Digital PCR for nucleic acid quantification

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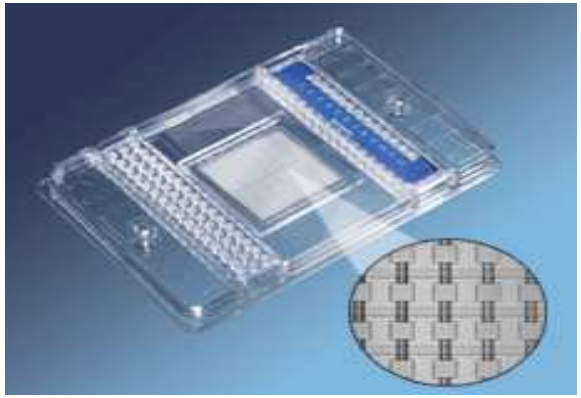
Joint Research Centre

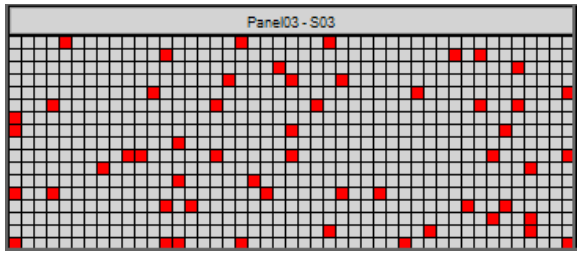
6th qPCR & Digital PCR Congress, 4Bio Summit: Europe, Rotterdam, 27 November 2018

European Commission

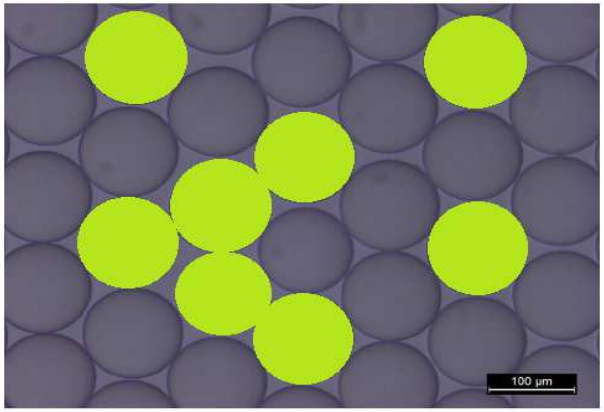
Digital PCR: a rising technique

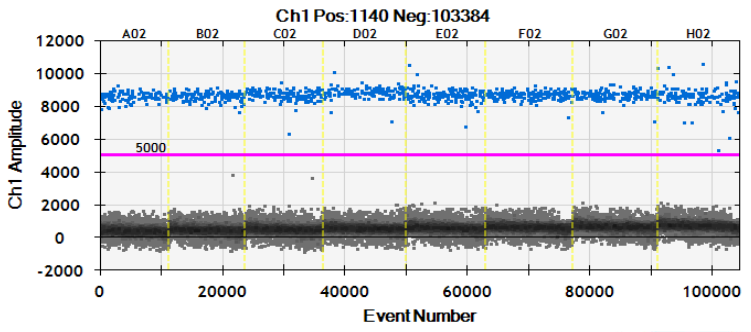
cdPCR





ddPCR





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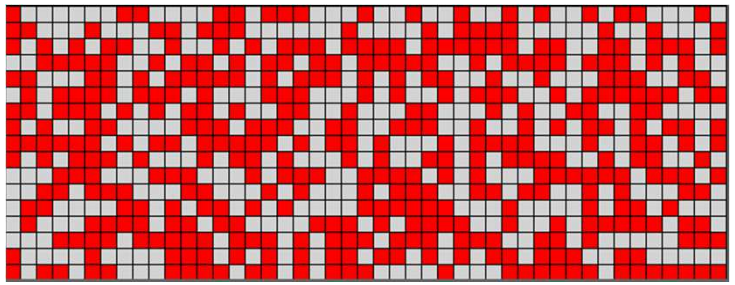
'Black box' creation of data ?



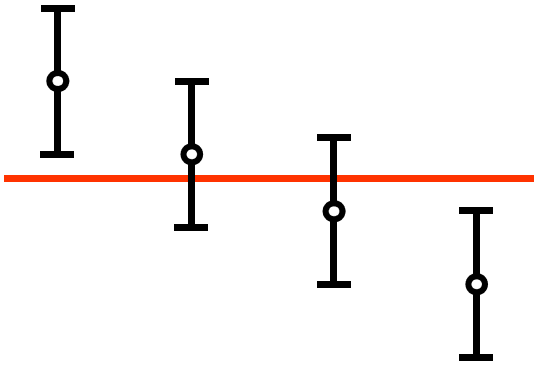
DNA concentration
in injected solution:

$$T_c = D \times \left(\frac{1}{C \times V_p} \right) \times \frac{\left(\log\left(1 - \frac{H}{C}\right) \right)}{\left(\log\left(1 - \frac{1}{C}\right) \right)}$$

Regulation (EU) 2017/625, Art. 34**:** *"Official laboratories shall use … relevant methods …validated in accordance with internationally accepted scientific protocols"*

Critical performance characteristics:

- Selectivity
- Robustness
- Precision & trueness (= accuracy)
- *Measurement uncertainty*
- Working range (incl. *LOD, LOQ*)



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Exploring dPCR limits: *Measurement uncertainty*

Test material: Solutions of a double-stranded linearized plasmid (ERM®-AD623)

Deprez et al.: *Biomol. Detect. & Quantif.* 9 (2016) 29-39

Negligible sources (< 1 %):

- Accuracy weighing (gravimetric dilutions)
- Density of sample mix
- Quality of HPLC purified primers
- Different assays
- Threshold settings

$$U_{meas,rel} = 2 \times \sqrt{\frac{s_{repeat,pooled,rel}^{2}}{n_{meas}} + \frac{s_{run,pooled,rel}^{2}}{n_{run}} + u_{V_d,rel}^{2} + u_{bias,rel}^{2}}$$

U_{exp} = 14.2 %

25 - 450 cp/µL

- o *intactness ds DNA ?*
- o *inhibitors ?*
- o *secondary structures ?*

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Exploring dPCR limits: *LOD and LOQ*

Test material: Solutions of a double-stranded linearized plasmid (ERM®-AD623)
Deprez et al.: *Biomol. Detect. & Quantif.* 9 (2016) 29-39

LOD

sample with 0.5 cp/µL measured, repeatability conditions (n = 64)

→ All positive (mean: 0.56 cp/µL; RSD 34.4 %)

→ **LOD < 0.5 cp/µL**

LOQ

= *Lowest cp/µL with acceptable* U_{exp}

- tested at 3.5 cp/µL → U_{exp} = 28.9 % (n = 4)

→ **LOQ = 3.5 cp/µL**

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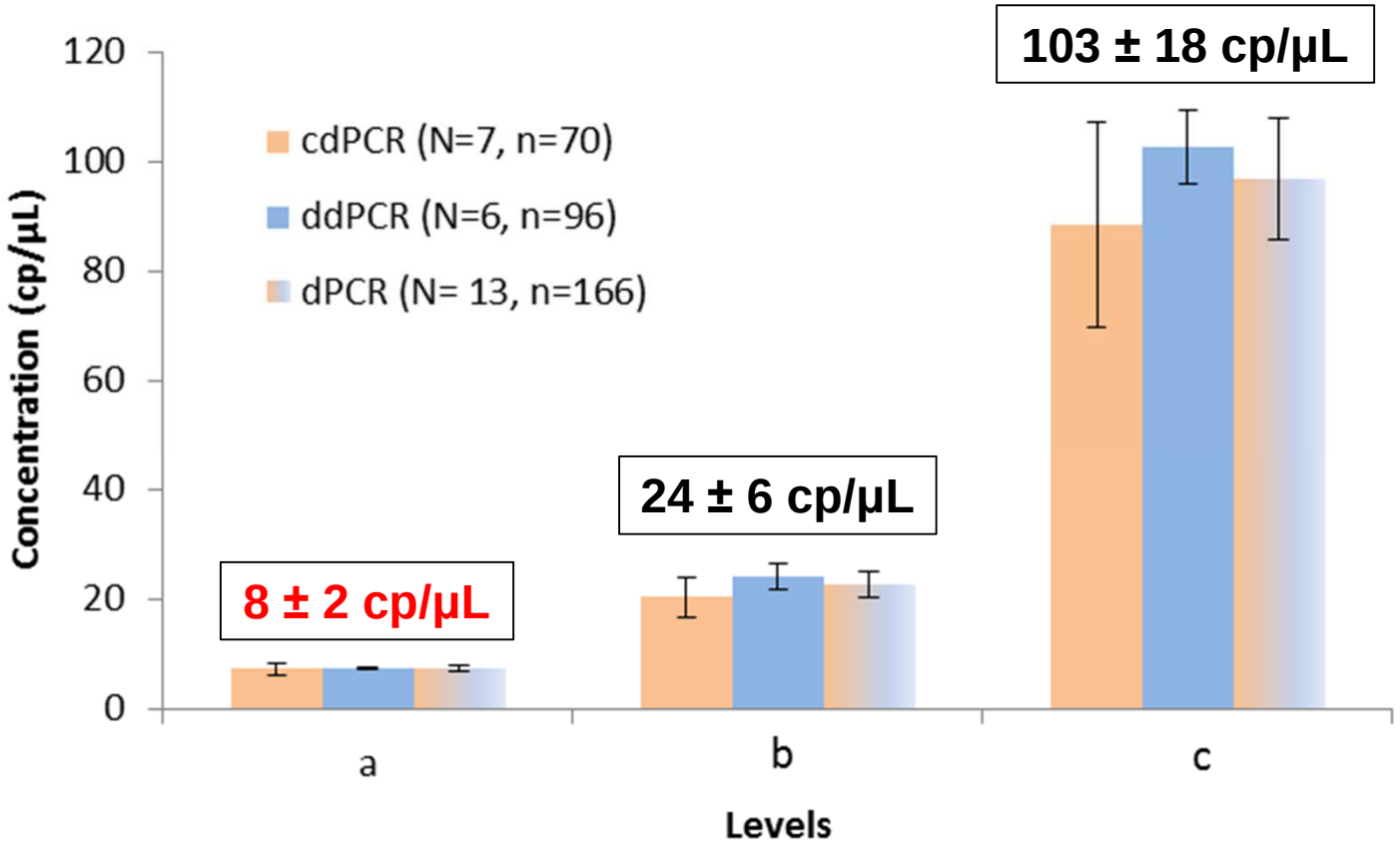
Example: *'ultratrace' PCR calibrant*

Commission Regulation (EU) No 51/2013 … as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed

candidate CRM: Plasmid DNA with poultry-specific sequence



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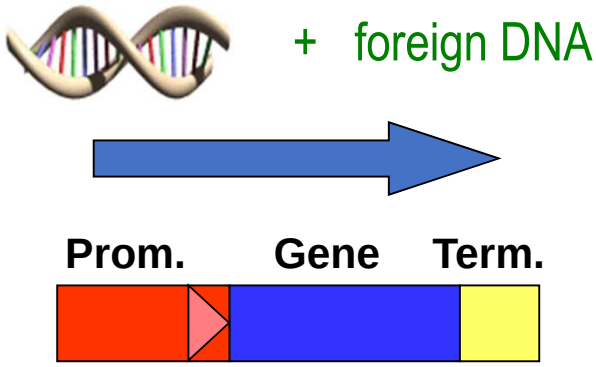
Establishing Reference Measurement Systems

Example: Food/feed labelling regarding Genetically Modified Organisms (GMO)

non-GMO



GMO

EU Member States decided to inform consumers on the presence of GMOs in food and feed

- **Regulation (EC) No 1829/2003** specifies that food and feed products containing more than 0.9 % authorised GMO have to be labelled
- **Regulation (EU) No 619/2011** defines the possible low level presence of GM material in feed with pending or expired authorisation (0.1 % GM mass fraction)

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Challenges for comparability

GMO quantification results

are ratios:

$$\frac{\text{content of transgenic DNA}}{\text{content of species-specific DNA}}$$

Exploiting digital PCR for:

1) **harmonising species-specific DNA reference fragments**
2) **establishing universal calibration systems**

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Identifying optimal reference fragments

Reference genes should be:

- ✓ **SPECIFIC: targeting only one species**
- ✓ **STABLE across varieties: primers and probe in conserved regions**
- ✓ **SINGLE COPY: amplify a single target in the genome**

Using digital droplet PCR because:

- ➢ **Bioinformatics analyses need to be verified experimentally** (allopolyploid species problematic)
- ➢ **Real-time qPCR quantification requires a corresponding DNA calibrant**

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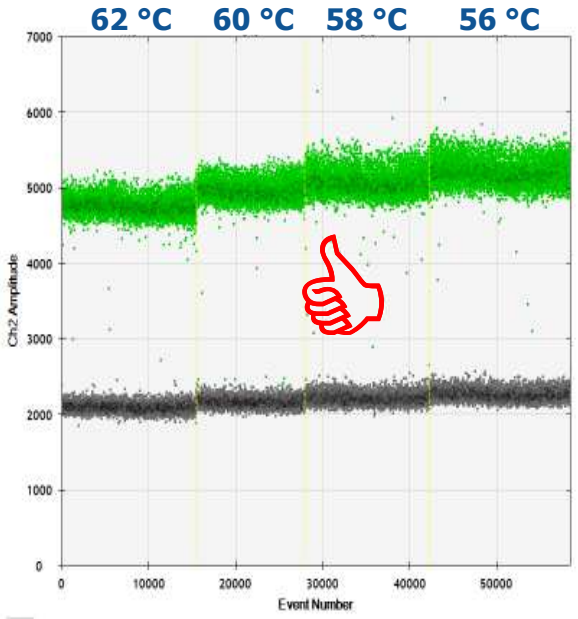
ddPCR with potential reference fragments

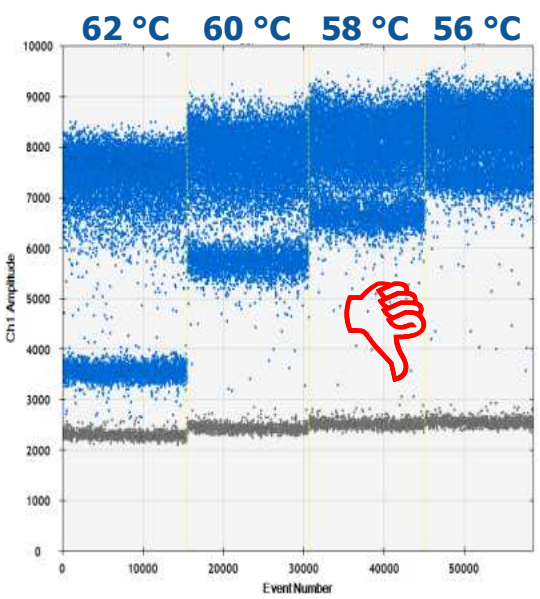
Soybean

Le1 B ✓



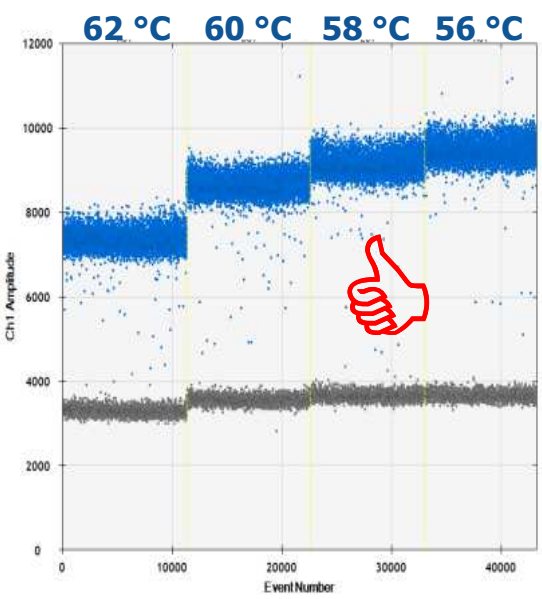
Maize

aldolase ✗



secondary target with mismatches in primer and probe

hmg ✓



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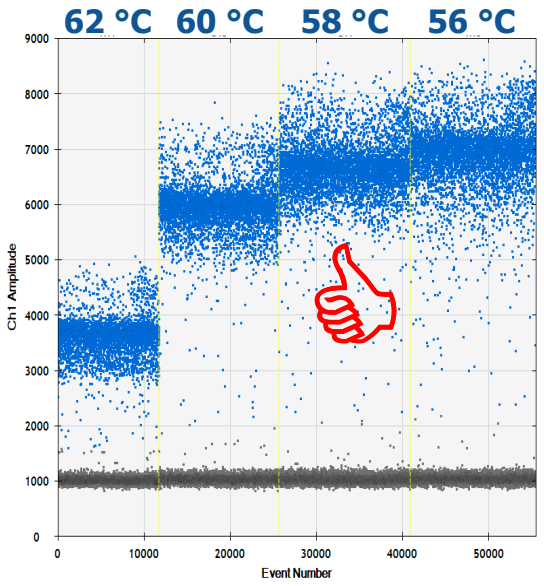
ddPCR with potential reference fragments

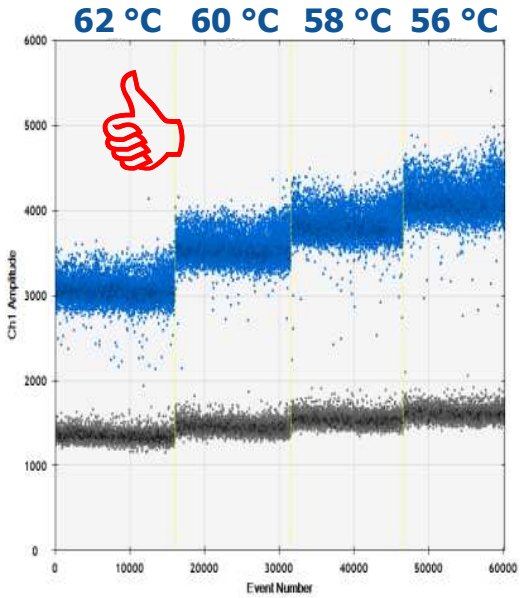
Oilseed rape

FatA(A) ✓



Cotton

AdhC ✓



S. Jacchia et al.: Food Control 93 (2018) 191-200

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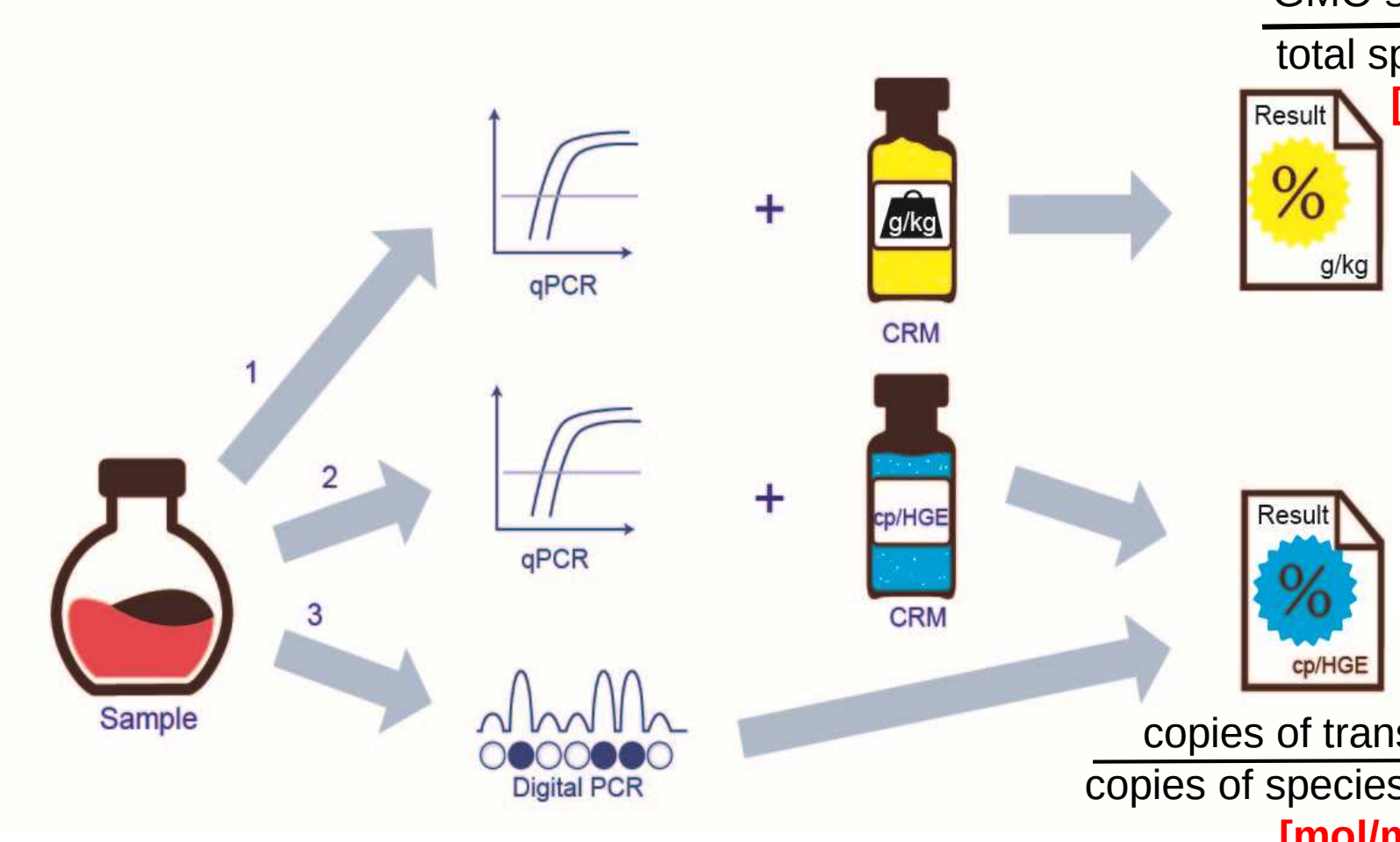
Currently used calibration systems



GMO species mass / total species mass **[g/kg]**

copies of transgenic DNA / copies of species-specific DNA **[mol/mol]**

Comparable results?

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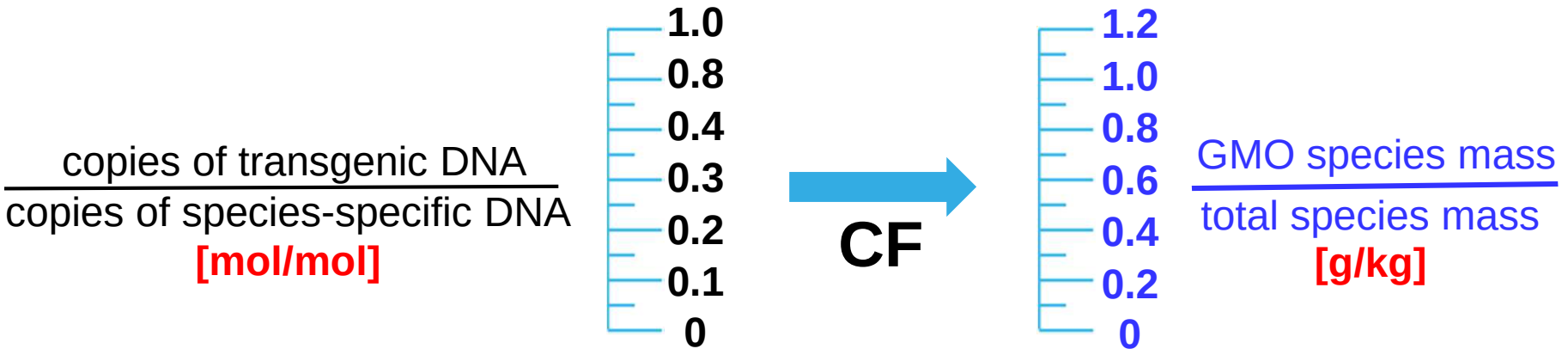
Towards a single Reference system

Conversion factor between measurement units influenced by:

- *Biological features (species, DNA/GM content in tissues, zygosity…)*
- *Biotechnological process (parental origin of transgene…)*



Experimental determination of conversion factor per material

Digital PCR
(no DNA calibrant required)

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Recent dPCR results

$$\frac{\text{copies of transgenic DNA}}{\text{copies of species-specific DNA}}\ \textbf{[mol/mol]} \xrightarrow{\frac{1}{\textbf{CF}}} \frac{\text{GMO species mass}}{\text{total species mass}}\ \textbf{[g/kg]}$$

a cotton CRM **1.05 ± U**

a maize CRM (1) **0.58 ± U** (maternal GM origin)

a maize CRM (2) **0.34 ± U** (paternal GM origin)

a maize CRM (3) **0.57** (lab A) or **0.93** (lab B) –

CRM claimed to be homozygous

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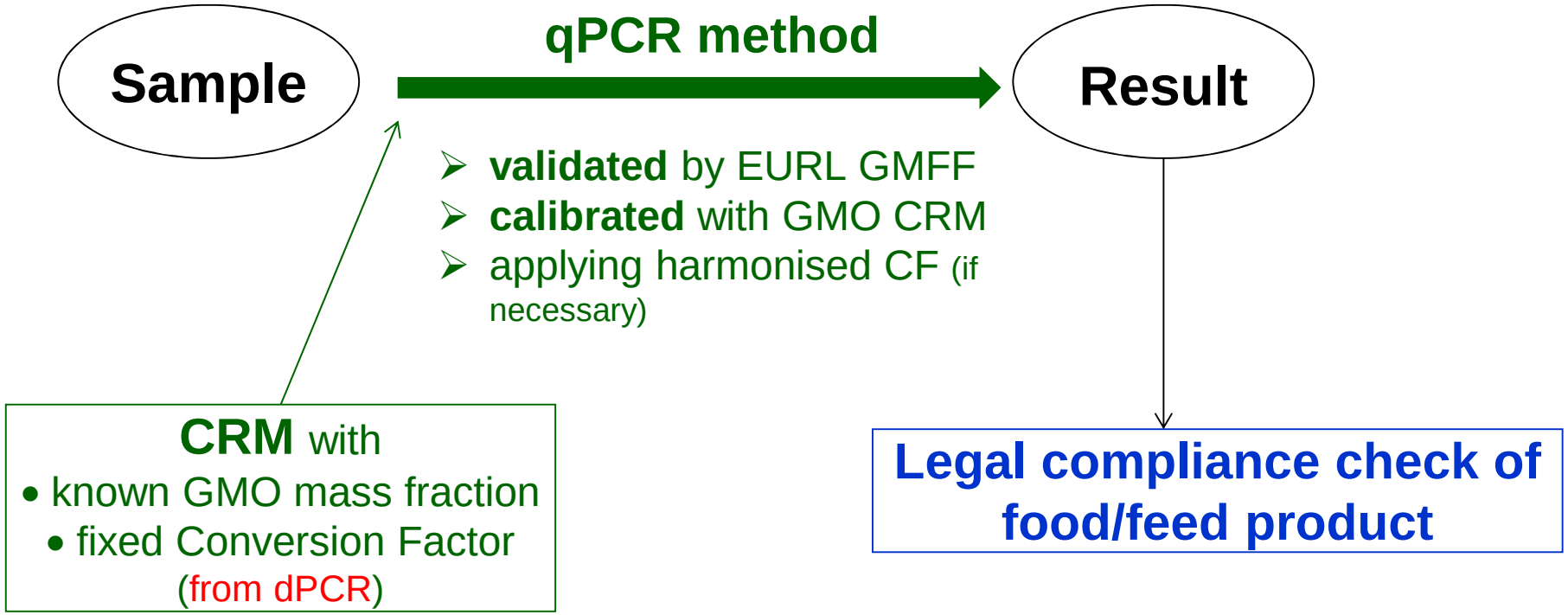
Reliable & comparable GMO quantification

EU legislation specifies GMO ***measurement method*** & GMO ***CRM*** during market authorisation



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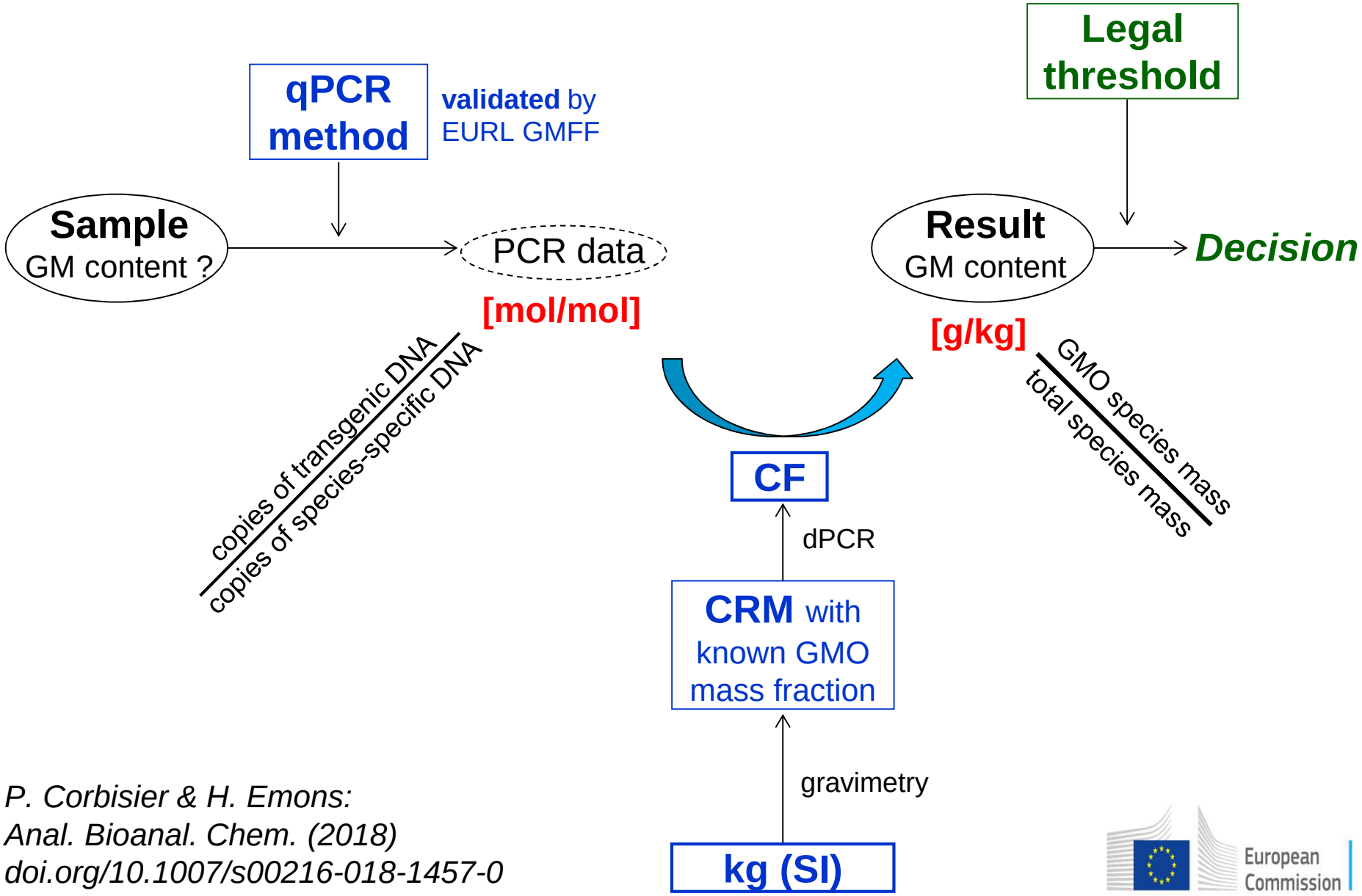
Reliable & comparable GMO quantification



P. Corbisier & H. Emons:
Anal. Bioanal. Chem. (2018)
doi.org/10.1007/s00216-018-1457-0

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Take-home messages

Digital PCR:

- **is an attractive option for quantifying low DNA concentrations (down to a few copies/µL)**
- **does not require a DNA calibrant**
- ***however,* the validity of the underlying evaluation model has to be checked case-by-case**
- **can be used for identifying plant-specific reference genes**
- **offers the opportunity to determine conversion factors between PCR measurement units**
- **facilitates the establishment of reference measurement systems for GMO quantification**

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RM Processing

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