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Droplet digital PCR

use of the third generation PCR in GMO testing

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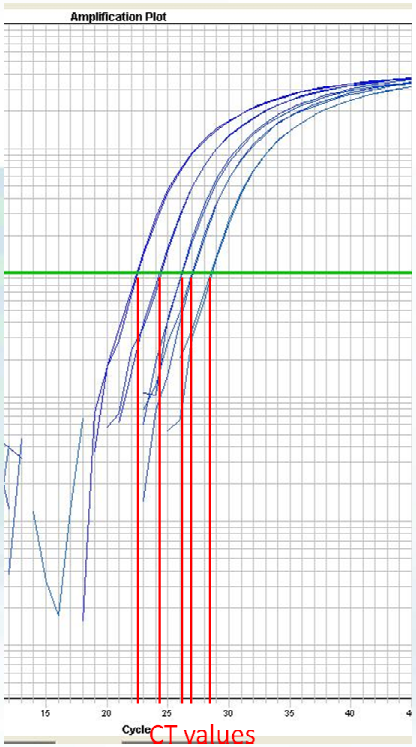


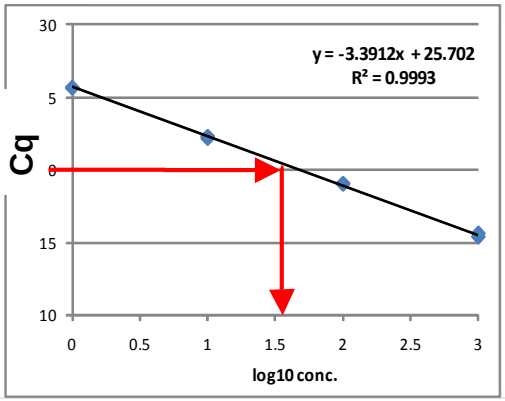
CT values

Sample: **determination of target copy number**

DNA quantity?
Endogene copy nb?
Transgene copy nb?

Standard: known (approximate) target copy number

Sample: observation of Cq values



Copy nb = f (Cq value)

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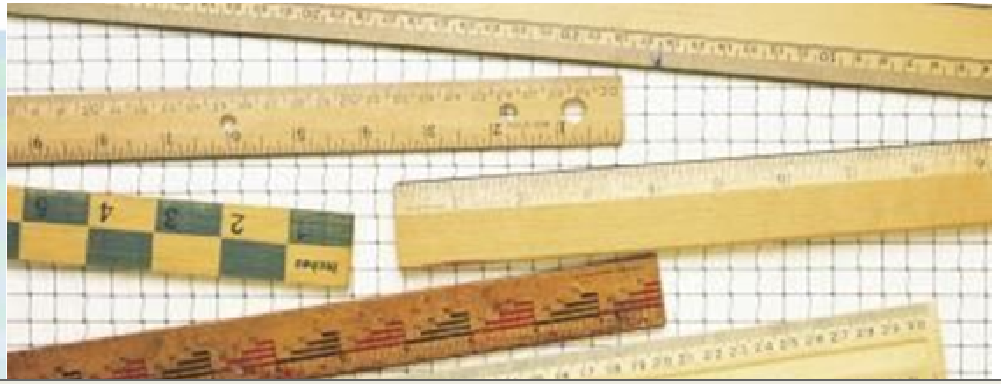

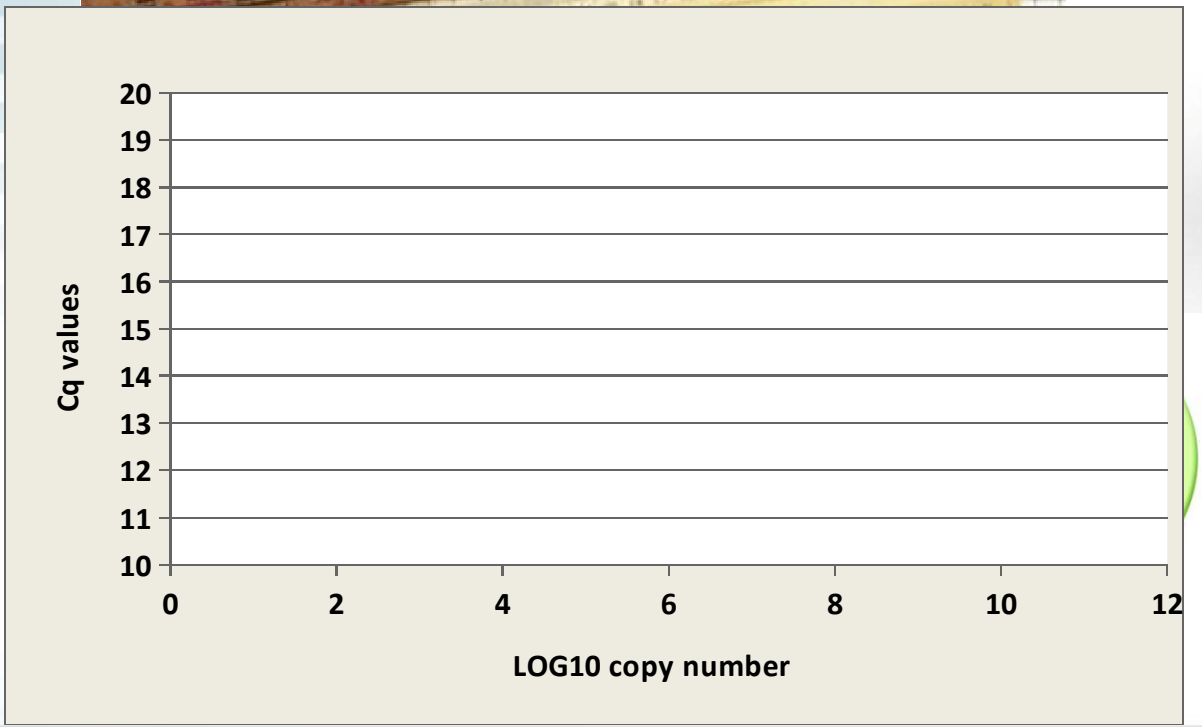

large MU

opies

Testing digital PCR for GMO quantification

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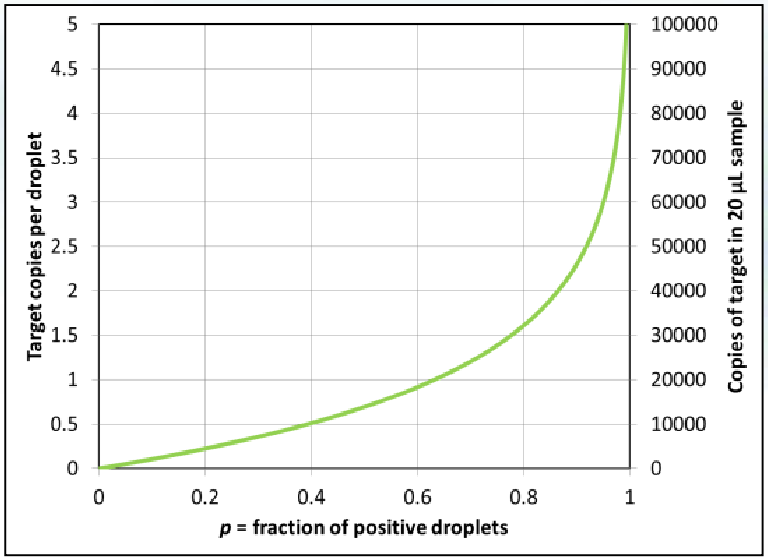
Testing digital PCR for GMO quantification

Number of positive partitions is directly related to concentration

Fraction of negatives is fit to a Poisson algorithm to determine absolute copy number, results in copies per input µl of sample

Siméon Denis Poisson (1781-1840)



Modeling as Poisson
copies per droplet = - ln (1 – p)
where p = fraction of positive droplets

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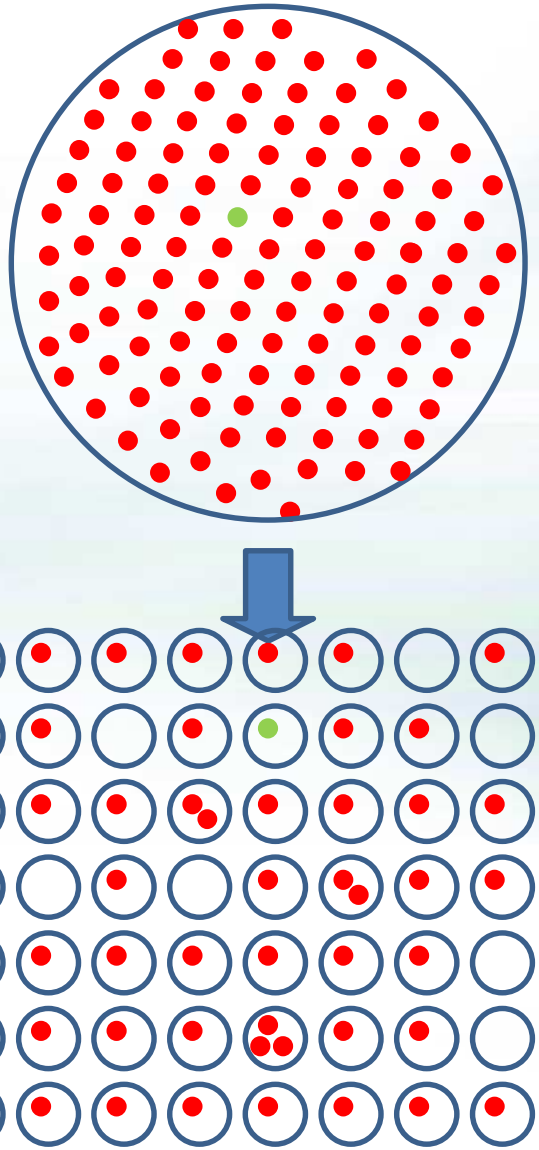
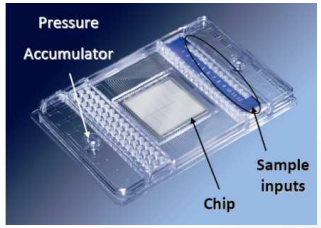
What is digital PCR (dPCR)?

- Number of positive partitions is directly related to concentration
- Fraction of negatives is fit to a Poisson algorithm to determine absolute copy number, results in copies per input µl of sample

Siméon Denis Poisson (1781-1840)

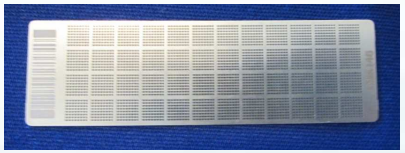


Two commercial approaches



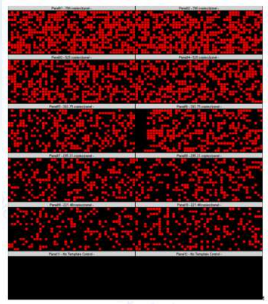
Arrays

- Digital Array™ (Fluidigm)
- QuantStudio™ 12K, QuantStudio™ 3D (Life Technologies)

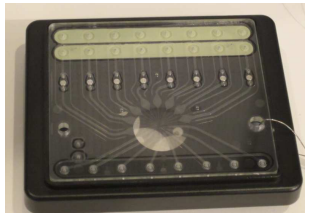


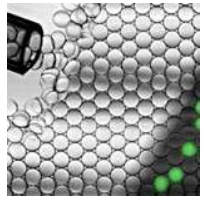
Droplets

- QX100 ™ droplet digital ™ (Bio-Rad)
- RainStorm™ (RainDance)



Why going digital?





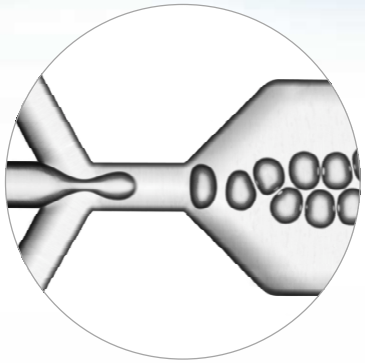
Digital PCR and GMOs

- GMO quantification already evaluated in chamber digital PCR:
  - Duplex possible: direct transgene/endogene ratio determination, lower uncertainty
  - Good sensitivity: <10 copies
  - Acceptable limit of quantification: 15-65 copies
  - 765 partitions (microfluidics).
    - Limited dynamic range: 2-3 logs.
    - Need to pre-determine concentration
    - Less room for duplex
    - ↑ replicates for ↓ uncertainty
  - High cost

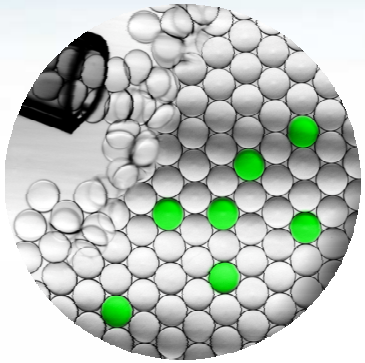
ddPCR for GMO quantification

Droplet Digital PCR Workflow (QX100, Bio-rad)

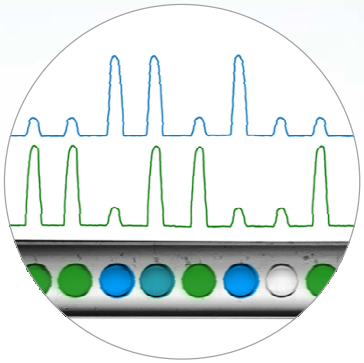
- Partition reagents and sample into 20,000 droplets
- Perform PCR on thermal cycler
- Quantify target nucleic acid by counting sample partitions with a positive PCR product (fluorescent) and a negative PCR product
- Digital readout provides absolute measure of target DNA



Make Droplets



PCR Droplets

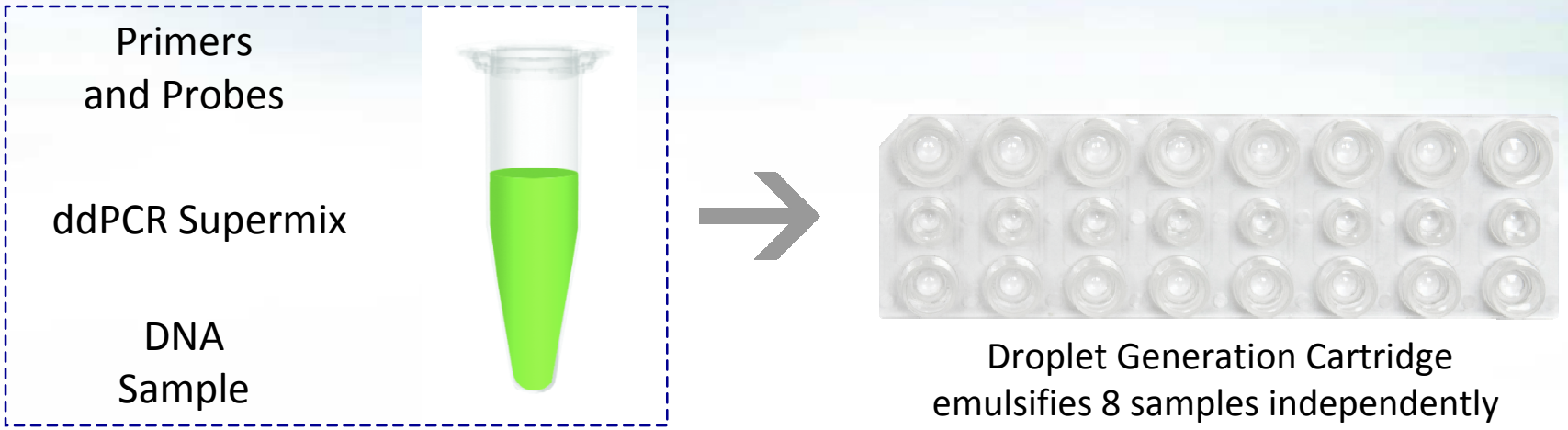


Read Droplets

Results

- Simple assay conversion
  - Same sample preparation as a real-time PCR assay
  - Same concentrations as for validated qPCR (optimization may be necessary)
  - FAM and VIC channels. BHQ1 as quencher

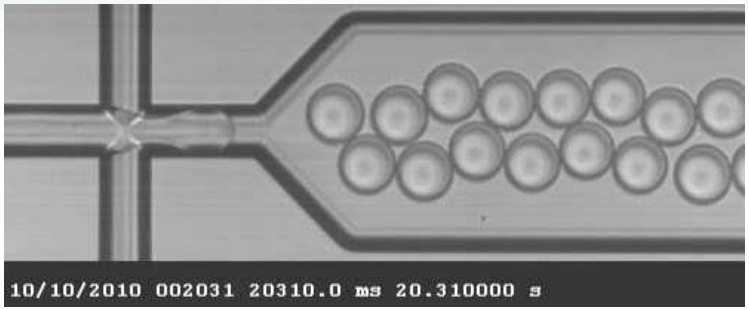
Prepare Sample and Reagent Mixture



Droplet Generation Cartridge
emulsifies 8 samples independently

Making Droplets

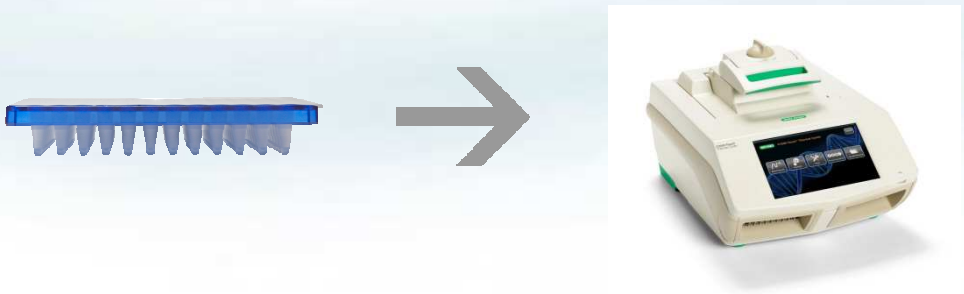
- Place disposable cartridge loaded with sample and droplet generation oil into QX100 droplet Generator
  - 20,000 droplets generated per sample



Uniform droplet generation

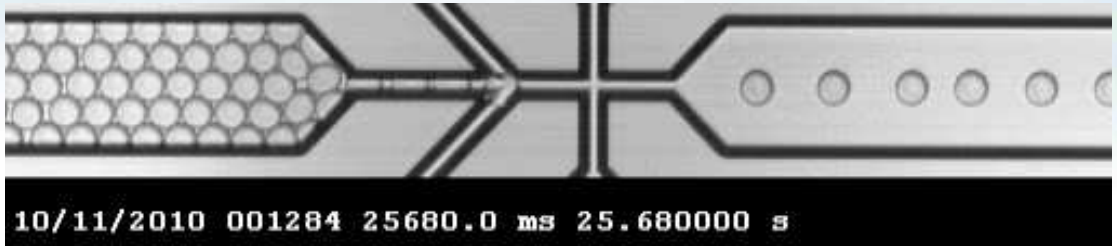
- Same thermo profile as for validated qPCR modules
- Using thermal gradient PCR may be needed

PCR Droplets on Thermal Cycler



Read Droplets

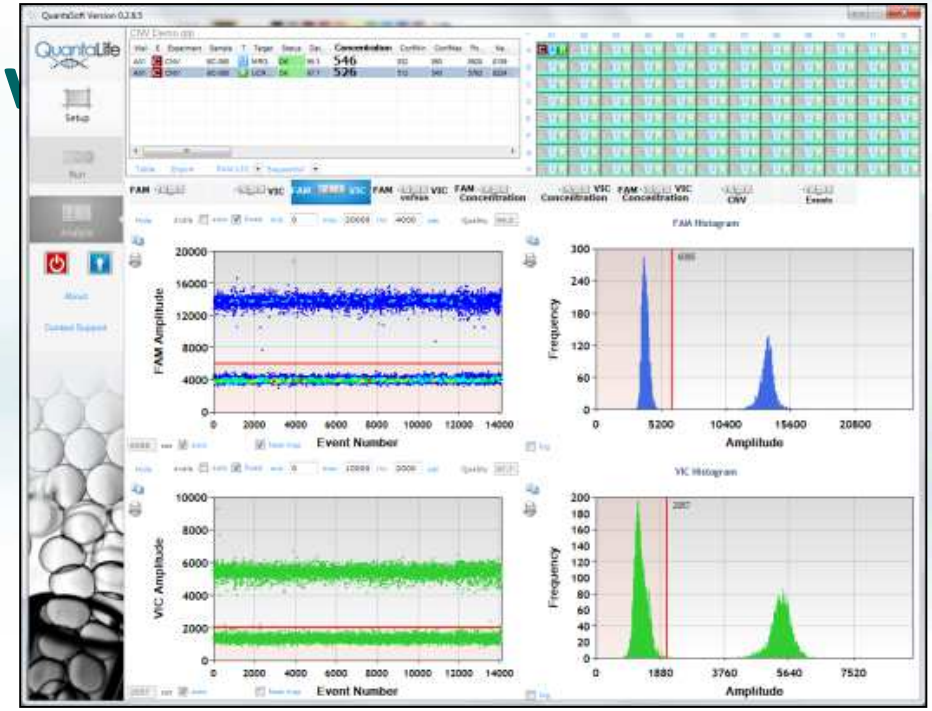
- Load the plate in the QX100 Droplet Reader
  - Each sample processed independently
- Droplets stream single-file past the optical detector
  - Detects fluorescence from each droplet



Droplet Readings Conv

Signal

- Positive droplets have increased fluorescence vs. negatives
- Positives contain at least one copy of the target DNA
- Software measures number of positive and negative droplets per fluorophore (FAM and VIC) per sample
- Software calculates the concentration of target/ µl
- Export to xls, and divide transgene cp/endogene cp!



Why ddPCR?

- 20,000 to 10,000,000 partitions (droplets). Higher dynamic range: $\geqslant$ 5 logs
  - Probably no need to pre-determine concentration
  - Flexibility for duplex
  - Lower uncertainty
- Lower cost (3US$/reaction, US$90,000 for instrument)

ddPCR evaluation

Method performance parameters (CODEX alimentarius and EURL-GMFF)

- Linearity
- Trueness
- Repeatability
- LOD, LOQ
- Applicability
- Practicability
- Specificity (false positive/negative results)
- PCR efficiency
- Reproducibility

Material

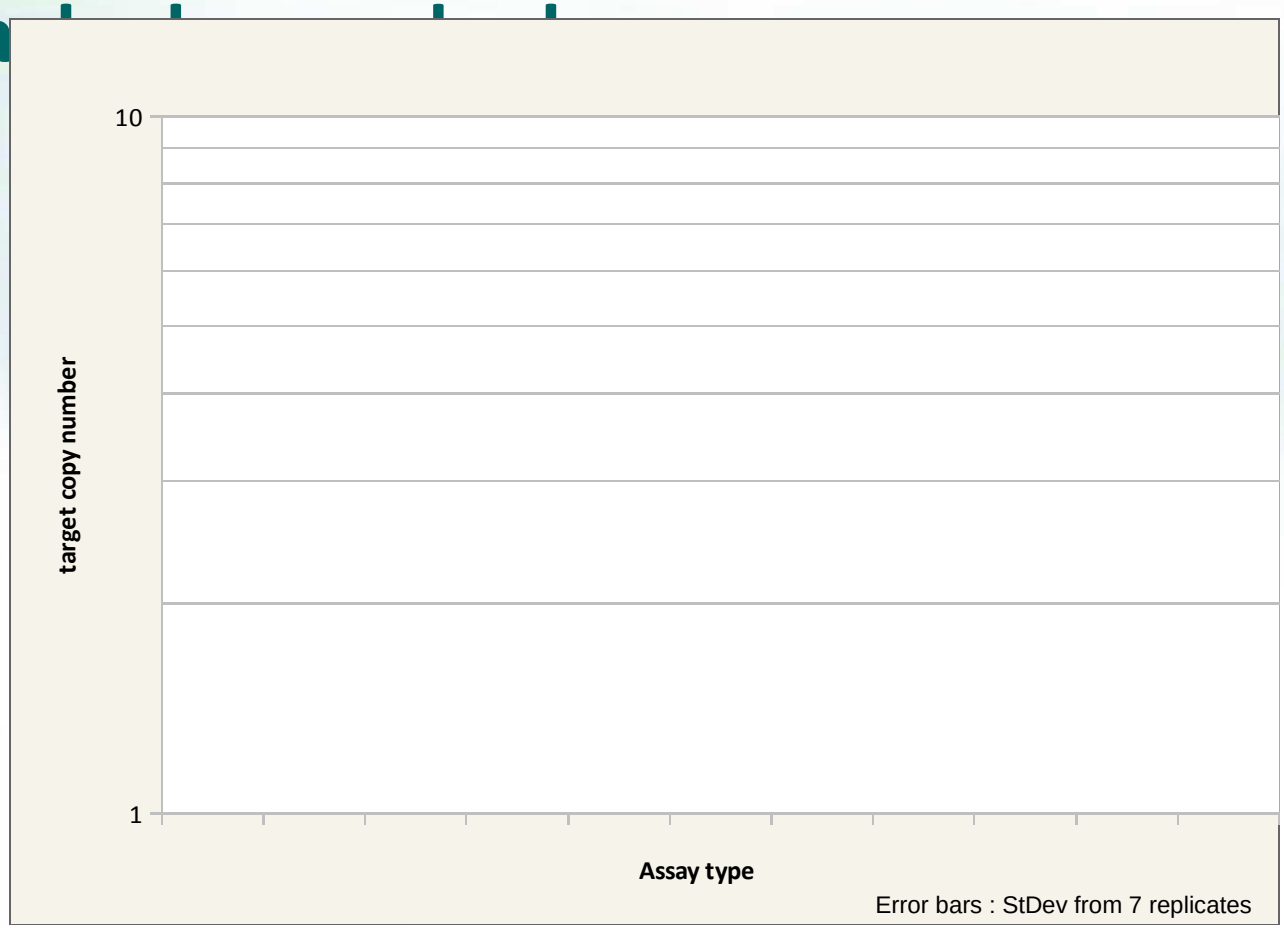
- GM MON810 maize : flour from ground seeds
  - Certified reference materials (CRM) with mass/mass (m/m) certificate, sometimes also copy/copy (cp/cp) certificate
- Other samples with different % of the same GM maize event (routine and PT)

Singleplex *vs.* duplex

- All official qPCR methods validated by the EU Reference Laboratory are combinations of two singleplex assays: one specific for the endogene, one for transgene
- Decrease costs and uncertainty by duplexing

No significant difference in copy number estimates (bias -1.8% for the *hmg* copy number and 3.7% for MON810) and …

Sin

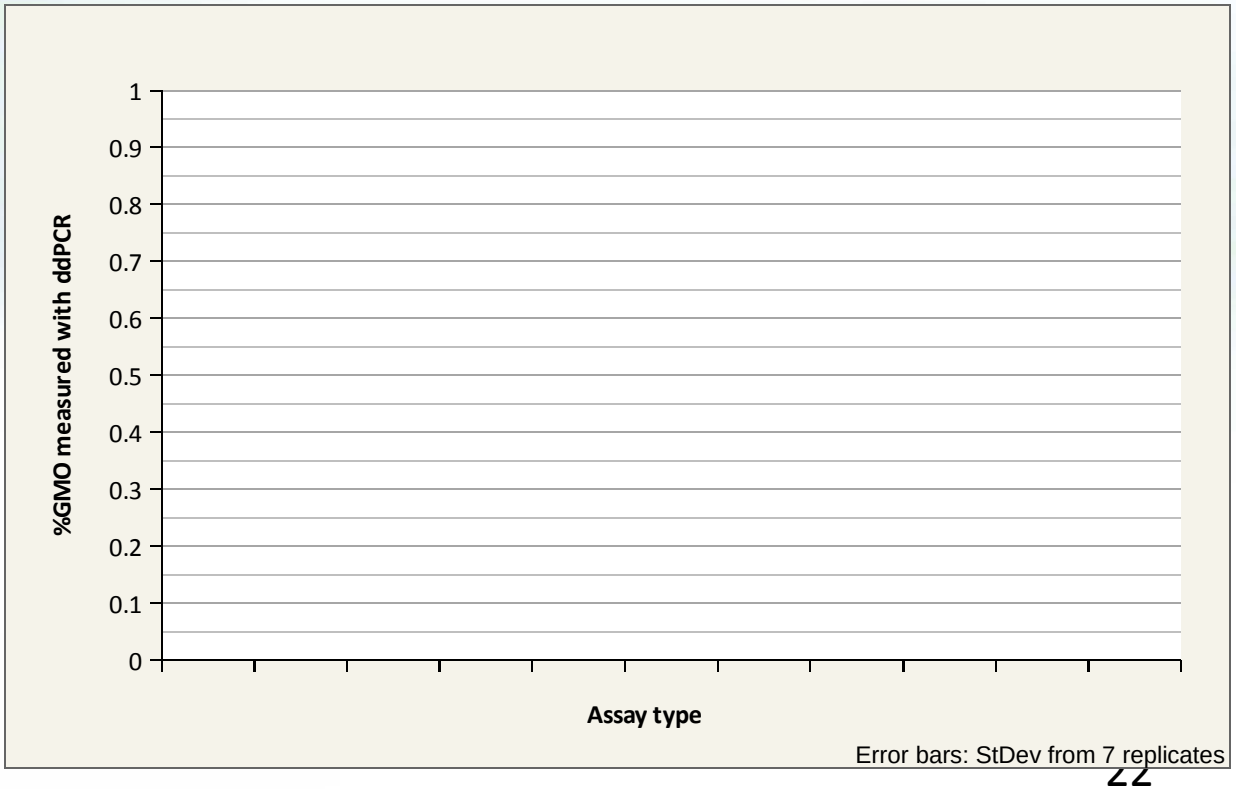


Singleplex *vs.* duplex

Similar GMO content estimates between singleplex and duplex assays.

Bias duplex *vs.* singleplex = 5.8%

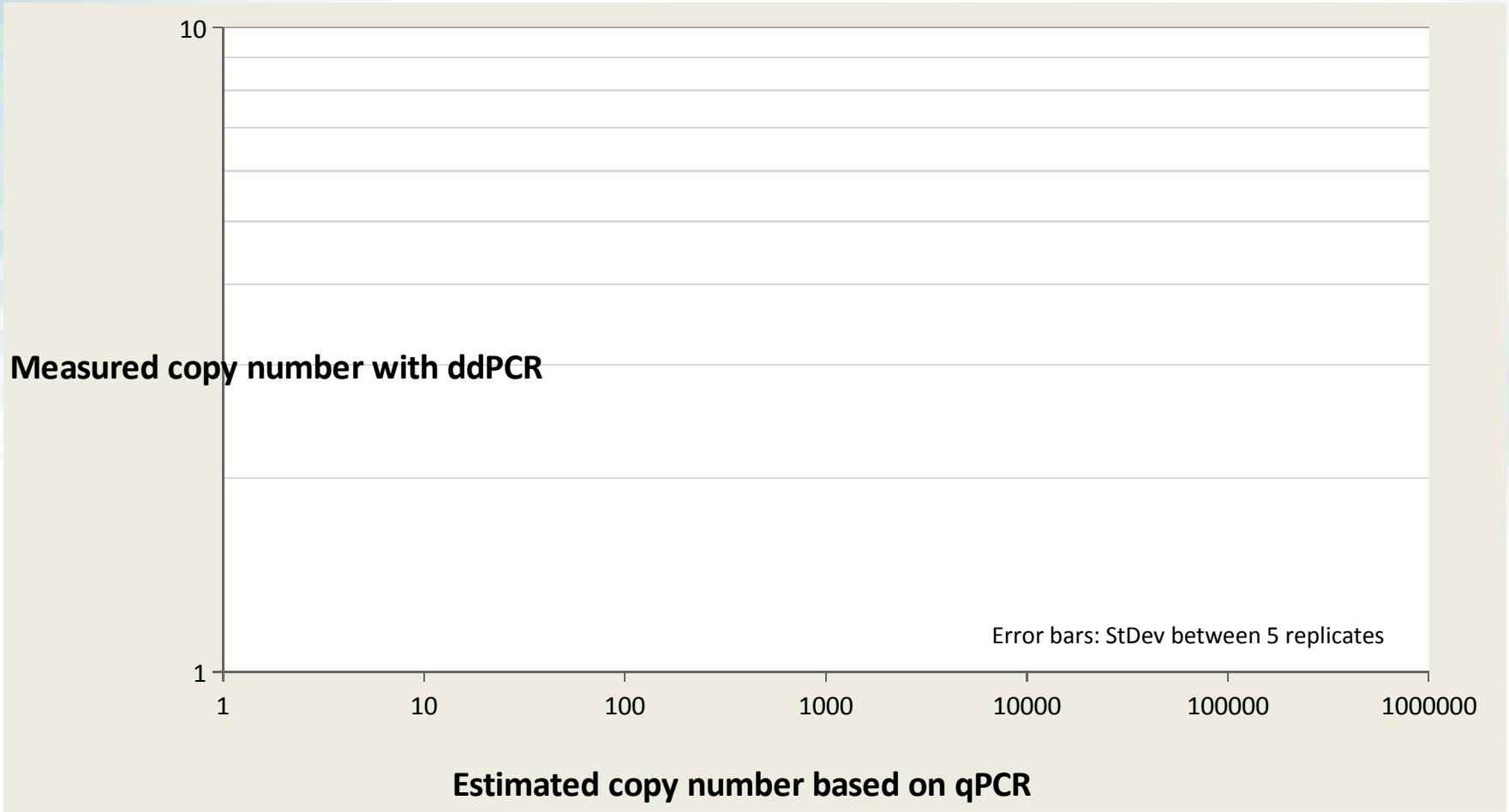
Measurements can be done using duplex.



Linearity

- Should cover at least 3 logs (for quantification limit of 0.1%)
- Wider range is preferred for more flexibility with DNA concentrations

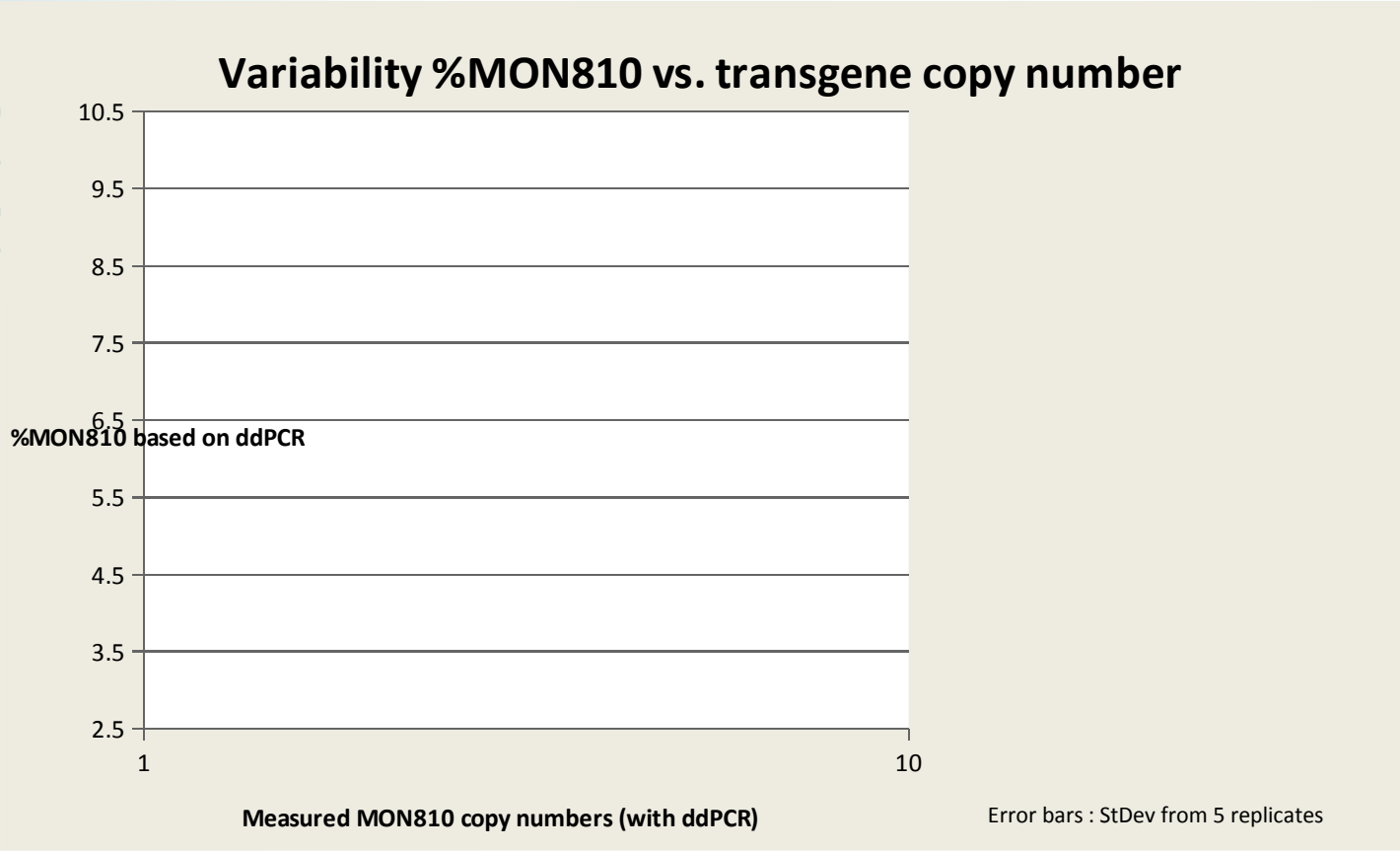
- **Good linearity beyond 10 copies with R2> 0.999 over 5 logs**



- Followed criterion: the measured value < ±25% of the accepted reference value over the whole dynamic

Trueness along linear range

- 10



Absolute LOQ and LOD

- Criteria:
  - Absolute limit of quantification (aLOQ): coefficient of variation (Cv) copy number between repeats and within dynamic range $\leqslant$ 25%
  - Absolute limit of detection (aLOD): target detected in all 5 replicates (100,000 droplets).
- Results:
  - **aLOQ – between 5 copies for *hmg* and 18 copies for transgene MON810**
  - **aLOD – 5 copies for *hmg* and 6 copies for transgene MON810**

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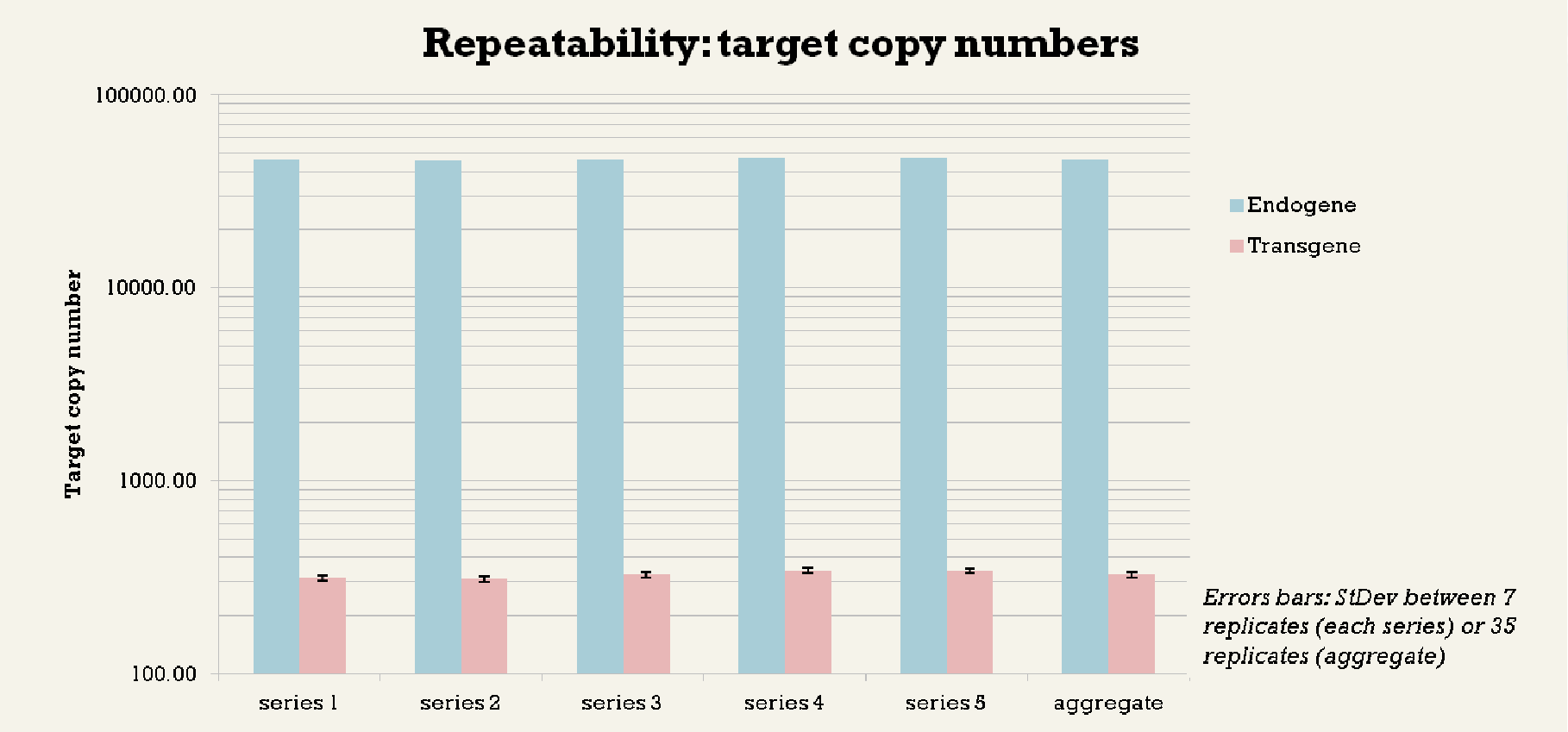
Repeatability

- **Low variability between repeats**

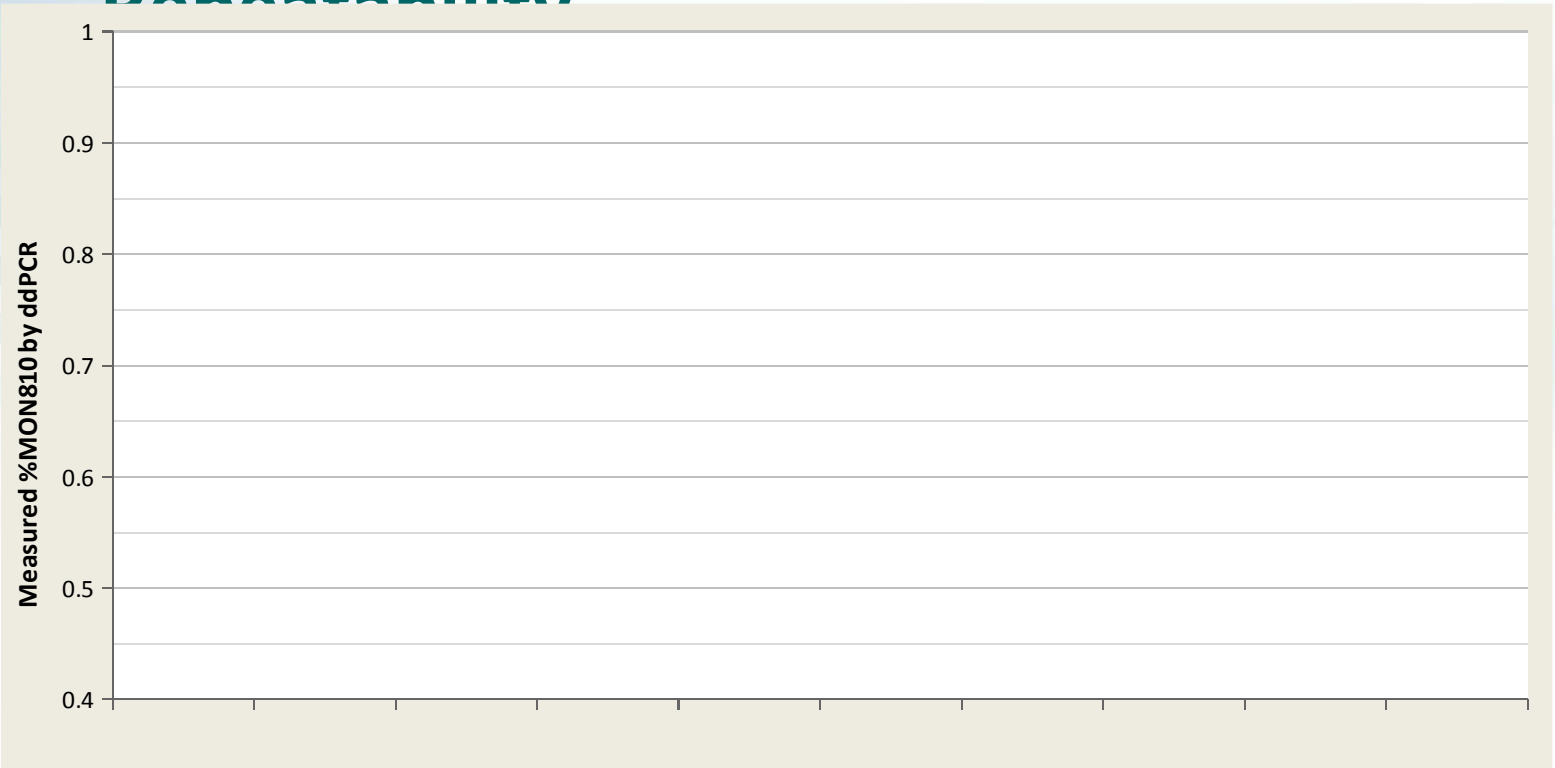
  copy numbers 1.3% to 2.1% for *hmg*, 4.2% to 8.2% for MON810



- **Consistent measurement of GMO content**

cv %GMO from 4.2% to 8.0%, 7.3% in average

Repeatability



Error bars from 7 replicates (each series) and 35 replicates (aggregate)

Specificity

- Not fully assessed (validated qPCR modules)
- All NTC negative
- Assessed on milk sample w/o maize, feed sample containing maize and RRS

	hmg				MON810			
Sample	**Positive reactions**	**% positive reactions**	**Positive droplets**	**% positive droplets**	**Positive reactions**	**% positive reactions**	**Positive droplets**	**% positive droplets**
Milk	0/8*	0%	1/106,133	9e-4%	0/8*	0%	1/106,133	9e-4%
Feed	n.a.	n.a.	n.a.	n.a.	0/8	0%	0/110,903	0%
TOTAL	0/8*	0%	1/106,133	9e-4%	0/16*	0%	1/217,036	5e-4%

Applicability

- "Ability to perform with different sample matrices and within a range of concentrations relevant for GMO testing should be demonstrated"1
- Samples and certified reference materials (CRM) with different GMO% tested

1 Codex Committee On Methods Of Analysis And Sampling *Guidelines On Performance Criteria And Validation Of Methods For Detection, Identification And Quantification Of Specific DNA Sequences And Specific Proteins In Foods*

- **ddPCR ensures precise measurement of GMO content through a large range corresponding to routine laboratory use (<0.1% - 30%)**

Applicability and trueness

Sample	Source	Matrix	Target value(cp/cp)	ddPCR (bias)	qPCR (bias)
ERM-BF413d	CRM	Seed-powder flour	0.57% ±0.17%	0.62% (8.0%)	0.46% (-19.3%)
ERM-BF413f	CRM	Seed-powder flour	2.85% a	2.92% (2.5%)	2.29% (-19.6%)
ERM-BF413ek	CRM	Seed-powder flour	0.77% ±0.08%	0.70% (-9.0%)	0.58% (-24.7%)
ERM-BF413gk	CRM	Seed-powder flour	3.85% a	3.68% (-4.1%)	3.66% (-4.9%)
G0009/04	USDA/GIPSA PP	Seed-powder flour	0.29% ±0.13% b	0.26% (-11.7%)	/
G0180/07	USDA/GIPSA PP	Seed-powder flour	0.04% ±0.02% b	0.04% (2.9%)	/
G211/10	ILC-EURL-GMFF PP	Seed-powder flour	0.45% ±0.098% c	0.46% (-1.8%)	0.50% (11.1%)
G212/10	ILC-EURL-GMFF PP	Seed-powder flour	2.10% ±0.35% c	2.32% (10.4%)	2.30% (9.5%)
G147/08	Gemma PP	Seed-powder flour	29.6% ±8.9% b	21.7% (-26.7%)	/
G231/11	Routine	Corn flakes	2.64% ±0.8% b	2.31% (-12.4%)	/
G254/11	Routine	Feed	3.82% ±1.1% b	3.47% (-9.2%)	/

Applicability: Matrix and inhibition effect

- Samples from different matrices and with known inhibition in qPCR tested

- **ddPCR is less sensitive to inhibition than qPCR**
  - **Use of only one dilution is possible**
  - **Stock not inhibited so more concentrated DNA = better sensitivity**
- **ddPCR works in different matrices**
- **Constant measurement within dilution**

Applicability: inhibition effect

Sample	Matrix	Dilution	Average %GMO cp/cp (qPCR)	Inhibition qPCR	Average %GMO cp/cp (ddPCR)	Inhibition ddPCR	bias %GMO ddPCR vs. qPCR
G147/08 NSF	Wheat flour + maize traces	1x	50.22	Yes	20.3	No	-59.53
		3x	27.34	No	19.9		-27.02
G147/08 CTAB	Wheat flour + maize traces	1x*	29.6	No	21.7	No	-26.70*
		3x	30.96	No	21.4		-30.79
G254/11	Maize feed	1x	5.64	Yes	3.41	No	-39.61
		4x*	3.82	No	3.47		-9.20*

Practicability

- Codex Alimentarius suggests to “conside parameters such as: the quantity of samples that can be processed within a given time, estimated fixed costs to implement the method and the approximate cost per sample, practical difficulties on daily use or under particular conditions, as well as other factors that could be of importance for the operators1

Practicability

- Based on a 4 samples (DNA extracted, mix prepared).
- 2 test portions, 2 repeats/test portion (ISO 24276)
  - qPCR: NTC, standard (at least 5 points), two dilutions/data point, positive control
  - ddPCR: NTC, one dilution/data point, positive control

	ddPCR	qPCR (96 well plate)
Time needed	185 min	160 min
Hands-on time	31 min	44 min
Number reactions	20	96
Cost/sample	16€	17€

Practicability

- Based on a 23 samples (DNA extracted, mix prepared).
- 2 test portions, 2 repeats/test portion (ISO 24276)
  - qPCR: NTC, standard (at least 5 points), two dilutions/data point, positive control
  - ddPCR: NTC, one dilution/data point, positive control

	ddPCR	qPCR (96 well plate)
Time needed	6 hours	13 hours (less if several simultaneous instrument)
Hands-on time	65 min	310 min
Number reactions	96	400
Cost/sample	11.4€	12.7€

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Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR

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Abstract

In this study, the applicability of droplet digital PCR (ddPCR) for routine analysis in food and feed samples was demonstrated with the quantification of genetically modified organisms (GMOs). Real-time quantitative polymerase chain reaction (qPCR) is currently used for quantitative molecular analysis of the presence of GMOs in products. However, its use is limited for detecting and quantifying very small numbers of DNA targets, as in some complex food and feed matrices. Using ddPCR duplex assay, we have measured the absolute numbers of MON810 transgene and *hmg* maize reference gene copies in DNA samples. Key performance parameters of the assay were determined. The ddPCR system is shown to offer precise absolute and relative quantification of targets, without the need for calibration curves. The sensitivity (five target DNA copies) of the ddPCR assay compares well with those of individual qPCR assays and of the chamber digital PCR (cdPCR) approach. It offers a dynamic range over four orders of magnitude, greater than that of cdPCR. Moreover, when compared to qPCR, the ddPCR assay showed better repeatability at low target concentrations and a greater tolerance to inhibitors. Finally, ddPCR throughput and cost are advantageous relative to those of qPCR for routine GMO quantification. It is thus concluded that ddPCR technology can be applied for routine quantification of GMOs, or any other domain where quantitative analysis of food and feed samples is needed.

Citation: Morisset D, Štebih D, Milavec M, Gruden K, Žel J (2013) Quantitative Analysis of Food and Feed Samples with Droplet Digital PCR. PLoS ONE 8(5): e62583. doi:10.1371/journal.pone.0062583

ddPCR for routine GMO quantification

- ddPCR satisfies all parameters listed by current (and future) EURL-GMFF guidelines: Precision, accuracy, LOD, LOQ, dynamic range.
- It is applicable for routine quantification and practical (throughput, price, complexity)
- No standard curve:
  - Easier/faster to calculate %GMO
  - Better harmonization!
- Combined with qPCR (screening/identification)