

# Joint Research Centre

## Application of the Low Level Presence Regulation (EU) No 619/2011

[www.jrc.ec.europa.eu](http://www.jrc.ec.europa.eu)

*Serving society  
Stimulating innovation  
Supporting legislation*



## Salient points of Regulation (EU) 619/2011

- Harmonises sampling and testing controls in all EU countries
- Limited to GM feed material
- GM material must comply with the following criteria:
  - be authorised for commercialisation in a non-EU country;
  - have a valid EFSA application under Article 17 of Regulation EC 1829/2003 or have an expired authorisation under Regulation EC 1829/2003;
  - authorisation pending for more than 3 months, provided that:
    - have not been identified by EFSA as susceptible to have adverse effects on health or the environment when present under 0.1%;
  - **quantitative method of analysis validated and published by the EU-RL;**
  - **certified** reference material must be available to EU countries and third parties

## Salient points of Regulation (EU) 619/2011

- Sets a technical zero at a level of 0.1% - the lowest level of GM material considered by the EU-RL for the validation of quantitative methods
- In practice Member States will have to declare a product as non-compliant when, taking into account the margin of error in the results (measurement uncertainty), the level of 0.1% is exceeded.
- The 0.1% is related to mass fraction of GM material
- When results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL.

## Method precision at 0.1% (1/2)

- The EU-RL GMFF accepts only methods when the applicant proves that the RSDr at the level of 0.1 % related to mass fraction of GM material  $\leq 25\%$ ; this value will be published in the validation reports
- The EURL GMFF will determine in-house the RSDr at the level of 0.1% related to mass fraction of GM material and will publish these data in the validation report.
- Following a ring-trial, the EU-RL GMFF calculates again the RSDr, this time according to ISO standard 5725. This value has been and will continue to be published in the validation reports.
- In order to be fit for the purpose of meeting the requirements of the LLP regulation, all RSDr values mentioned above have to be below 25%.

## Method precision at 0.1% (2/2)

If the method allows to quantify at the 0.1% level within the stated level of precision (i.e.  $RSDr \leq 25\%$ ) at all previous steps, the method may be considered to be included in the list of GMOs that can benefit from the LLP legislation (SANCO);

otherwise

the method will not be included in that list; however, it will be part of the EFSA overall opinion in the context of the authorisation of GMOs for food and feed (Reg. (EC) No 1829/2003)

# Establishing method precision at NRLs

ISO 17025:

“The laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations”

At time zero ( $t_0$ ):

- CRM is available
- NRLs test the RSDr of the method on the 0.1%
- RSDr on CRM should be  $\leq 25\%$

# Testing of feed samples (1/3)

To ensure a level of confidence of approximately 95%, the outcome of the analysis shall be reported as  $x \pm U$  whereby  $x$  is the analytical result and  $U$  is the expanded measurement uncertainty.

$$U = k * u(x) \quad 1)$$

The standard uncertainty  $u(x)$  corresponds to the relative repeatability standard deviation (RSDr) of test results;  $U$  is obtained by multiplying  $u(x)$  by a coverage factor  $k$ .

The coverage factor  $k$  is a function of the number of replicates and can be approximated to 2 when the number of test replicates is at least 10 (the EU-RL applies 15).

## Testing of feed samples (2/3)

A feed shall be considered as non compliant with Regulation (EC) No 1829/2003 when the analytical result ( $x$ ) minus the expanded measurement uncertainty ( $U$ ) equals or exceeds the level of 0.1 % related to mass fraction of GM material:

$$\begin{aligned} x - U &\geq 0.1\% \\ \text{or} \\ x &\geq 0.1\% + U \end{aligned} \quad (2)$$



# Testing of feed samples (3/3)

In practice, assuming the maximum RSDr allowed of 25% and an applied coverage factor of 2, the rejection criterion for low level presence of unauthorized GMO in feed concerns any GMO concentration larger than 0.15%, since:

$$0.1\% + U = 0.1\% + (50\% * 0.1\%) = 0.15\%$$

(expressed in mass)

## Guidance on conversion factor

Considering that the maize commodity market consists mainly of hybrid maize, and considering the uncertainty related to the biology estimates to vary approximately between 40 to 60%, the proposed relationship to deal with the *biological* uncertainty for heterozygous single inserts in maize is:

**GM % in DNA copy number = 50% [GM% in mass fraction]**

The proposed relationship to deal with the *biological* uncertainty for homozygous single inserts in soya is:

**GM % in DNA copy number = 100% [GM% in mass fraction]**

This is similar to the equation accepted by the European Network for GMO laboratories in the case of seeds.

- The EU-RL GMFF will have to request the applicant more information on the control samples provided for the validation
- Sample homogeneity, description of the of zygosity of GM-target(s), parental contribution for hybrids, copy number of reference system
- The EU-RL GMFF will verify the GM-target to reference-target copy number ratio

# Changes in the EU-RL GMFF validation procedures and in the reporting

# Regulation (EU) No 619/2011

## Repeatability Standard Deviation (%)

	RSDr %	GM %
<b>Applicant' method optimisation</b>	<b>15 %</b>	<b>0.1 %</b>
<b>EU-RL GMFF tests *</b>	<b>18 %</b>	<b>0.1 %</b>
<b>Collaborative study</b>	<b>19.8 %</b>	<b>0.1 %</b>

\* (SD / mean) \* 100 of 15 PCR replicates

<http://gmo-crl.jrc.ec.europa.eu/doc/Technical%20Guidance%20from%20EURL%20on%20LLP.pdf>

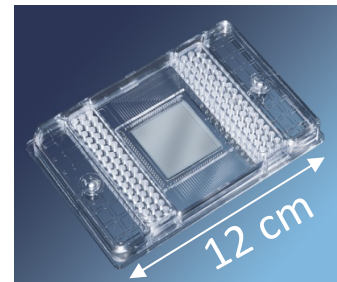
# Digital PCR

## BioMark™ System and IFC Technology

BioMark System



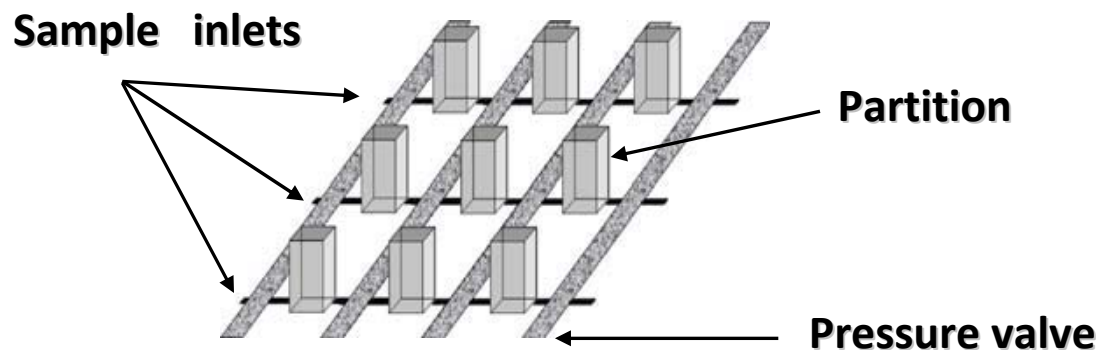
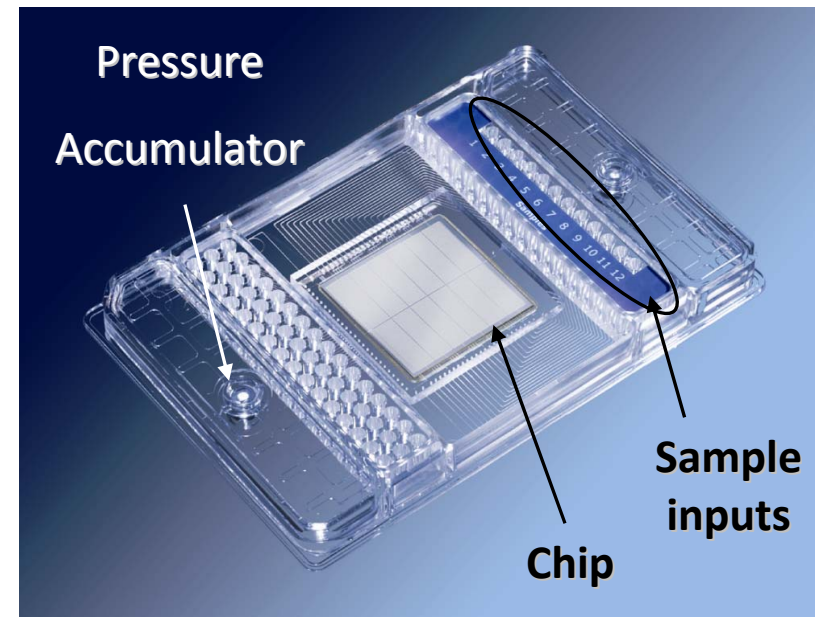
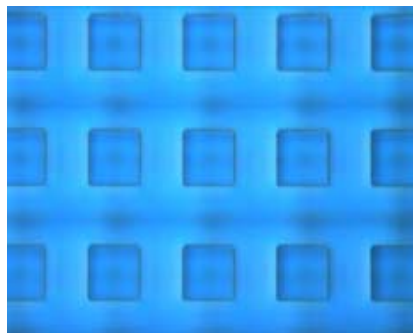
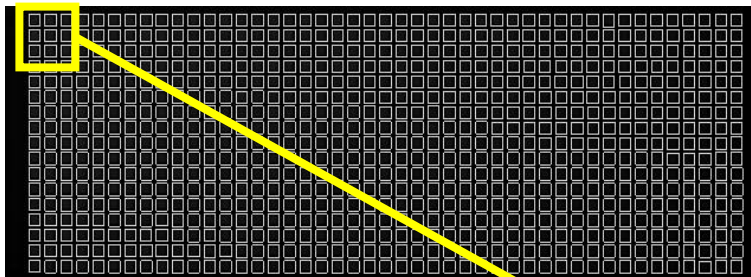
IFC Controller



Digital Arrays Chip: Integrated Fluidic Circuits

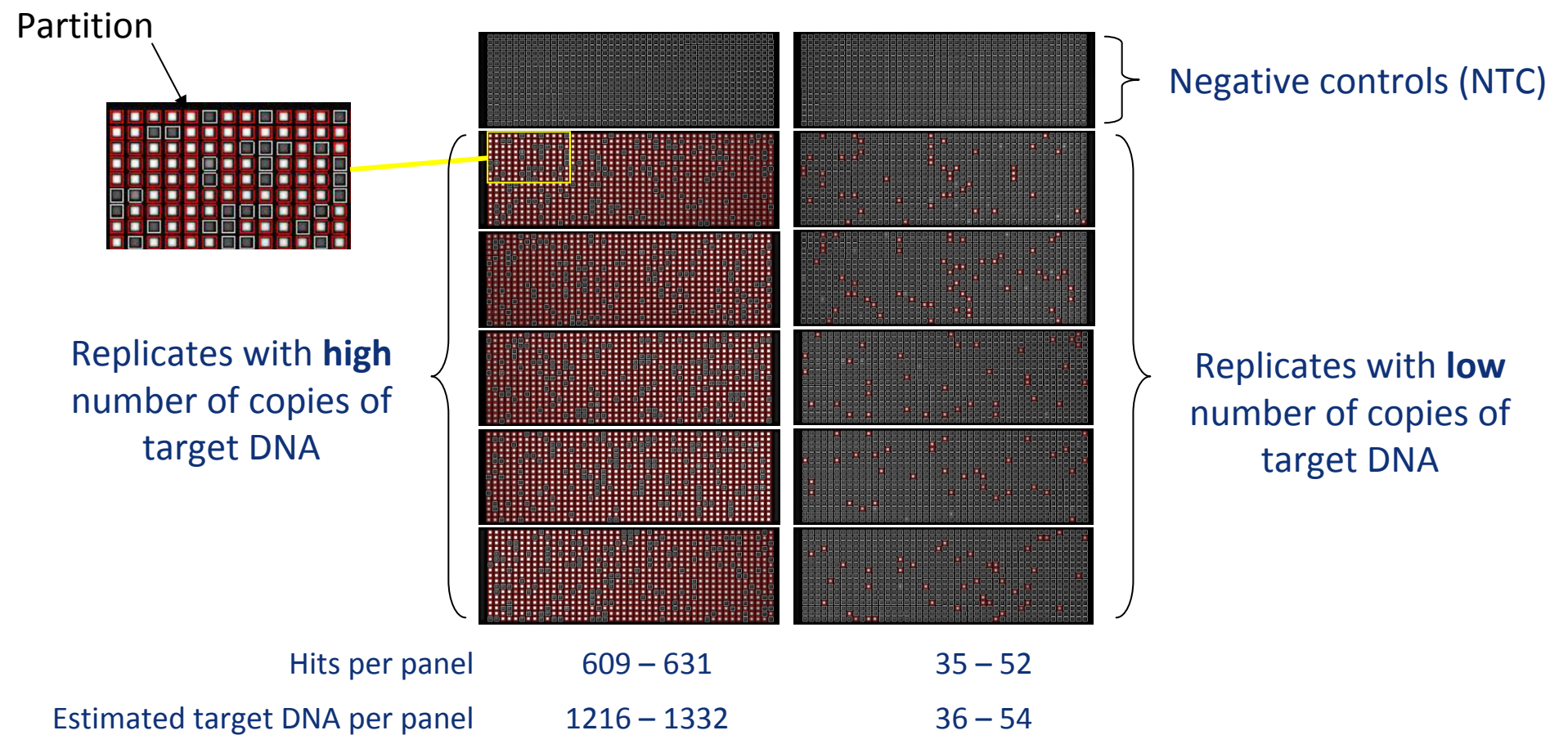
# Digital Array Chip: integrated fluidic circuit

- 12 sample input ports and **panels**
- 765 **partitions** per panel





# Digital PCR



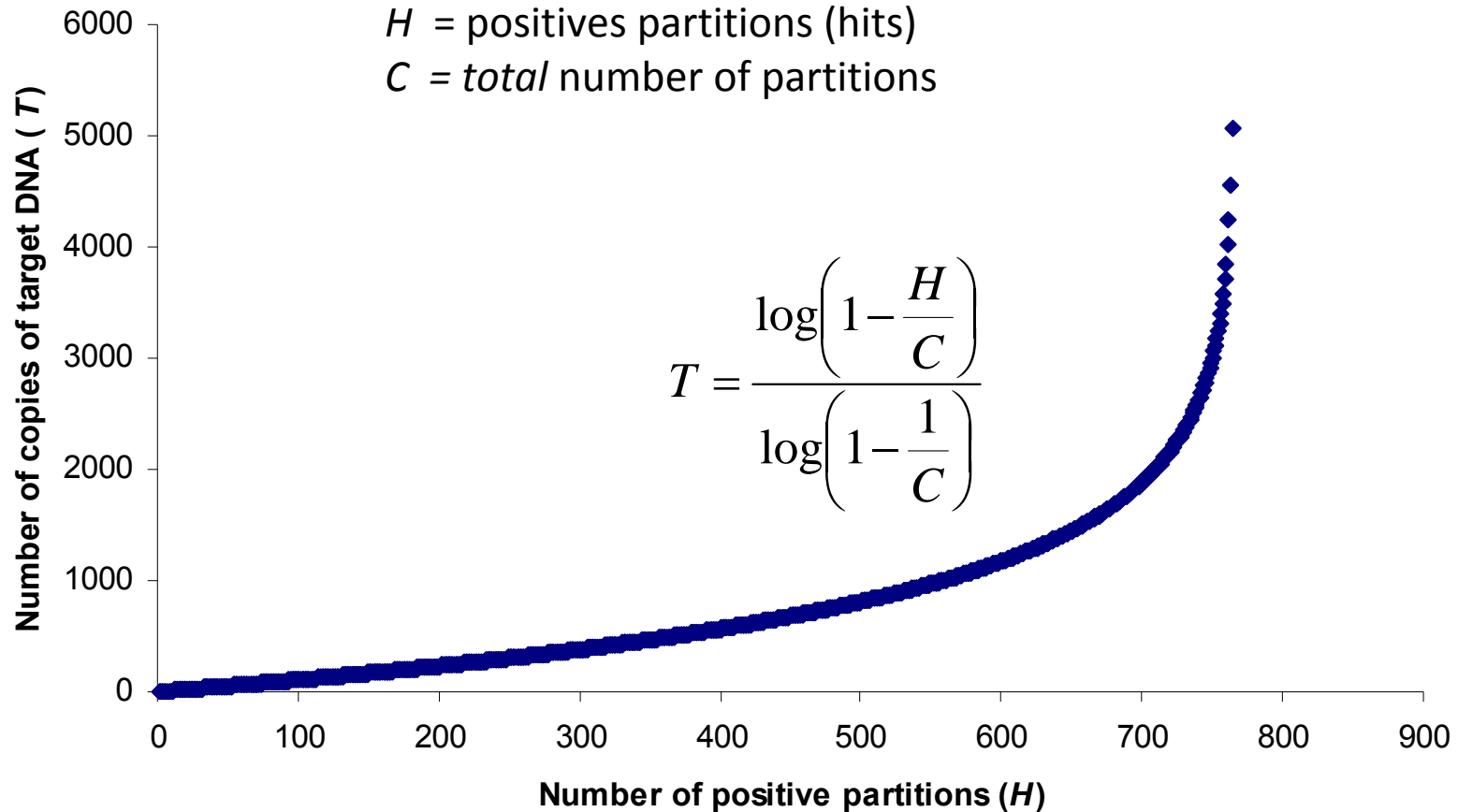


## Binomial approximation

$T$  = number of copies of target DNA


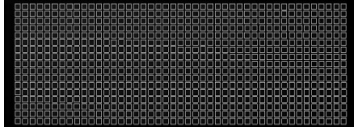
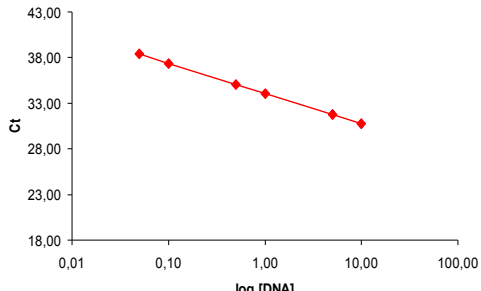
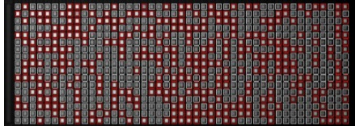
$H$  = positives partitions (hits)

$C$  = *total* number of partitions



As  $H$  increases, there is an increase in the number of partitions containing more than one copy of the target DNA

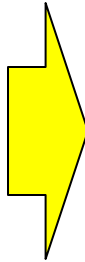
# Traditional vs digital real-time PCR

Feature	Traditional real-time PCR	Digital PCR
Assay format	Single tube/well 	Panel containing 765 individual partitions 
Assay volume	5 – 50 $\mu$ L	6 nL per partition (12.765 arrays) <i>1.5 nL per partition (48.770 arrays)</i>
Dynamic range	Theoretically, 1-10 <sup>10</sup> copies target DNA (exponential signal)	1 – 4000 copies target DNA (concentration of DNA adjusted to ensure some partitions do not contain DNA)
Determination of unknown	Calibrant 	Binomial approximation based on - the number of partitions containing amplified products; and - the total number of partitions analysed 

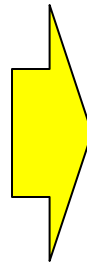
# BioMark™ Digital Array Workflow



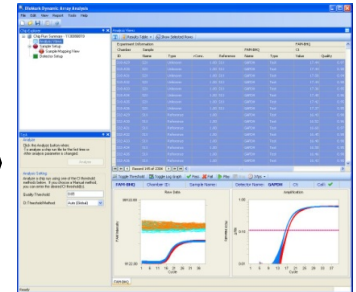
Pipette  
1-4



Load  
5



Run  
6



Analyze  
7

1. Extract the DNA
2. Quantify the ~ [DNA]
3. Convert [DNA] into number of DNA molecules  $f(\text{size of DNA})$
4. Dilute the [DNA] to obtain +/- ve partitions per panels
5. Load the biochip w/ the IFC controller
6. Run the digital PCR
7. Analyse the results

# Measurement equation

$T_c$  : concentration of target DNA in original solution (copy number per  $\mu\text{L}$ )

**The measurement is independent of a calibrant**

$$T_c = D \times \left( \frac{1}{C \times V_p} \right) \times \frac{\left( \log \left( 1 - \frac{H}{C} \right) \right)}{\left( \log \left( 1 - \frac{1}{C} \right) \right)} \quad \text{(copy number/ } \mu\text{L)}$$

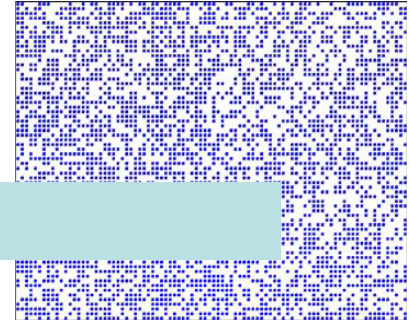
Binomial approximation (copy number/panel)

$V_p$  is partition volume ( $\mu\text{L}$ )  
 $C$  is total number of partitions  
 $\rightarrow$  Panel volume ( $\mu\text{L}$ )

Dilution factor from original solution to PCR reaction

# Critical assumption (1)

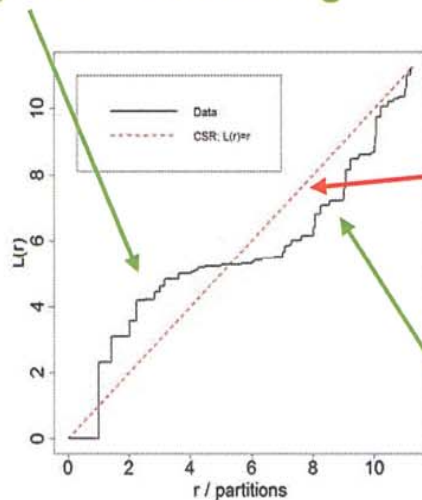
$$L(r) = \sqrt{\frac{K(r)}{\pi}}$$



No evidence of clustering or ordering across panels

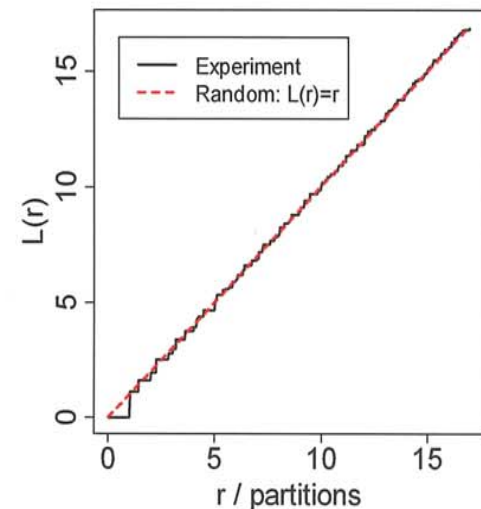
- Assessed the spatial distribution of positive partitions across 8 panels
- used the Ripley's  $K$  function,  $K(r)$
  - average number of extra points within radius  $r$  of a randomly chosen point, normalised by the number of points per unit area for complete spatial randomness

$L(r) > r$  clustering



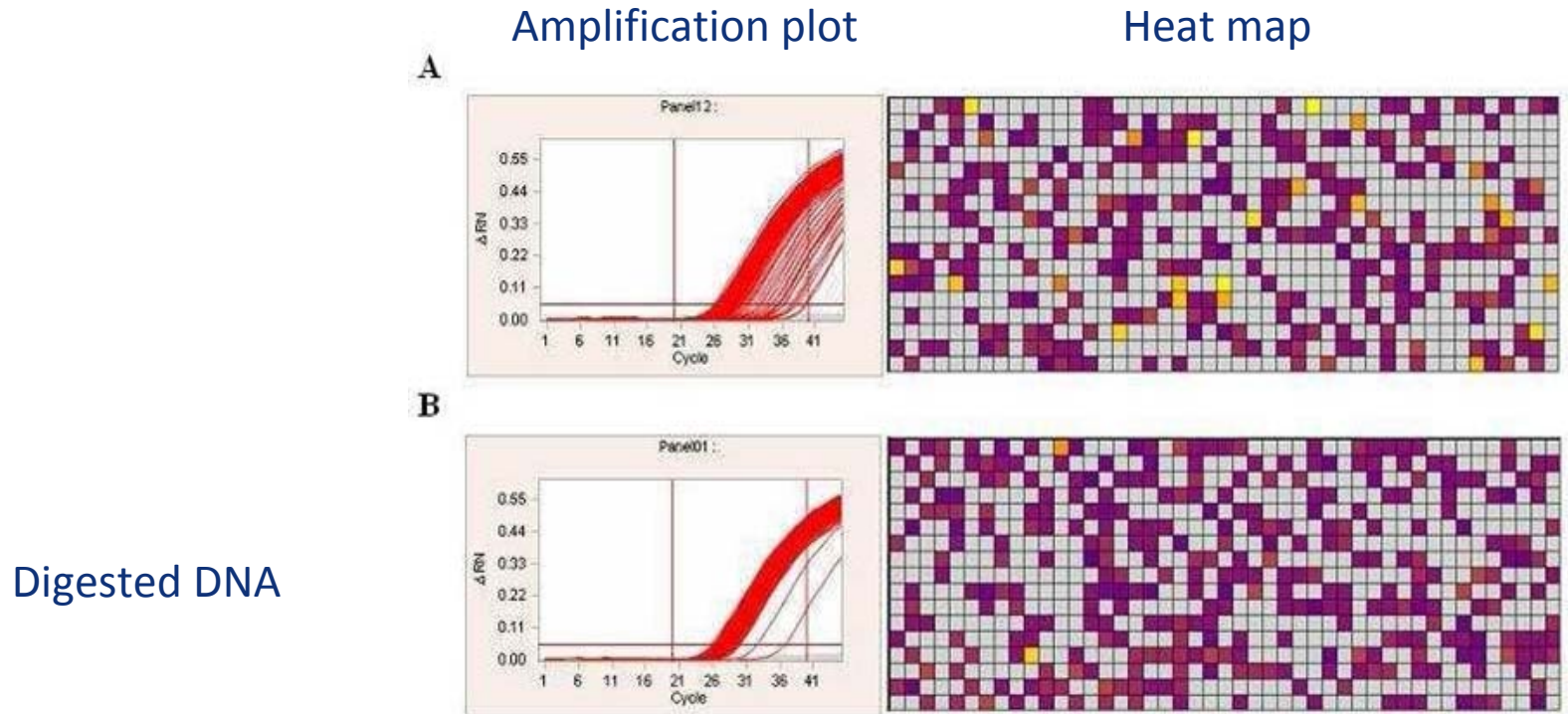
$L(r) = r$  complete randomness

$L(r) < r$  ordering

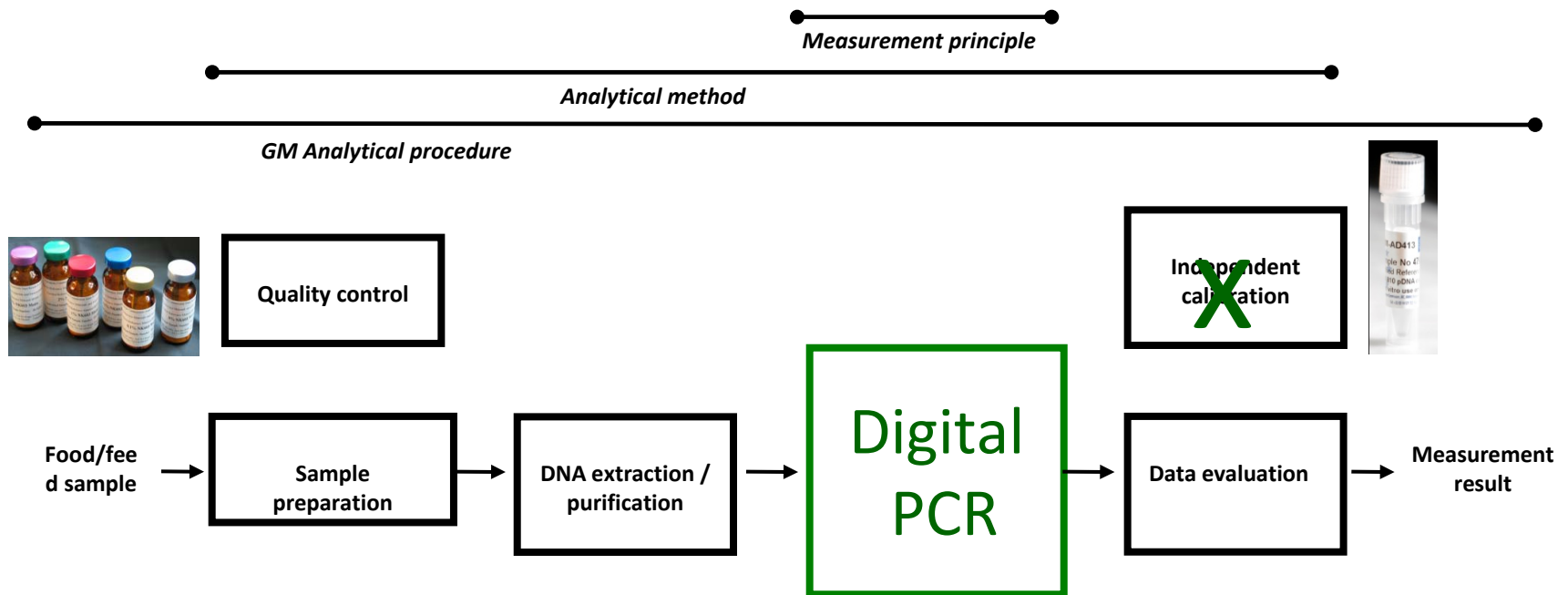


## Critical assumption (2)

- All target molecules are amplified



Digestion of DNA reduced potential bias due to underestimation of partitions containing target DNA



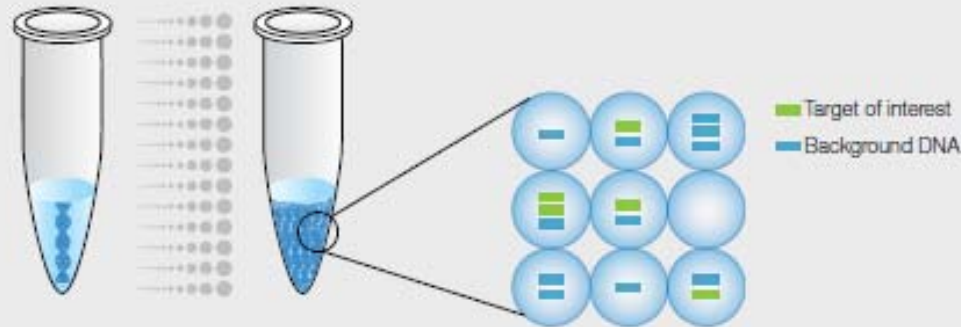
$$\frac{\text{Absolute number of transgene copies / } \mu\text{L}}{\text{Absolute number of reference copies / } \mu\text{L}} \times 100 = (\text{cp/cp}) \%$$

Absolute quantification of DNA targets without the need of external calibrant



A microscopic image of a sample emulsified into tens of 1,000s of nanoliter volume droplets. Only droplets that contain a copy of the target molecule will "light up" after PCR.

## Droplet Digital PCR



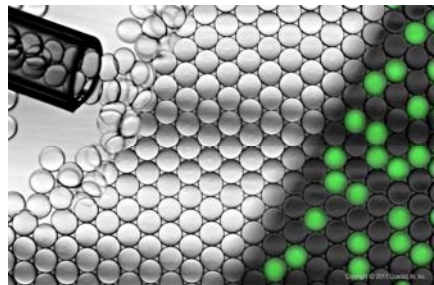
The sample is partitioned into 20,000 droplets, with target and background DNA randomly distributed among the droplets.



After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target DNA was present or not present after partitioning. Each droplet provides an independent digital measurement.

**"X" target copies**

Positive and negative droplets are counted for the sample and the software calculates the concentration of target DNA as copies per microliter.



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# Cotton GHB119

Positive control sample declared homozygous  
Undigested gDNA      Digested (Dral) gDNA

*Does not cut within  
the amplicons and  
several tens nt far  
from amplicon  
edges*

Number of plates	3
Number of samples	15
<b>GM-/ref- target mean ratio</b>	<b>1.03</b>
Standard deviation	0.094
Pooled RSDr (%)	9.2
Standard error of mean	0.024
t-value	2.13
<b>Upper 95% CI of mean</b>	<b>1.08</b>
<b>Lower 95% CI of mean</b>	<b>0.98</b>

*In relation  
to N=15*

Number of plates	3
Number of samples	15
<b>GM-/ref- target mean ratio</b>	<b>0.97</b>
Standard deviation	0.095
Pooled RSDr (%)	9.8
Standard error of mean	0.025
t-value	2.13
<b>Upper 95% CI of mean</b>	<b>1.02</b>
<b>Lower 95% CI of mean</b>	<b>0.92</b>

*Precision does not  
improve  
necessarily with  
digestion*

\* Ct window = 15-40

The 95% CI spans around 1, therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

No difference in zygosity ratio between testing undigested and digested DNA in GHB119

# Soybean FG72

Homozygous positive control sample (applicant's source)

**Undigested gDNA\***

**Digested (TaqI) gDNA\***

*Does not cut within  
the amplicons and  
several tens nt far  
from amplicon  
edges*

Number of plates	3
Number of samples	15
<b>GM-/ref- target mean ratio</b>	<b>1.00</b>
Standard deviation	0.089
Pooled RSDr (%)	8.9
Standard error of mean	0.023
t-value	2.13
<b>Upper 95% CI of mean</b>	<b>1.05</b>
<b>Lower 95% CI of mean</b>	<b>0.95</b>

\* Ct window = 15-35

Number of plates	3
Number of samples	15
<b>GM-/ref- target mean ratio</b>	<b>0.98</b>
Standard deviation	0.064
Pooled RSDr (%)	6.6
Standard error of mean	0.017
t-value	2.13
<b>Upper 95% CI of mean</b>	<b>1.01</b>
<b>Lower 95% CI of mean</b>	<b>0.94</b>

*In relation  
to N=15*

The 95% CI spans around 1, therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single copy reference target, for an alpha = 0.05.

No difference in zygosity ratio between testing undigested and digested DNA in FG72

# Regulation (EU) No 619/2011

## Copy number ratio (GM/taxon)

Mean ratio (FG72/Le1)	0.976
Standard deviation	0.064
RSDr (%)	6.6
Standard error of the mean	0.017
Upper 95% CI of the mean	1.011
Lower 95% CI of the mean	0.941

**GM % in DNA copy number ratio = 100% [GM % in mass fraction]**

## Regulation (EU) No 619/2011

- Monitor the real applicability and impact of the LLP
- Establish a link between conversion factor established on control sample and that found in real life samples