Digital PCR for nucleic acid quantification

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Digital PCR: a rising technique

cdPCR

ddPCR
DNA concentration in injected solution:

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

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)
Exploring dPCR limits: Measurement uncertainty

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

Negligible sources (< 1 %):
- Accuracy weighing (gravimetric dilutions)
- Density of sample mix
- Quality of HPLC purified primers
- Different assays
- Threshold settings

\[
U_{\text{meas,rel}} = 2 \times \sqrt{\frac{s_{\text{repeat,pooled,rel}}^2}{n_{\text{meas}}} + \frac{s_{\text{run,pooled,rel}}^2}{n_{\text{run}}} + u_{\text{V,rel}}^2 + u_{\text{bias,rel}}^2}
\]

\[
U_{\text{exp}} = 14.2 \%
\]

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

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Exploring dPCR limits: *LOD* and *LOQ*

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

- **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**  
  \[ \text{LOQ} = \text{Lowest } \text{cp/µL with acceptable } U_{\exp} \]

  - tested at 3.5 cp/µL  
    \[ U_{\exp} = 28.9 \% \ (n = 4) \]
  → LOQ = 3.5 cp/µL
**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

![Graph showing concentration levels](image)
Establishing Reference Measurement Systems

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

- 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).

EU Member States decided to inform consumers on the presence of GMOs in food and feed.
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
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
ddPCR with potential reference fragments

Soybean

Le1 B ✓

Maize

aldhase ❌

hmg ✓

secondary target with mismatches in primer and probe
ddPCR with potential reference fragments

**Oilseed rape**

*FatA(A)*

![Graph for Oilseed rape](image1)

**Cotton**

*AdhC*

![Graph for Cotton](image2)

*S. Jacchia et al.: Food Control 93 (2018) 191-200*
Currently used calibration systems

GMO species mass

[\text{g/kg}]

total species mass

copies of transgenic DNA

[\text{mol/mol}]
copies of species-specific DNA

Comparable results?
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)
Recent dPCR results

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

- **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
Reliable & comparable GMO quantification

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

Sample

qPCR method

Result

- validated by EURL GMFF
- calibrated with GMO CRM
- applying harmonised CF (if necessary)

CRM with
- known GMO mass fraction
- fixed Conversion Factor (from dPCR)

Legal compliance check of food/feed product
**Reliable & comparable GMO quantification**

- **Sample**
  - GM content?
  - qPCR method validated by EURL GMFF

- **PCR data**
  - [mol/mol]

- **Result**
  - GM content [g/kg]
  - CRM with known GMO mass fraction
  - Legal threshold
  - Decision

- **CF**
  - dPCR

- **CRM**
  - gravimetry

**P. Corbisier & H. Emons:**
doi.org/10.1007/s00216-018-1457-0
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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