

# Digital PCR and its role in GMO analysis

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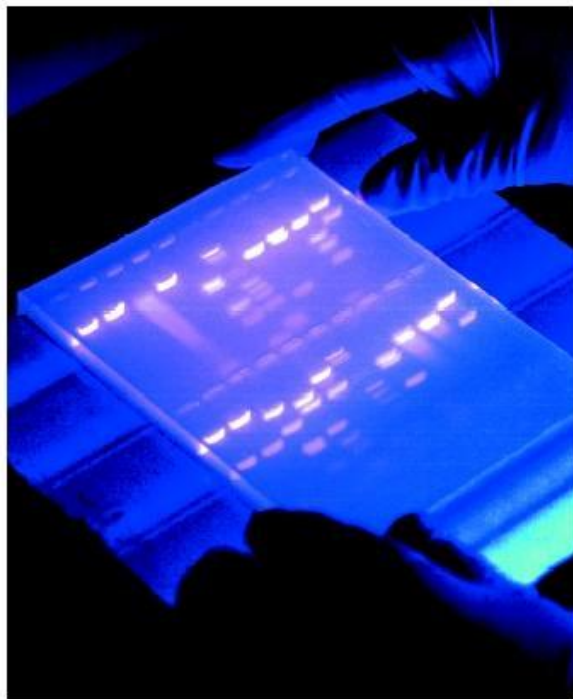
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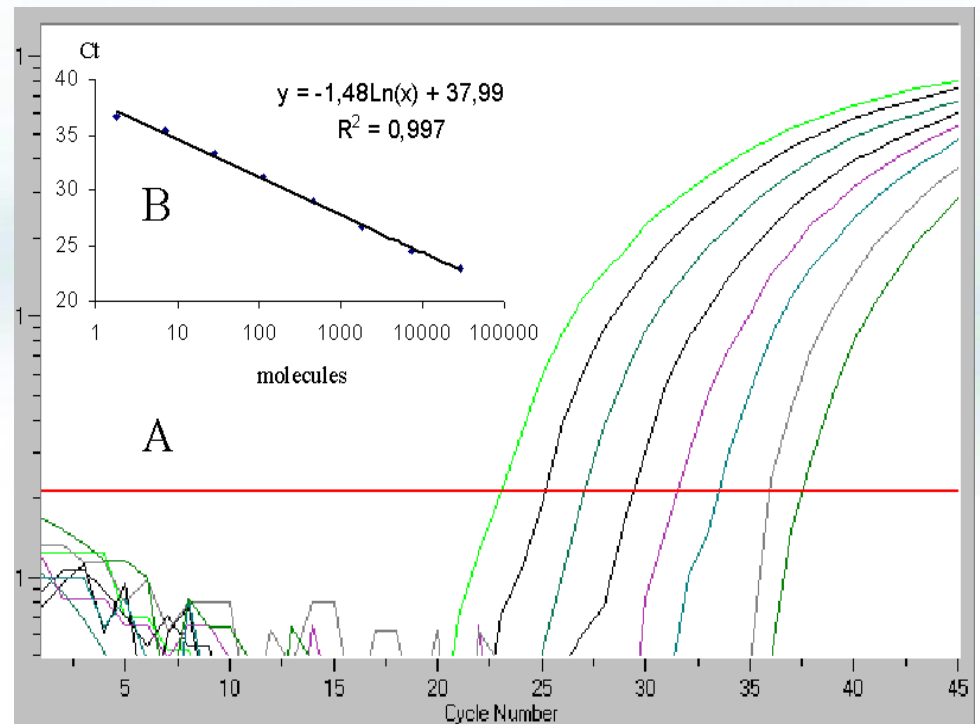


## Current DNA-based GMOs detection

### Conventional PCR



### Real-time PCR



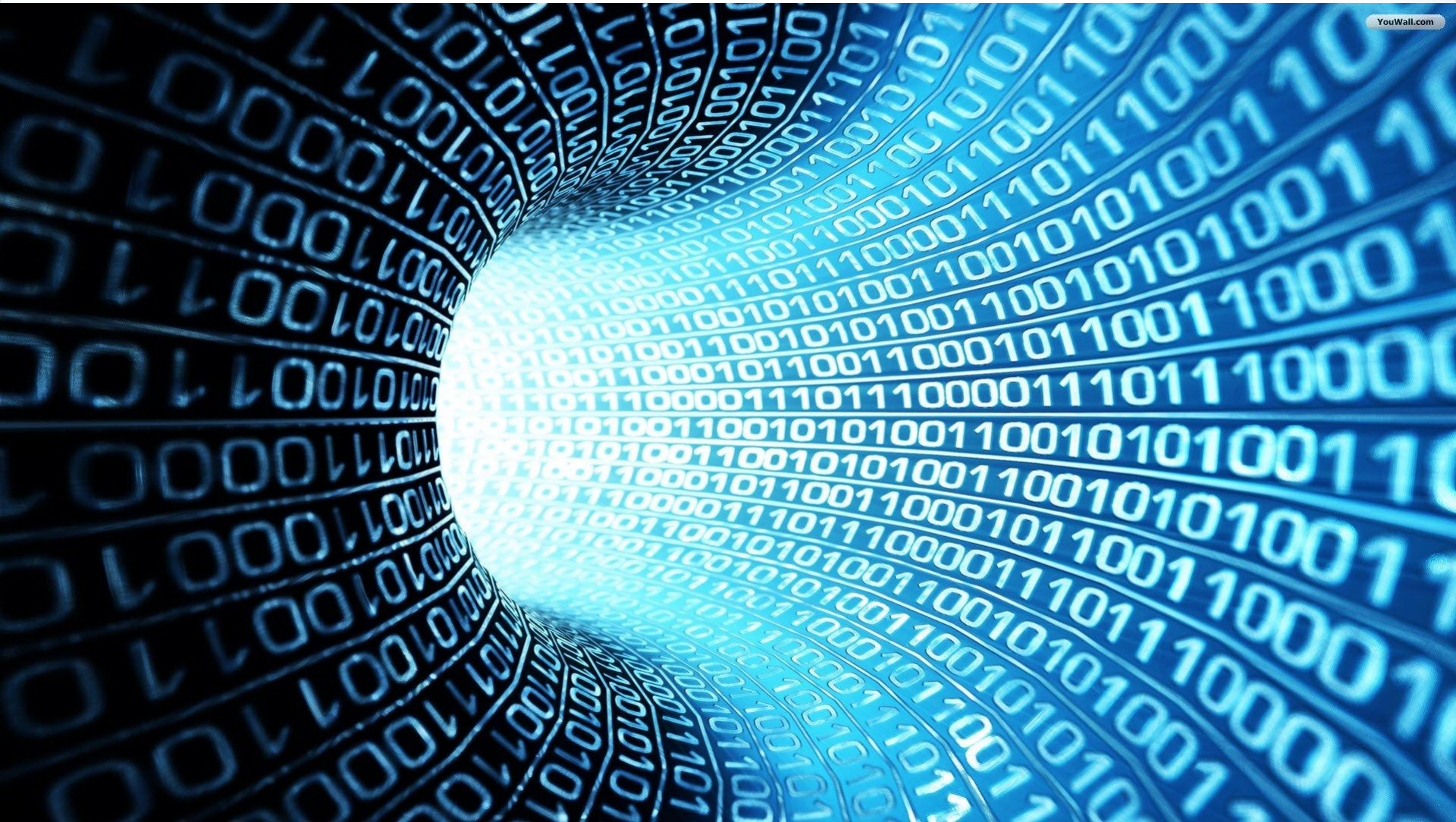
## Challenges in GMO detection

- More and more GMOs available (and authorized in EU)
- Quantification of low levels
- Cost-efficiency



**new technologies, multiplexing**

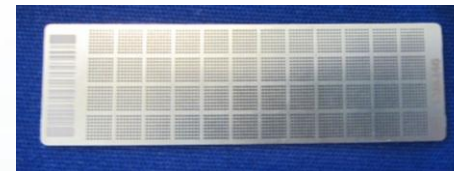
## The digital era



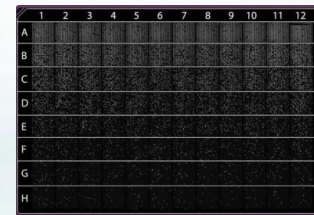
## dPCR – two approaches

- **Microfluidic/chip based**

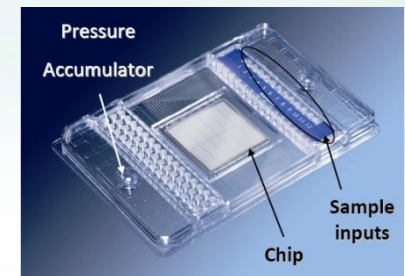
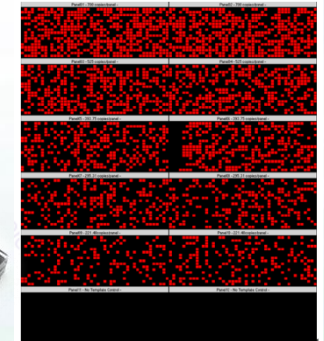
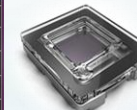
- Digital Array™ (Fluidigm)
- OpenArray®, QuantStudio® 3D (Life Technologies)
- Constellation (Formulatrix)



lifetechnologies.com



formulatrix.com

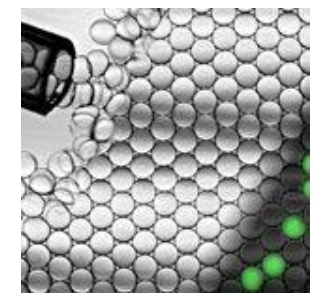


- **Droplet based**

- QX200™ droplet digital™ (Bio-Rad)
- RainStorm™ (RainDance)



raindancetech.com



www.bio-rad.com

# Advantages of dPCR

- **Determination of absolute copy numbers**
- **Accurate estimations at low copy numbers**
- **Tolerance to inhibitors**
- **Easy transfer of assays from qPCR to dPCR**



# dPCR and GMOs

- Copy number estimation
- Absolute and relative quantification of GM targets

Anal Bioanal Chem (2014) 406:6485–6497  
DOI 10.1007/s00216-014-8077-0

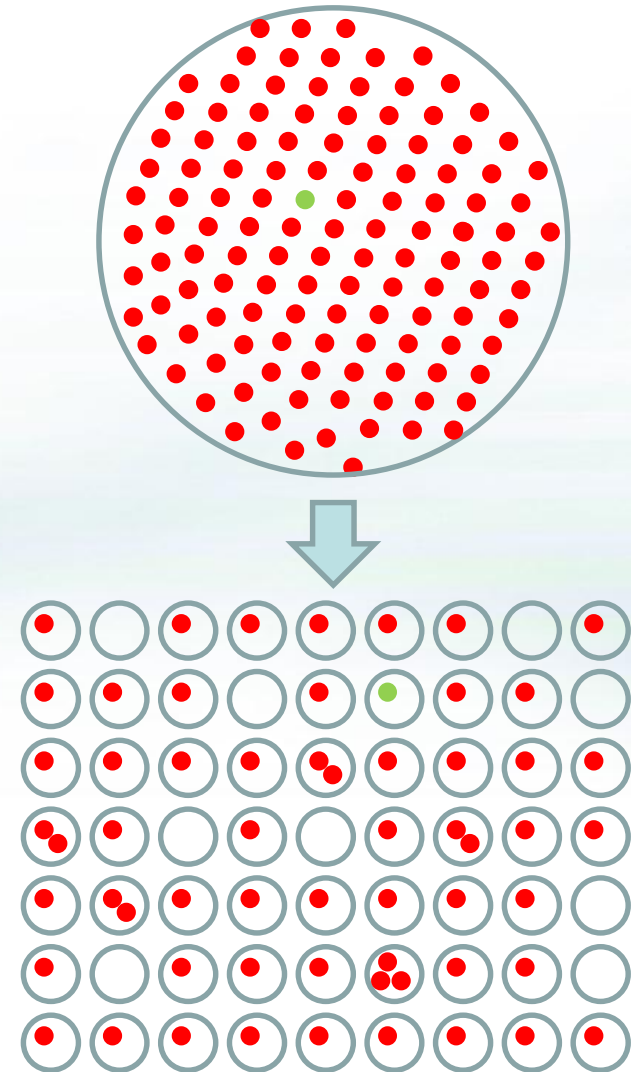
REVIEW

**GMO quantification: valuable experience and insights for the future**

Mojca Milavec • David Dobnik • Litao Yang •  
Dabing Zhang • Kristina Gruden • Jana Žel

## Digital PCR (dPCR)

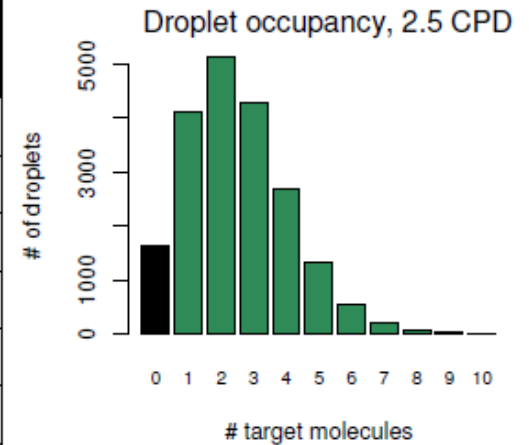
- Reaction mixture is divided in several partitions
- All partitions are subjected to a standard PCR
- DNA background is diluted
- Reactions scored as positive (1) or negative (0)



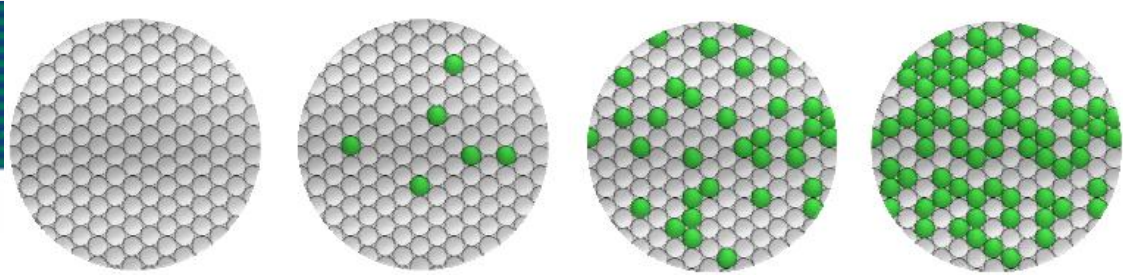
- Random distribution in partitions



# of target molecules	Count of droplets	Percent of total droplets
0	1642	8.21%
1	4104	20.5%
2	5130	25.7%
3	4275	21.4%
4	2672	13.4%
5	1336	6.68%
6	557	2.78%
7	199	0.99%
8	62	0.31%
9	17	0.086%
10	4	0.022%
11	1	0.0049%



*When we package 50,000 molecules into 20,000 droplets, on average 1642 droplets will have 0 targets, 4104 droplets will have 1 target, 5130 droplets will have 2 targets, etc.*



No target

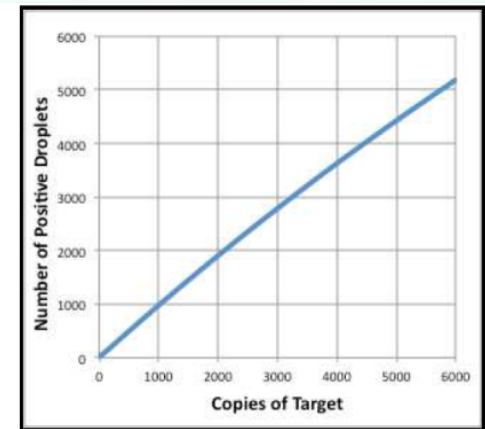
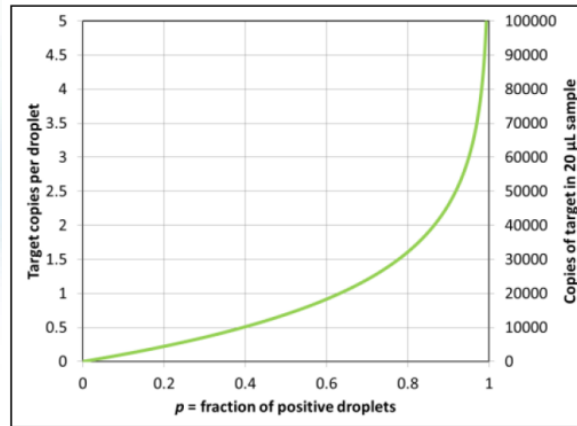
Low concentration

Medium concentration

High concentration

- Number of positive droplets is directly related to concentration

- Absolute copy number is determined using Poisson statistics



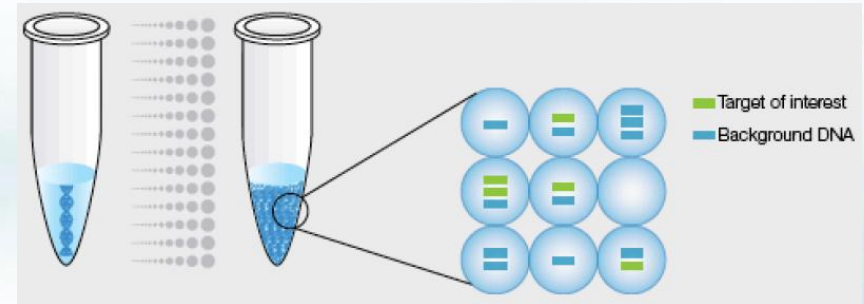
Modeling as Poisson  
 $\text{copies per droplet} = -\ln(1 - p)$   
 where  $p$  = fraction of positive droplets

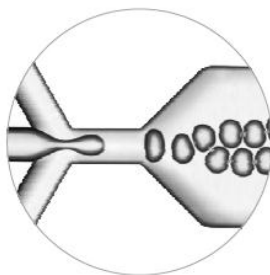
At 20,000 droplets per reaction



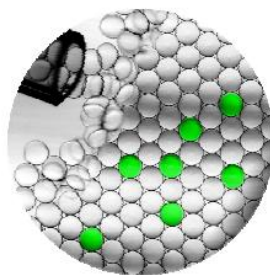
Siméon Denis Poisson  
 (1781-1840)

## Droplet digital PCR

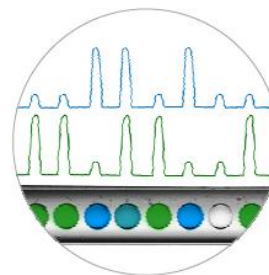




Make Droplets



PCR Droplets



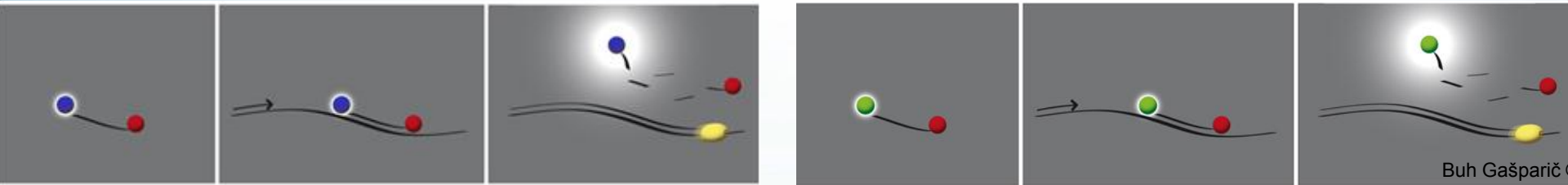
Read Droplets



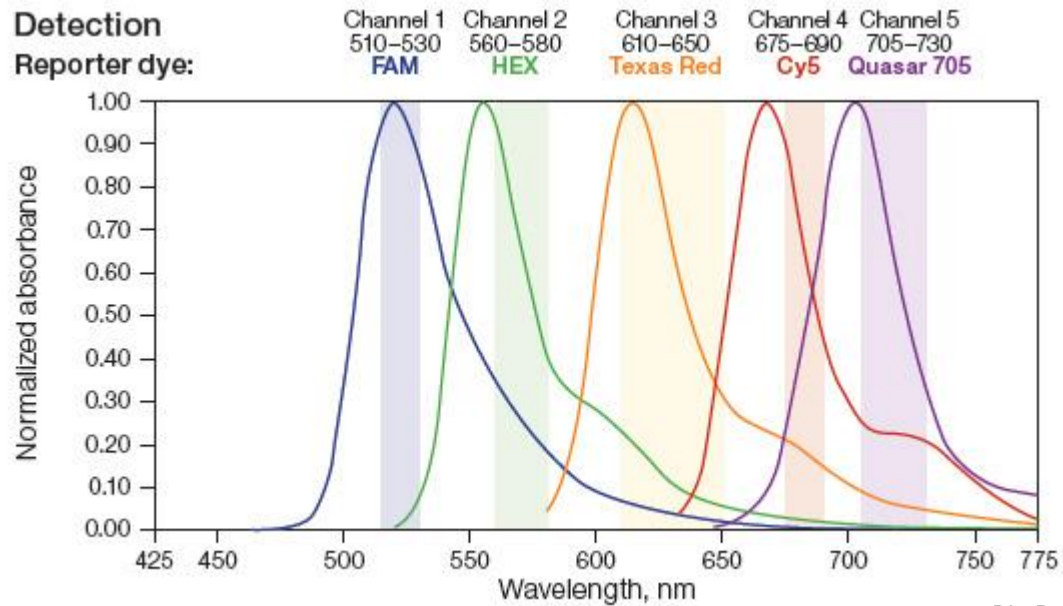
"X" target  
copies

Results

## Fluorescent reporters



Buh Gašparič



# Duplex ddPCR for MON810

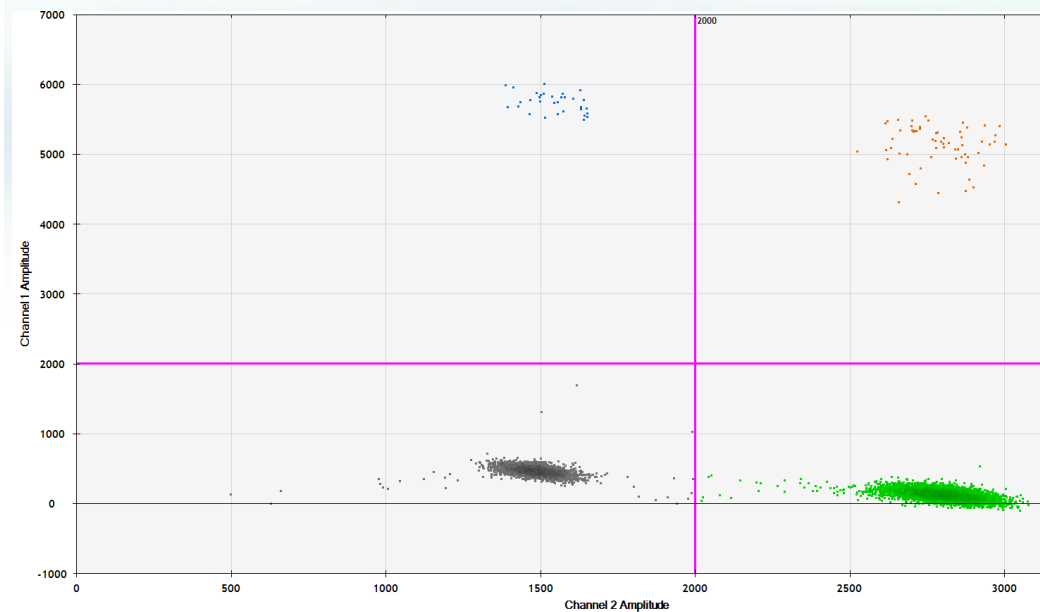
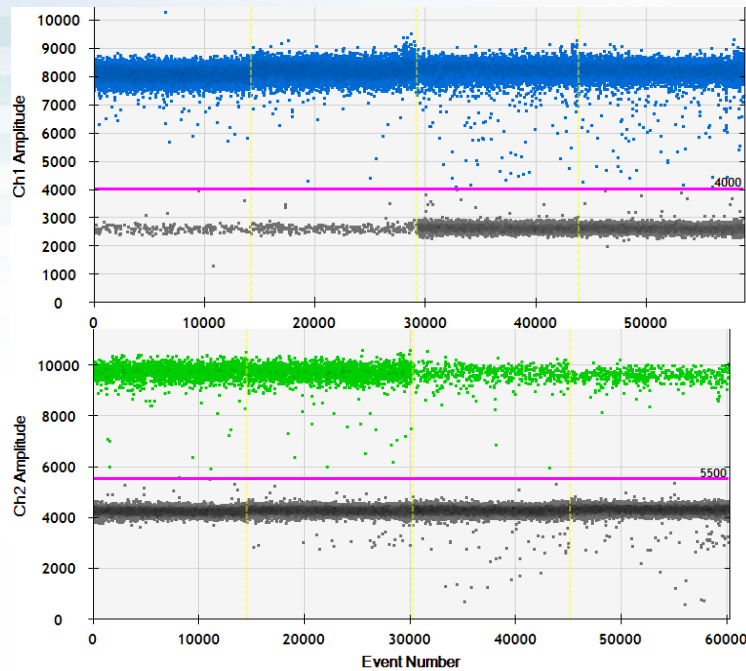
OPEN ACCESS Freely available online

PLOS ONE

## Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR

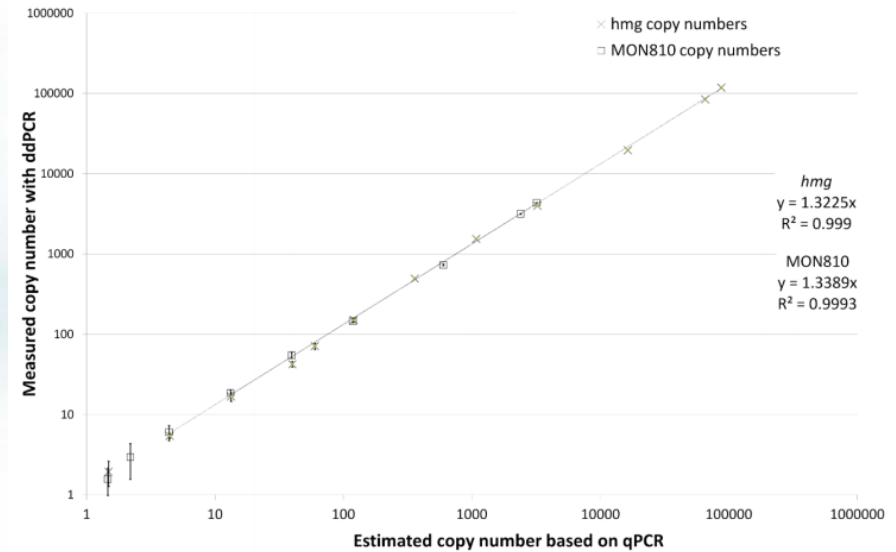
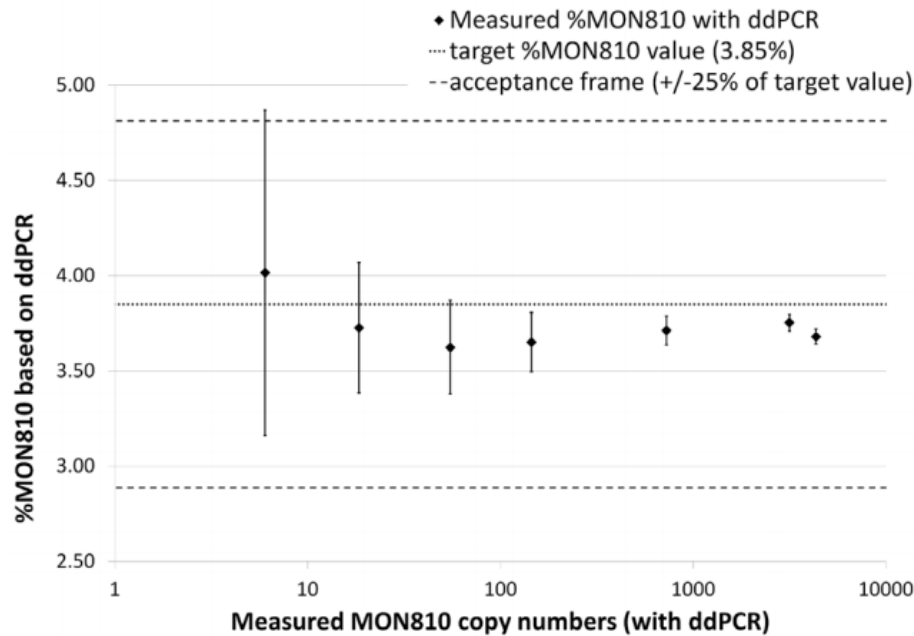
Dany Morisset\*, Dejan Štebih, Mojca Milavec, Kristina Gruden, Jana Žel

Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia



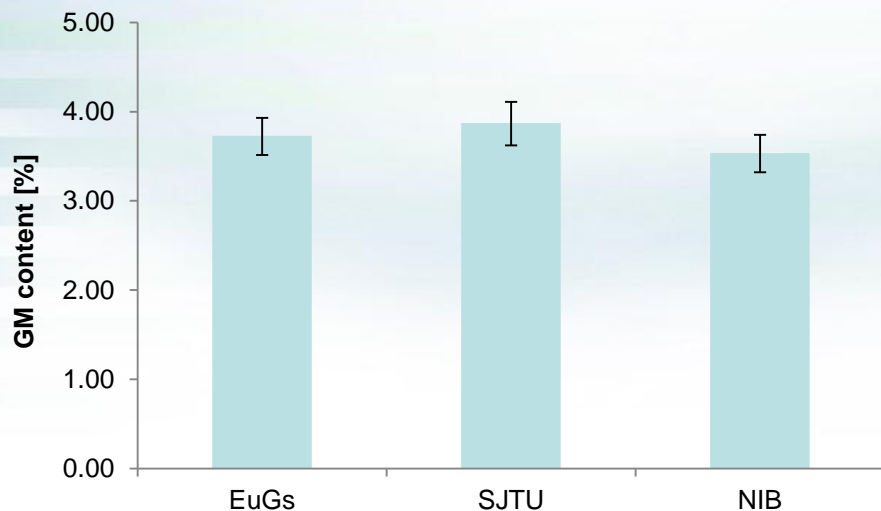


## Duplex ddPCR for MON810



# Duplex ddPCR for MON810

## Interlaboratory comparison of the protocol



GM quantity (GM%) determined in three laboratories with 95% Poisson confidence intervals

Target	EuGs	SJTU	NIB	cv%
MON810	2768	2775	2951	9
	2282	2409	2599	
hmgA	76874	71213	80616	11
	58566	62665	76312	

Target concentration (copies/ $\mu$ l in stock DNA) determined in three laboratories

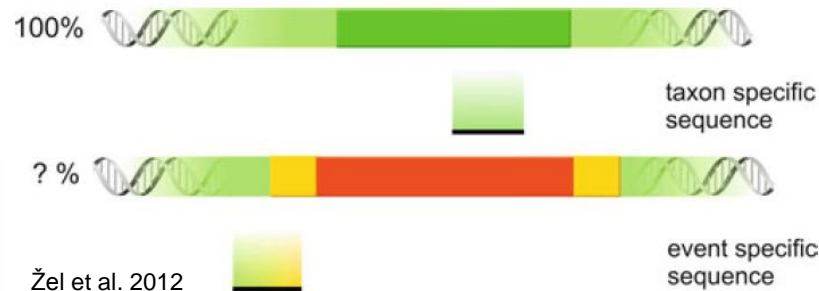
## EU regulations and guidances

- **1829/2003**

Section on labelling “*..shall not apply to foods 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...*”

- **Technical guidance for sampling and detection of GMOs**

*"The results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes".*



## Multiplex ddPCR – basic idea

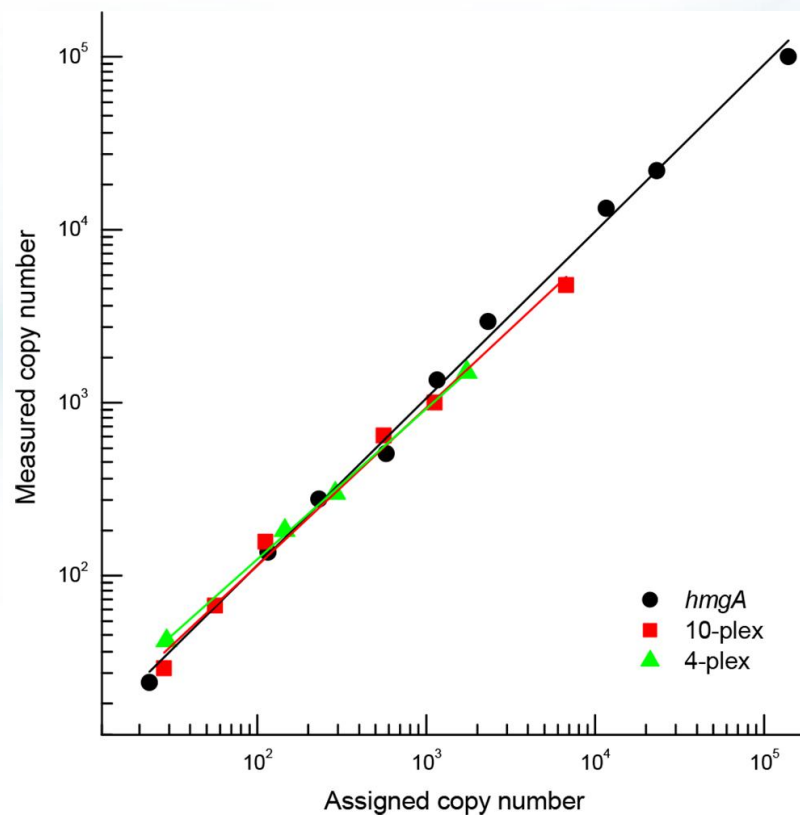
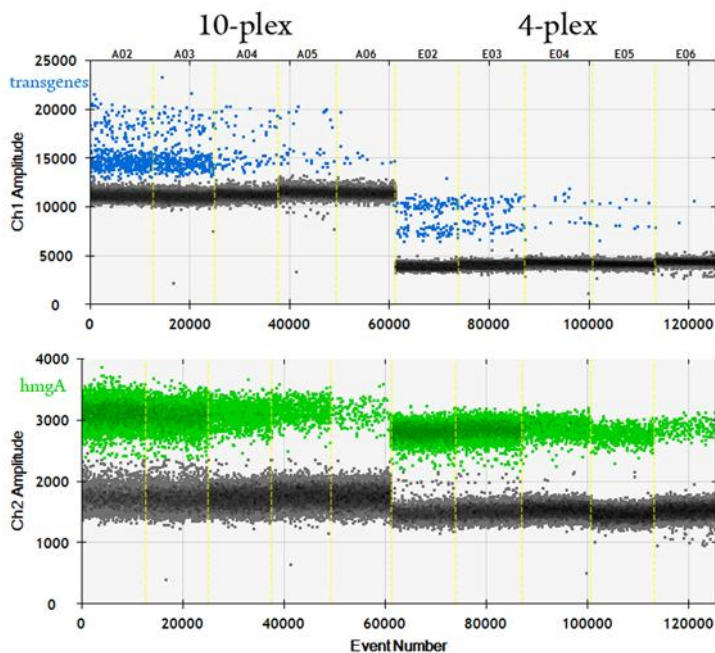
- **Quantify all EU authorized GM maize lines in multiplex assay with ddPCR (quantification per ingredient)**
  - Transgenes labelled with FAM
  - Endogene (hmgA) labelled with HEX

# Multiplex ddPCR for maize quantification

- 2 multiplex assays (4-plex and 10-plex) selected due to interactions of some primers/probes:
  - GA21, MON88017, MON89034, MIR162, MIR604, T25, MON810, MON863, DAS1507, NK603
  - NK603, Bt11 in DAS59122 (**MON87460**)

## Dynamic range and correlation between measured and assigned copy number

Mixture of equal amounts (target copies) of each of the 12 maize events was used



# Multiplex ddPCR – tested parameters

- aLOQ: 42 cp/reaction, 4-plex  
29 cp/reaction, 10-plex  
24 cp/reaction, hmgA
- rLOQ: 0.068 %, 4-plex  
0.058 %, 10-plex
- Specificity: no false positive reactions
- Trueness : all results within  $\pm 25\%$  of “true” value  
|z-scores| below 1.4



## Comparison of ddPCR multiplex to qPCR approaches

Testing pipeline	Steps prior quantification	Number of tested samples	Estimated hands-on time (hours)	Working days until final result (days)	Relative final price per sample (%)
Direct quantification of twelve approved GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	/	1	3	0.8	<b>100</b>
		11	5	1.0	<b>100</b>
Direct quantification of twelve approved GM maize lines in simplex reactions with qPCR	/	1	8	2.0	272*
		11	25	7.5	300*
Initial screening with qPCR, all samples negative	5x simplex screening	1	2	0.5	83
		11	8	1.5	105*
Initial screening with qPCR, all samples negative	Pentaplex screening <sup>a</sup>	1	1	0.4	<b>31</b>
		11	5	1.0	<b>39*</b>
Initial screening, identification of six GM maize lines, quantification of one GM maize line with qPCR	Pentaplex screening, 6 specific lines	1	8	1.5	175*
		11	20	4.4	159*
Direct identification of twelve approved GM maize lines, quantification of two GM maize lines with qPCR	12 specific lines	1	10	1.8	226*
		11	18	4.8	185*
Initial screening with qPCR and quantification of twelve approved GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR	Pentaplex screening	1	4	1.1	<b>131*</b>
		11	10	2.0	<b>139*</b>

\* more than one 96-well plate must be run



# Validation

- **Multiplex ddPCR method will be validated by international collaborative study within DECATHLON project**
- **Method has a potential to become a widely applied in its field**



## Acknowledgements



# Thank you for your attention!

