Feasibility and optimization of ddPCR for gene copy number quantification in large gene families: Case study of α-gliadin in hexaploid wheat

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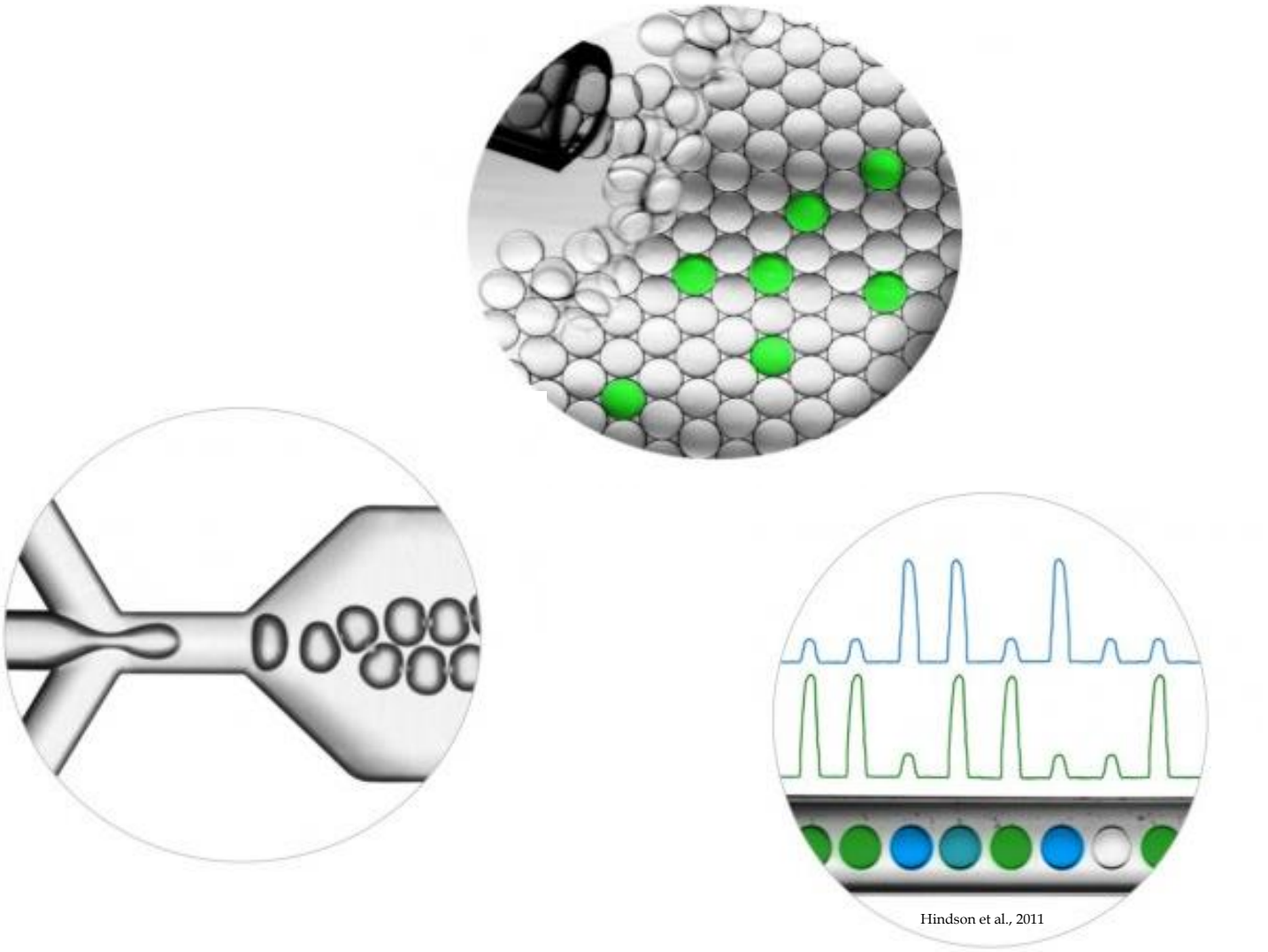

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Abstract

Droplet digital PCR (ddPCR) is a novel technique able to accurately quantify target nucleic acids. This technique has been proposed as a rapid and cost-efficient tool for high-throughput copy number variation (CNV) screenings, which is a vital step in the genetic analysis of large gene families. This approach is also used to screen mutant populations for gene editing. However, the sensitivity of ddPCR for gene copy number quantification and assessment of edited gene copy number in large families in complex polyploid organisms has yet to be shown.

Hexaploid wheat and the α-gliadin gene family were used as a case study to validate this technique. To prove the feasibility and accuracy of the approach, three synthetic hexaploid wheat lines and their respective parental lines were analysed. In addition, a set of deletion and nullisomic-tetrasomic lines from 'Chinese Spring' cultivar was studied and compared. Afterwards, the technique was validated by analysing large mutations in 'Paragon' gamma-irradiated lines. As a direct application of the technique, several α-gliadin targeted CRISPR/Cas9 lines in cultivar 'Fielder' were analysed. Here, it has been described in detail the validation and optimization of ddPCR for CNV estimation in hexaploid wheat.

Using the results of the synthetic and 'Chinese Spring' mutant lines as a proof of concept, the feasibility and reliability of ddPCR to quantify gene copy number was confirmed. Gamma-irradiated lines proved the possibility of using ddPCR to detect deletions and served as a direct validation of the technique. Lastly, analysis of CRISPR/Cas9 allowed not only to screen for α-gliadin CNV but also to discriminate small and large mutations. Here, feasibility and reliability of ddPCR has been proven in hexaploid wheat, opening the possibility to be adapted for genetic analysis in other polyploid organisms.

Keywords: **CNV, ddPCR, CRISPR/Cas9, polyploid, optimization**

Introduction

Gene copy number variation (CNV) research has gained considerable interest in the last decade as a type of genetic variation. However, current high-throughput CNV screenings approaches are very limited. Copy number is usually estimated either by Southern Blot hybridization analyses or quantitative polymerase chain reaction (qPCR) (Collier *et al*., 2017). However, these techniques are not reliable enough or too tedious to be used in high-throughput CNV screenings. Currently, qPCR is the predominant method to detect CNV in large populations despite having a low accuracy (Karlen *et al*., 2007) and requiring many replicates to achieve fine discrimination (Weaver *et al*., 2010). Southern blot analysis is more reliable, but time consuming, expensive and, due to the limited gel resolution, not reliable to estimate copy numbers greater than 20 (Collier *et al*., 2017). New methods for a reliable and efficient method to measure gene copy number have been of great interest in the last decades. Digital Polymerase chain reaction (dPCR) is a novel technique more sensible and precise than real-time PCR and has been suggested as a reliable alternative to absolute or relative gene copy number quantification (Hindson *et al*., 2011, Manoj 2016).

In dPCR, a certain amount of target DNA is distributed across multiple replicated reactions and relies on a binary end threshold. One of the techniques developed to split the samples in multiple partitions is the droplet digital PCR (ddPCR). This technique generates water-in-oil droplets as replicates and allows the use of high throughput dPCR in a practical and low-cost format (Hindson *et al*., 2011). Recently, feasibility of ddPCR for gene editing detection has been reported in the tetraploid *Medicago sativa* (Gao *et al*., 2018). However, due to the novelty of this technique, its feasibility has yet to be proven on organisms with simultaneously higher levels of ploidy and large number of gene copies. In this project, hexaploid wheat and the α-gliadin gene family were utilized as the case study to establish the feasibility of ddPCR for high-throughput screening of large populations.

Hexaploid wheat (*Triticum aestivum*, 2n = 6x = 42, AABBDD) is a staple food crop which belongs to a special group called gluten-containing grains. Gluten includes two major storage protein families, the glutenins and the gliadins. Both are in the grain endosperm and are important for several food industrial applications. However, exposure to these proteins may cause several diseases which affect a considerable part of the global population, of which Coeliac disease (CD) is the most common. CD is an autoimmune reaction triggered by the ingestion of gluten proteins in humans and it affects around 1-2 % of the population (Koning, 2012). This reaction produces a chronic inflammation of the small intestine, leading to other symptoms like bowel disorders and malnutrition. Gluten proteins contain immunogenic epitopes which are responsible for the reaction. These epitopes are recognised by antigen presenting cells (APC) and T cells, triggering the immune reaction (Koning, 2012). Gliadins are divided into three gene families: α-, γ- or ω- gliadins, among which α-gliadins are the most important due to its high immunogenicity (Lammers *et al*., 2014). In wheat, γ- and ω-gliadins are encoded by Gli-1 and Gli-3 loci respectively, located on the short arm of homoeologous chromosomes 1. The α-gliadin gene family is encoded by the Gli-2 loci located on the short arm of homoeologous chromosome 6 (Payne, 1987). In these loci, it is estimated that approximately 100 gene copies are present in *T. aestivum* (Ozuna *et al*., 2015).

Since following a gluten-free diet is relatively hard due to the high amounts of wheat derived products on the market (Atchison *et al*., 2010), other approaches aim to develop gluten-safe products with reduced immunogenicity, allowing their consumption by CD patients. Gluten immunogenicity is mainly caused by a small part of the protein, the epitope region. Therefore, mutation breeding became an interesting approach to eliminate or modify this region without affecting the rest of the protein, which is crucial to develop gluten-safe wheat variety while maintaining the properties of the protein. This study focusses on two mutant populations: (a) 'Paragon' gamma-irradiated population and (b) 'Fielder' mutant lines generated by CRISPR/Cas9 targeting α-gliadin genes. Gamma-irradiation causes oxidative DNA lesions and double strand breaks (DSB) which usually lead to large deletions (Morita

et al., 2009). CRISPR/Cas9 generates targeted DSB and the repair mechanisms may produce errors, which eventually might lead to random substitutions, inversions and small or large deletions. This technique has previously been used to generate small indels and/or large deletions in different gliadin gene variants. Nowadays, high-throughput screening is the major bottleneck in gluten-safe wheat development. ddPCR has been proposed for the screening of mutation lines by quantifying the relative number of α-gliadin gene copies per line. Thus, there is great interest on developing and optimizing ddPCR as a reliable high-throughput screening method, not only to estimate the initial α-gliadin gene copy number but also to evaluate the number of intact gene copies post-mutations. These two approaches aim to reduce the immunogenicity of wheat without affecting the quality of the product. Mutating the epitope region without affecting the rest of the protein is crucial to maintain the properties of the protein.

Using the α-gliadin gene family in hexaploid wheat case study, the accuracy and reliability of ddPCR technique will be proven in two dimensions: (a) estimation of the absolute gene copy number in a large family of genes in complex polyploid organism and (b) estimation of the relative number of mutated or deleted copies of the targeted genes. However, setting up this method in this complex scenario requires optimization. CS mutant lines were studied as a proof of concept to validate the feasibility of this approach. Afterwards, several Paragon gamma-irradiated lines and Fielder CRISPR/Cas9 lines were analysed to validate and as a direct application of the technique. This report contains a detailed description of the design, optimization, validation and application of ddPCR for the relative quantification of α-gliadin CNV in hexaploid wheat.

Optimization

In the last decade, ddPCR has been suggested as a rapid, accurate and efficient method for high-throughput validation of gene CNV (Hidson *et al*., 2011). However, it is still a novel technique that has never been applied to estimate the copy number or the editing efficiency of a large family of genes in polyploid organisms. Therefore, it is crucial to establish an optimal protocol prior to using ddPCR for experimental approaches in these conditions. As a new level of complexity is added to the ddPCR assay by multiplexing targets, several trials were conducted to set the optimum conditions, improving the reliability, accuracy and output visualization of the method.

As in qPCR, ddPCR is based on target amplification using probes or primers with fluorescent dye. However, the power of ddPCR relies on the partition of the targets into droplets, enabling multiple simultaneous PCR reactions per sample and increasing the reliability and precision of the quantification. To ensure that only one copy of the target gene is present per reaction, restriction enzymes are used to digest and separate the multiple copies. In the first step of the ddPCR workflow (Figure 1), the PCR reaction and droplet generation oil are loaded into individual wells of an injection moulding cartridge. Around 20.000 droplets are generated in each sample due to the emulsion created by a vacuum. Afterwards, the samples are transferred into a thermocycler to undergo a standard PCR. Each droplet constitutes a partition or replicate and a PCR reaction will occur simultaneously in all the droplets containing the target DNA. Some of those partitions containing a copy of the template and others belonging to the non-template replicates. After the amplification, samples are transferred to the reader. Here, droplets are aspirated, separated and aligned toward the detector. Imperfect quenching produces an intrinsic fluorescence in the droplet, allowing to detect the droplets without template. These droplets form the background which is essential to estimate the concentration of the target. The positive droplet generates a strong fluorescence signal that is detected by the reader, establishing a threshold between them and the negative droplets. The concentration is determined by measuring the number of fluorescently positive and negative droplets in the sample. The random distribution of the templates across the droplets allows to correct and calculate the confidence limits of the concentration using Poisson statistics (Hindson *et al*., 2011). To calculate the CNV, the concentration of the target must be compared to the concentration of another target whose number of copies in the genome is previously known. In this study, the gene TaPFT1, an orthologue of the flowering regulatory and disease susceptibility gene PFT1 in *Arabidopsis*, which is present once per haploid genome (Fitzgerald *et al*., 2010) was used as an internal reference.

Droplet fluorescence can be provided by fluorogenic probes or by DNA-Binding dye chemistries. Probes have been widely used in the last decade, namely FAM, VIC and HEX, and allow discrimination of the target by the signal emitted at different wavelength. Usage of Eva Green as a dye has also been proven to be as reliable as using probes, maintaining the advantages of the practicality of designing primer pairs for standard PCR (McDermott *et al*., 2013). Since there is only one dye used, several strategies can be followed to separate the amplitude intensity of the signal of different targets.

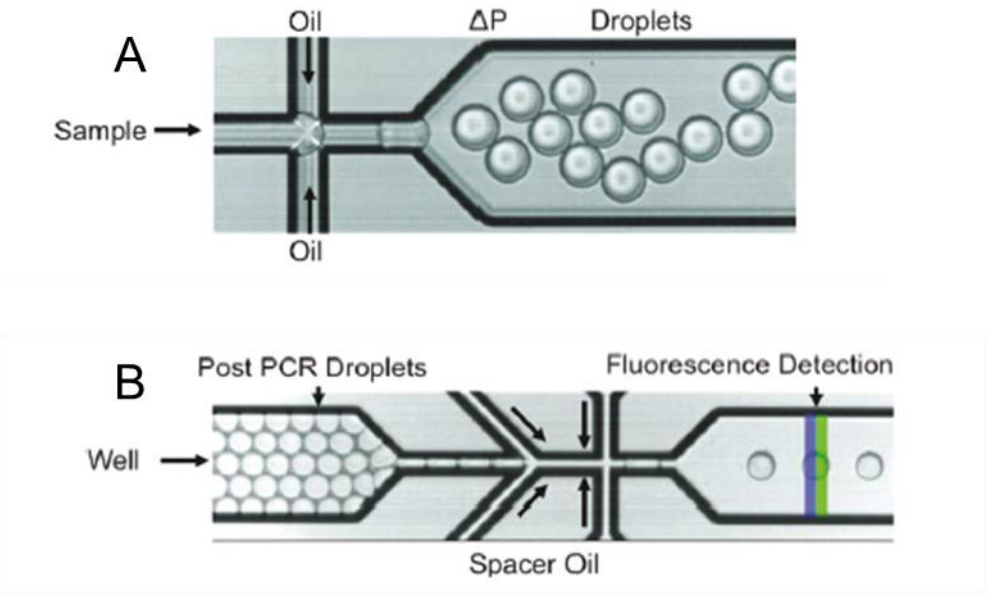

Figure 1. Main steps of droplet digital PCR workflow. (A) Droplet generation: A vacuum is applied to the well, aspirating the sample and the oil and generating around 20.000 surfactant-stabilized droplets. (B) Read droplet fluorescence: After PCR, droplets are extracted from each well and individually streamed through a fluorescence detector. Adapted from Hindson et al.*, 2011.*

DNA CONCENTRATION

DNA input optimization is crucial for the reliability and accuracy of the assay. At least one genomic copy of the reference gene is required for the PCR reaction. A very low DNA input hampers the visualization of the output. Additionally, the sample will be saturated if the DNA input is too high, not only deteriorating the visualization of both populations, but also reducing the number of negative droplets, which are necessary to conduct statistical analysis. Several studies were already performed, recommending an amount of input depending on an approximate number of copies of the gene expected in the sample. This point is called "sweet spot" and has been determined to be reached with 100ng DNA, representing approximately 1,500 target gene copies per assay in human genome (Bio-Rad, 2018). In the case of α-gliadin genes, which copy number is estimated to be around 100 (Ozuna *et al*., 2015), which is much higher than the number of copies found in human. Therefore, a lower amount of DNA is necessary to avoid saturation. A range of DNA input between 2 and 50 ng per assay was tested. Two criteria were analysed to evaluate the optimum: (a) there are enough gene copies of the reference to enable a correct visualization of the output and (b) the maximum number of gene copies were covered. It was possible to distinguish both targets in all the concentrations tested, but extremely low or high DNA concentration hampered the visualization of the output. According to the number of CNVs measured, the optimum range of DNA input was established between 10 and 30 ng per assay (Table 1). Due to the low concentration of some of the available samples, consistency was maintained by selecting a DNA input of 10 ng.

Table 1. Comparison of measured gene copy number at different amounts of DNA input.

DNA (ng)	***CNV***
50	50.9
40	54.3
30	58.2
20	61.5
10	58.2
5	56
2	41

PRIMER SELECTION – TARGET MULTIPLEX VS SINGLEPLEX

This study focused on multiplexing targets, which refers to the analysis of two or more targets in the same sample. This approach is, despite the longer optimization and preparation time, more accurate and cost efficient than singleplex assays. To discriminate between two or more targets in the output it is necessary to separate them by the fluorescence amplitude. Multiplexing experiments are usually performed using different probes to separate targets. In this case, different fluorophores bound to each probe provide different lengths for each target, allowing their separation However, many requirements need to be fulfilled to design these probes (Bio-rad. 2018). Due to the high variability of the gene sequences within α-gliadin family, the use of probes was not feasible in this study.

Besides probes, ddPCR offers another alternative to distinguish multiplexed targets that involves the use of DNA binding dye chemistry, giving more flexibility to the design of the assays. Using this alternative, modifying the primer concentration from the different targets is one of the possibilities to perform target multiplexing. This strategy is based on the separation of the target amplitude signals by modifying the concentration of each primer pair in the reaction (McDermott *et al*., 2013). By increasing the concentration of one of the primer pairs the amplitude of the correspondent target signal will also increase, allowing distinction of each target in the output. Nevertheless, this strategy was not optimal since an increase on the concentration of the primer would also lead to an increase of the amplitude of the background, which might overlap with the signal of one of the positive targets due to their size variability. A second approach of single-dye ddPCR multiplexing is based on varying the annealing temperature of the different primer targets. The output of the primers designed with a lower annealing temperature provides a lower fluorescence, separating the population (Bio-Rad, 2018) However, the rigidity to design primers due to the high variation of the sequences restrained the use of this method. The last method is based on the higher signal of the amplitude given by a longer amplicon. The fluorescence of the positive droplets is increased with amplicon length since more dye molecules bind the same target (McDermott *et al*., 2013). Amplicons with different length give distinct amplitude signals, allowing the differentiation of two or more targets in a single reaction. Primers were designed following this method. However, a proper optimization is still required to ensure enough signal discrimination between the targets. A reference target gene whose number of copies is already known is necessary to compare and allow the quantification of the number of copies of the actual target gene. In this study, the PFT1 gene was chosen as only a single copy is present on each chromosome 5 (Fitzgerald *et al*., 2010). Once the concentrations of the target gene and the reference are measured, the copy number of the unknown target is calculated by comparing the concentration of the target to the concentration and previously known copy number of the reference gene.

A maximum of one copy of the target gene must be present in each droplet for accurate copy number determination. Since digestion is recommended to reduce viscosity and to separate the α-gliadin genes located in the same locus (Bio-Rad, 2018), ApoI restriction enzyme was used for fragmenting DNA. ApoI restriction site is present in the conserved part of α-gliadins, which ensure the separation of the copies but is not located within the epitope region, or at the primer sites. Additionally, three pair of primers were designed to generate amplicons of three different sizes, two on the target gene and one in the reference PFT1. In the α-gliadin genes one primer pair (EPI) was designed to amplify the part of the epitope region aligned with the PAM in the CRISPR/Cas9 experiments (Figure 2). This primer was designed to be able to distinguish between small mutations produced after the PAM. The second pair (NRD) was designed downstream the epitope region to be able to detect large deletions downstream the PAM. A third primer pair (REF) amplifies a conserved region of the PFT1 gene, the control gene located in the fifth chromosome.

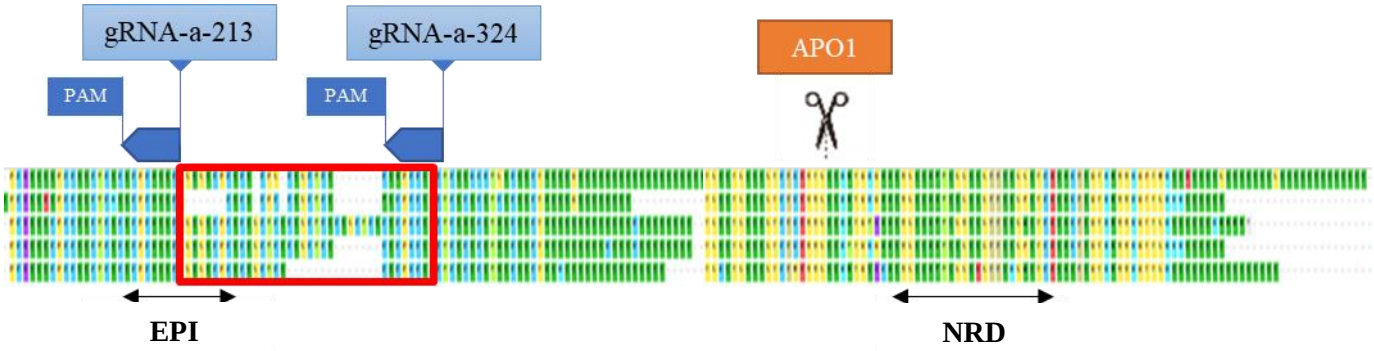

Figure 2. Fragment of the amino acid sequence of α-gliadin (90-600 nt). Location of the epitope region (framed) and the two primer pairs used (EPI and NRD). Position of the gRNA complementary sequences (blue) from the CRISPR/Cas9 experiments.

For the primer design two criteria were followed. First, ddPCR requires amplicon lengths between 60 and 200 bps. Afterwards, a difference of 60 bps is required between the products of EPI, NRD and REF to separate the populations (Bio-Rad, 2018). Following these criteria and adapting to the sequences given, primers lengths of 61 bps, 136 bps and 181 bps respectively were designed. Due to the variability in the α-gliadin family of genes, it was not possible to multiplex three targets together and manually discriminate each target signal population (Figure 3). However, it was possible to distinguish each population when multiplexing the reference with only one of the unknown targets. Therefore, duplexing the reference target and one of the actual targets was the approach used in this study.

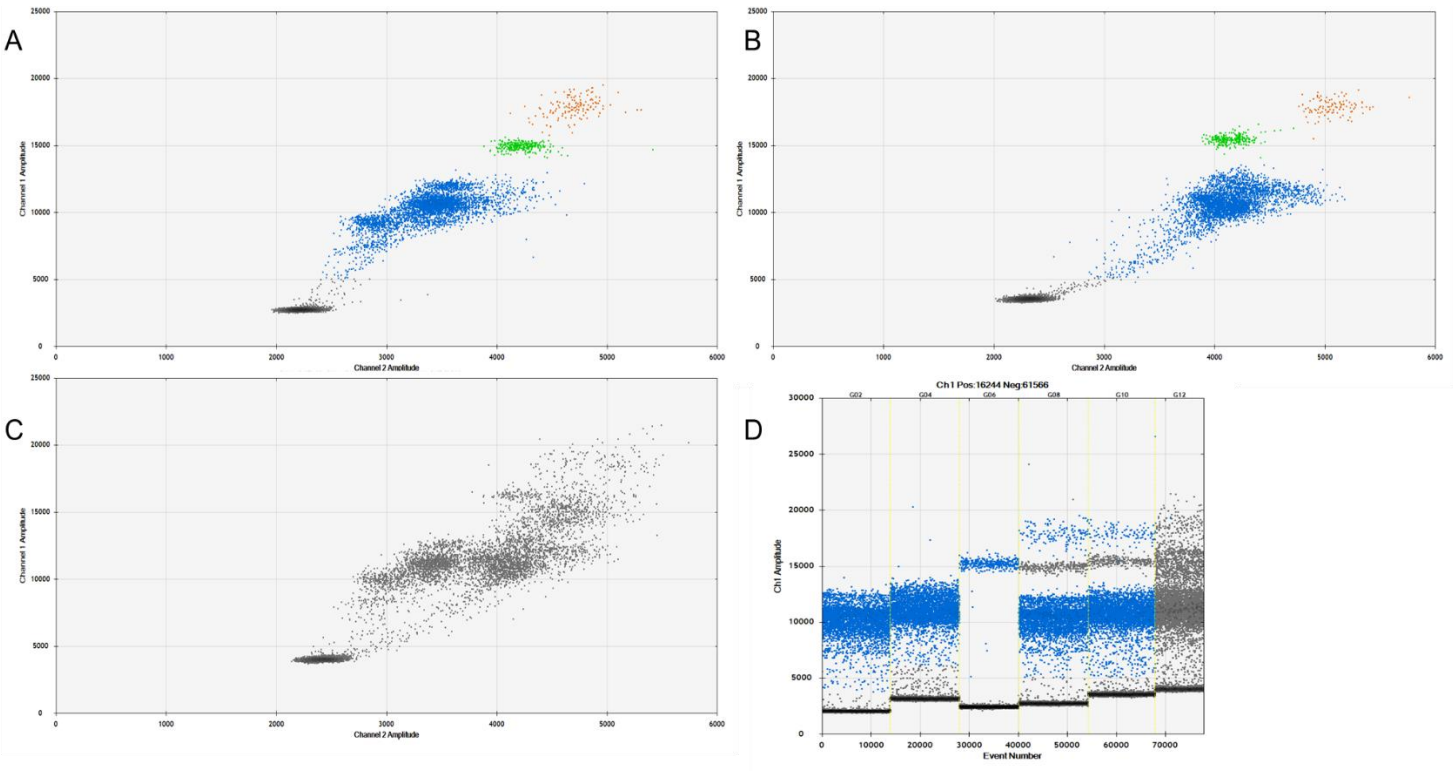

Figure 3. Multiplexing assays. Representing the negative droplets (grey), target positive droplets (blue), reference positive droplets (green) and double positives (orange) (A) 2D view plot of duplexing EPI (blue) and REF (green). (B) 2D view plot of duplexing NRD (blue) and REF (green). (C) 2D view plot of multiplexing EPI, NRD and REF. (D) 1D view plot of EPI, NRD, REF, EPI+REF, NRD+REF and EPI+NRD+REF from left to right. The lower cluster belongs to the non-template droplets. The clouds above represent the positive droplets, the amplitude of these droplets depends on the length of the amplicon. In multiplexing assays the double positive droplets provide the highest amplitude.

PRIMER THERMAL GRADIENT OPTIMIZATION

A mixture of genomic DNA from different hexaploid wheat cultivars was used as a template to assess on the optimal annealing temperature from the individual and multiplexed assays. The standard ddPCR cycling program was modified and eight different annealing temperatures were tested in a gradient between 55 °C and 64 °C. An optimum annealing temperature was obtained between 55 °C and 57 °C (Figure 4). A fixed temperature of 56 °C was selected for further experiments.

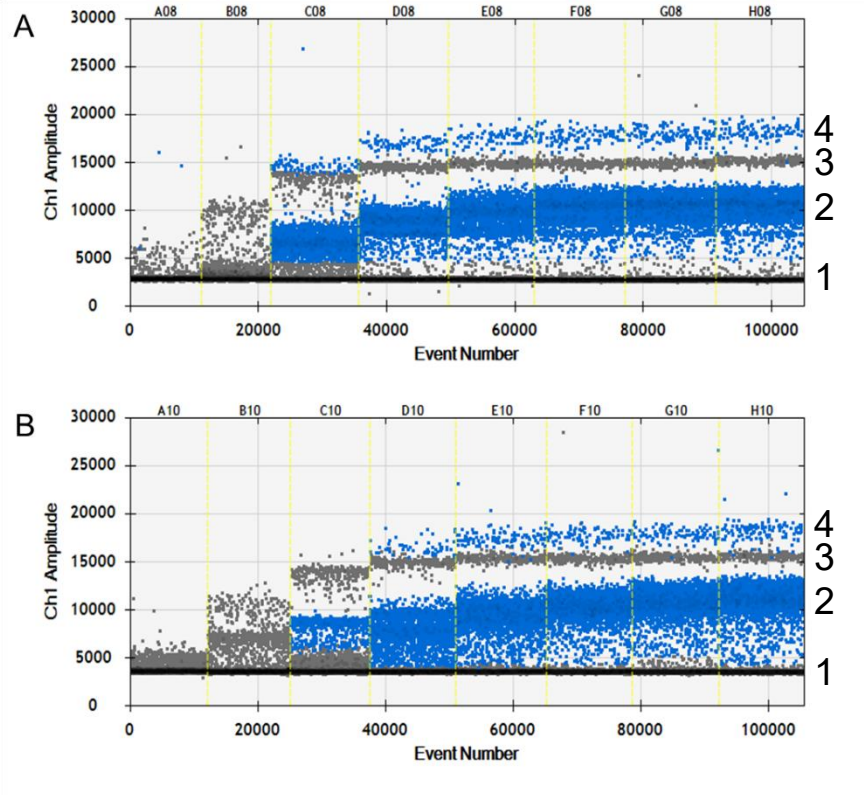

Figure 4. 1D plot view of the annealing temperature thermal gradient. Representing the negative droplets (1), target positive droplets (2), reference positive droplets (3) and double positive droplets (4). Each sample annealed at 64, 63.3, 62.2, 60.5, 58.4, 56.7, 55.6 and 55 º C, from left to right, duplexing with REF and EPI (A) or NRD (B).

PRIMER CONCENTRATION

The only requisite that needs to be fulfilled is the presence of at least one primer pair per droplet. A concentration of 100 nM is enough to fulfil this requirement (McDermott *et al.*, 2013). As previously mentioned, increasing the concentration of the primer also increases the amplitude of the respective population. This approach was implemented together with different amplicon size to improve the visualization in the output when NRD and REF were multiplexed. The corresponding populations of these primers were overlapping in some cultivars due to a higher amplicon size variability of the unknown target. Increasing the final concentration of REF leads to a larger separation of the populations. Various REF concentrations were tested. Increasing the concentration of REF from 100 to 150 nM was enough to improve the discrimination of the populations (Figure 5). It was not further increased so overlapping of the NRD with the background signal could be avoided. Increasing the concentration of the target did not have significant effects on the output.

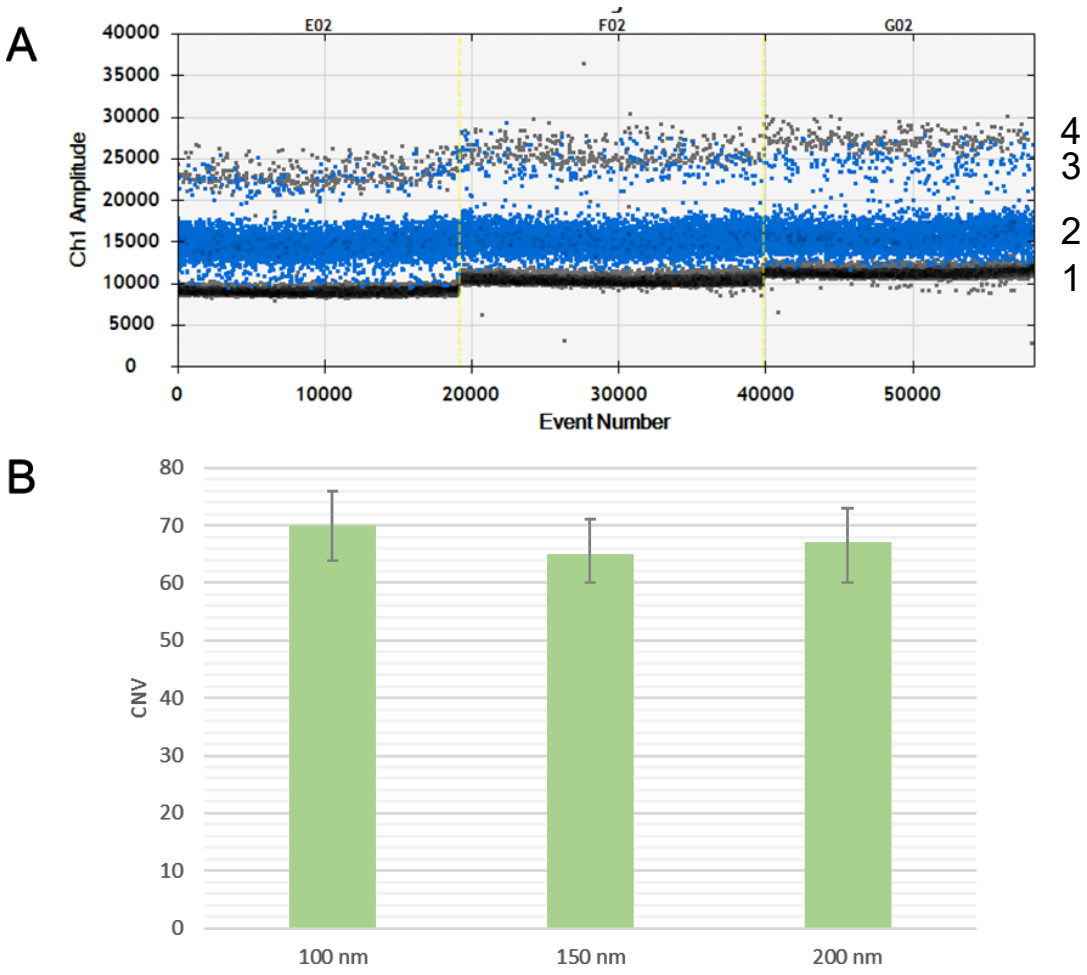

Figure 5. Primer concentration optimization. (A) 1D plot view of the effect of increasing REF concentration from 100nM (left) to 150 nM (middle) and 200 nM (right). Representing negative droplets (1), NRD positive droplets (2), REF positive droplets (3) and double positive droplets (4). Increasing REF concentration increase the separation between NRD and REF positive droplets. Increasing REF concentration also increase the amplitude of the negative droplets. (B) CNV of the concentration tested. No significant differences were found between the samples. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

Materials and Methods

Plant Material

In this study, genomic DNA extracted from five Nullisomic-tetrasomic lines (Sears, E. R. 1966) and four deletion lines (Endo & Gill, 1966) obtained from CS cultivar were used. Additionally, three synthetic lines derived from the cross of *Aegilops Taushii* (DD) and *Triticum Turgidum* (AABB) from the National Institute of Agricultural Botany (NIAB) (Ogbonnaya *et al*., 2013) were analysed, along with their respective parental lines. Finally, four gamma-irradiation Paragon accessions (Shaw *et al*., 2013) and 18 CRISPR/Cas9 Fielder lines produced by this group (Jouanin, in prep.), along with Paragon and Fielder control lines, were studied. All the accessions are listed in Table 2.

Table 2. Description of the lines.

Species	Line	Description	Species	Line	Description
***Triticum Aestivum* 'Chinese Spring'**	WT	Control	***Triticum Aestivum* 'Fielder'**	WT	Control
	6AS-1	Deletion lines (Endo & Gill, 1966)		A14_S1	(1gRNA, α87)
	6BS-4/5BS-2			C1_S10	(2gRNA, α213_ α324)
	6DS-4			C1_S15	(2gRNA, α213_ α324)
	6DS-4/1BS-19			C15_S1	(2gRNA, α213_ α324)
	6DDB	Nullisomic-tetrasomic lines (Sears, E. R. (1966)		C19_S9	(2gRNA, α213_ α324)
	6BBD			C19_S12	(2gRNA, α213_ α324)
	6AAB			C21_S10	(2gRNA, α213_ α324)
	6BBA			C26_S14	(2gRNA, α213_ α324)
	6DDA			C28_S7	(2gRNA, α213_ α324)
***Triticum Aestivum* 'Paragon'**	WT	Control		E1_S6	(3gRNA, γ86_γ274_γ603)
	P3-75	Gamma-irradiatied lines (Shaw *et al*., 2013)		E12_S4	(3gRNA, γ86_γ274_γ603)
	P6-43			E13_S7	(3gRNA, γ86_γ274_γ603)
	P6-57			E17_S8	(3gRNA, γ86_γ274_γ603)
	P6-59			E18_S2	(3gRNA, γ86_γ274_γ603)
Aegilops Taushii	54	DD parental		F38_S1	(5gRNA, α213_ α324_ γ86_γ274_γ603)
	75			F38_S4	(5gRNA, α213_ α324_ γ86_γ274_γ603)
	76			F38_S7	(5gRNA, α213_ α324_ γ86_γ274_γ603)
Triticum Turgidum	54	AABB parental		F38_S8	(5gRNA, α213_ α324_ γ86_γ274_γ603)
	75				
SHW (Ogbonnaya *et al*., 2013)	54	DD54xAABB54			
	75	DD75xAABB75			
	76	DD76xAABB75			

DIGESTION

Prior to the ddPCR reaction, DNA was digested to separate α-gliadin copies and fragment the DNA using the restriction enzyme ApoI-HF (New England BioLabs). Digestion reactions was performed in a working volume of 20 µl of aqueous reaction containing 0.5 µl of enzyme, 2 µl of 10XNEBuffer and 500 ng of genomic DNA. DNA was incubated at 37 ºC between 2 and 24 hours and inactivated at 65 ºC for 20 minutes, following the ddPCR protocol (an inactivation temperature of 80 ºC is recommended by the enzyme protocol, however a maximum temperature of 65 ºC is suggested by the ddPCR protocol (Bio-Rad)). After the digestion all the samples were diluted ten times to reduce the salt concentration.

GENE SEQUENCES

α-gliadin sequences were obtained from the gDNA, cDNA and ESTs data of diploid, tetraploid and hexaploid wheat sequences published on NCBI on September 2014 and from ESTs of Salentijn *et al*. (2009) (available via WUR network). In total, more than 1300 sequences, previously manually aligned, were analysed.

TaPFT1 sequence was obtained from the DNA sequencing in cultivar Chara (*T. Aestivum*) of Fitzgerald *et al* (2010).

PRIMERS USED FOR DDPCR AMPLIFICATION OF TARGET AND REFERENCE GENE

Sequences of the unknown and control targets primers are shown in (Table 3). Primers were designed following the ddPCR technique recommendations (Hindson *et al*., 2011) (Bio-Rad, 2018) specifically designed for ddPCR ("QX200 Droplet Digital PCR"). Designed primers must have a length between 17 and 25 nucleotides, Tm between 50 and 70 ºC (Optimal 65 ºC) and an amplicon length between 50 and 200 nt. Each primer was checked, ensuring that there was no 3' end complementarity to avoid primer dimer formation, using the tool Multiple Primer Analyzer (Thermofisher).

Table 3. ddPCR primers used for CNV detection

Name	*Sequence*	*Tm (°C)*	*Length*	*CG%*	*Amplicon length (bp)*
F_PAM	GCAACCATTTCCATCACAACA	59.9	21 bp	42.9	61
R1_PAM	AAGGCGTCGGCGTTGAT	61.5	17 bp	58.8	61
R2_PAM	AAGGtGTCGGCGTTGAT	58.1	17 bp	52.9	61
F1_END	TTGCAACAACACAGCATAGC	59.8	20 bp	63.0	136
F3_END	TTGCAgCAACACAaCATAGC	59.8	20 bp	63.0	136
R2_END	GGTcCGGTAgGTgTTACAaC	61.7	20 bp	55.0	136
R3_END	GGTtCGGTAgGTgTTACAaC	59.9	20 bp	50.0	136
F_REF1	GCAGAGTGGAGTGGACATTT	60.1	20 bp	50.0	181
R_REF1	CTCTATGGTGGCTGTTGCTA	59.4	20 bp	50.0	181

DDPCR EXPERIMENTS

The ddPCR reaction mixture was prepared using 2 or 3 µl (depending on the primer combination) of 1 nM forward and reverse primer, 12 µl of ddPCR EvaGreen Supermix (Bio-Rad), 10 ng of digested DNA and miliQ water in a final volume of 20 µl. Droplets were generated loading 20 microliters of each reaction mix using the QX200 droplet generator (Bio-Rad). A final volume of 40 microliters of droplet- partitioned samples, containing approximately 20,000 droplets, were generated loading 70 µl of Droplet Generation Oil for EvaGreen (Bio-Rad) into the oil well for each reaction. Each sample was transferred to a 96- well plate (Bio-Rad) and sealed. Subsequently, samples were cycled in a Thermocycler (Bio-Rad). The thermal cycling profile was: DNA polymerase activation of 95°C for 5 min, followed by 40 cycles of 95°C of denaturation for 30 s and 56°C annealing temperature for 1

min. Post-cycling steps are 5 min at 4°C, 5 min at 90°C and an infinite 12°C hold. After the thermal cycling, the plate was transferred and read in the QX200 reader (Bio-Rad).

DATA ACQUISITION

After amplification, the plate was transferred and read in the QX200 reader (Bio-Rad). Quantasoft Software™ (Bio-Rad) that accompanied the droplet reader was used for data acquisition and posterior analysis. For the quality rejection criterium, samples with lower than 10.000 droplets were excluded. The given output corresponds to the fluorescence amplitude of each droplet. In the output, droplets are clustered in clouds or populations, corresponding to the presence of absence of a specific target gene. For each well, the Experiment Type was set to Copy Number Variation and indicating the ploidy for the reference.

ANALYSIS OF DDPCR OUTPUT

After acquiring the data, the threshold for the fluorescence values of the reference and unknown target were manually determined in the 2D plot view using Quantasoft Software™ (Bio-Rad) to separate the different populations. Four populations can be observed in the output separated by the amplified amplicon length (Figure 11). The dense cloud giving the lowest amplitude corresponds to the negative population, droplets with no amplification that are necessary for the statistic calculations. A second cloud, more dispersed due to the variability, corresponds to the unknown target population. The reference population is represented by the small and condensed cloud above. A fourth cloud giving the higher amplitude represents the double positives, those droplets where both targets were amplified. In the experiments where it was not possible to discriminate the double positives, this population was considered part of the reference population. Since most of the target population is included in the second cloud, not considering the double positive population just reduced the number of copies by one or two copies. 1D view plot view was used as a double check and the thresholds were corrected if necessary. Once the populations are separated, Poisson statistics are applied and yield an absolute measure of the nucleic concentration, necessary to calculate the number of copies of the sample. The confidence limits are applied using statistical models (Hindson *et al.*, 2011).

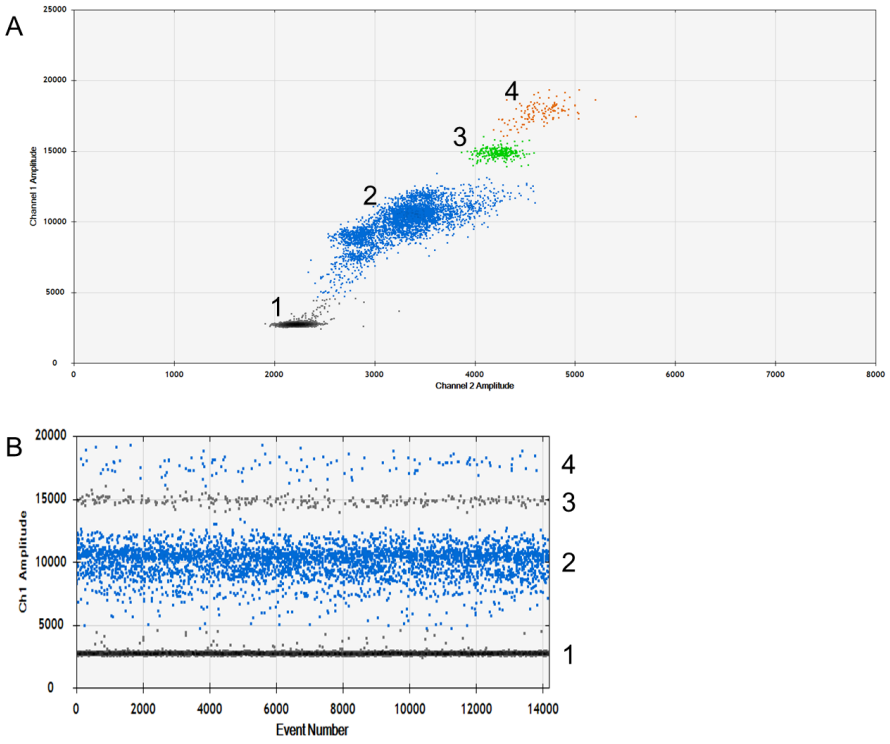

Figure 6. Multiplex assay using EPI and REF. (A) 2D plot view. (B) 1D plot view. Effect of amplicon length on the amplitude of non-template (1), EPI (2), REF (3) and EPI+ REF (4) droplets.

Results

PROOF OF CONCEPT: FEASIBILITY OF DDPCR TO ESTIMATE CNV IN HEXAPLOID WHEAT

Several experiments were conducted to demonstrate the feasibility and accuracy of the method. Since ddPCR is a novel technique and the exact gene copy number of the target remains unclear, it is necessary to proof the feasibility of the method before the implementation. Gene copy number was estimated by multiplexing the target EPI and the reference REF.

SYNTHETIC LINES

Synthetic Hexaploid Wheat (SHW) lines were created by crossing tetraploid T*riticum turgidum (*AABB) with diploid *Aegilops tauschii* (DD) and doubling the chromosomes of the offspring to obtain the hexaploid synthetic line (AABBDD) (Ogbonnaya *et al*., 2013). Three pairs of parental lines and their respective SHW lines generated were analysed. All the combinations were originated from unique lines except for the third combination which used the same tetraploid line as the second combination. No amplification of the reference was found in *A. tauschii* (DD) line 76. The average reference concentration the other two *A. Tauschii* was used to manually estimate the gene copy number. The expected gene copy number of each Synthetic Hexaploid Wheat line was calculated as the sum of the copies found in the respective parental. The number of gene copies is not constant across different lines of the same species. No significant differences were found between the gene copy number found in the synthetic lines and the sum of the gene copy number in the respective parental lines (Table 4).

Table 4. Comparison of measured and expected CNV of the SHW lines (ABD).

SHW	*AB*	*D*	*ABD*	*Expected ABD*
54	76	15.5	95	91.5
75	70	17.2	86	87.2
76	70	13.5	87	83.5

Expected ABD calculated as the sum of the respective parental AB and D lines.
** CNV calculated using the average REF concentration of other lines.*

DELETION LINES

Four lines generated from Chinese Spring and lacking a large region in the short arm of chromosomes 6, where the α-gliadin family of genes are located, were analysed. These lines (6AS-1, 6BS4/5BS-2, 6DS-4 and 6DS-4/1BS-19) carry a deletion in one of the pair of homoeologous chromosome 6. The relative copy number present in each diploid genome is estimated as the difference between the number of copies present in the WT and the respective deletion line. Significant copy number reduction was found in all the mutant lines (Figure 6). 61 gene copies were found in CS WT, while the deletion lines ranged between 41 and 52 copies. A different reduction degree was found depending on the homoeologous genome affected. Gene copy numbers of the diploid genomes were calculated as the difference of copies between the WT and each deletion line, 6AS-1, 6BS4/5BS-2, 6DS-4 and 6DS-4/1BS-19, obtaining the number of copies of AA, BB and DD respectively (Table 5). 44 copies were estimated in 6AS-1, with a deletion in the short arm of the sixth A chromosome, meaning that at least 23 copies were present in the A diploid genome. Correspondingly, 17 and 15 copies were measured for the BB and the DD genome respectively. 26 missing copies were estimated in the line 6DS-4/1BS-19, which carries another deletion in the short arm of the first BB chromosome.

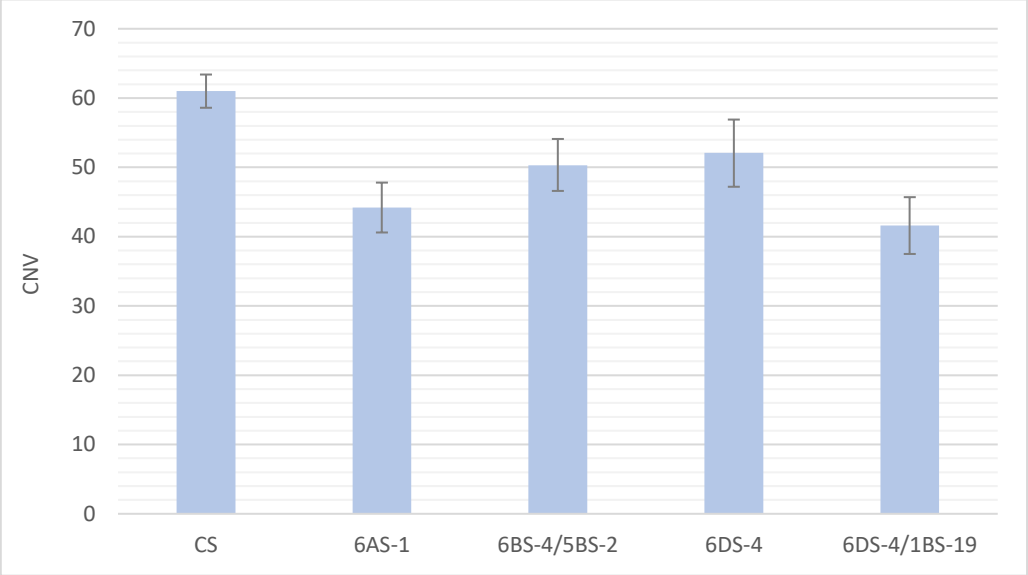

Figure 7. Gene copy number in WT and four deletion lines. Each mutant line carrying a deletion in one of the homoeologous sixth chromosome pair. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

Table 5. Comparison of missing gene copies on the deletion lines to WT

Deletion Line	***Missing copies***
6AS-1	23.2
6BS-4/5BS-2	17.1
6DS-4	15.3
6DS-4/1BS-19	25.8

Missing copies calculated as the difference between the number of copies in the WT and the deletion lines

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NULLISOMIC TETRASOMIC LINES

Four nullisomic-tetrasomic lines, which lack one of the homoeologous pairs of chromosome 6 (e.g 6A) and possess two pairs of another homoeologous chromosome 6 (e.g 6D), were analysed in this experiment (e.g. 6DDB). 61 copies were measured in the WT. In the nulli-tetra lines 47, 50, 66 and 111 gene copies were estimated in 6DDB, 6BBD, 6AAB and 6DDA respectively (Figure 7). Once the gene copy number of each line was estimated, the gene copy number of each diploid genome is calculated for each combination (Table 6). Diploid gene copy number is calculated as a function of three variables, using the number of copies obtained from three different combinations. Gene copy number of the diploid genomes was calculated comparing the individual results of the lines 6DDB, 6BBD and 6AAB as a sum of the individual diploid genomes (2D+b, 2B+D and 2A+B respectively). 6DDA showed a considerably higher number of copies and was not considered in this calculation. The gene copy number of each diploid genome was calculated and compared to the results obtained from the deletion lines to analyse the reliability of the method in two different experiments (Table 6). Not significant differences were found between the results of both experiments, except when compared to the results obtained from 6DS-4/1BS-19.

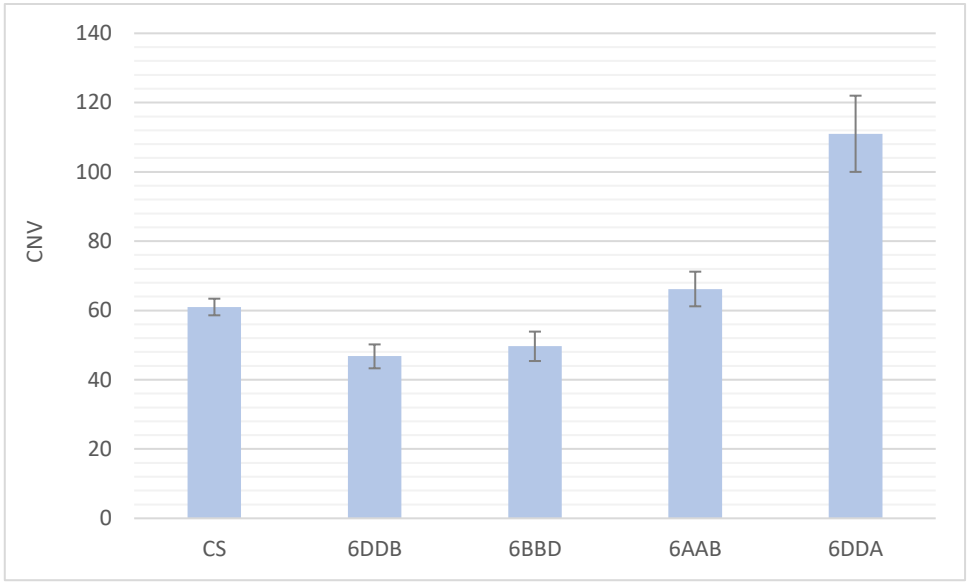

Figure 8. Gene copy number in WT and four Nullisomic-tetrasomic lines. Each line lacking one of the homoeologous pairs of chromosome 6 and possessing two pairs of another homoeologous chromosome. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

Table 6. Comparison of missing gene copies estimated in two independent experiments.

Diploid genome	***Nulli-Tetra lines***	***Deletion lines***
AA	24.6	23.2
BB	17.6	17.1
DD	14.6	15.3 (25.6*)

** Includes mutations in other parts of the genome.*

ddPCR VALIDATION - IRRADIATED LINES

Gamma-irradiation treatments can lead to random substitutions, inversions and small or large deletions (Morita *et al*., 2009). Four gamma-irradiated 'Paragon' lines, previously identify as having an altered gliadin protein expression profile, were analysed, using WT 'Paragon' as a reference. In this experiment, both primer pairs EPI and NRD were used and compared. All the screened lines presented an overall significant reduction between 15 and 30 copies independently to the primer used (Figure 8). The number of copies found in Paragon WT was 61 when either using EPI or NRD. A slightly difference of 6 copies was found in the line P6-43. No significant differences were found when using EPI or NRD primer pairs.

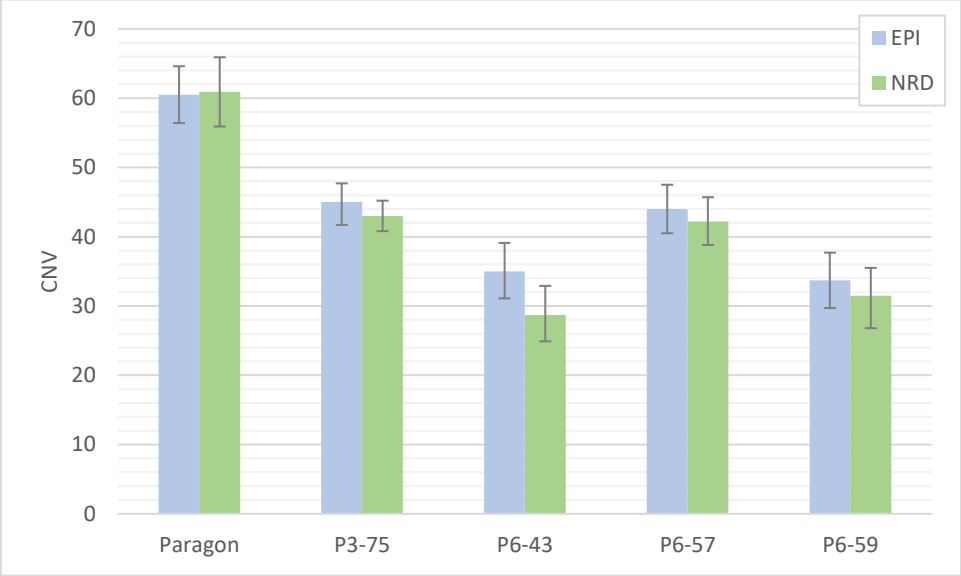

Figure 9. Gene copy number in WT and four gamma-irradiation lines. CNV was measured using two different primer pairs, EPI and NRD in different regions of the α-gliadin sequence. All the mutant lines missed a significant number of copies due to large deletions. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

ddPCR APPLICATION - CRISPR/Cas9 LINES

A total of 18 CRISPR/Cas9 lines from 'Fielder' cultivar, previously identified as having an altered gliadin protein expression profile, were screened using the ddPCR technique. Mutations were induced by gRNA targeting the α-gliadin and/or γ-gliadin family of genes. The repair mechanisms of the DNA breaks induced three bases upstream the protospacer adjacent motif (PAM) can produce indels or larger deletions. One of the primer pairs, EPI, targets the same sequence as the PAM located upstream of the epitope region. Comparison of both primer pairs allows screening of the mutation-type generated. Different number of copies were detected in Fielder WT when using EPI or NRD, 87 and 82 respectively. The number of gene copies of the samples were standardized to 82 copies of both primer pairs in the WT control, for easier comparison with the mutants. The reduction of 5 copies applied to NRD for this standardization was also applied to all results from this primer pair. All the lines showed a significant copy number reduction when EPI, at the PAM location, is used. A significant reduction when using NRD was only observed in the four F38 derived lines (Figure 9). Significant differences between the number of gene copies when using different primer pairs were found in all the CRISPR/Cas9 lines except C21_S10 and C26_S14. Moreover, all the lines studied presented a significant copy number reduction when the sequence in the epitope region was studied. This reduction was more noticeable in the lines derived from F38, in which γ-gliadins on the first chromosomes were also targeted.

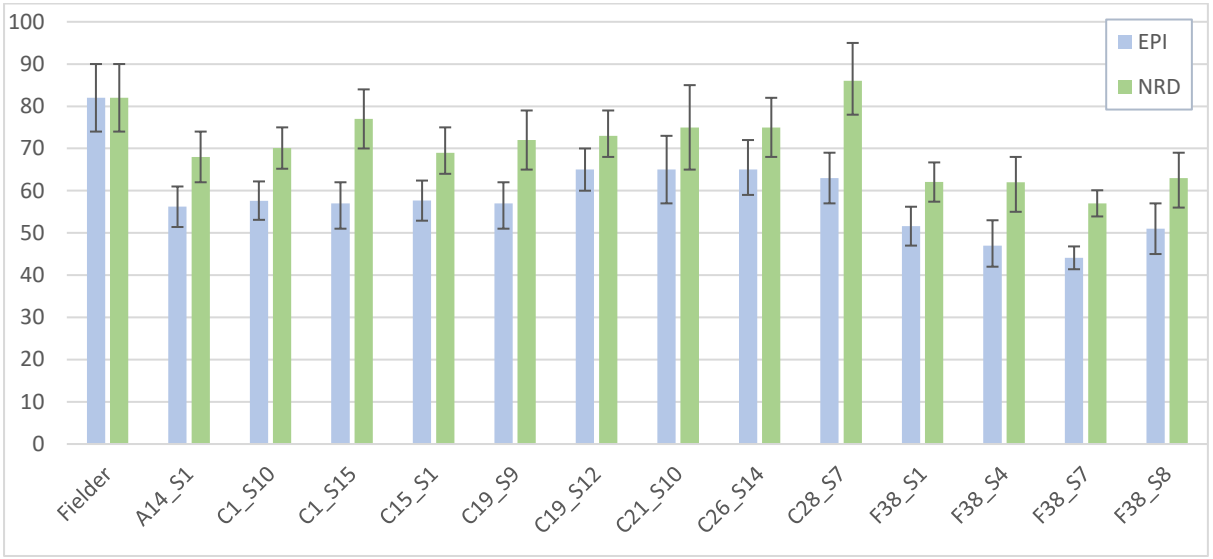

Figure 10. Gene copy number in WT and 13 CRISPR/Cas9 lines. CNV was measured using two different primer pairs, EPI and NRD in different regions of the α-gliadin sequence. α-gliadin genes were targeted in all the lines screened. γ-gliadin were also targeted in the line F. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

A second set of mutant lines, where only the γ-gliadins were mutated instead of the α-gliadin, was analysed. Significant copy number reductions were found between the WT and the mutant lines when EPI was applied (Figure 10). An average of 20 copies were missing in the mutant lines. Only the line E1_S6 significantly missed copies when NRD primer pair was used.

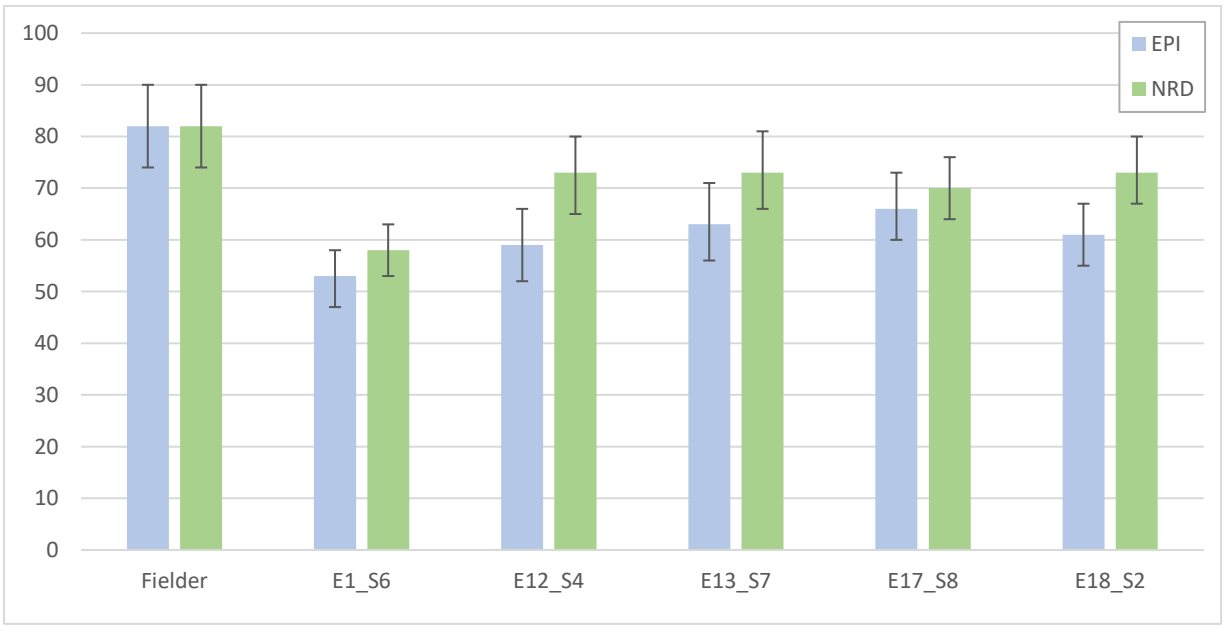

Figure 11. Gene copy number in WT and five CRISPR/Cas9 lines. CNV was measured using two different primer pairs, EPI and NRD, in *different regions of the α-gliadin sequence.* γ-gliadin were targeted in all the lines screened. Error bars indicate the Poisson 95% confidence intervals for each copy number determination.

Discussion

An efficient and reliable method to assess high-throughput gene copy number quantification in polyploid organisms has been of interest in the last decades. Screening for gene copy number in multiple samples has been the major bottleneck for the development of molecular plant breeding programs. Therefore, a multiplexed ddPCR-based approach that accurately estimates gene copy number in hexaploid wheat was suggested in this study. Recent attempts have proven the feasibility of this technique in other organisms with a lower level of ploidy, as in tetraploid *Medicago Sativa* (Gao *et al*., 2018). The feasibility and optimization of ddPCR to estimate the relative number of α-gliadin copies in hexaploid wheat was assessed in this study.

Due to the variability of the sequences and the unawareness of the exact number of copies, relative quantification was aimed. The sequences were obtained from the list of sequences available, which might not be representative for all the species and cultivars. The complete list of available sequences could not be covered with the primers designed, therefore an absolute quantification is not possible due to possible underestimations of the target. However, it was possible to perform a relative quantification using the WT as a control. One of the strengths of this study relies on the possibility to multiplex the targets. Multiplexing allowed not only to increase the accuracy of the method by, for example, minimizing the effects of pipetting errors, but also incrementing the cost-efficiency by reducing the number of samples needed. However, multiplexing requires longer preparation and optimization time. In a first attempt of multiplexing, three samples were combined in the same well. However, this was unsuccessful due to the high variability of the target sequences. Nonetheless, duplexing with the internal reference as common target was possible and enough to increase the accuracy of the approach.

To validate the technique a proof of concept, involving the use of several lines, was provided. Firstly, analysis of Synthetic Hexaploid Wheat (SHW) and the respective parental lines yielded positive results. To prove the accuracy of the technique, the number of gene copies present in the synthetic lines should be equal to the sum of copies of both parental lines. Even though a small variation was found, expected and measured results were not significantly different (Table 6). These results demonstrated the feasibility and accuracy of ddPCR to estimate the copy number not only from hexaploid wheat, but also from the tetraploid and diploid lines. Intriguingly, in *A. tauschii* (DD) line 76 the reference gene was not amplified. These results suggest that a variation of the reference sequence, whose sequences was obtained from *T. Aestivum*, potentially occurred. Since the same DNA input was added to every sample, the average copy number from the PFT1 reference found in other lines was successfully used to re-calculate the α-gliadin gene copies. These results demonstrated the feasibility and accuracy of ddPCR to estimate the copy number not only from wheat, but also from the tetraploid and diploid lines.

The deletion and nulli-tetra lines experiments are two individual experiments that assess the same issue: trying to elucidate the relative number of α-gliadin gene copies belonging to each diploid genome. Both experiments kept consistency in the number of copies. In these experiments the reliability of the technique was proven in two different ways, independently calculating the gene copies present on each diploid genome in both experiments. However, the line 6DS-4/1BS-19 provided a larger reduction which was not consistent with the results obtained in the nulli-tetra experiments. This line carries a second deletion in the BB homoeologous pair of the first chromosome. Intriguingly, the sum of each diploid genome (AA+BB+DD) was only consistent with the number of copies measured in WT CS (AABBDD) when the DD copies are calculated from 6DS-4/1BS-19. Moreover, number of DD copies calculated from 6DS-4/1BS-19 is not consistent with the number of copies estimated in the nulli-tetra lines. Based on this observation the presence of a sequence in the first chromosome that is being amplified has been hypothesized.

One of the nulli-tetra lines, 6DDA, presented a surprisingly high number of copies, mainly due to a low reference concentration. Measurement of reference concentration was less than half of the average concentration measured in the other lines. This overestimation was corrected by using the average reference concentration of the other copies, giving 52 α-gliadin gene copies. The estimated copy number is consistent with the expected gene copy number (A+D+D=53.8). Gene copy number of the diploid genomes was obtained from the nulli-tetra CNV calculation (Table 6). Remarkably, variability of the reference gene is higher than expected as observed in two lines, a mutant CS line of T. *Aestivum* and a *A. tauschii* line. Moreover, this variability is not linked to species or cultivars, but is randomly distributed across lines instead. Data from PFT1 sequences was obtained from cultivar 'Chara' in *T. Aestivum* (Fitzgerald *et al*., 2010). Further studies to determine the conservation of the sequences in different species and cultivars are recommended.

Once the feasibility and accuracy of ddPCR was proven in the proof of concept, gamma irradiated lines served as a validation of the technique. As expected, based on the pre-screening of these lines for α-gliadin protein profile alteration, random mutation generated deletions in the Gli-2 loci. In this experiment, both primer pairs of the α-gliadin target gene were studied and compared. This served as a control since significant differences between both targets were not expected. Gamma irradiation causes larges deletion that affect the whole gene, sweeping both primer pairs without distinction. Small deletions could also happen (Morita *et al*., 2009), but would occur randomly across the genome, making it impossible to detect. The significant differences found between the number of gene copies of the mutation lines and Paragon WT validate ddPCR as a feasible method to efficiently screen for large deletions.

The next step of the project was a direct application of the technique and the possibility to detect small targeted mutations. With one primer pair that aligns to the gRNA sequence of the epitope region and another aligning a sequence downstream this region, it is possible to distinguish small mutations and large deletions. Significant differences were found when using EPI in all the lines, accounting for both small and large deletions. Since the lines used were pre-screened for α-gliadin protein profile alteration, significant reductions were expected in all the samples. Using NRD to measure the CNV, less significant differences were found since only the large deletion were being screened. It was possible to distinguish in some lines the number of small mutations as the difference between the product of both primer pairs, which enables to select lines were mainly small indels have occurred. Lines with small indels on the epitope region would be of interest for two reasons: (a) those copies would be considered 'safe', retaining the properties given by the α-gliadins but not causing immunogenic reaction. (b) Having small mutations might avoid compensation of expression by other gene families, caused when genes are deleted.

A second set of lines, where the CRISPR/Cas9 line in which only the γ-gliadins in the first chromosome were targeted was analysed. Surprisingly, significant gene copy number differences were found when they were screened for mutations in α-gliadins. These observations are in line with the higher gene copy number decrease in the F38 derived lines, in which both α- and γ-gliadin were targeted. These results suggest that there is indeed a sequence in the first chromosome that is being annealed by the primers used. Firstly, analysis of 6DS-4/1BS-19 deletion line resulted in 10 extra copies of this sequence located in the first BB chromosome. Additionally, CRISPR/Cas9 lines where the γ-gliadins of the first chromosome were targeted, accounted for at least 20 sequences missing. Two hypotheses might explain these abnormalities: (a) previous studies already reported the presence of α-gliadin in other chromosomes (Huo *et al*., 2018), probably due to translocation. (b) Another possibility involves the nonspecific amplicon formation caused by sequence similarities between α-gliadin from chromosome 6 and γ- and ω-gliadins from chromosome 1. The presence of genetically related genes (Chen *et al*., 2014), such as the γ-gliadins encoding genes in Gli-1 loci, in the first chromosome (Wang *et al*., 2017), in addition, the low annealing temperature applied during thermal cycling might induce non-specific reactions.

It has been proven that ddPCR is a valuable tool to estimate the number of gene copies in the large α-gliadin gene family, which counted over 60 gene copies, in the complex hexaploid wheat genome. Besides, this technique also enables the detection of small mutations and larger deletions generated by CRISPR/Cas9 and gamma-irradiation. ddPCR becomes thus a relevant tool for high-throughput analysis of gene copy number and gene editing in large gene families, in polyploids. However, gene copy number and expression of the gene are not directly related. Information about the relation of gene copy number and expression of α-gliadins is yet to be elucidated. Therefore, further studies concerning the relation between the gene copy number present in cDNA and the original DNA are recommended.

Conclusion

Results of this study demonstrate the feasibility and reliability of ddPCR to quantify the gene copy number of a large family of genes in a complex polyploid organism, wheat. A protocol to relatively quantify α-gliadin gene copy number in hexaploid wheat has been developed and optimized. Through a direct application of the technique using CRISPR/Cas9 lines, it has been validated not only the potential of this approach in high-throughput screenings but also to discriminate the type of mutation. Feasibility of ddPCR has been validated in the study case of wheat and α-gliadin gene family, opening the possibility to be adapted in any other gene family or polyploid organism.

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