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Original Research Article

Development of a droplet digital Polymerase Chain Reaction assay for peanut detection of food allergens

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ABSTRACT

Food allergies are a risk for patients, especially peanut-allergy, even at low concentration it is still effective. As a consequence, a regulation based on the precautionary principle requires the labelling to declared allergenic ingredients. This enforces an importance to develop and validate the appropriate methods in accurately detection of peanut DNA. Therefore, the aim of the present work was to validate the method in order to detect and quantify peanut DNA by using droplet digital PCR (ddPCR). Peanut DNA was extracted with DNeasy mericon Food Kit (Qiagen) and was used as the template to optimise method. The assay was designed to target *Arachis hypogaea allergen II* gene based on hydrolysis probe. ddPCR was used to detect and quantify peanut DNA without the requirement of calibration curve. The limit of detection (LOD) was 0.015 ng/µL or 3.04±0.82 copies/µL and the limit of quantification (LOQ) was 0.03 ng/µL or 9.21±1.11 copies/µL. It can be said that the ddPCR assay was sensitive, reliable and could be used as a quantitative tool for detection of allergenic-peanut.

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INTRODUCTION

Allergens in foods even in small quantity can be serious, life threatening. Many allergens such as shellfish, wheat or other grains, soya, sesame and peanut, present in food products, however, undeclared those allergens as contaminants in food products affect a major risk for sensitive person on food. Manufacturers, therefore, should be aware of the presence of the labels for the allergens on food product. It has been reported that approximately 2-4% of the world populations can get allergic to food (Tortajada-Genaro *et al.* 2011) particularly, peanuts which are widely used in food industry such as peanut butter, confection or as ingredient in cookies and biscuits (Hird *et al.* 2003). Food allergies are characterised by disorders of the body after eating certain such as dyspnoea, chills, itching, vomiting, diarrhoea, and mouth swelling. Some may be fatal to shock, unconsciousness and death (Walker *et al.* 2016). By the mechanism of allergic reactions, the proteins or allergens stimulate the immune system (Immunoglobulin E, IgE, mediated reaction) causes allergic reactions (Walker *et al.* 2016). Currently, the method of treatment for food allergies is only symptomatic treatment and there is no real treatment. Doctors can only recommend that the patients should avoid eating allergic foods. Hence, the display of allergenic details on the label is very important. A correct ingredient in food label can help people with allergic problem be able to choose and consume the food without any harm. So that, it is crucial to have fast, reliable methods of allergen detection in processed food products to ensure compliance with food labelling and improve consumer protection.

The traditional peanut allergen detection was protein detection methods such as Enzyme-Linked Immunosorbent Assay (ELISA), however, the disadvantage of ELISA assay is that the cost is expensive and also found that using this method with the processed food can be less effective due to the protein degradation with high temperature (Prado *et al.* 2015). In contrast, using the genetic material of an allergen can give more benefit in measurement due to high precision, sensitivity and specificity as genomic DNA are highly stable (Prado *et al.* 2015). Quantitative real-time PCR (qPCR) has been used in allergen measurements, such as peanuts (Hird *et al.* 2003, Stephan *et al.* 2004), gluten (Cheng *et al.* 2016, Martın-Fernandez *et al.* 2016) and soybean (Cheng *et al.* 2016). The qPCR technique is high sensitivity method; however, it requires reference material to create a standard curve to measure the amount of allergen samples (Sanders *et al.* 2011).

This project aims to introduce droplet digital PCR technology (ddPCR) to quantify the amount of peanut allergen. The advantage of this method is no need of the standard curve in quantification of the target molecule and also has a high precision, specificity and sensitivity in detection even at low concentrations of DNA targets. The method to detect and quantify the peanut by ddPCR was developed and validated in order to be a standardised method to measure the amount of peanut allergens. This leads to accurate display of information on food labels. They also reached agreements with their trading partners on the export of food in accordance with the rules in the display of allergenic details on food products.

MATERIALS AND METHODS

DNA extraction

Raw peanuts were milled and blended to be a powder. 200 mg of peanut powder was gravimetrically weighed. The peanut DNA was extracted and purified by the DNeasy mericon Food Kit (Qiagen, Hilden, Germany) following the manufacture's protocol. Genomic DNA was eluted in 100 µL of TE buffer then the quantity and purity of DNA was determined by measured absorbance A260/A280 and A260/A230 ratios using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific). The extracted DNA was kept at -20˚C until use and for long-term storage.

Primer and probe design

Primers and hydrolysis probe were designed by Primer Express 3.0 (Applied Biosystem) to target *Arachis hypogaea allergen II* (*Arah2*) gene (accession no. AY007229). The potential cross-reactivity of primers and probe were tested *in silico* using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST). To optimise the assay, primers and probe were varied in concentrations. The concentrations of forward and reverse primers were 50nM, 300nM, and 900nM in a matrix. Then the probe concentration was subsequently optimised with three different probe concentrations (50nM, 100nM and 200nM). The optimisation of the assay was performed in qPCR (ABI7500, Applied Biosystems). Then the optimum annealing temperature was determined using gradient PCR (from 53 to 63°C) of ddPCR (QX200, Bio-rad).

Quantitative real-time PCR

The reaction mixtures of qPCR contained TaqMan Universal PCR mastermix (2x), 200 nM of probe, 300 nM of each primers (sequences for the primers and probe were shown in Table 1 and the concentrations were varied when optimisation), 2 uL of sample DNA and water to make up 20 µL. For no template control (NTC) reaction, water was used as the template. The real-time PCR was performed with an ABI7500 (Applied Biosystems) using thermal profile of 50°C UNG incubation step for 2 minutes, then 95°C AmpliTaq Gold activation for 1 minute, followed by 45 cycles of a two steps 95°C for 15 seconds and 60°C for 1 minute. The data was analysed by the 7500 software version 2.0.5 (Applied Biosystems).

Droplet Digital PCR analysis

The peanut DNA was determined by QX200 AutoDG Droplet Digital PCR (Bio-Rad, Hercules,CA, USA). The total volume of ddPCR reaction mixtures was 25 uL including 2x ddPCR supermix for Probes, 200 nM of probe, 300 nM of each primers, 5U of *HindIII* restriction enzyme, 2.5 uL of DNA samples (from 0.1-10,000 copies/µL) and water to make up 25 µL. For NTC reaction, water was used as the template. Then 20 uL of ddPCR mixture was used and partitioned to generate the droplets (one PCR reaction = one droplet) by Automated Droplet Generator (Bio-Rad, Hercules, CA, USA). Then the thermal cycling PCR including a cycle of 95 °C enzyme activation step for 10 minutes, then 40 cycles of a two-step cycling protocol, 94°C for 30 seconds and 60 °C for 1 minute (for optimisation annealing temperature was varied), followed by 98°C enzyme deactivation step for 10 minutes and hold at 4°CPCR cycles were performed in a T100 thermal cycler (Bio-rad). After PCR, the droplets were read by QX200 Droplet Reader (Bio-rad).

Limit of detection (LOD) and Limit of quantification (LOQ)

A pure peanut DNA was used to determine the LOD and LOQ of ddPCR. Peanut DNA was calculated back into copy number and then was diluted into 10,000, 1,000, 100, 10, 5, 1, 0.5 and 0.1 copies/µL (30, 3, 0.3, 0.03, 0.015, 0.003, 0.0015, and 0.0003 ng/µL). Each dilution was done in ten replicates. The concentration of DNA was measured by ddPCR (QX200, Bio-rad).

Data analysis

The data was analysed by QuantaSoft software (Bio-Rad) version 1.7.4 without the requirement of standard curve. The software

measured the number of positive, negative and total droplets. Therefore, ddPCR can measure the number of the target molecules (positive droplets) directly in copies/uL by correcting the number with Poisson statistic using equation (1) (Deprez *et al.* 2016).

$$C_{sample} = Df_{sample} \times Df_{PCR} \times \left(\frac{1}{A \times V_d}\right) \times \frac{\left(\log\left(1-\frac{P}{A}\right)\right)}{\left(\log\left(1-\frac{1}{A}\right)\right)} \quad \text{.....(1)}$$

Where

C_{sample} : the copy number concentration of the undiluted sample

Df_{sample} : dilution factor of the DNA sample before adding to the PCR mix

Df_{PCR} : dilution factor of the DNA solution in the PCR mix

A : number of analysed droplets

P : number of positive droplets

V_d : droplet volume

The combined uncertainty was calculated by combining the measurement repeatability of PCR with the variation in the volume of droplets and the dilution factor associated with volumetric dilution effects (uncertainty from pipette) of sample using equation (2) (Pinheir *et al.* 2012). The expanded uncertainty (U) was calculated (U=u_c x 2, when the coverage factor *k*=2 with 95% confidence interval, CI).

$$u_c = C\sqrt{\left(\frac{u_M}{M}\right)^2 + \left(\frac{u_{vd}}{V_d}\right)^2 + \left(\frac{u_{Df_{PCR}}}{Df_{PCR}}\right)^2 + \left(\frac{u_{Dfsample}}{Df_{sample}}\right)^2} \quad \text{.....(2)}$$

Where

u_c : combine uncertainty

M : Mean of copies

u_M : uncertainty of precision method (obtained from standard error of the mean)

u_{Vd} : uncertainty of the droplet volume (Corbisier *et al.* 2015)

u_{DfPCR} : uncertainty of volumetric dilution of the DNA solution in the PCR Mix

$u_{Dfsample}$: uncertainty of volumetric dilution of the DNA sample before adding to the PCR mix

RESULTS AND DISCUSSION

DNA Extraction

As peanuts contain with the high levels of fats and oils known as PCR inhibitors, the method used in peanut DNA isolation should remove these from the extracted DNA to get a good quality of DNA. In this study, the DNeasy mericon Food Kit (Qiagen, Hilden, Germany) was used to isolate and purify peanut DNA with performed in triplicate for repeatability and duplicate for reproducibility. The results showed that the averages of DNA concentrations in the first and second extractions (reproducibility) were 150 and 108.27 ng/µL, respectively. The average of A260/A280 ratios were 1.79. The ratio of A260/A280 indicated (O'Neill *et al.* 2011) that the extracted peanut DNA in this study was purity.

Assay design

The primers and hydrolysis probe were designed to *Arah2* gene showed in Table 1. This gene has been used as the specific target gene for detection of peanut DNA by qPCR (Hird *et al.* 2003, Tortajada-Genaro *et al.* 2011, Stephan and Vieths 2004). In this study, a short oligonucleotide targeting sequences between primers, was introduced because it can improve the assay specificity (Kutyavin *et al.* 2000). Mikeska and Dobrovic (2009) have reported that the performance of PCR can be improved by the optimisation of the primer and probe concentrations and annealing temperature (Mikeska and Dobrovic 2009). Therefore, to obtain a robust PCR assay, the conditions of PCR were tested. The optimal primer and probe concentrations were at 300 nM of both forward and reverse primers and 200 nM of probe in the final concentration of PCR reaction. The optimal annealing temperature was 60°C. With the optimal condition, PCR efficiency was 103% and R2=0.9949 (Figure 1).

Table 1. Primer and probe sequences for detection of peanut DNA.

Primer/ probe name	Oligonucleotide sequence (5' to 3')	Conc. (nM)	Target
Peanut_F	5'-GCAACAGGAGCAACAGTTCAAG-3'	300	*Arah2* gene (AY007229)
Peanut_R	5'-CGCTGTGGTGCCCTAAGG-3'	300	
Peanut_P	5'-FAM-AGCTCAGGAACTTGCCTCAA-CAGTGCG-BHQ1-3'	200	

F=Forward, R=Reverse, P=Probe, FAM=6-Carboxyfluorescein, BHQ1=Black Hole Quencher-1

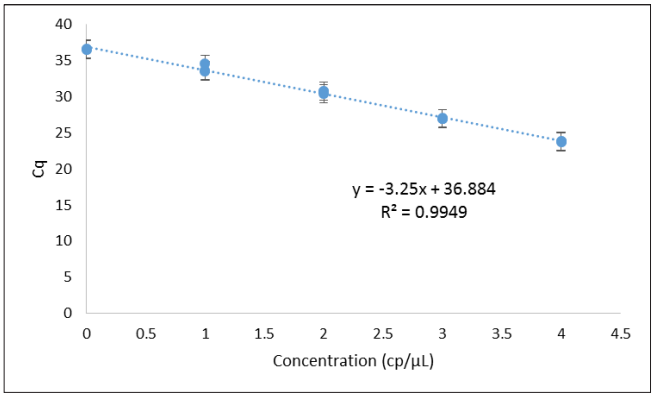

Figure 1. Calibration curve of serial copy number dilutions (from one up to 10,000 copies in log scale with SD as an error bar) determining PCR efficiency (PCR efficiency=103%).

Droplet digital PCR

ddPCR has been developed based on qPCR. ddPCR technique uses a limited sample dilution by partitioning the target molecules into many PCR reactions. As a result, some reactions contain a target molecules while other have no target molecules accorded by the Poisson distribution. After the PCR cycling, the PCR reactions with a target molecule yield positive droplets, while those with no template result in negative droplets. The counting of positive and negative droplets can calculate the absolute quantification of the target molecule (Sanders *et al.* 2011).

Therefore after optimisation of peanut assay with qPCR, it was

transferred to perform on ddPCR. The prelim experiments to test the assay in detection of peanut DNA were performed on ddPCR. The template peanut DNA were range from 1 to 10,000 copies/µL and performed in triplicate. The results showed that at these ranges concentrations, the positive droplets of target peanut DNA were clearly separated from negative droplets (Figure 2) and this guided the concentrations of peanut DAN to be used to determine limit of detection (LOD) and limit of quantification (LOQ).

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of ddPCR was defined as the lowest concentration (copy number) that can be detected with a level of confidence of 95%. In this study, LOD was obtained by measuring the dilution series of peanut DNA in 10 replicates using ddPCR (Table 2). The LOD was determined to be as low as 0.015 ng/µL or approximately 5 copies/µL of peanut DNA or with 100% of detection rate (above 95%, Table 2). At this concentration, ddPCR can quantify peanut DNA at 3.04±0.82 cp/µL.

The LOQ of ddPCR was defined as the lowest concentration (copy number) with an acceptable uncertainty (Deprez *et al.* 2016). In this study, the acceptable expanded measurement uncertainty was considered less than 25% (U(x)/x %) (Table 3). Therefore, the LOQ of ddPCR for detection of peanut DNA was 0.03 ng/µL or 10 copies/µL in the PCR mix with approximately 15,860 analysed droplets (Table 3). According to the Poisson distribution, if it has a high number of analysed droplets, it will increase the minimum theoretical LOD as well as LOQ (Deprez *et al.* 2016). At this concentration, ddPCR can measure the target peanut DNA at 9.21 ± 1.11 copies/µL with a coefficient of variation (CV) 18.63%. Cai *et al.* (2017) also used %CV in indication of the LOQ. They used the lowest concentration which gave the %CV less than 25% (Cai *et al.* 2017).

Table 2. Limit of detection for peanut DNA by ddPCR (n=10).

Peanut DNA (ng/µL)	Expected Peanut DNA (cp/µL)	Peanut Positive	Peanut Negative	Detection Rate (%)
30	10,000.00	10	0	100
3	1,000.00	10	0	100
0.3	100.00	10	0	100
0.03	10.00	10	0	100
0.015	5.00	10	0	100
0.003	1.00	8	2	80
0.0015	0.50	7	3	70
0.0003	0.10	1	9	10
NTC	NTC	0	10	0

NTC = no template control

Table 3. Limit of quantification for peanut DNA by ddPCR (n=10).

Peanut DNA (ng/µL)	Expected Peanut DNA (cp/µL)	Average of Peanut DNA ± U (cp/µL) by ddPCR	U(x)/x (%)	CV (%)	Average number of positive droplets	Average number of negative droplets	Average number of total droplets
30	10,000.00	11931.25 ± 689.96	5.78	7.32	7,577	4,332	11,909
3	1,000.00	1047.36 ± 63.23	6.51	9.46	1,409	15,171	16,580
0.3	100.00	100.77 ± 9.27	9.20	13.97	121	14,222	14,343
0.03	10.00	9.21 ± 1.11	12.06	18.63	12	14,805	14,817
0.015	5.00	3.04 ± 0.82	26.95	42.42	4	15,856	15,860
0.003	1.00	1.51 ± 0.70	46.31	73.11	2	14,404	14,406
0.0015	0.50	0.62 ± 0.31	49.53	78.21	1	14,479	14,479
0.0003	0.10	0.09	NC	NC	0	13,717	13,718
NTC	NTC	ND	NC	NC	0	17,368	17,368

U=Expanded uncertainty by the coverage factor k=2 with 95% CI, x=average of peanut DNA copies, ND = not detected, NC = not calculated

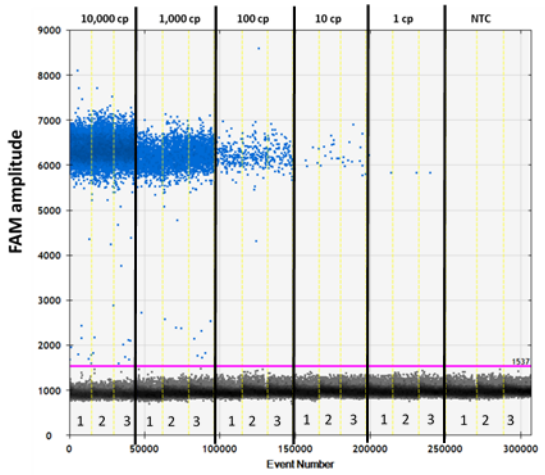

Figure 2. The graph was plotted between fluorescence intensity versus droplet numbers. Blue dots represent the positive droplets for target peant DNA, while black dots were the negative droplets. Each sample was separated by yellow lines. NTC: No template control

Peanut DNA detection by ddPCR

The serial dilutions of peanut DNA as mention above with known concentration (measured by Nanodrop and converted from ng/µL to copies/µL) were quantified by ddPCR. The result showed that each dilution had droplets over 13,000 analysed droplets. The relationship between expected concentration and measured copies of peanut DNA by ddPCR demonstrated a linear relationship (R^2=0.9998) (Figure 3). However, the expanded uncertainty increased in a low sample concentration (Figure 3). That can be explained with sampling effect as a low concentration of DNA can increase more variable (Bhat *et al.* 2009).

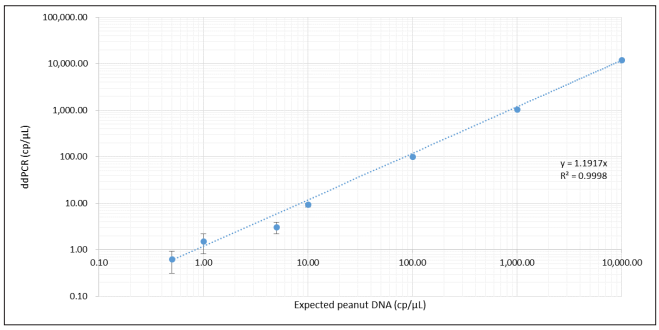

Figure 3. Linearity of ddPCR assay when measuring the peanut DNA range of 0.5 to 10,000 copies/µL.

The results also showed that ddPCR can reliably quantify even low levels of DNA concentration with high precision and accuracy as comparison between expected values (Nanodrop) and observed values obtained from ddPCR (Table 3). With ddPCR, the standard curve is not needed. Thus, the quantification of routing samples using ddPCR is practical and has potential to improve for detection peanut DNA in food allergy.

CONCLUSIONS

As food allergens, peanut, causes health risks to sensitive individual patients, for food safety this study showed an important works in development of a novel method, ddPCR, in detection and quantification of peanut DNA. This method is high sensitivity and reliability. Without the requirement of calibration curve, the peanut DNA was detected and quantified at 0.015 ng/µL (3.04±0.82 copies/µL) for LOD and 0.03 ng/µL (9.21±1.11 copies/µL) for LOQ. Hence, ddPCR is the promising techniques to be used in detection and quantification of peanut in food.

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