scope*

Področja preskušanja glede na vrsto preskušanja / *Testing fields with reference to the type of test:*
- biologija, biokemija (določanje GSO) / *biology, biochemistry (GMO detection)*
- mikrobiologija (molekularne metode) / *microbiology (molecular methods)*

Področja preskušanja glede na vrsto preskušanca / *Testing fields with reference to the type of test item:*
- živila / *foodstuffs*
- kmetijski proizvodi / *agricultural products*
- biološki vzorci / *biological samples*

NACIONALNI INŠTITUT ZA **BIOLOGIJO**
NATIONAL INSTITUTE OF **BIOLOGY**

Plant Pathogens

Fire blight (*Erwinia amylovora*)

Potato brown rot (*Ralstonia solanacearum)*

Anal Bioanal Chem
DOI 10.1007/s00216-014-8084-1

PAPER IN FOREFRONT

Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot

Tanja Dreo · Manca Pirc · Živa Ramšak · Jernej Pavšič · Mojca Milavec · Jana Žel · Kristina Gruden

Received: 19 June 2014 / Revised: 28 July 2014 / Accepted: 29 July 2014

Human Pathogens

Human cytomegalovirus (HCMV)

Anal Bioanal Chem (2016) 408:107–121
DOI 10.1007/s00216-015-9107-2

PAPER IN FOREFRONT

Assessment of the real-time PCR and different digital PCR platforms for DNA quantification

Jernej Pavšič[1,2] · Jana Žel[1] · Mojca Milavec[1]

Anal Bioanal Chem (2016) 408:67–75
DOI 10.1007/s00216-015-9109-0

RAPID COMMUNICATION

Digital PCR for direct quantification of viruses without DNA extraction

Jernej Pavšič[1,2] · Jana Žel[1] · Mojca Milavec[1]

Acknowledgement

Department of Biotechnology and Systems Biology

Andrej Blejec
Alexandra Bogožalec Košir
Tina Demšar
David Dobnik
Kristina Gruden
Mojca Milavec
Dany Morisset
Dejan Štebih
Jana Žel

Funding

Research core funding No. P4-0165
Ministry for the Environment and Spatial Planning
Ministry of Agriculture, Forestry and Food
Metrology Institute of the Republic of Slovenia - MIRS

Thank you for your attention