EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

EURL
European Union Reference Laboratory
for GM Food & Feed

Report
on the TRAINING on DIGITAL PCR and DROPLET DIGITAL PCR
for National Reference Laboratories (NRLs)
assigned under Regulation (EC) No 882/2004
19-21 November 2014, Ispra, Italy

The training was organised on request of the NRLs/882 who had indicated this topic as one of the main training topics of interest during the NRL/882 workshop in 2013. The EU-RL GMFF organised a two and a half day training programme and invited all NRLs/882 to the training on a first-come-first-served basis, with a maximum capacity of 20 registered participants. A few additional NRLs not assigned as NRL under Regulation 882/2004 had also showed an interest in such training and were allowed to participate. A total of 19 participants registered to the training, one per NRL, originating from 15 countries (including 14 EU Member States and Serbia). One participant cancelled his participation at the last minute, therefore, 18 registered participants actually participated to the training event. In addition, representatives from three companies, all selling digital PCR instruments, were invited and accepted to give a presentation on their platforms during the first day of the training. A 4th company was invited but was unable to participate. The other presentations provided during the training were from JRC-IRMM (through video conference), and from several colleagues from JRC-IHCP. IHCP-colleagues were also involved in the laboratory phase of the training on day 2.

The programme of the training included the following general topics:
- day 1: basics of digital PCR and platforms used
- day 2: hands-on training and user experiences
- day 3: applications of digital PCR and discussion

Further details on the programme can be found in Annex 1.

A number of presentations are included in Annex 2. Additional documentation on digital PCR was provided after the training through a weblink (Annex 3).

Details on the social dinner provided on day 1 and day 2 are shown in Annex 4.

At the end of the training, a feedback form was filled in by the participants. The results of this feedback are shown in Annex 5.

ANNEX 1. Detailed programme of the training

TRAINING on CHAMBER DIGITAL PCR and DROPLET DIGITAL PCR

for National Reference Laboratories (NRLs)
assigned under Regulation (EC) No 882/2004
19-21 November 2014, Ispra, Italy

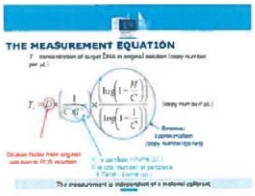
AGENDA

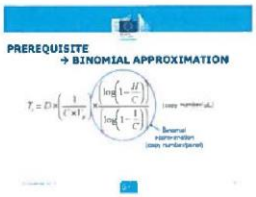
DAY 1 - 19 November 2014 (meeting room building 20) *Chairperson: Wim Broothaerts*		
TIME	**TOPIC**	**STAFF INVOLVED**
09:30	Welcome by MBG HoU and Introduction to the training	J. Kreysa, W. Broothaerts
10:00	Basics of droplet digital PCR	Alessandro Martino (BioRad representative)
11:00	*Coffee Break (Building 20)*	
11:30	Droplet digital PCR with RainDrop	Viviane Sternkopf (RainDance representative)
12:30	*Lunch: cold buffet (Building 20A)*	
13:30	Digital PCR with QuantStudio 3D	Jan Ghyssaert (Life Technologies representative)
14:30	Experiences with applying ddPCR for GMO analysis	Philippe Corbisier (by video conference)
15:30	*Coffee Break (Building 20)*	
16:00	Statistical principles of ddPCR	Antoon Lievens
16:30	Discussion	
17:00	***End of day 1***	
19:00-21:30	***Social dinner – Ristorante Il Melograno, Angera***	

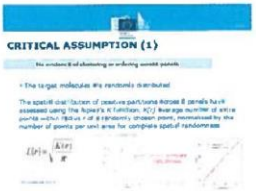
DAY 2 - 20 November 2014 (meeting room building 20 & Laboratory building 20A) *Chairperson: Wim Broothaerts*		
TIME	**TOPIC**	**STAFF INVOLVED**
09:00	Introduction to experimental part. Hands-on training/demonstration in the laboratory in two groups: setting up a chamber dPCR and a droplet dPCR experiment	Antoon Lievens Valentina Paracchini
09:30	First lab session	Antoon Lievens Gregor Pinski
11:00	*Coffee Break (Building 20A)*	
11:30	Second lab session	Antoon Lievens Gregor Pinski
13:00	*Lunch: cold buffet (Building 20A)*	
14:00	Reading of the runs and data analysis	Antoon Lievens Valentina Paracchini
14:45	Experiences with restriction, inhibition, multiplexing	Antoon Lievens
15:30	*Coffee Break (Building 20)*	
16:00	Comparison of rtPCR, cdPCR and ddPCR	Christian Savini
16:30	Discussion	
17:00	***End of day 2***	
19:00–21:30	***Social dinner – Pizzeria Il Capriccio, Ispra***	

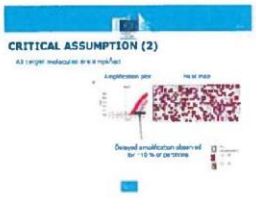
DAY 3 - 21 November 2014 (meeting room building 20) *Chairperson: Wim Broothaerts*		
TIME	**TOPIC**	**STAFF INVOLVED**
09:00	Using dPCR data in support of GMO detection method validation	Elena Nardini
09:45	Test: what did you (not) learn?	Marco Mazzara
10:15	*Coffee Break (Building 20)*	
10:45	Discussion, wrapping-up and closure of meeting	J. Kreysa, Marco Mazzara
12:00	***End of day 3***	
12:00-13:00	*Lunch: sandwiches (Building 20A)*	

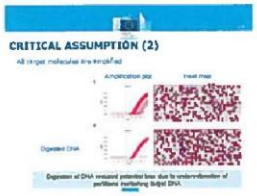
ANNEX 2. Presentations provided during the training

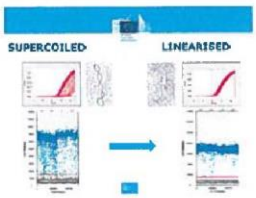


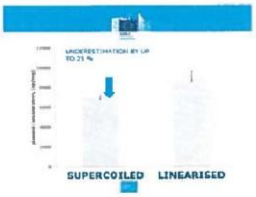


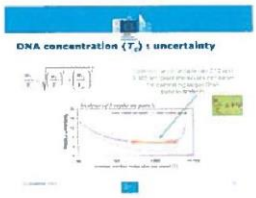


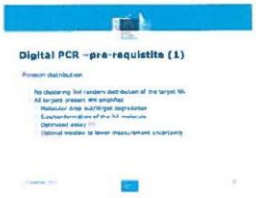


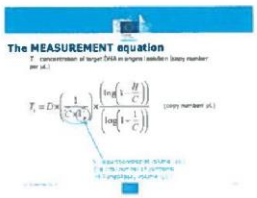


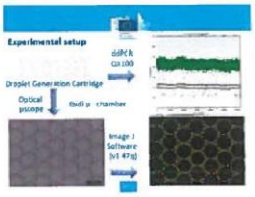


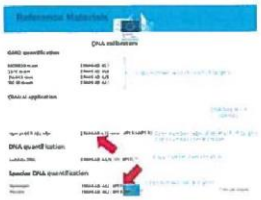


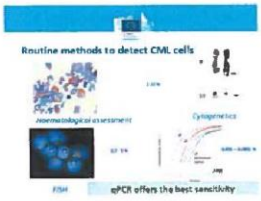


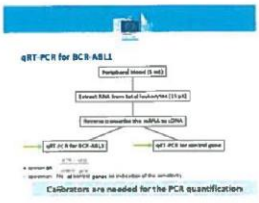







Reference Materials
DNA quantitation
DNA quantitation
Genetic DNA quantification
Genetic DNA quantification

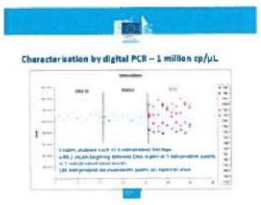

Routine methods to detect CML cells
Haematological assessment
Cytogenetics
FISH
qPCR offers the best sensitivity

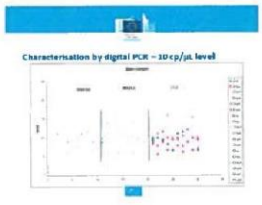

qRT-PCR for BCR-ABL1
Peripheral blood (5 ml)
Extract RNA from buffy coat/whole blood (10 µl)
Reverse transcribe the mRNA to cDNA
qRT-PCR for BCR-ABL1
qRT-PCR for control gene
Calibrators are needed for the PCR quantification

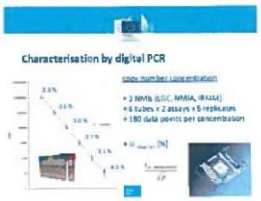

ERM-AD623
• Set of 6 ready-to-use calibrants over a 5-log dynamic range
• EcoRV linearised plasmid in Tris-EDTA pH 8.0
50 ng µL-1 of tRNA from E. coli
• Almost commonly recommended internal control genes


Certification
Sequence identity
Copy number concentration

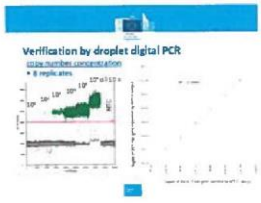

Characterisation by digital PCR
Measurement performed by National Metrological Institutes
Copy number concentration (cp/µL)
Biorad system (32 765 beads/ch)
Validated standardized assay –BCR ABL and ABL targets
Gravimetrical dilutions


Characterisation by digital PCR – 1 million cp/µL


Characterisation by digital PCR – 10 cp/µL level

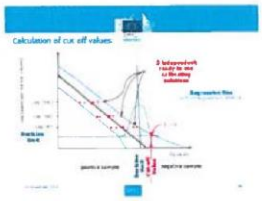

Characterisation by digital PCR
copy number concentration
• 3 NMIs (LGC, NMIA, IRMM)
• 6 tubes x 2 assays x 5 replicates
• 180 data points per concentration


Certified values and their uncertainties


Verification by droplet digital PCR
copy number concentration
• 8 replicates

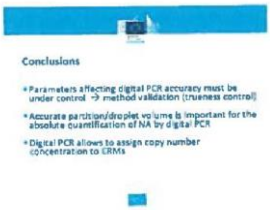

Some applications of d(d)PCR at IRMM
2- Value assignement of DNA calibrators
2b Ruminant calibration kit


Certified Reference Materials
Commission Regulation (EU) No 51/2013 of 16 January 2013
methods of analysis


Calculation of cut off values.


Calibrating solutions
• 5 mL solutions containing 128, 32 and 8 cp/µL
• Values assigned by digital PCR
• Further characterisation


IRMM-AD482
ERM-AD482


Conclusions
• Parameters affecting digital PCR accuracy must be under control → method validation (trueness control)
• Accurate partition/droplet volume is important for the absolute quantification of NA by digital PCR
• Digital PCR allows to assign copy number concentration to CRMs


Acknowledgments

Inhibition, restriction and multiplexing

Antoon Lievens

JRC digital PCR Training 2014

Outline

Inhibition
Inhibitors
Inhibition effects on dPCR
Inhibition effects on ddPCR

Restriction

Multiplex
Multiplexing Probes
Conclusion

Inhibition

Inhibition

Inhibitors

What are inhibitors?

- Anything interfering with your reaction
- Sources: sample extraction, matrix
- examples: KCl, NaCl, ionic detergents, alcohols, phenolic compounds, organic acids

Effects on the reaction

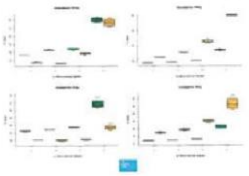
- Binding to DNA
- Interfere with polymerase
- Change ionic conditions

Inhibitors and qPCR

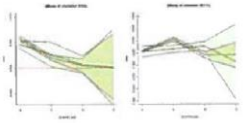
Violation of assumptions

- Not all targets are amplified at the same rate
- amplification curves reach the threshold later than expected
- Especially problematic are differences in efficiency between sample and standard curve
- Typically the variability of the results increases

Inhibitors and qPCR



Effects on qPCR quantification



Inhibition effects on ddPCR

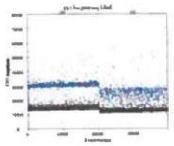
dPCR advantages

- due to the compartmentalisation, DNA exposure to inhibitors is reduced
- digital PCR is essentially binary, it is only necessary to distinguish positives from negatives

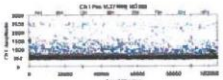
Effects on the reaction

- more difficult to set the threshold
- more "rain" due to increased variability between amplifications
- an increase in variability between repeats is to be expected

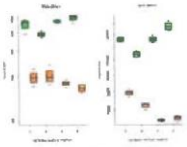
Inhibition effects on ddPCR



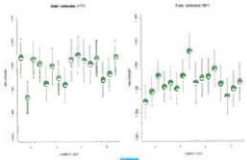
Inhibition effects on ddPCR



In House Experiment



In House Experiment



Restriction

Restriction

dPCR assumptions

Definition

The Poisson Distribution Expresses the probability of a given number of events occurring in a fixed interval of time and/or space if these events occur with a constant average rate and **independently** of the time since the last event

Violation of assumptions

- if targets are physically located in close proximity they will not be distributed independently over the reaction vessels
- this will create bias in the quantification

dPCR assumptions

Solutions

- Sonication: fast, random, targets may be lost
- Restriction: slower, additional buffers, choice enzymes wisely

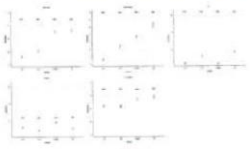
In House Experiment

- Measure the ddPCR to Endogene ratio
- Measure the SSR to Endogene ratio

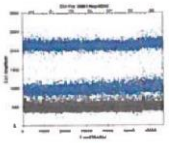
target events

- MON1445: expected ratio 1 1 1 (35x ddPCR error)
- MON15985: expected ratio 1 2 1 (35x ddPCR error)
- Bt11: expected ratio 2 2 1 (35x ddPCR error)
- GA21: expected ratio 1 2 1 (35x ddPCR error)
- GTS-40-3-2: expected ratio 1 1 1 (35x ddPCR error)

In House Experiment



In House Experiment



Multiplex

Multiplex

Spectral Issues

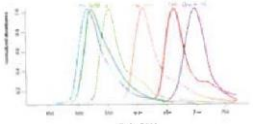
Spectral Issues

- Spectral separation between assays
- ddPCR 2 dimensional separation allows more accurate gating of positive reactions
- possibility of mixing dyes to obtain higher plexing

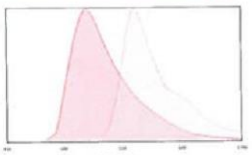
Cent/cary quenchers and fluorophores

- TAMRA is a popular quencher
- TAMRA has an emission spectrum that is close to HEX/VIC
- negative FAM-TAMRA droplets may show up among the positives in the second channel
- Using dark quenchers is pretty much a must

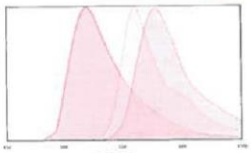
Spectral Issues



Spectral Issues



Spectral Issues



Conclusions

Concluding remarks

- dPCR has several advantages
- but may also require additional steps in sample preparation
- Multiplexing conditions are straightforward than for qPCR
- depending on your system, special care has to be taken to select the correct dyes

Mathematics and Statistics of digital PCR

Ambion Lievens

JRC digital PCR Training 2014

Outline

qPCR Refresh course

qPCR Refresh course

Measuring DNA

Basic principles

- The fluorescence is equivalent to DNA mass
- reactions with more initial copies accumulate DNA more quickly

qPCR Core idea

- if a threshold is set, reactions with more initial copies will reach it sooner than reactions with fewer copies
- The difference in time (cycles) between two reactions reaching the threshold is proportional to their ration of target copies

Measuring DNA

Principle

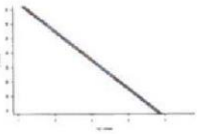
$$\log(F_R - F_B) = \log(\alpha) + \log(\lambda) + n\log(2)$$

$$C_q = \frac{-(\log(\alpha) + \log(\eta) - \log(F_R - F_B))}{\log(2)}$$

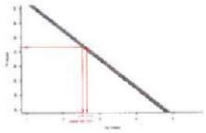
$$C_{q,1} - C_{q,2} = \frac{\log(\eta_{,1}) - \log(\eta_{,2})}{\log(2)}$$

$$\Delta C_q = \frac{\lambda_1}{\eta_1}$$

Standard Curves



Standard Curves



real time PCR

What to remember?

- Logarithmic relation between C_q and copy number
- C_q values are approximately normal (copy numbers are log-normal)
- exponential accumulation of uncertainty
- Uncertainty of quantification strongly dependent on the quality calibration curve

digital PCR

Digital PCR

The Poisson Distribution

Definition

The Poisson Distribution Expresses the probability of a given number of events occurring in a **fixed interval** of time and/or space if these events occur with a constant **average rate** and **independently** of the time since the last event

dPCR Core idea

- fragment the reaction into a large number of (identical) reaction vessels
- the ratio of positive to negative vessels allows to estimate the actual concentration of targets

The Poisson Distribution

$$P(k) = e^{-\lambda}\frac{\lambda^k}{k!} \quad (2)$$

- k = number of events
- λ = average number of events per interval

Definition

applied to dPCR The event is amplification of a single target, the interval is the reaction vessel, and λ is the average number of targets per reaction vessel

Example

Example if there are on average 2 targets per vessel, the chance of having 2 targets in a vessel is $P(3) = e^{-2}\frac{2^3}{3!} \approx 0.18$

From counts to lambda

From counts to copies

- Using Poisson statistics we can convert the ratio of positive to negative vessels into the number of targets present
- It only works if you have **both** positive and negative vessels

$$P(k) = e^{-\lambda}\frac{\lambda^k}{k!}$$

$$P(0) = e^{-\lambda}$$

$$\hat{p}_0 = \frac{n_{neg}}{n_{tot}}$$

$$\hat{\lambda} = -\ln(\hat{p}_0)$$

From lambda to copy number

λ n_{tot} = number of targets in analysed

$\frac{Vol_i}{Vol_t}$ n_{tot} = fraction of sample analysed

$\frac{Vol_i}{Vol_t}$

λ = number of targets in the sample

$\frac{\lambda}{Vol_i}$ = concentration of targets

Reaction Versus Volume

- the more identical the volume of each reaction vessel is, the more reliable the result
- For BNQ quantification the percentage can be directly calculated from the λ of each reaction

Uncertainty

Uncertainty

Uncertainty in qPCR

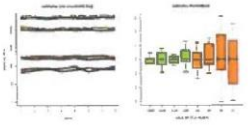
- C_q distribution is approximately normal, the confidence interval around the mean is 1.96 $\cdot$ δ
- The prediction of copy numbers from the calibration curve adds a level of uncertainty

$$s_{conc} = \frac{RSE}{\alpha}\sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(\bar{y}_0 - \bar{y})^2}{\beta_1^2 \sum_i (x_i - \bar{x})^2}} \quad (3)$$

where: RSE is the root mean square error, β_1 is the slope of the calibration curve, n is the number of source calibration points, N is the number of repeat measurements made on the sample (do not vary from sample to sample and can equal 1), $\bar{y}_0$ is the mean of N repeated measurements of y for the sample, $\bar{y}$ is the mean of the y values for the calibration standards, x_i is value on the x-axis, and $\bar{x}$ is the mean of the x values.

A confidence interval is obtained by multiplying s_{conc} by the 2-tailed Student t value for the appropriate level of confidence and $n - 2$ degrees of freedom

Uncertainty in qPCR



Uncertainty in dPCR

- the count distribution is **binomial**
- via the population proportion test we can derive a confidence interval

$$\delta_p = \sqrt{\frac{\hat{p}_0(1 - \hat{p}_0)}{n_{tot}}} \quad (4)$$

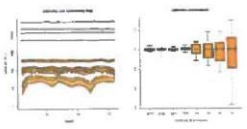
A confidence interval is obtained by multiplying δ_{count} by the 2-tailed z-score value for the appropriate level of confidence (i.e. 1.96 for the 95% confidence interval)

From this value we can then obtain confidence bounds on λ

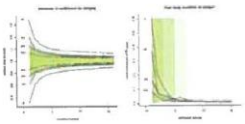
$$\lambda_{high} = -\ln(\hat{p}_0 - \delta_{high})$$

$$\lambda_{low} = -\ln(\hat{p}_0 + \delta_{low})$$

Uncertainty in dPCR

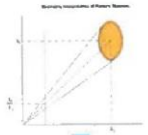


replicating dPCR reactions

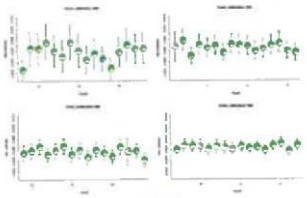


Ratio Confidence Intervals

Fieller's theorem allows the derivation of confidence intervals for arbitrary distributions



Ratio Uncertainty in ddPCR



Conclusions

Concluding remarks

- there is a large difference in error structure between dPCR and qPCR
- dPCR does not need a standard curve, and thus looses a source of variance
- replicating reactions is **always** useful
- Statistical calculations for dPCR are fairly straightforward compared to qPCR

References

- S Dube, J Qin, R Ramakrishnan. Mathematical Analysis of Copy Number Variation in a DNA sample using Digital PCR on a Nanofluidic Device. PLoS One. e2876. 2008.
- L.B. Pinheiro, V.A. Coleman, C.M. Hindson, J. Herrmann, B.J. Hindson, S. Bhat, K.R. Emslie. Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Calculation. Analytical Chemistry. **84**. 1003-1011. 2012.

Using dPCR data in support of GMO detection method validation

JRC The European Commission's in-house science service

www.jrc.ec.europa.eu

Elena Nardini
EU-RL GMFF team

Serving society
Stimulating innovation
Supporting legislation

dPCR applications

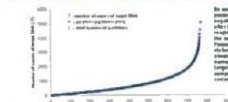
- dPCR is a method that works by *partitioning* the sample so that only a small number of DNA molecules of interest (targets) are present in each partition, with numerous reactions run in parallel. This partitioning happens either by dividing the sample *into chambers* (cdPCR) or *droplets* (ddPCR). Determining the starting number of template molecules in the sample (absolute measure) is possible by counting the *number of positive and negative reactions* at the end of the PCR.
- **cancer mutations, copy number variation analysis and rare event detection.**

Traditional *vs* digital real-time PCR (P. Corbisier)

Feature	Traditional real-time PCR	Digital PCR
Assay format	Single tube/well	Panel containing 765 individual partitions
Assay volume	5 - 50 µL	6 nL per partition (12.765 Array); 1.5 nL per partition (48.770 Array)
Dynamic range	Theoretically: 1-10[?] copies target DNA (dependent on signal)	1 - 4000 copies target DNA (concentration of DNA adjusted to ensure some partitions do not contain DNA)
Determination of unknown sample	Calibrant	Binomial approximation based on: the number of partitions containing amplified products, and the total number of partitions analysed

Binomial approximation

(Warren L.A. (2006) The digital array response curve)



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

Legal requirements

- **Reg. (EC) 1829/2003**: 0.9% labelling threshold (the measurement unit was not explicitly specified).
- **EU Recommendation 2004/787/EC** proposed that this should be expressed as the percentage of event-specific DNA copy numbers in relation to the target taxon-specific DNA copy numbers, calculated in terms of haploid genomes.
- Annex II of **Regulation (EU) No 619/2011** requires that "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 GMFF."
- Majority of certified reference materials (CRMs) are certified for *mass fraction*

dPCR at the EU-RL GMFF: Determination of GM-target to Reference Target Ratio via digital PCR

- In order to satisfy legal requirements, the EU-RL GMFF performs an assessment of the **zygosity** (GM-target to Reference-target ratio) in the positive control samples submitted by the applicant
- Quantification of the absolute number of targets that are present in a sample is achieved partitioning the PCR mix into a large number of separate reactions that contain zero, one or a few copies of the target nucleic acid.

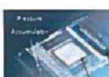
Zygosity

- the state of a cell or individual in regard to the alleles determining a specific character, whether identical (**homozygosity**) or different (**heterozygosity**)
- degree of similarity of the alleles for a trait in an organism. If both alleles of a diploid organism are the same, the organism is homozygous at that locus. If they are different, the organism is heterozygous at that locus. If one allele is missing, it is hemizygous

Steps

1. DNA extraction
2. DNA quantification
3. Conversion into genome copy number considering the size of DNA haploid genomes
4. Dilution of the DNA to obtain both positive/ negative partitions per panels
5. Loading the chip with IFC controller
6. Run of the digital PCR
7. Analysis of the results

Digital Arrays Chip: Integrated Fluidic Circuits



- 12 panels, each with 765 partitions (or chambers)
- One chip= 5 panels/chambers for GM event and 5 for Reference target
- 3 chips= in total 15 data sets for GM event and Reference target

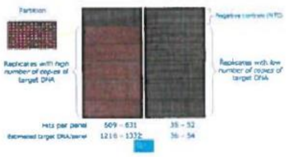
dPCR at the EU-RL GMFF

- Loading of digital chip according to the manufacturer's instructions: 4.6 µL/9 µL of reaction mix are distributed into the 765 partitions (or chambers) constituting one panel
- **Three runs:** five replicates of the same dilution are loaded in five panels for both the GM- and reference assay, with a total number of **fifteen data sets** for both targets amplification conditions of the Validated Method.
- Data analysis and copy number calculation is performed using the BioMark digital PCR Analysis software
- Range of Cq retention usually from 20 to 40
- Calculations of means and variances were carried out according to the **procedure outlined for random variables** in Annex 4 of the ENGL guidance document "Verification of analytical methods for GMO testing when implementing inter laboratory validated methods"

Example of Calculation of the Number of Targets/panel

- 12 panels per chip
- 765 partitions/panel
  - Between 100 and 700 positive partitions/panel
  - between 100 and 700 genome copies/panel
- Rapeseed haploid genome size: 1.15 pg
- 500 genome copies/panel = 1.15*500 = 575 pg (0.57 ng)
- Only around 45% of mastermix prepared is loaded on partition
  - Actual DNA amount needed in mastermix: 1.1 ng

Different panels saturation



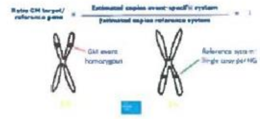
Reaction Mix Preparation

BioMark HD System, 12.765 digital arrays (Fluidigm)

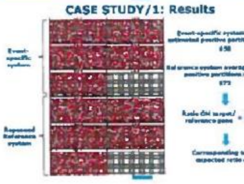
Genomic DNA (digested or NOT)	X ng (calculated as shown, 200<pos partitions<700)
TaqMan Universal PCR Master Mix	1X
Loading reagent	1X
Primers/probes	Concentration as in Validated Method
TOT volume	9 µL

CASE STUDY/1 determination of the ratio between GM and reference targets with digital PCR: HOMOZIGOUS GM event

$$\text{Ratio GM target/reference gene} = \frac{\text{Estimated copies event-specific system}}{\text{Estimated copies reference system}} = 1$$



CASE STUDY/1: Results



Event-specific system average estimated positive partitions/panel: 658

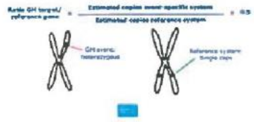
Reference system average estimated positive partitions/panel: 672

Ratio GM target/reference gene = 0.979

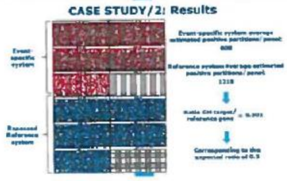
Corresponding to the expected ratio of 1

CASE STUDY/2: determination of the ratio between GM and reference targets with digital PCR: HETEROZIGOUS GM event

$$\text{Ratio GM target/reference gene} = \frac{\text{Estimated copies event-specific system}}{\text{Estimated copies reference system}} = 0.5$$



CASE STUDY/2: Results



Event-specific system average estimated positive partitions/panel: 608

Reference system average estimated positive partitions/panel: 1218

Ratio GM target/reference gene = 0.501

Corresponding to the expected ratio of 0.5

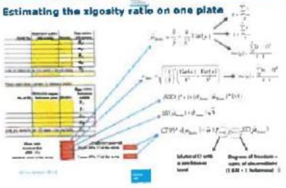
dPCR analysis conducted on a *cotton* GM event and reference targets in the positive control sample

Mean ratio (GM/Ref)	1.0
Standard deviation	0.085
RSDr (%)	8.5
Standard error of the mean	0.022
Upper 95% CI of the mean	1.1
Lower 95% CI of the mean	0.94

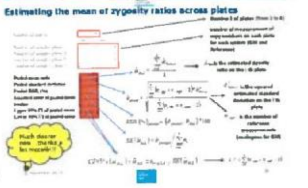
Our statistician will help to understand

GM % in DNA copy number ratio = GM % in mass fraction

Estimating the zigosity ratio on one plate



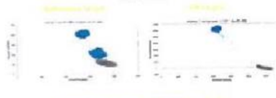
Estimating the mean of zygosity ratios across plates



Strange results and troubleshooting: cotton *Gossypium hirsutum*

Putative Cotton Reference amplicons

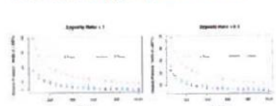
ddPCR



Remarks

- dPCR, through the assessment of the zygosity, allows the expression of measurement results on GM samples in DNA copy numbers or mass fraction
- Parental origin of GM traits can be identified: GM copy numbers in maize are influenced by parental contribution in endosperm tissue: male origin (33% GM), female origin (66% GM)
- Real-time PCR values are typically based on measurements relative to a calibration curve, necessitating the generation and availability of suitable reference materials which could exhibit matrix differences with the sample for evaluation

Precision and zygosity...a simulation



dPCR analysis conducted on a *rapeseed* GM event and reference targets in the positive control sample

Mean ratio (GM/Ref)	0.501
Standard deviation	0.036
RSDr (%)	7.2
Standard error of the mean	0.009
Upper 95% CI of the mean	0.521
Lower 95% CI of the mean	0.481

2 x GM % in DNA copy number ratio = GM % in mass fraction

dPCR analysis conducted on a *soybean* GM event and reference targets in the positive control sample

Mean ratio (GM/Ref)	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

Summary of dPCR analysis conducted on a *maize* GM event and reference targets in the positive control sample

Mean ratio (GM/Ref)	0.991
Standard deviation	0.067
RSDr (%)	6.8
Standard error of the mean	0.017
Upper 95% CI of the mean	1.028
Lower 95% CI of the mean	0.954

Conclusions

- Digital PCR enables quantification of target DNA (GM/reference ratio) without reference to a calibrant
- It avoids the bias of amplification efficiency between samples and reference material
- It reduces the bias of the sample matrix that is frequently observed with qPCR
- Measurement uncertainty can be reduced by
  - ensuring that the number of target molecules fall within an optimal range
  - increasing the number of partitions analysed
  - fragmenting the DNA into short segments containing the intact target DNA sequence

Some References

- Corbisier P *et al.* (2010). Anal. Bioanal Chem 396 2143
- Bhat S *et al.* (2009). Anal Bioanal Chem 394 457
- M. J. Burns *et al.* (2010). Eur Food Res Technol (2010) 231 353–362
- Whale AS, Cowen S, Foy CA, Huggett JF (2013) Methods for applying accurate digital PCR analysis on Low copy DNA samples PLoS ONE 8 e58177

Thanks!

Quantifying GMOs

EU-RL experiences with the digital PCR

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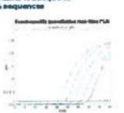
Stimulating innovation
Supporting legislation

Quantitative PCR is the golden standard technique to estimate the amount of target DNA sequences



In EU, compliance to labelling and to LLP requires to measure GMO concentration relative to the ingredient

Two standard curves are built with calibrators to determine the absolute number of GM target and of reference target

Further, the % GMO is determined

$$\%GMO = \frac{\text{GM copy number}}{\text{Reference copy number}} \times 100$$

Several factors are of difficult control and contribute to inaccuracies and uncertainties

Importantly, absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards using a calibration curve. It is therefore essential that the PCR of the sample and the standard have the same amplification efficiency

The amplified fluorescence signal from low amounts of target DNA molecules may not reach amplification to detectable or quantifiable levels

In the exercise of relative quantification the two amplification systems (GM- and reference-systems) can be affected by different efficiencies.

Digital PCR: overcoming critical factors of qPCR

The EU-RL study: to compare performance of qPCR and digital PCR (cdPCR and ddPCR) in GMO quantification

Technologies: qPCR, cdPCR, ddPCR

Materials: CRM 4.29% GA21 and CRM 4.9% NK603

The EU-RL study: to compare performance of qPCR and digital PCR (cdPCR and ddPCR) in GMO quantification

Experimental Design: DNA extracted from GA21 was mixed with DNA extracted from NK603 in order to produce a dilution curve in a constant amount of NK603 DNA

Technical challenges

The zigosity ratio

Zigosity tests carried out on NK603 and GA21 CRM to establish the ratio in copy number between the GM target and the respective reference system

Restricted NK603/HMG = 0.53

Restricted GA21/ADH1 = 0.61

These data are used to build the samples at different levels of GA21 in fixed NK603 maize DNA content

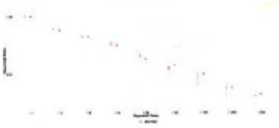
In practice, 100 ng of GA21 CRM 4.29% and 100 ng of NK603 4.9% contain 952 copies of GA21 and NK603 respectively

Sample preparation

qPCR results

qPCR results

qPCR results



The chamber digital PCR assay

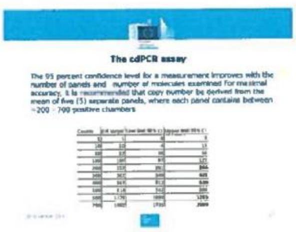
- In cdPCR a reduced amount of DNA can be loaded per reaction from 1 ng to few hundreds of nanograms but this has to be verified
- Preliminary tests to verify what is the amount of DNA to load per cdPCR
- Preliminary tests to verify whether to restrict DNA matters

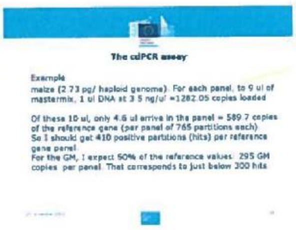
The cdPCR assay

Up to 97 ng of undigested DNA can be assayed per partition with the GA21 and ADH1 system but below 40 ng with the NK603 and HMG systems (not shown). We used maximum 40 ng DNA per sample

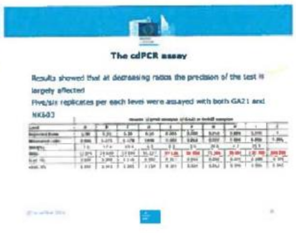
The cdPCR assay

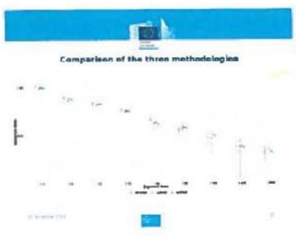
'sweet spot' of 200-500 positive partitions, acceptable between 200 and 700 positive partitions

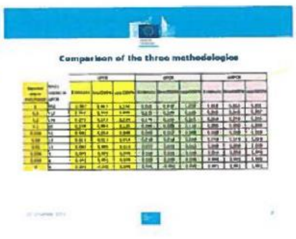

The cdPCR assay
The 95 percent confidence level for a measurement improves with the number of panels and number of molecules examined for maximal accuracy. It is recommended that copy number be derived from the mean of five (5) separate panels, where each panel contains between ~200 - 700 positive chambers


The cdPCR assay
Example
maize (2.73 pg/ haploid genome). For each panel, to 9 ul of mastermix, 1 ul DNA at 3.5 ng/ul =1282.05 copies loaded
Of these 10 ul, only 4.6 ul arrive in the panel = 589.7 copies of the reference gene (per panel of 765 partitions each)
So I should get 410 positive partitions (hits) per reference gene panel
For the GM, I expect 50% of the reference values: 295 GM copies per panel. That corresponds to just below 300 hits


The cdPCR assay
The raw data: an example

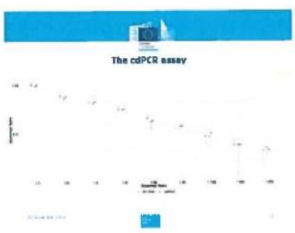

The cdPCR assay
Results showed that at decreasing ratios the precision of the test is largely affected
Five/six replicates per each level were assayed with both GA21 and NK603


Comparison of the three methodologies


Comparison of the three methodologies


Comparison of the three methodologies
The LOQ area

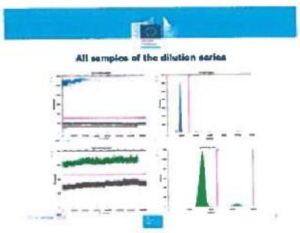

Comparison of the three methodologies
The LOD for qPCR is below 10 copies
The LOD for ddPCR

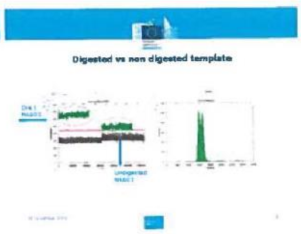

The cdPCR assay

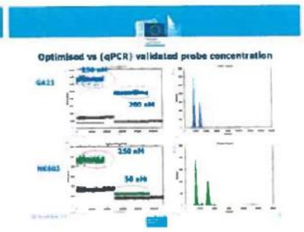

Droplet digital PCR assay
In ddPCR the relevant changes are in the
supermix (reaction buffer)
in the requested concentration of probe (around 250 nM)
the use of black quencher probe
Up to 300 nanograms can be loaded per reaction
easy protocol for DNA restriction

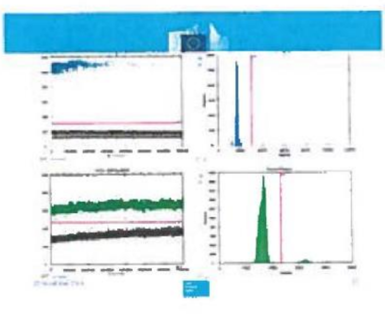

ddPCR assay
Descriptive statistics for droplets

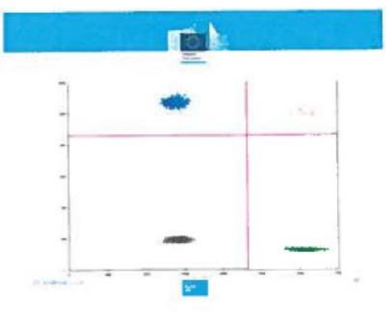

ddPCR assay
ddPCR

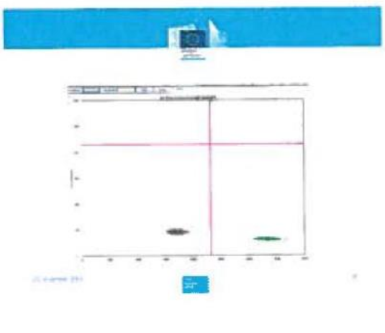

All samples of the dilution series


Digested vs non digested template


Optimised vs (qPCR) validated probe concentration
GA21
NK603
250 nM
50 nM







ANNEX 3. Documentation on digital PCR made available through weblink Links

to documentation on (chamber and droplet) digital PCR

Links to instruments:
http://www.bio-rad.com/it-it/applications-technologies/droplet-digital-pcr-ddpcrtechnology
http://www.fluidigm.com/biomark-hd-system.html
http://www.lifetechnologies.com/it/en/home/life-science/pcr/digital-pcr.html
http://raindancetech.com/digital-pcr-tech/

Links to some articles:
Milavec, M., Dobnik, D., Yang, L., Zhang, D., Gruden, K., Žel, J. (2014) GMO quantification: valuable experience and insights for the future. Anal. Bioanal. Chem. 406, 6485–6497
http://www.gene-quantification.de/milavec-et-al-gmo-digital-pcr-2014.pdf

Morisset, D., Štebih, D.S., 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
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3642186/pdf/pone.0062583.pdf

Li, L., Zhang, X., Wan, Y., Jin, W. (2013) Development of a Novel Reference Plasmid for Accurate Quantification of Genetically Modified Kefeng6 Rice DNA in Food and Feed Samples. BioMed Research International, Volume 2013, Article ID 134675, 7 pages http://dx.doi.org/10.1155/2013/134675
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3845723/pdf/BMRI2013-134675.pdf

Dube, S., Qin, J., Ramakrishnan, R. (2008) Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device. PLoS One 3(8), e2876; doi:10.1371/journal.pone.0002876

Pinheiro, L.B., Coleman, V.A., Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R. (2012) Evaluation of a Droplet Digital Polymerase Chain Reaction Format for DNA Copy Number Quantification. Anal. Chem. 84, 1003−1011; dx.doi.org/10.1021/ac202578x

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R.D., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., Bustin, S.A. (2013) The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin. Chem. 59, 892–902

Corbisier, P., Bhat, S., Partis, L., Rui, V., Xie, D., Emslie, K.R. (2010) Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction (2013) Anal. Bioanal. Chem. 396, 2143–2150; DOI 10.1007/s00216-009-3200-3

ANNEX 4. Details on the social dinners provided

Ristorante Il Melograno

19 Novembre 2014

Antipastino Misto Melograno
mixed appetizer Melograno

Risotto Con Zucca Mantovana Orecchiette Fresche Alle Cime Di Rapa
Bis of rice with pumpkin and fresh orecchiette with turnip tops

Aletta Di Vitello Arrosto Con Funghi e Patate Al Forno
Roasted Veal with mushrooms and baked potatoes

Sorbetto Al Limone
Lemon sorbet

Vino, Acqua, Caffè
Wine, Water,
Coffee

Pizzeria Il Capriccio

20 Novembre 2014

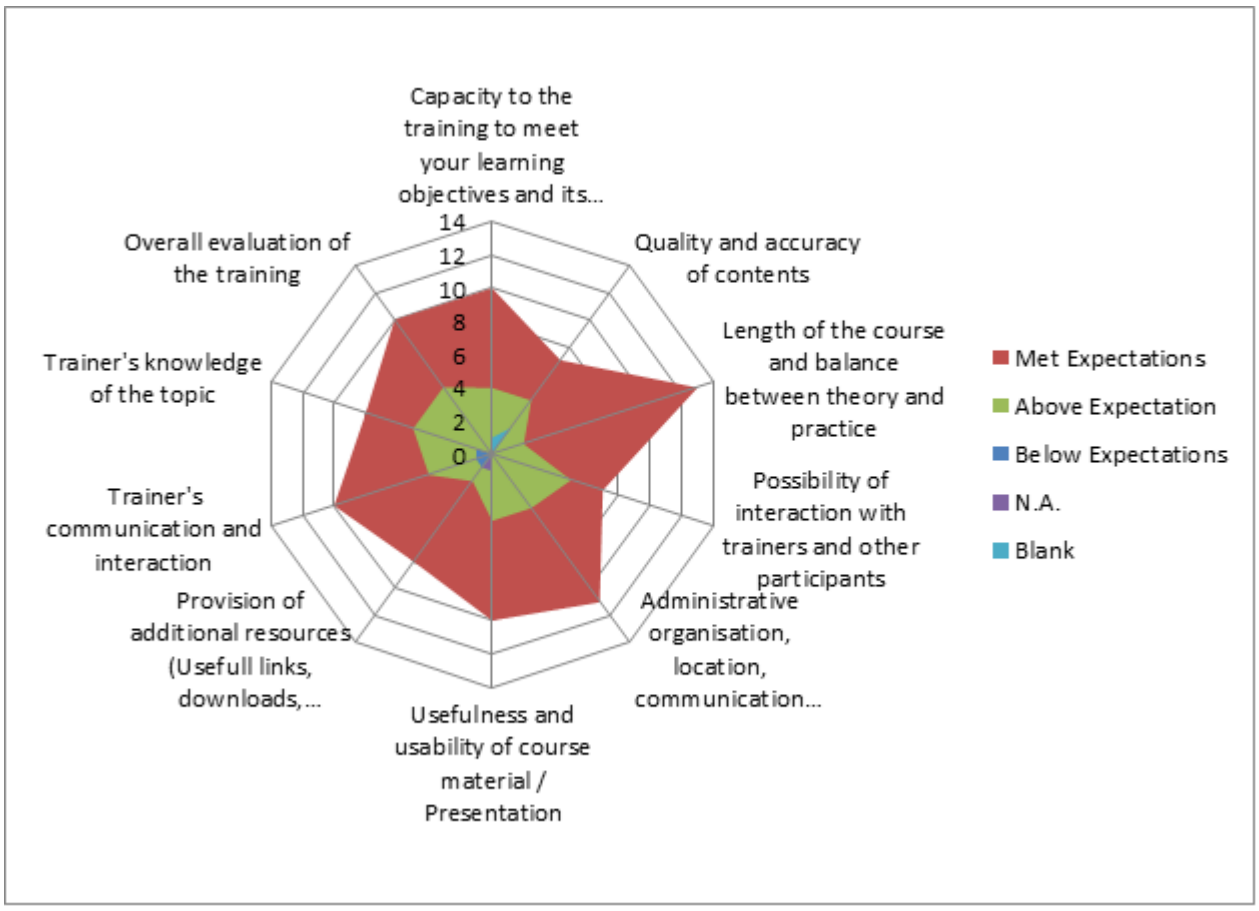
ANTIPASTI
salumi misti, tris di formaggi, olive marinate, verdure alla zingara, focaccia all'origano
Mixed appetizer

GIROPIZZA
Tell the chef which pizza you would like to taste

DOLCE A SCELTA
Dessert at your choice

Acqua, Vino, Birra, Bibite E Caffè
Water, Wine, Beer and Coffee

ANNEX 5. Feedback results



Additional feedback: Availability of paper copy of presentations: I find it useful, but depends on authorization by external speakers. Maybe serve lunch in block 20 as more room which would allow capacity to interact with fellow delegated & networking.