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DIGITAL PCR FOR GENOTYPE QUANTIFICATION: A CASE STUDY IN A PASTA PRODUCTION CHAIN

Document prepared by an expert from Italy

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Digital polymerase chain reaction (dPCR) is a breakthrough technology able to provide an absolute quantification of the target sequence through the compartmentalization of the sample and independent amplifications of the numerous separate compartments. Such technology has recently found several applications in plant science; however, to the best of our knowledge, it has never been applied until now for the detection and quantification of a specific plant variety along a production chain. As proof of concept, a dPCR assay targeted to the quantification of a durum wheat variety routinely used in an Italian premium pasta. The actual applicability of this analytical technique to quantify the presence of a specific plant genotype, in both raw materials and transformed products, by exploiting a point polymorphism has been evaluated. As proof of confirmation, an Italian premium pasta production chain was considered and a dPCR assay based on a durum wheat target variety private point mutation was designed and evaluated in supply-chain samples. From the results obtained, the assay can be applied to confirm the presence of a target variety and to quantify it in raw materials and transformed products, such as commercial grain lots and pasta. The performance, costs, and applicability of the assay has been compared to analytical alternatives, namely simple sequence repeats (SSRs) and genotype-by-sequencing based on Diversity Arrays Technology sequencing (DArTseqTM).

The Annex to this document contains a copy of a presentation on “Digital PCR for Genotype Quantification: A Case Study in a Pasta Production Chain”, prepared by an expert from Italy, to be made at the first session of the TWM.

[Annex follows]

Digital PCR for Genotype Quantification: A Case Study in a Pasta Production Chain

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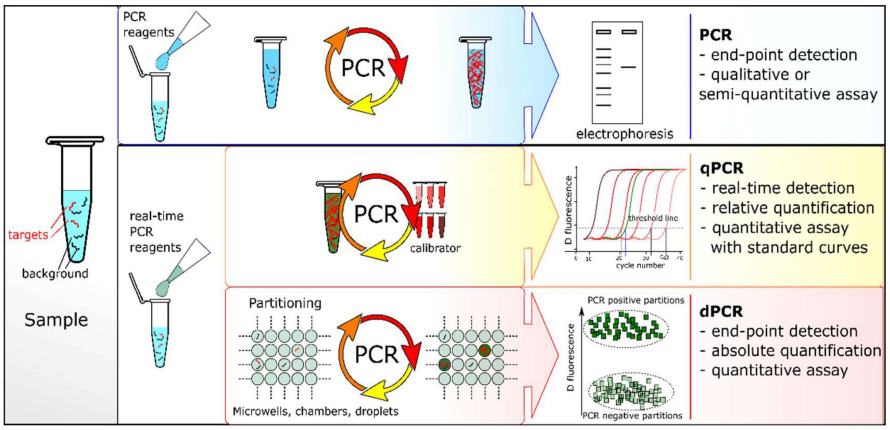
UPOV Technical Working Party on Testing Methods and Techniques, September 19-23, 2022.

Outlook

- Short description of the technique
- Overview of the fields of application in plant science
- Is Digital PCR a useful tool for Genotype Quantification?
- Case study presentation

Short description of the technique

Digital PCR is a third-generation technology



Sensors **2018**, *18*(4), 1271; https://doi.org/10.3390/s18041271

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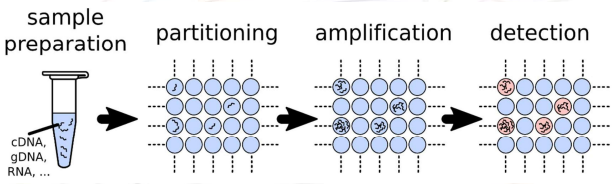
Short description of the technique

Principle

Compartmentation of the samples and independent amplification of one or few DNA fragments contained in the numerous separate compartments

- End point detection and counting of the fluorescent partition (target DNA) based on Poisson distribution

Absolute quantification of the target sequence



Sensors **2018**, *18*(4), 1271; https://doi.org/10.3390/s18041271

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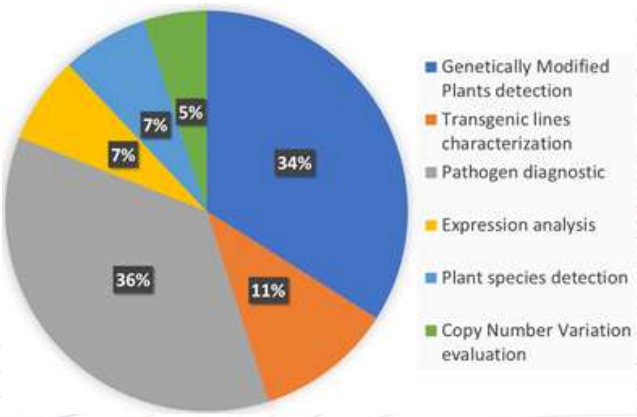
Plant studies: field of application

Data from the literature of the last five years, 81 papers published in the peer-reviewed international journals presenting the development and application of Digital PCR protocols



Biology 2020, 9(12), 433; https://doi.org/10.3390/biology9120433

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Case study presentation

1. Variety identification and traceability are key issues for the evaluation of heterogeneous materials
2. digital PCR potentialities for the rapid description and traceability of genotype content in wheat autogamous landraces, composite crosses, and mixtures are evaluated starting from the "simplified model" of pasta supply chain

Biology **2021**, *10*(5), 419; https://doi.org/10.3390/biology10050419

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Variety traceability in pasta supply chain

- wide variability durum wheat cultivars from the technological and qualitative points of view
- some cultivars are legacies of the past, linked to traditional uses, including bread and pasta
- commercial interest in bread and pasta made from a single variety or with a prevalence of one or few specific varieties

Interest in the tracking of specific genotypes along the supply chain, from seeds to grains and transformed products.

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Variety traceability in pasta supply chain

DNA-based technologies have been evaluated for plant-variety protection and registration and for wheat genetic-diversity estimation

- Simple sequence repeats (SSRs),
- SNP array
- DArTseq

Useful for varietal fingerprinting but

time consuming in a routine work to track one or more specific genotypes

Digital PCR could be a solution?

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Study Workflow

- Establishment of a **working collection** of 28 durum wheat varieties cultivated in Italian environments.
- Selection of a durum wheat target variety (**TV**) protected by requirements of sensitive industrial data.
- Genotype-by-sequencing through DArTseq analysis of the DNAs extracted from certified seeds of **TV** and **working collection.**

Establishment of an SNPs profile database.

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Study Workflow

- Screening SNP database by custom R script directed to the identification of a **private allele** of the target variety (**TV**).
- Development of a **chip digital PCR assay** designed **for such private** allele to identify and quantify the target variety.

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Development of a dPCR assay chip

- A G/T polymorphism was identified on SNP 12876 localized on the 7A chromosome of durum wheat. The homeologous chromosome 7B holds a monomorphic G/G locus.
- The **T** allele present only in the **Target Variety** was marked with **FAM** whereas the alternative, **NON-Target Varieties allele G** was marked with **VIC.**

Expected genotype		
Crh	**Target Var. Genotipe**	**Non Target Var. Genotipe**
7A	FAM-T	VIC-G
7B	VIC-G	VIC-G
Dye/genotype combination	FAM/VIC	VIC/VIC

- Primer and probes were designed on SNP 12786 sequence (Taqman® SNP assay procedure). A chip digital PCR was performed on Quant studio™
- End-point fluorescence data were elaborated according to the equipment's software. Each sample was analyzed in triplicate.

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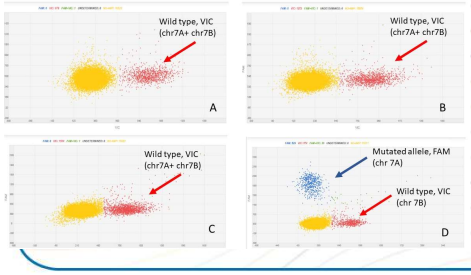
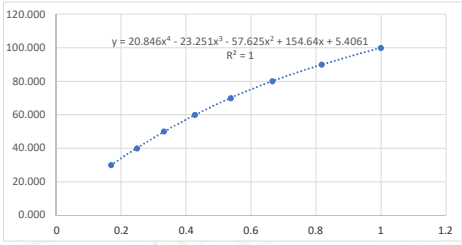
Digital PCR results

Two-dimensional scatter graphs obtained by chip digital PCR analysis of DNAs from the durum wheat varieties.



Theoretical correlation between TV percentage and FAM/VIC ratio.



Actual TV % in flour	Mean TV % in flour	Std Dev	Absolute error	Relative error
100%	96.6	0	3.4	0.03
90%	90.9	0.07	0.95	0.01
80%	84.22	0.47	4.2	0.05
70%	70.3	0.56	0.3	0.004
60%	55.7	2.48	4.25	0.07
50%	48.7	1.63	1.25	0.025
40%	39.7	2.62	0.25	0.006
30%	31.4	1.84	1.4	0.04
20%	26.1	0.92	6.15	0.3
0%	0	-	-	-

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SSR fingerprinting

- 14 SSR markers were used as described in the ISTA Rules 2021 *International for Seed Testing association* for wheat varieties divided into two multiplex PCR reactions.
- Each SSR forward primer was labeled with a fluorescent dye on the 5' ends. The PCR products were separated by capillary electrophoresis on the 3500 Genetic Analyzer
- For each fragment, size (bp), height, and peak area were measured by v5 GeneMapper software (Applied Biosystems by Life Technologies, Monza, Italy).
- TV is quantified based on the relative quantities of its specific allelic fragment vs. all amplified alleles for the concerned locus, in terms of peak area.
- The estimated percentage was calculated as the average of the relative Pick Area value obtained from the polymorphic loci.

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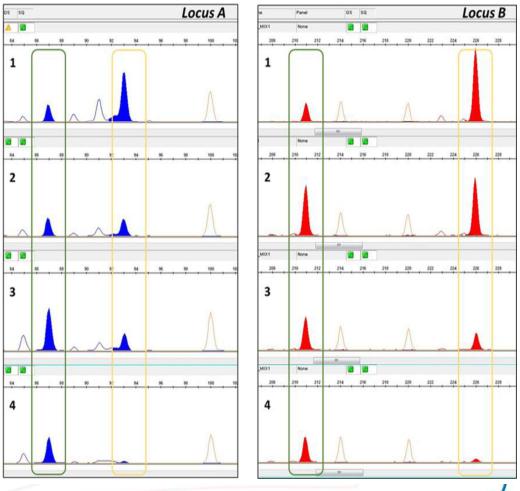
SSR Results

- The **green line** highlights the **TV alleles**, while the **yellow line** highlights the **Odisseo alleles**.
- Plots of Locus A and Locus B from 1 to 4 show the fragment obtained from the pasta samples:

1- TV 20%–Odisseo 80%,
2- TV 50%–Odisseo 50%;
3- TV 70%–Odisseo 30%;
4- TV 90%–Odisseo 10%).

Electropherograms showing amplicons at two polymorphic loci between the **TV** and **Odisseo**.



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DArTseq analysis

- DNAs extracted from 15 single seeds of five commercial grain samples, and 15 single seeds sampled from the Target Variety certified seed lots, were sequenced (http://www.diversityarrays.com), and SNP marker identification was done by DArTseq genotyping.
- The data were filtered to include only SNP markers with NA <5% and MAF >5%. The final data set included 6249 SNPs. The final data set included 6249 SNPs.
- Euclidean genetic distances were calculated between each pair of samples and further used in clustering analysis (R stats: hclust, method = "average").

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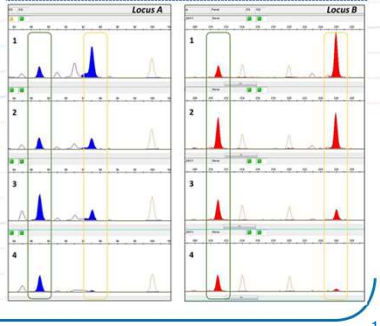
Digital PCR validation on Reference Pasta and comparison with SSR

- Certified seed samples of the Target Variety were genotyped using the 14 SSR (ISTA Method).
- TV and Odisseo showed two different polymorphic alleles at two SSR Loci, considered "specific marker alleles"
- 4 reference Pasta samples obtained by mixing TV and Odisseo were genotyped with the SSR technique and were screened at the two Loci with the "specific marker alleles".
- The percentage of the two varieties was calculated as the average of the relative Pick Area value obtained from the polymorphic loci

Sample	dPCR	DArTseq	SSR
Working collection of certified seeds	+	+	+
Pasta 100% TV	+	+	+
Pasta 90% TV	+	+	+
Pasta 70% TV	+	+	+
Pasta 50% TV	+	+	+
Pasta 20% TV	+	+	+
Grain commercial lots	+	+	+



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Results

The same reference pasta samples were analyzed with dPCR.

Actual TV % in pasta	Mean TV % in flour (dPCR)	Std Dev	Absolute error	Relative error
90%	88.7	1.34	1.25	0.01
70%	63.4	2.69	6.6	0.09
50%	48.4	2.05	1.55	0.03
20%	26.1	0.92	6.15	0.31
Actual TV % in pasta	**Mean TV % in flour (SSR)**	**Std Dev**	**Absolute error**	**Relative error**
90%	89	0,02	1	0.01
70%	66	0,01	4	0.06
50%	49	0,03	1	0.02
20%	20.5	0,01	0.5	0.025

dPCR: The Pearson's r between the actual and the experimentally determined percentages in pasta samples were of 0.991.

SSR: The Pearson's r between the actual and the experimentally determined percentages in the pasta samples was 0.998 with SSR analysis

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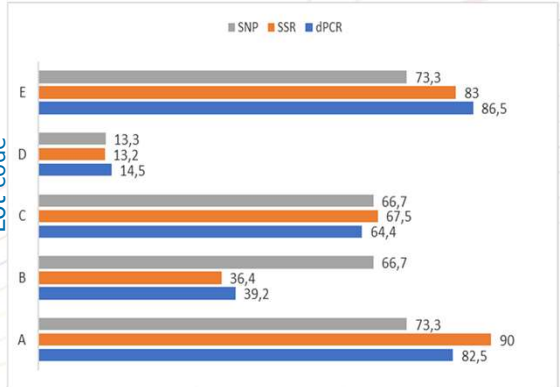
Evaluation of commercial seed lots

Commercial seed lots of the Target variety suspected to be contaminated by non-Target variety were evaluated. The quantification of Target variety was performed by the dPCR, SSR, DArTseq

Pearson's value
- dPCR vs SSR 0,991
- dPCRvs GBS 0.852
- SSR vs GBS 0,834

GBS analysis seems to overestimate the TV percentage in commercial lot B.



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Conclusion

- dPCR technique has been demonstrated to be usefully applied for varietal quantification not only in grains and flours but also in processed products, in our case pasta.

- SSR varietal fingerprinting, the CE (capillary electrophoresis) quantification method could give results close to those obtained with more innovative technologies. The quantification is the result of the qualitative-quantitative evaluation of the total polymorphic alleles obtained

- Lower correlations among SNP-based results and dPCR or SSR based ones in commercial lots were observed, in comparison with the very high correlations found between dPCR and SSR data on the same samples. On the other hand, SNP based analysis is the key step for the identification of private alleles needed for dPCR assay development

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Conclusion

Starting from the pilot work developed here, it can be said that the dPCR has a useful role in verifying and confirming the genetic traceability from seed to agro-food products.

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biology

MDPI

Article

Digital PCR for Genotype Quantification: A Case Study in a Pasta Production Chain

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