Optimised multiplex droplet digital PCR is more precise, but not more sensitive, than real-time PCR for the detection of allergenic peanut

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ABSTRACT
The United States requires labelling of food products containing major allergens, such as peanut, through the Food Allergen Labeling and Consumer Protection Act. Accurate labelling requires sensitive, specific and robust detection methods, and PCR-based techniques have proven highly effective. This article describes the transition of a previously developed multiplex real-time PCR assay for allergenic peanut to a droplet digital PCR format. The triplex droplet digital PCR assay was developed in a probe mixing format and directly compared to the established real-time PCR assay. Data are provided for thorough optimisation in the digital format, including the effects of primer and probe concentration, cycle number and annealing/extension time. Optimisation parameters influenced relative location and separation of droplet clusters but not final copy number. The droplet digital PCR assay was linear over five orders of magnitude; its lower limit of detection was 0.05 pg DNA per reaction, more sensitive than published digital PCR allergen assays. It was more precise, but not more sensitive, than the previously established real-time PCR assay.

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Introduction
The United States’ Food Allergen Labeling and Consumer Protection Act (FALCPA) mandates that products containing any of eight major allergenic foods or food groups, such as peanut, be labelled accordingly. DNA-based methods such as PCR and real-time PCR have proven to be excellent confirmatory techniques which are highly sensitive, specific and robust in their detection of DNA as an indicator for the presence of allergenic foods. Recent years have seen increased application of a third generation of PCR technology, digital PCR. Digital PCR has several advantages over real-time PCR, but there are relatively few published studies which report its use for detection of allergenic foods or compare it directly to real-time PCR. Further study of digital PCR technology is an important next step in DNA-based allergen detection.

Digital PCR (dPCR) is the partitioning of a ‘bulk’ aqueous PCR sample into a high number of smaller samples prior to target amplification. It is based on the concept of partitioning that bulk sample to the point of limiting dilution (Sykes et al. 1992; Vogelstein and Kinzler 1999), at which some partitions contain no copies of target DNA and yield a negative result while some partitions contain one or more copies and yield a positive result. Absolute copy number is determined through application of a Poisson correction to positive and negative partition counts (Sykes et al. 1992). Digital PCR’s performance depends on factors such as number, volume and size uniformity of partitions, which were generated and analysed manually in initial studies (Sykes et al. 1992; Vogelstein and Kinzler 1999). Adoption of digital PCR has increased dramatically with advances in microfluidics that enable rapid generation of large numbers of consistently sized partitions at benchtop scale (Hindson et al. 2011; Baker 2012; Pinheiro et al. 2012; Quan et al., 2018). One prevalent approach is droplet digital PCR (ddPCR), in which the aqueous PCR
sample is emulsified as droplets in oil, and data are acquired via a droplet flow cytometer (Hindson et al. 2011).

ddPCR employs fluorescent probe-based target detection approaches like real-time PCR, but differs in other respects. Previously developed real-time PCR assays can be transferred directly to a digital format using the same primer and probe sequences. In digital PCR, quantification based on Poisson statistics produces data in the form of a target copy number which is absolute, rather than inherently relative like the $C_T$ value obtained from real-time PCR. Digital PCR uses data generated at end-point, after all cycles of amplification are complete, so it is less sensitive to the effects of reaction efficiency. However, targets within a given droplet still undergo the same process of amplification as those in real-time PCR, so assay optimisation, which ensures consistently high reaction efficiency within each droplet is important to ensure robust performance. At a minimum, ddPCR assays must be optimised to enable a clear distinction between positive and negative populations, or minimisation of ‘rain’ droplets which have intermediate fluorescent amplitudes (Huggett et al. 2013; Lievens et al. 2016). Optimisation is especially important in multiplexing, as comparable efficiencies for different targets within a given droplet are important for accurate results (Whale et al. 2016). Multiplexing approaches in ddPCR are significantly different from those used in real-time PCR. ddPCR is most commonly performed on a system with two fluorescent channels (Hindson et al. 2011). Higher level multiplexing is accomplished by varying not just the fluorophores themselves but also the final fluorescent amplitude in each channel (Whale et al. 2016). Comparisons to real-time PCR have shown generally good agreement, though authors have emphasised the need for more direct comparisons between established real-time PCR assays and the newer technology of digital PCR (Hindson et al. 2013; Quan et al. 2018).

Digital PCR for allergen detection has consisted of singleplex assays with varying degrees of optimisation in the digital format and limited direct comparison with real-time PCR (Daga et al. 2018; Pierboni et al. 2018; Mayer et al. 2019; Temisak et al. 2019; Cau et al., 2021). This article details the extension of a previously developed real-time PCR assay for peanut (Puente-Lelievre and Eischeid 2018) to a ddPCR format. Unlike previous reports, this paper describes a multiplex digital PCR assay, which employs three different targets in the peanut chloroplast genome located in regions coding for rpl16, matK and the trnH-psbA spacer region (Yin et al. 2017). These targets were chosen based on their performance in cross-reactivity, linearity and reaction efficiency tests with real-time PCR (Puente-Lelievre and Eischeid, 2018). The use of multiple targets provides internal confirmation of results and minimises the chance of false negatives or false positives. The current manuscript also includes a more thorough analysis of the transition to and optimisation in a digital PCR format. Data are presented for ddPCR-based optimisation of annealing and extension temperature, annealing and extension time, cycle number and primer and probe concentrations as well as specificity. The newly developed ddPCR assay was directly compared to the established real-time PCR assay in terms of linearity, dynamic range, LOD, LOQ and precision.

Materials and methods

Sample preparation and DNA extraction

Peanut, tree nut and legume samples were obtained from Nuts.com (www.nuts.com, Cranford, NJ) and stored at $-80\,^\circ{\rm C}$ until use. Peanuts and tree nuts were obtained as ‘in-shell’ varieties and removed from shells under controlled laboratory conditions to prevent cross-contamination. DNA extraction was carried out using a DNeasy Plant Kit according to manufacturer’s instructions (Qiagen, Valencia, CA). DNA quantification was carried out using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific) and DNA samples were diluted to desired concentrations in Buffer AE (Qiagen, Valencia, CA) and stored at $4\,^\circ{\rm C}$ until use.

Droplet digital PCR

ddPCR was carried out according to manufacturer’s instructions using a QX200 Droplet
Digital PCR System (BioRad, Hercules, CA; Hindson et al. 2011). Aqueous PCRs were set up using ddPCR Multiplex Supermix from BioRad (Hercules, CA) with primers and probes from Integrated DNA Technologies (Coralville, IA). Primers and probes were included in the final optimised version of the assay as follows, trnH-psbA spacer region (trnH): 500 nM forward primer (5'-AGG AGC AAT AGA AAC TGC GT-3'), 500 nM reverse primer (5'-TTT TTG TCT TAA GGG ATA CGA GT-3'), 150 nM FAM probe (5'-FAM-TGA TAT TGC/ZEN/TCC TTT ACT TTC AAA A-IowaBlackFQ-3'); matK: 500 nM forward primer (5'-AAG CGA AAT TTT GTA ATA TAT TAG G-3'), 500 nM reverse primer (5'-CTG CAT ATC CGC AAA TAC-3'), 150 nM HEX probe (5'-HEX-CAT CCC ATT/ZEN/AGT AAG CCC GTT TG-IowaBlackFQ-3'); rpl16: 500 nM forward primer (5'-GCG ATG GGA ACG ACG AAA AC-3'), 500 nM reverse primer (5'-TTA TTA GAT TGC ZZT ACC ATC CC-3'), 75 nM FAM probe + 75 nM HEX probe (5'-FAM/HEX-ACC TAA GAT/ZEN/TCA TTT GAC GGG A-IowaBlackFQ-3'). Optimisation and LOD/LOQ tests were carried out using quantities of DNA specified in ‘Results and discussion’ section. Cross-reactivity testing was conducted using 100 pg DNA per reaction. This amount of DNA is high enough to provide reliable cross-reactivity data and falls within the linear range of the assay, near optimal values for copy number and λ (Supplementary Table S2; Lievens et al. 2016). Cross-reactivity tests included: (1) the following tree nuts: brazil nut, pistachio, pili nut, macadamia, pecan, English walnut, black walnut, pine nut, hazelnut, almond, cashew and (2) the following legumes: navy bean, soybean, lima bean, adzuki bean, split peas, red lentils, red kidney bean, fava bean, black bean.

Droplets were generated on a QX200 Droplet Generator using a DG8 cartridge loaded with 70 μL of droplet generation oil and 20 μL of aqueous PCR per well. From each well, 40 μL of droplets were transferred to a ddPCR 96-well plate and heat sealed using a PX1 PCR Plate Sealer with a pierceable foil heat seal (BioRad, Hercules, CA). Thermal cycling was conducted on a C1000 Touch thermal cycler. The fully optimised version of the assay consisted of initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s plus annealing/extension at 57°C for 2.5 min, with a final cycle of droplet stabilisation at 98°C for 10 min. After amplification, reactions were held at 4°C until they were read and counted on a QX200 Droplet Reader. Data were analysed using the probe mix triplex assay option in QuantaSoft Analysis Pro (BioRad, Hercules, CA) and Excel software (Microsoft, Redmond, WA).

**Real-time PCR**

Real-time PCR was carried out as previously described (Puente-Lelievre and Eischeid 2018). Each 12.5 μL PCR setup contained 1× PCR buffer, 7 mM Mg, 2 mM dNTPs, 0.625 units of Platinum Taq Polymerase (Invitrogen/Life Technologies, Grand Island, NY), 200 nM each primer + 200 nM probe for the internal control, 5 × 10^5 copies of internal control template and 500 nM each primer + 200 nM probe for each peanut target. Primer and probe sequences for real-time PCR were the same as listed above for digital PCR. Probes were labelled as follows: matK/Cy5, rpl16/FAM, trnH/TexasRed. Cycling was conducted on a CFX96 Touch Real Time PCR system (BioRad, Hercules, CA) and consisted of 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, and extension at 65°C for 2 min. Fluorescent signal was read at the end of the annealing step. Data were analysed using CFX Manager (BioRad, Hercules, CA) and Excel software (Microsoft, Redmond, WA).

**Limit of detection, limit of quantification and precision**

Limit of detection (LOD), limit of quantification (LOQ) and precision were determined using 12 technical replicates at each of eight DNA concentrations, ranging from 0.0005 to 5000 pg, for both ddPCR and real-time PCR (Tables 1 and 2). Precision was assessed based on relative standard deviation of the technical replicates (RSD_{repeatability}; U.S. Food and Drug Administration 2019). LOQ was determined to
be the lowest concentration at which real-time PCR $C_T$ values had a standard deviation less than or equal to 0.5 $C_T$, and at which ddPCR copy numbers had a CV of 25% or less. LOD was determined to be the lowest concentration at which all 12 technical replicates yielded a positive result (U.S. Food and Drug Administration 2019).

Results and discussion

**Singleplex optimisation: annealing and extension temperature**

Annealing and extension temperature was optimised for each target in singleplex to ensure clear separation of positive and negative droplets prior to working with the assay in multiplex. The optimal temperature for all three targets, that which gave the greatest separation of positive and negative droplets with the least rain, was found to be 56–58°C. Complete loss of amplification occurred above approximately 61°C (Figure 1 and additional unpublished data). Of all parameters optimised, annealing/extension temperature had the most significant effect on ddPCR performance, and it differed significantly from the previously established optimal temperature of 65°C for the real-time PCR assay (Puente-Lelievre and Eischeid 2018). Other researchers have also reported optimal temperatures in the 55–60°C range and loss of amplification above 61–62°C, with optimal temperatures tending to

| Table 1. LOD, LOQ and precision tests: real-time PCR. |
|-----------------|-----------------|-----------------|
| pg DNA | matK-Cy5 | rpl16-FAM | trnH-TexRed |
| $C_T$ | SD | # pos/12 | $C_T$ | SD | # pos/12 | $C_T$ | SD | # pos/12 |
| 5000 | 14.11 | 0.18 | a | 18.52 | 0.09 | a | 15.25 | 0.16 | a |
| 500 | 17.25 | 0.18 | a | 21.62 | 0.09 | a | 18.48 | 0.27 | a |
| 50 | 20.19 | 0.3 | a | 24.64 | 0.08 | a | 21.64 | 0.25 | a |
| 5 | 23.69 | 0.34 | a | 27.72 | 0.08 | a | 24.82 | 0.26 | a |
| 0.5 | 27.27 | 0.34 | a | 31.89 | 0.11 | a | 26.05 | 0.92 | a |
| 0.05 | 30.79 | 0.54 | a | 35.11 | 0.25 | a | 29.54 | 0.88 | a |
| 0.005 | 33.81 | 2.44 | a | 37.53 | 0.61 | a | 35.56 | 1 | a |
| 0.0005 | 36.37 | 2.29 | 6/12 | 38.85 | 0.58 | 11/12 | 36.97 | 1.4 | 10/12 |

*aAll replicates positive; quantitative range: 0.05–5000 pg/reaction, $R^2$ values: 0.997 (matK), 0.998 (rpl16) and 0.988 (trnH).*

| Table 2. LOD, LOQ and precision testing: ddPCR. |
|-----------------|-----------------|-----------------|
| Pg DNA | matK | rpl16 | trnH |
| Copies/μL | RSD | # pos/12 | Copies/μL | RSD | # pos/12 | Copies/μL | RSD | # pos/12 |
| 5000 | 340.608 | 142.98 | a | 340.325 | 143.16 | a | 340.608 | 142.98 | a |
| 500 | 6281.33 | 5.67 | a | 6509.47 | 8.31 | a | 6693.7 | 6.62 | a |
| 50 | 634.45 | 8.66 | a | 741.47 | 7.25 | a | 679.33 | 8.79 | a |
| 5 | 68.19 | 7.45 | a | 80.05 | 5.92 | a | 72.72 | 6.57 | a |
| 0.5 | 7.44 | 13.71 | a | 7.52 | 13.43 | a | 7.81 | 11.14 | a |
| 0.05 | 0.73 | 27.40 | a | 0.9 | 20.00 | a | 0.87 | 21.84 | a |
| 0.005 | 0.14 | 35.71 | 10 | 0.14 | 57.14 | 10 | 0.15 | 66.67 | 8 |
| 0.0005 | 0.1 | 50.00 | 5 | 0.08 | 12.50 | 5 | 0.1 | 40.00 | 5 |

*aAll replicates positive; Quantitative range: 0.05–500 pg/reaction, $R^2$ values: matK = 1.00, rpl16 = 0.999, tmH = 1.00.*

Figure 1. Optimisation of annealing/extension temperature. For each target, annealing/extension temp from left to right: 70–69.2–67.5–64.5–60.9–58–56°C.
be lower for ddPCR than for real time (Daga et al. 2018; Cau et al. 2021). Differences in optimal temperature are not unexpected, since the thermodynamics of heating the water-in-oil emulsions used for ddPCR differ from the thermodynamics of heating bulk aqueous reactions used for real-time PCR. These results clearly indicate that annealing and extension temperatures for a ddPCR assay should be thoroughly optimised in the digital format and not transferred directly from real-time PCR.

**Triplex assay establishment**

The triplex assay was established using ratio-based multiplexing with complete cluster identification (Whale et al. 2016) as follows: the trnH probe was labelled with 100% FAM and produced a droplet population located entirely on the FAM axis of a 2-dimensional (2D) plot (Figure 2). The matK probe was labelled with 100% HEX and produced a droplet population located entirely on the HEX axis. The rpl16 probe was labelled with 50% FAM + 50% HEX and produced a droplet population located at intermediate fluorescence on both axes. As expected, the assay produced droplet populations for all possible combinations of 1, 2 or 3 targets, and resulting fluorescence levels were in accordance with probe labels (Figure 2). Separate, independent tests with all possible combinations of 1, 2 or 3 targets in the ddPCR reaction were carried out to confirm the identities of droplet populations (data not shown). Copy numbers were nearly identical between targets, and copy numbers for the triplex assay were nearly identical to those obtained in singleplex (data not shown). Optimisation was carried out to determine conditions which resulted in the tightest clusters and best separation of droplet populations.

**Triplex optimisation**

The triplex assay was optimised for rpl16 primer and probe concentration, annealing/extension time, and cycle number. Data were analysed for copy number as well as location and separation of droplet populations. Similar copy numbers were obtained across DNA concentrations with rpl16 primer/probe concentrations of 300/100 nM, 500/150 nM and 750/200 nM (Figure 3(a)), annealing/extension times of 1, 2.5 and 4 min (Figure 3(b)), and cycle numbers of 35, 40 and 45 cycles with or without a final 10-min extension step at 57°C (Figure 3(c)). These results agree with other published reports that ddPCR copy numbers are robust to variation in run parameters (Temisak et al. 2019; Pierboni et al. 2018; Schulze et al. 2021). However, all parameters tested did affect relative locations.

![Figure 2. Triplex ddPCR assay: 2D plot showing all droplet populations. y-axis: Channel 1/FAM, x-axis: Channel 2, HEX.](image-url)
and separation of droplet clusters, especially when evaluated together. The final, optimised set of conditions chosen was 500/150 nM rpl16 primers and probe, 2.5 min annealing/extension and 45 cycles. When all three parameters were outside this optimal set, triple positive droplet clusters were not effectively separated on a 2D plot (Figure 4). These results show that optimisation should be thorough to ensure unambiguous separation of droplet clusters so that all populations can be correctly identified, especially at higher levels of multiplexing in which numerous droplet clusters are analysed.

Figure 3. Copy number as a function of DNA quantity and (a) primer and probe concentration, (b) annealing and extension time and (c) cycle number. Plots show copy number average ± SD for all three targets. Full conditions for each optimisation test were: (a) 2.5 min annealing/extension, 45 cycles, (b) 300/100 nM rpl16 primer/probe, 40 cycles, (c) 2.5 min. annealing/extension, 300/100 nM rpl16 primer and probe.

Figure 4. Triplex ddPCR assay, 2D plot, all parameters suboptimal: y-axis: Channel 1/FAM, x-axis: Channel 2/HEX. Assay conditions: 40 cycles, rpl16 300/100, 1 min anneal/extend.
**Cross-reactivity testing**

The peanut ddPCR assay did not cross-react with 23 different nuts and legumes tested (Figure 5). These cross-reactivity data show some improvement over the original real-time PCR assay, for which late amplification was observed in one to two targets with pecan, soybean and pistachio (Puente-Lelievre and Eischeid 2018).

**LOD, LOQ, precision and comparison to real-time PCR**

Limits of detection and quantification, as well as precision, were evaluated in side-by-side comparisons for ddPCR and real-time PCR. Variation both within and between targets was higher, and therefore precision was lower, for the real-time PCR assay, which had an LOD of 0.005 pg/reaction for all targets but differing LOQs of 0.05 pg/reaction for matK and rpl16 versus 5 pg/reaction for trnH (Table 1 and Supplementary Figure S1a). Previous work did not indicate a significant difference between trnH and the other targets in this real-time PCR assay (Puente-Lelievre and Eischeid 2018). However, full LOD/LOQ experiments using 12 technical replicates were not conducted, and in this work $C_T$ values for trnH were within 1 $C_T$ for all but the lowest DNA level (Table 1). $C_T$ values between targets in the real-time PCR assay differed by up to four cycles, with rpl16 yielding consistently higher $C_T$ values across all DNA concentrations. In contrast the ddPCR assay had lower variation, and higher precision, both within and between targets, yet a higher LOD. For the ddPCR assay, both LOD and LOQ were approximately 0.05 pg DNA per reaction for all targets (Table 2 and Supplementary Figure S1b). These results are consistent with previous work demonstrating that ddPCR is less variable but not uniformly more sensitive than real-time PCR (Hindson et al. 2013). The relative performances of real-time and digital PCR in this work may also be affected by assay-specific factors: primers and probes were chosen based on their performance in real-time PCR. The real-time PCR assay was optimised for additional parameters, such as Mg$^{2+}$ and dNTP concentrations (Puente-Lelievre and Eischeid 2018), which were not optimised here: the manufacturer requires use of a pre-fabricated master
mix compatible with the microfluidics and oil phase of the ddPCR system.

Additional parameters evaluated for the ddPCR assay include droplet counts, sample compartmentalisation, $\lambda$ and resolution. For LOD–LOQ tests, total droplet counts were approximately 12,000–16,000 and sample compartmentalisation was 54–72% (Supplementary Table S1), well above the levels required for quantification down to 0.5% (Lievens et al. 2016). Calculations of $\lambda$ (Huggett et al. 2013) show that the optimal target concentration for this assay is between 50 and 500 pg DNA (Supplementary Table S2). Manual estimation of resolution for the peanut samples used in singleplex cross-reactivity testing yielded values of: 9.8 for the rpl16 target, 5.5 for the trnH target, and 7.9 for the matK target (Supplementary Figure S2; Lievens et al. 2016).

The linear range, LOD, and LOQ determined for this ddPCR assay are in general agreement with published single-target allergen assays in terms of copy number but not in terms of DNA quantity. The linear range determined here, 0.05–500 pg per reaction, is five orders of magnitude, in agreement with previous work and with the theoretical range of the QX200 ddPCR system. Published assays report LOQs and LODs ranging from 0.2 to 9 copies/µl and 0.2 to 5 copies/µl, respectively, but corresponding DNA quantities per reaction are approximately 75–90 pg for LOQ and 4–40 pg for LOD (Daga et al. 2018; Pierboni et al. 2018; Mayer et al. 2019; Temisak et al. 2019; Cau et al. 2021; Deconinck et al. 2021). The LOD/LOQ found in this work was 0.7–0.9 copies/µL and occurred at 0.05 pg/reaction, orders of magnitude lower (Table 2 and Supplementary Figure S1b). Optimisation in the ddPCR format likely makes a significant contribution to these differences. Previous reports include assay conditions taken directly from manufacturer recommendations or existing real-time PCR assays. Data presented here underscore the importance of thoroughly optimising a new ddPCR assay in the digital format. These data also highlight that assay limits should be evaluated in terms of DNA quantity rather than copy number, since copy number limits are largely defined by number of partitions.

Copy number is calculated from the Poisson distribution using fraction of positive droplets: a given fraction of positives in a given ddPCR system will always yield the same copy number. The true measure of sensitivity, therefore, is how much DNA was required to generate that fraction of positive droplets.

Conclusions

This work describes the transition of a previously established real-time PCR assay to a multiplex droplet digital PCR format. It includes detailed information on thorough optimisation in the digital format as well as direct comparison to the original real-time PCR assay. This work shows that while final ddPCR copy number data were robust to changes in assay conditions, separation of droplet populations was less so. In this work, the primary advantage of ddPCR was higher precision. While the ddPCR assay was not shown to be more sensitive than real-time PCR, its greater precision does make it better at detecting differences between samples with statistical significance. This makes ddPCR a more powerful and quantitatively accurate allergen detection method. Furthermore, the ddPCR assay was compared to a real-time PCR assay which already works well in the conditions tested. The partitioning of reactions can be expected to confer additional advantage in cases where real-time PCR does not already perform well. While this article describes work on the detection of allergenic peanut, the data and information presented here should be more broadly applicable to the development and optimisation of ddPCR assays as well as the performance of ddPCR compared to real-time PCR.

Disclosure statement

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