OPTIMIZATION OF REAL TIME PCR PARAMETERS BY CENTRAL COMPOSITE DESIGN IN GMO ANALYSIS OF CROP PLANT[1] [2]

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ABSTRACT | In total quality management, the main goal is to solve the problems encountered in a way that will not occur again. Experimental design is a statistical technique widely applied in every field and makes many contributions to the improvement of quality. This study aims to perform GMO (Genetically Modified Organism) analysis quickly, reliably and at the lowest cost by providing multi-response optimization of real-time PCR (Polimerase Chain Reaction) parameters to search and properly amplify the GMO gene region in maize using experimental design techniques. In this study, the ct value, which plays a role in the detection of genetically modified products, which are thought to have many risks, and the factors affecting the PCR product concentration and the levels of these factors were determined. The effects of these factors on the response values were determined by the central composite design method, one of the surface response methods, in Minitab.18 program and the factors were optimized. The model was validated by performing validation experiments using optimum factor levels.

Keywords: *GDO, real time PCR, central composite design*
JEL Codes: *M11, C44, C51*

Scope: *Business administration*
Type: *Research*

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[1] It has been declared that the relevant study complies with the ethical rules.
[2] It is derived from PhD thesis.

MISIR BİTKİSİNİN GDO ANALİZİNDE GERÇEK ZAMANLI PCR PARAMETRELERİNİN MERKEZİ KOMPOZİT TASARIM İLE OPTİMİZASYONU

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ÖZ | Toplam kalite yönetiminde, karşılaşılan problemleri bir daha ortaya çıkmayacak şekilde çözmek temel hedeftir. Deney tasarımı, her alanda geniş bir şekilde uygulanan istatistiksel bir teknik olup kalitenin geliştirilmesinde birçok katkıda bulunmaktadır. Bu çalışma, deney tasarımı tekniklerini kullanarak mısırda GDO (Genetiği Değiştirilmiş Organizma) gen bölgesinin aranması ve düzgün bir şekilde çoğaltılması için gerçek zamanlı PCR (Polimeraz Zincir Reaksiyonu) parametrelerinin çok yanıtlı optimizasyonu sağlanarak çabuk, güvenilir ve en düşük maliyetle GDO analizinin gerçekleştirilmesini amaçlamaktadır. Bu çalışmada birçok riske sahip olduğu düşünülen genetiği değiştirilmiş ürünlerin tespitinde rol oynayan c_t değeri ve PCR ürün konsantrasyonuna etki eden faktörler ve bu faktörlerin seviyeleri tespit edilmiştir. Bu faktörlerin yanıt değerlerine olan etkileri Minitab.18 programında yüzey yanıt yöntemlerinden merkezi kompozit tasarım metodu ile belirlenmiş ve faktörler optimize edilmiştir. Optimum faktör seviyeleri kullanılarak doğrulama deneyleri gerçekleştirilerek oluşturulan model doğrulanmıştır.

Anahtar Kelimeler: *GDO, gerçek zamanlı PCR, merkezi kompozit tasarım*
JEL Kodları*: M11, C44, C51*

Alan: *İşletme*
Türü: *Araştırma*

1. INTRODUCTION

GMO (Genetically Modified Organism) studies are at the forefront of these biotechnological studies and these studies are increasing rapidly. As GMO studies become more widespread, the risks it brings become the subject of more discussion. Since there are many question marks in the minds of people about GMO products, their use, placing on the market, importing, exporting etc. strict controls and measures are being taken on many issues and many countries bring legal regulations in this regard.

All these inspections and determinations are possible with GMO analyzes made under laboratory conditions. Real-time PCR (Polymerase Chain Reaction) is the most widely used method in GMO detection and quantification analysis.

In this time of increasing competition, the production of higher quality products at lower costs and faster can only be achieved by increasing quality awareness. Since quality awareness has increased a lot today, quality is an activity that needs to be developed and produced continuously rather than control.

It has become a necessity to carry out R&D studies by using statistical methods to increase quality and minimize losses by preventing errors. Statistical analysis is a method that finds answers to the questions to be answered by processing the data. For this purpose, many statistical methods have been developed and applied.

Experimental design is a technique that determines the most important factors affecting a product or process and their optimum values. Thanks to this method, the effect of more than one factor can be investigated at the same time. With the use of regression analysis, the relationship between the factors and the response is converted into a mathematical equation and the differences in the product or process in a random way are examined by statistical techniques.

The experimental design approach aims to increase customer satisfaction and the reliability of the system by optimizing the changes in the output value of different levels of the factors in question and by investigating the underlying causes of these changes. Although the response surface method is one of the most effective statistical experimental design methods used today, its cost is much lower and its advantages are much higher than classical methods.

Considering the studies in this field; Cobb and Clarkson (1994), using the Taguchi method, studied the effect of primer, DNA, Mg++ and dNTP concentrations on PCR (Polymerase Chain Reaction) optimization. Factors are selected at 3 levels. With this study, it was seen that the Taguchi method is a fast and reliable method in reaction optimization and gave an idea to subsequent studies (Cobb & Clarkson, 1994).

Boleda et al. (1996) aimed for an easy, fast and inexpensive PCR optimization using the experimental design methods Fractional Factorial design and Central Composite design. In this study, DNA from human blood cells was used and DNA extraction methods, factors such as DNA amount, Mg++ concentration and interactions between them were studied (Boleda, Briones, Farrés, Tyfield & Pi, 1996).

In a study (Caetano Anollés, 1998) on DNA amplification fingerprint (DAF= DNA Amplification Fingerprint), which is very similar even though PCR method is not used, 6 factors including primer, DNA, enzyme, dNTP, $MgCl_2$, concentrations and temperature were examined with Taguchi Design (Caetano-Anollés, 1998).

Niens et al. (2005) used the factorial design method for the optimization of PCR conditions in genotyping. Two experiments were designed in this study. In the first experiment, the buffer, primers, DNA, $MgCl_2$, taq polymerase, coupling temperature and cycle number were examined at 2 levels. In this experiment, buffer, taq polymerase, temperature, $MgCl_2$ and the interaction between them were investigated again with a second experiment. With this study, very important results were obtained at very low cost (Niens, Spijker, Diepstra & Meerman, 2005).

Fattah and Gaballa (2006) optimized the PCR conditions by using the Box Behnken surface response method in their study. In the study; primer concentration, Taq polymerase, Mg++, bonding temperature, number of cycles and elongation time were examined as factors (Abdel-Fattah & A. Gaballa, 2006).

Kitchen Robert et al. (2010) designed an experiment on quantitative real-time PCR using the Powernest program and used statistical methods such as nested ANOVA analysis and power analysis (Kitchen Robert, Kubista, & Tichopad, 2010).

Souza et al. (2011) tried to determine the best PCR parameters using the Taguchi experimental design, while detecting the genes that have a key role in the diagnosis of venous thromboembolism with real time PCR (real time PCR). He examined the effects of master mix, primer, probe concentrations and cycle number parameters at 3 levels (Souza et al., 2011).

Thanakiatkrai and Welch (2011) used the Taguchi experimental design method for real-time PCR optimization with SBR Green I dye. In this study, the results obtained from Taguchi, factorial design and regression analysis were compared. They saw that the results obtained with the factorial design with 27 experiments could be obtained faster and at a lower cost with the Taguchi design method by performing only 9 experiments (Thanakiatkrai & Welch, 2011).

Zhang et al. (2012) used central composite design, one of the surface response methods, for the detection of three different microorganisms (Salmonella, S. aureus and E. coli) in selectively enriched broth in multiplex PCR. NaCl, LiCl and KSCN ratios were considered as 3 factors, and the detection results of 3 different microorganisms were considered as responses (Zhang et al, 2012).

Camacho et al. (2013), Salmonella spp. He worked for the optimization of multiplex PCR on the DNA of his bacterium. Using the factorial design method, one of the experimental design techniques, they focused on the effect of MgCl2 concentration at 4 levels and the bonding temperature factor at 5 levels on the reaction and the interaction between these 2 factors. They also calculated the PCR efficiency with this study. They concluded that factorial design can also be used in other PCR methods (Camacho et al, 2013).

Nabi et al. (2016) used the Fractional Factorial design method to make optimization faster, easier and cheaper in the diagnosis of GMOs (Genetically Modified Organisms) in maize with multiplex PCR method. 4 different genes, which are indicators for the detection of GMO, and separate primers for each of them were used. One of these genes was used as a reference from a previous study, and the other 3 were first studied in this study. GMO gene regions have been investigated in processed food product (Nabi, Zellama, Hafsa & Chaouachi, 2016).

Wadle et al. (2016) applied an experimental design on RNA in multiplex real-time PCR, examined 7 factors at different levels and considered the ct value as a response variable (Wadle, Lehnert, Rubenwolf, Zengerle & von Stetten F., 2016).

Lafrance et al. (2021), in their study, aimed to optimize PCR parameters in the detection of TG178, TG105A and P6-25 molecular markers linked to Ty-1, Ty-2 and Ty-3 genes, respectively, that tomato provides resistance against yellow leaf curl virus (TYLCV). He examined four factors, temperature, annealing, amount of DNA, $MgCl_2$ and primer concentration, with a central compound design (CCD) (Lafrance et al., 2016).

Due to the limited number of previous studies on experimental design and response surface method in the field of PCR and GMO analysis, this study was needed and it was aimed to contribute to previous studies.

This study was conducted to search for and properly amplify the DAS 40278-9 GMO gene region in maize DNA using experimental design techniques, optimizing Real Time PCR parameters (DNA concentration, primer concentration probe concentration, master mix concentration) quickly, reliably and at the lowest level. It aims to carry out GMO analysis at a cost. In this study,

the factors affecting the c_t value and PCR product concentration, which play an important role in gene screening and GMO amount determination in the analysis of GMO products, which are thought to have many risks, and the levels of these factors were determined, and the levels of these factors (DNA concentration, primer concentration, probe concentration, master mix concentration) and their effects on both their interactions and response values (c_t value and PCR product concentration) were tried to be determined and examined by the central composite design method, one of the surface response methods.

Since the production, import, export and placing on the market of GMO products for food purposes is prohibited in our country, the production of GMO products for feed purposes is prohibited and their import and placing on the market is limited by some legal regulations. Import of feed products with approved GMO gene regions above the threshold value is allowed, provided that it is stated on the label that they are GMO. Confirmed genes below the threshold are considered contagious. In these respects, the screening of genes in GMO analysis and then obtaining the c_t value, which is effective in quantification, is of great importance for the correct evaluation of the GMO product.

The optimization of the created model was again made with the central composite design, and the optimum factor levels at which the lowest c_t value and the highest PCR product concentration were obtained were tried to be determined.

2. THEORETICAL BACKGROUND

2.1. Genetically Modified Organisms (GMO)

By using recently developed biotechnological methods, plant and animal varieties with higher quality and yield, more resistant to pests and diseases have been developed by transferring genes between different species. The product or living things developed in this way are called Genetically Modified Organisms (GMO) or Transgenic Product (Haspolat, 2004).

If the product has declared on its label that it contains GMOs to be used for feed purposes, only non-approved genes are directly examined, regardless of terminator gene regions (p35S, tNOS, pFMV, etc.). If an unconfirmed gene is found, a quantification analysis is performed. If the amount is below the threshold value of 0.9%, contamination is accepted and the product is allowed to be used as feed. However, if the result is above the 0.9% threshold, it is decided that the product contains unapproved GMOs and no use of the product is allowed. If an approved gene is found, it is considered appropriate for use as feed, since it is stated on the product label that it contains GMOs. However, if an approved GMO gene is detected in the feed product that does not have a declaration on the product

label, its use is allowed on the condition that the product is labeled (Anonimous, 2018).

2.2. Real Time Polimerase Chain Reaction (RT-PCR)

Polymerase Chain Reaction (PCR) is a method consisting of reactions that enable the synthesis of copies of a specific region of DNA belonging to various living things in vitro with the help of primers, oligonucleotides and enzymes (Persing, 1991).

Real-time PCR application is a PCR method that can monitor DNA synthesis simultaneously by adding fluorescent dyes to the PCR solution and a computer directly to the thermal cycler. Fluorescent dye that allows visualization of the proliferating target region; free SYBR Green I, primer-bound Scorpion probes or probe-bound TaqMan probes can be used (Pryor & Wittwer, 2006).

Basic components of PCR; template DNA, primers that bind to DNA designed according to the sequence of DNA and unchained, deoxyribonucleotide triphosphate (dNTPs), DNA polymerase, MgCl2 and buffer liquid. Taq DNA polymerase enzyme; It is obtained from Thermus aquaticus bacteria and is the most used DNA polymerase enzyme in PCR (Siqueira & Roças, 2003).

2.3. PCR Product and Cycle Threshold (c_t)

The Real Time PCR process consists of the baseline value where the proliferation is too low to be visualized, the exponential phase in which the logarithmic growth is observed, and the plateau phase in which the materials required for the proliferation in the PCR solution are depleted. The level of growth observed at the end of the reaction is directly proportional to the amount of product formed. The number of cycles ct (cycle threshold) in which the product begins to multiply and crosses the threshold is inversely proportional to the product concentration. The higher the amount of product, the earlier it will start to multiply, so the threshold value is crossed at a lower number of cycles and the lower the c_t value. With the Real Time PCR method, quantitation can be made through standard samples that have been quantified (Kanturvardar Tütenyurd, 2013).

2.4. Response Surface Design (RSM)

The Response Surface Method consists of an experimental strategy examining the process and independent variables, an empirical statistical modeling that appropriately expresses the relationship between output and variables, and optimization methods used to find the level or values of the variables that cause the response at desired values.

In most RSM problems, the relationship between response and independent variables is unknown. In the first step, a low-order polynomial equation is used to find the functional relationship between y and the independent

variables. If the response is modeled with a linear function by the independent variables, the approximation function is first order.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 \dots\dots + \beta_k x_k + \varepsilon$$

If the system has a curve, a second-order polynomial function is used.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon$$

ε denotes the observed error for the response. If the estimated answer is E (y) = f (x_1, x_2)= η, then η = f (x_1, x_2). In this equation, η is called the response surface. The response surface is usually represented by graphs drawn between η and the levels of x_1 and x_2 (İnan, 2009).

2.5. Central Composite Design (CCD)

Central Composite Designs (CCD) is one of the most frequently used methods in second order designs. Box and Wilson introduced this method to the literature for the first time in 1951. The design can be created as full or fractional experiments with multiple factors defined for two-level experiments. In fractional experiments, Solution-V (Resolution-V) occurs. As shown in Figure 1, the points where the experiment will be created occur in 2k number of axes, 2k number of cube points and n_c number of center points (Myers & Montgomery, 2002).

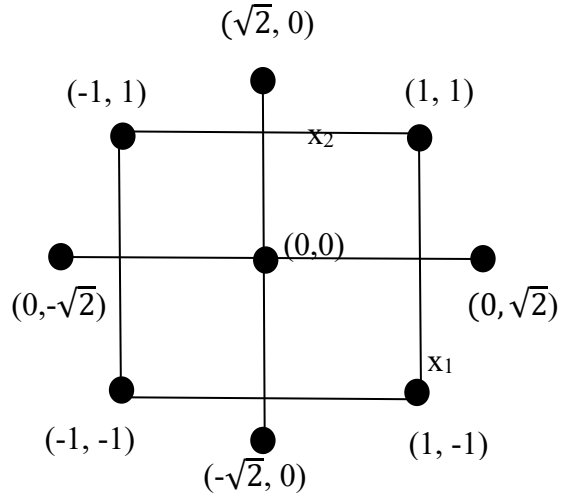

Figure 1: k = 2 and $\boldsymbol{\alpha = \sqrt[4]{2^2}}$ Central Composite Design for the Values

The α value that provides the rotatability changes depending on the number of experiments.

α =[Deney Sayısı]$^{1/4}$

Tam faktöriyel ise :

α = [2^k] ¼

k, represents the number of factors to be used (Myers & Montgomery, 2002).

Central composite design method, which is one of the response surface methods, was used in this study due to its ability to allow rotatability and at the same time to predict quadratic variables in the quadratic model. Orthogonally blocked designs allow block effects to predict variables in the model and reduce the variation between regression coefficients. In addition, examining the α value and factors in a wider range, the excess of central trials and the correct identification of the error and the creation of a stable model are other reasons for choosing the method. Thus, with this method, it has been tried to obtain a lot of information with a small number of experiments and low cost.

3. METHOD

3.1. Ethical permissions of the research

In this study, all the rules specified to be followed within the scope of "Higher Education Institutions Scientific Research and Publication Ethics Directive" were complied with. None of the actions specified under the title of "Actions Contrary to Scientific Research and Publication Ethics", which is the second part of the directive, were not carried out.

3.2. Material

The DNA used in the study was isolated from forage corn. The DAS 40278-9 GMO gene region was studied. This gene cannot be detected by promoters or terminators, but can be detected by a specific primer. Primers are considered as the total concentration of forward and reverse primers. Mycrosynth brand primer was used. Mycrosynth brand SYBR Green I was used as probe.

The Sequences of Primers and Probes of the Target Gene used in the experiment are shown in Table 1 below.

Table 1: Sequences of Primers and Probes of the DAS 40278 Target Gene in the Experiment

DAS40278 Gene Target Sequence	
DAS-40278-9_5'-f1	5' – CAC GAA CCA TTG AGT TAC AAT C– 3'
DAS-40278-9_5'-r3	5' – TGG TTC ATT GTA TTC TGG CTT TG– 3'
DAS-40278-9_5'-S2	5' 6FAM- CGT AGC TAA CCT TCA TTG TAT TCC G TAMRA 3'
DAS40278 hmg Reference Gene Sequence	
MaiJ-F	5' – TTG GAC TAG AAA TCT CGT GCT GA– 3'
mhmg-R	5' – GCT ACA TAG GGA GCC TTG TCC T– 3'
mhmg-P	5' 6FAM- CAA TCC ACA CAA ACG CAC GCG TA -TAMRA 3'

The master mix is a mixture that includes the factors that significantly affect the PCR mentioned in the previous sections. Master mixes used in PCR can be found in the market with different contents and brands. In this study, FluoCycle IITM Master mix was used.

FluoCycle IITM Master mixin contains: 100 mM KCl, 20mM tris HCl, 0.8 mM each dNTP (A, T, G, C), 20 units/ml Taq DNA polymerase enzyme, 8mM $MgCl_2$ and stabilizers.

Distilled water was used to complete the final mixture volume to 25 µl.

3.3. DNA Isolation

DNA isolation in the forage corn sample was performed with the Eurofins GeneSpin Protocol. The purity value of the DNA was measured in Micro UV Spectrophotometer (Nanodrop) using 1 ng/µl of the isolated DNA, the A260/A280 ratio was measured as 1.82 and the DNA concentration was adjusted to 40 ng/µl (Anonymous, 2013a; Anonymous, 2013b; Anonymous, 2018).

3.4. Central Composite Design

Previous studies on this subject (Souza, 2011; Thanakiatkrai and Welch, 2011) and sectoral experiences (Anonymous, 2013b; Anonymous 2016a) were used in the selection of the factors affecting the Real Time PCR and the parameters at the levels.

The α value used in the Central Composite Design method varies according to the number of factors (k).

Since 4 factors were used in the study, (k=4) : $\alpha = (2^k)^{1/4}$

$\alpha = 2$ was calculated and used. As a result, with the addition of α, the design has become 5-level.

Table 2: Factors and Levels Used in Central Composite Design

Factor No	Factors	Levels				
		- α(-2)	**-1**	**0**	**1**	**+ α(+2)**
X_1	DNA Conc. (µl/reak.)	3	4	5	6	7
X_2	Primer Conc. (F+R) (µl/reak.)	0,5	1	1,5	2	2,5
X_3	Prob Conc. (µl/reak.)	0,5	0,75	1	1,25	1,5
X_4	Master Mix Kons. (µl/reak.)	7,5	10	12,5	15	17,5

The c_t value is important for the correct calculation of the 0.9% threshold value in determining whether the product is GMO or GMO contaminant in the quantification after the type determination in the GMO analysis. It also gives information about the effectiveness of the reaction. A low ct value indicates a high suitability of PCR conditions. Therefore, in this study, the ct value is considered as "the smallest best".

The product concentration, on the other hand, expresses the concentration of replicated DNA sequences obtained as a result of PCR, that is, the amount of amplification. The more PCR product formed as a result of the reaction, the more suitable the reaction is. Therefore, the product concentration response variable was studied as “largest best”.

Central Composite Design Method Using the Minitab.18 program, a total of 93 experiments were designed, 31 experiments with 3 repetitions, as full factorial, 16 (2^k) cube points, 8 axis points (2k) and 7 center points (nc).

3.5. Experimental Procedure

As a result of the typing analysis, the DAS 40278-9 gene, which could not be detected with the promoter/terminator genes, was found (Anonymous, 2013b; Anonymous, 2016).

93 wells were filled with mixes prepared according to the levels in the experimental design plan, for 31 experiments with 3 repetitions to be analyzed in the above-mentioned 4 factors and 5 levels on the DAS 40278-9 gene whose presence was detected. The prepared mixes are made up to 25 µl with distilled water, that is, there is 25 µl of mixture in each well (Anonymous, 2013b; Anonymous, 2016).

Experiments were carried out in the Molecular Biology Laboratory of Balıkesir Food Control Laboratory Directorate. The settings of the Real Time PCR used are shown in Table 3.

Table 3: Real Time PCR Tempature-Time Settings

	Step	TempetureC)	Time (sn)	Cycle Number
1	Begining denaturation	95°C	600	1
2	Amplification:			
	Denaturation	95°C	15	45
	Annealing anda Extension	60°C	60	

4. RESULTS

The c_t values and PCR product concentrations obtained from 31 experiments with 3 replicates at the end of the thermal cycle from Real Time PCR are shown in Table 4.

Table 4: Results of Experiments with Central Composite Design

Exp. No	DNA X_1	Primer (F+R) X_2	Prob X_3	Master Mix X_4	C_t (number) Y_1			PCR Product Cons. (ng/µl) Y_2		
					Replic. 1	Replic. 2	Replic. 3	Replic. 1	Replic. 2	Replic. 3
1	-1	-1	-1	-1	28,57	28,61	28,54	856	853	858
2	1	-1	-1	-1	27,9	27,91	28,04	1090	1088	1095
3	-1	1	-1	-1	28,81	28,86	28,97	698	695	700
4	1	1	-1	-1	28,21	28,21	28,04	1108	1105	1112
5	-1	-1	1	-1	28,58	28,65	28,76	802	799	805
6	1	-1	1	-1	27,97	27,93	27,83	1104	1108	1101
7	-1	1	1	-1	28,92	29,12	29,07	602	605	798
8	1	1	1	-1	28,48	28,49	28,25	956	959	952
9	-1	-1	-1	1	28,81	28,87	28,85	694	698	690
10	1	-1	-1	1	28,33	28,44	28,32	982	986	979
11	-1	1	-1	1	29,19	29,28	29,32	581	583	580
12	1	1	-1	1	28,52	28,45	28,5	906	902	908
13	-1	-1	1	1	29,03	28,95	28,97	641	643	639
14	1	-1	1	1	28,55	28,29	28,47	938	940	935
15	-1	1	1	1	29,68	29,66	29,39	552	549	555
16	1	1	1	1	28,93	28,84	28,75	712	715	710
17	-2	0	0	0	29,36	29,56	29,68	567	571	565
18	2	0	0	0	28,27	28,33	28,08	1023	1026	1019
19	0	-2	0	0	28,08	27,92	27,94	1068	1071	1064
20	0	2	0	0	28,99	29,05	28,89	654	657	650
21	0	0	-2	0	28,59	28,51	28,14	952	948	955
22	0	0	2	0	28,94	28,9	28,87	658	663	652
23	0	0	0	-2	27,26	27,27	27,24	1146	1149	1141
24	0	0	0	2	28,12	28,15	28,05	1051	1053	1054
25	0	0	0	0	28,75	28,81	28,87	794	798	792
26	0	0	0	0	28,76	28,6	28,73	782	785	786
27	0	0	0	0	28,76	28,79	28,29	802	800	801
28	0	0	0	0	28,91	28,72	28,85	794	791	796
29	0	0	0	0	28,85	28,32	28,87	792	795	798
30	0	0	0	0	28,55	28,62	28,82	802	800	804
31	0	0	0	0	28,8	28,83	28,76	798	801	803

The amplification graph showing the c_t values of the experimental results is shown in Figure 2 below.

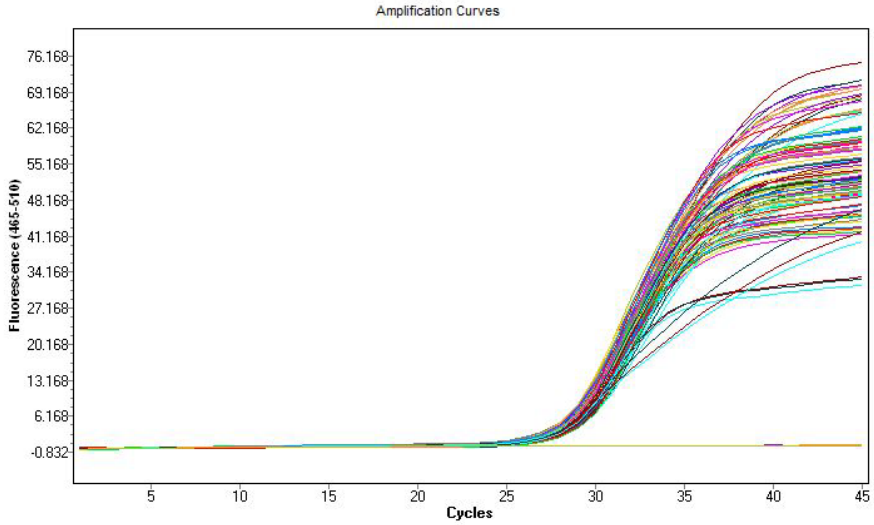

Figure 2: Amplification Chart of Experiment Results

When the variables that were found to be insignificant in the 95% confidence interval for both response variables were removed from the model, all variables became significant in the new model.

Table 5: Analysis of Variance Results for the ct value (Y_1) of the Model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	19,7978	2,82826	108,64	0,000
Linear	4	14,4908	3,62271	139,16	0,000
DNA	1	7,7684	7,76837	298,41	0,000
Primer	1	3,0217	3,02170	116,07	0,000
Prob	1	0,6709	0,67087	25,77	0,000
Master mix	1	3,0299	3,02990	116,39	0,000
Square	2	5,1840	2,59198	99,57	0,000
DNA*DNA	1	0,5064	0,50636	19,45	0,000
Master mix*Master mix	1	4,3766	4,37655	168,12	0,000
2-Way Interaction	1	0,1230	0,12302	4,73	0,032
Primer*Prob	1	0,1230	0,12302	4,73	0,032
Error	85	2,2128	0,02603		
Lack-of-Fit	17	1,1527	0,06781	4,35	0,000
Pure Error	68	1,0601	0,01559		
Total	92	22,0106			

S	R-sq	R-sq(adj)	R-sq(pred)
0,161346	89,95%	89,12%	87,84%

$$Y_1 = 28{,}7224 - 0{,}3285\,X_1 + 0{,}2049\,X_2 + 0{,}0965\,X_3 + 0{,}2051\,X_4 + 0{,}0760\,X_1^2 - 0{,}2235\,X_4^2 + 0{,}0506\,X_2X_3$$

If we look at the results for the ct response variable, the new model created after removing the quadratic variables and interactions with a p value greater than 0.05, that is, meaningless ones in the model, became meaningful with all its independent variables. The surface graph showing the interactions of the piner and probe concentrations, which is the only significant interaction in the model of the ct response variable, is as follows.

Figure 3: Surface Plot of the Effect of Primer and Probe Concentration on c_t

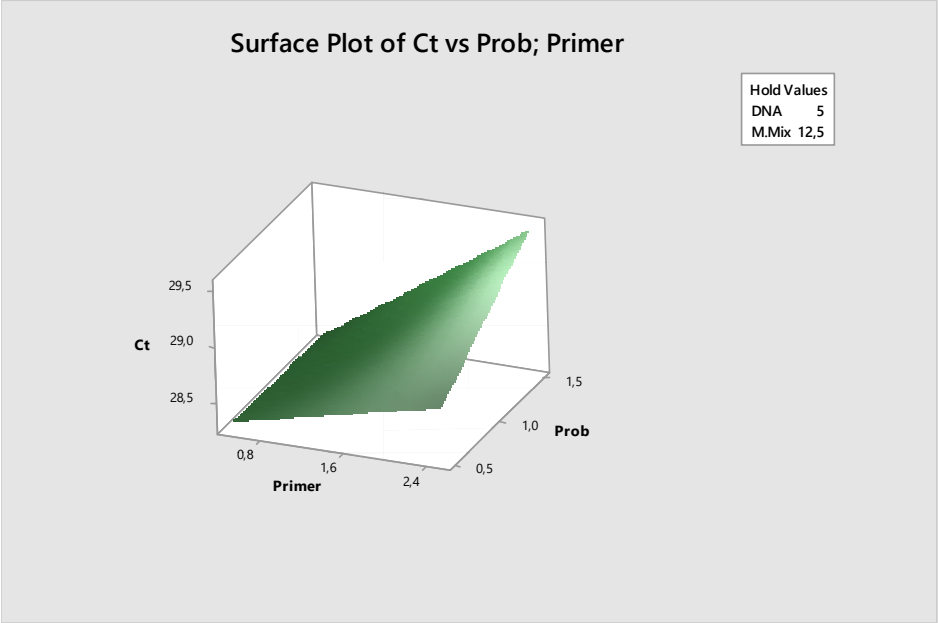

Figure 3 shows the effects of primer and probe concentrations on the c_t response value when DNA and master mix concentrations are kept constant at the center point. It is understood from the contour and surface graphs that the c_t value increases at high levels of primer and probe concentrations. There is a linear relationship between primer and probe concentration and c_t value. The effect of the primer concentration on the c_t value is higher than the probe concentration, that is, the change in the primer concentration affects the c_t value more than the probe concentration.

Table 6: Analysis of Variance Results for the Product Concentration (Y_2) of the Model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	6	2512274	418712	127,75	0,000
Linear	4	2104040	526010	160,49	0,000
DNA	1	1292028	1292028	394,21	0,000
Primer	1	385003	385003	117,47	0,000
Prob	1	159707	159707	48,73	0,000
Master mix	1	267302	267302	81,56	0,000
Square	1	394934	394934	120,50	0,000
Master mix*Master mix	1	394934	394934	120,50	0,000
2-Way Interaction	1	13300	13300	4,06	0,047
Primer*Prob	1	13300	13300	4,06	0,047
Error	86	281864	3277		
Lack-of-Fit	18	255387	14188	36,44	0,000
Pure Error	68	26476	389		
Total	92	2794138			

S	R-sq	R-sq(adj)	R-sq(pred)
57,2493	89,91%	89,21%	87,51%

$$Y_2 = 786,25 + 133,96\ X_1 - 73,13\ X_2 - 47,10\ X_3 - 60,93\ X_4 + 66,89\ X_4^2 - 16,65\ X_2X_3$$

If we look at the results for the product concentration response variable, the new model created after the quadratic variables and interactions with a p value greater than 0.05, that is, those that seem meaningless in the model, became meaningful with all its independent variables.

Considering the model equality and the coefficients of the variables; When all the other variables are kept constant, when the DNA concentration is increased by 1 unit, the product concentration increases by 133.96 units. The coefficients of (-) mean that when the interaction of primer, probe, master mix concentrations and primer and probe concentrations increase, the response variable product concentration will decrease. Since the coefficients of DNA concentration and master mix square are (+), the increase in these variables creates an increase in product concentration.

The independent variables in the model describe 89.91% of the variation in product concentration.

The surface graph showing the interactions of the primer and probe concentrations, the only interaction found significant in the model of the product concentration response variable, is as follows.

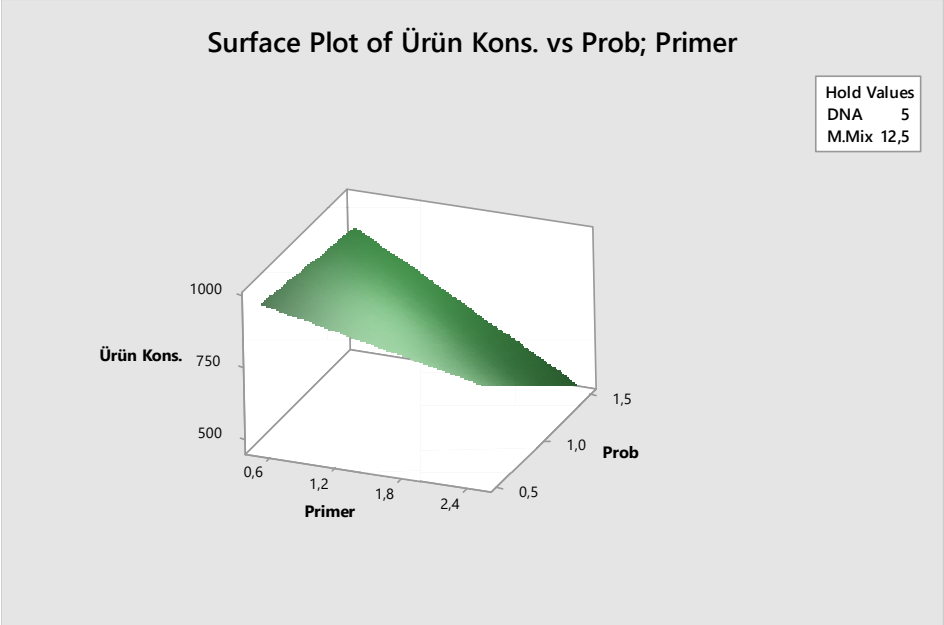

Figure 4: Surface Plot of the Effect of Primer and Probe Concentration on Product Concentration

Figure 4 shows the reciprocal effects of primer and probe concentrations on product concentration when DNA and master mix concentrations are held constant at the center point. It is seen from the contour and surface graphs that there is a decrease in the product concentration at high levels of primer and probe concentrations. There is an inverse relationship between the primer and probe concentration and the product concentration. While a significant decrease is observed in the product concentration as a result of the increase in the primer concentration when the probe concentration is at the lowest level, the decrease in the product concentration with the increase in the probe concentration at the lowest level of the primer concentration is lower than that of the high primer concentration.

4.1. Optimization of the Model

In this section, our latest quadratic model is optimized from the DOE/Response Surface/Response Optimizer tab of the minitab.18 program. The outputs obtained as a result of the optimization are as follows.

Table 7: Optimum Factor Levels and Responses Achieved by Central Composite Design

Solution	DNA	Primer	Prob	Master mix	Ürün Kons. Fit	Ct Fit	Composite Desirability
1	2	-2	2	-2	1562,25	26,6460	1

Table 8: Optimum Result Ranges of Response Values Obtained by the Central Composite Design Method

Response	Fit	SE Fit	95% CI	95% PI
Ürün Kons.	1562,2	47,4	(1468,1; 1656,4)	(1414,5; 1709,9)
Ct	26,646	0,147	(26,355; 26,937)	(26,213; 27,079)

According to the obtained optimization results, both the individual responses and the D values of the common function were obtained as 1 and it is the highest value it can get.

4.2. Validation of the Model

A confirmation experiment was conducted using the optimum factor levels obtained in the model, and the model was validated by comparing the predicted response values in the model with the results obtained from the confirmation experiments. The results obtained from the validation experiments and the values calculated from the model were found close to one, and the experimental results are included in the optimum response value range in the Minitab optimization result table.

Table 9: Comparison of the Results of the Confirmation Experiment with the Predicted Values of the Model

Responses	**Predicted Values**	**Experiment Values**	**Distinction**
Y_1 (c_t)	26,65	26,92	-0,27
Y_2 (Product Conc.)	1562,25	1483,26	78,99

5. CONCLUSION

Since there are many question marks about GMO products, the purpose of use, placing on the market, import, export etc. Strict controls and measures are taken in many issues and many countries bring legal regulations on this issue.

In this study, the factors affecting the c_t value and PCR product concentration, which play an important role in gene screening and GMO amount determination in the analysis of GMO products, which are thought to have many risks, and the levels of these factors were determined, and the levels of these

factors (DNA concentration, primary concentration (F+R)), probe concentration, master mix concentration) both their interactions among themselves and their effects on response values (c_t value and PCR product concentration) were determined and examined by the central composite design method, one of the surface response methods.

Considering the answers examined in the study; In the GMO analysis, the 0.9% threshold value is critical for the correct determination of whether the product is GMO or GMO contaminant in the quantification after the type determination analysis. Since the ct value is used in the calculation of the assay, it is important for accurate and reliable calculation. It is also inversely proportional to the efficiency of the reaction. A low c_t value indicates a high suitability of PCR conditions. Therefore, in this study, the c_t value was tried to be minimized.

Considering the studies in the field of PCR other than GMO; Experimental design studies with classical PCR: Cobb and Clarkson (1994), with Taguchi method, primer, DNA, $MgCl_2$ and dNTP concentrations were determined at 4 factors and 3 levels; Boleda et al. (1996), with the central composite design, DNA and $MgCl_2$ concentration at 2 factors and 5 levels; Niens et al. (2005), with factorial design, 7 factors at 2 levels; Fattah and Gaballa (2006) used the Box Behnken surface response method for a single response at 6 factors and 3 levels. Compared to this study, although it differs primarily in terms of the PCR method and the DNA source examined, it also differs in terms of the experimental design method used, the number of factors and levels, the response itself, and the number of responses. The study of Boleda et al. (1996) is the same only in terms of the experimental design method used (central composite design).

The study by Souza et al. (2011) with real-time PCR is the most similar to this study in terms of the factors used and their numbers. While 3 factors (primer, probe and master mix concentration) used in the study are the same, 1 factor (number of cycles) is different. Other differences in factors are the examination of the forward and reverse primer as two separate factors and the type of probe used. Apart from these, the DNA source examined, the experimental design method used, the response itself and the number of responses differ from this study. In the study, the melting curve response variable was investigated with the Taguchi design method. It is identical to this study in that only the real time PCR method is used.

In another study by Thanakiatkrai and Welch (2011) with real-time PCR, the Taguchi experimental design and the results of the regression analysis were compared and they were similar only in terms of the ct response value used.

Zhang et al. (2012) applied the central composite design as 3 factors and 5 levels in their study on three separate DNAs with multiplex PCR in the field of microbiology. Only the experimental design method used in this study is the same.

In another study conducted with Multiplex PCR (Camacho et al., 2013), $MgCl_2$ and temperature factor were examined by factorial design method and differs from this study in all respects.

A single study was conducted on the experimental design of GMOs (Nabi et al., 2016) and in the study, 4 different genes and their primers, temperature, DMSO and $MgCl_2$ factors were examined in multiplex PCR with the fractional factorial design method at 2 levels. The PCR method used in the study differs from this study in terms of the experimental design method, the factors examined, the number of levels, the DNA gene region investigated, and both the response itself and the number of responses.

In another experimental design study related to PCR (Lafrance et al., 2021), it was tried to optimize the PCR parameters for the detection of TG178, TG105A and P6-25 molecular markers linked to Ty-1, Ty-2 and Ty-3 genes. By examining four factors, temperature, annealing, DNA amount, $MgCl_2$ and primer concentration, with a central composite design (CCD), this study is similar to this study in terms of number of factors and method used.

This study, in the field of GMO; The use of real time PCR was made for the first time in terms of studying the DAS 40278-9 GMO gene region in forage maize, investigating 4 factors together at 5 levels with the central composite design method, examining and optimizing multiple responses.

With this study, it was concluded that central composite design, which is one of the surface response methods in the field of GMO analysis and real time PCR, is an effective method, can find wide use in this field, and low error margin and reliable results can be obtained. In addition to these, considering the number of factors and levels used in the study, when all trials are considered as full factorial design, each of the 4 factors should be examined in 5 levels, one by one. In other words, if all combinations were tried, $45 = 625$ trials were required, while the central composite design allowed the effect of 4 factors in 5 levels to be investigated and optimized with 31 trials.

In addition, the possible harm of GMO products to human health and people's concerns about GMOs increase the importance of this study. Especially with the current Covid pandemic, people became more sensitive about nutrition to able have stronger immune system and live healthy. Due to the possible risks such as weakening the immune system and increasing the risk of cancer, the use of GMO products as food, especially as a result of incorrect GMO analysis, and

the use of GMO products for food purposes is an unacceptable error in our country. With the permission to be used for feed purposes, it is possible that as a result of the wrong detection that can be made in the GMO analysis, the GMO product is labeled as non-GMO, causing the consumer to be misinformed, and the uninformed consumption of GMO feeds to indirectly threaten human health.

In future studies, different experimental design methods that have not been used in GMO analysis or real time PCR can be tried, the methods can be compared, the number of factors can be increased, different factors or different outputs can be investigated with central composite design, different plant or animal GMO gene regions can be studied. In the field of GMOs, different experimental design studies can be conducted on quantification. The factors examined by the experimental design can be optimized with different algorithms.

6. STATEMENT OF CONFLICT OF INTEREST

There is no conflict of interest between the authors.

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8. AUTHOR CONTRIBUTIONS

ÖZ, AK: İdea; design; interpretation; critical review; audit

ME, DE: Literature review; Collection and processing of resources; interpretation; who wrote the article; design

9. ETHICS COMMITTEE STATEMENT AND INTELLECTUAL PROPERTY COPYRIGHTS

Ethics committee principles were complied with in the study.

10. REFERENCES

Abdel-Fattah, Y. R., & Gaballa, A. A. (2006). Synthesis of DNA ladder by polymerase chain reaction and optimization of yield using response surface methodology. *Biotechnology*, 166-172.

Anonimous, (2013a). TS EN ISO 21571 Foodstuffs - Analysis Methods for the Determination of Genetically Modified Organisms and Derived Products - Nucleic Acid Extraction.

Anonimous, (2013b). TS EN ISO 24276 Foods - Analysis Methods for Detection of Genetically Modified Organisms and Derived Derivatives - Recipes and General Rules.

Anonimous, (2016). Event-specific method for the quantitation of Maize DAS-40278-9 using real- time PCR /EURL Metot.

Anonimous, (2018). Eurofins GeneSpin Catalogue, Kit for isolation of high-Quality DNA from Food and Feed Samples, Cat. No. 522440605.

Boleda, M. D., Briones, P., Farrés J., Tyfield, L., & Pi, R. (1996). Experimental design: a useful tool for PCR optimization. *BioTechniques*, 134-140.

Caetano-Anollés, G. (1998). DAF Optimization Using Taguchi Methods and the Effect of Thermal Cycling Parameters on DNA Amplification, Research Reports, *BioTechniques*, *25*(3), 472-480.

Camacho, J. L., Torres, E. M., Cadena, C., Prieto, J., Prieto, L. L., & Torregroza, D. A. (2013). The use of factorial design, ımage analysis, and an efficiency calculation for multiplex PCR optimization. *Journal of Clinical Laboratory Analysis*, 249-252.

Cobb, B. D., & Clarkson, M. J. (1994). A simple procedure for optimising the polymerase chain sreaction (PCR) using modified taguchi methods. *Nucleic Acids Research*, 3801-3805.

Haspolat, I. (2012). Genetiği değiştirilmiş organizmalar ve biyogüvenlik, *Ankara Üniversitesi Veterinerlik Fakültesi Dergisi, 59*, 75-80.

İnan, S. (2009). *Zirkonyum–mangan oksit / pan kompozit kürelerinin hazırlanması ve stronsiyum sorpsiyonunun incelenmesi*, Doktora tezi, Ege Üniversitesi, Fen Bilimleri Enstitüsü, İzmir.

Kanturvardar Tütenyurd, M. (2013). *Solunum yolu viral enfeksiyon etkenlerinin saptanmasında ın-house PCR optimizasyonu*, Doktora Tezi, İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü.

Kitchen Robert R., Kubista, M., & Tichopad, A. (2010). Statistical aspects of Quantitative Real-time PCR experiment design, *Methods*, *50*, 231-236.

Lafrance, R., Villicana, C., Vald´ez-Torres, J. B., Martínez-Montoya, H., Castillo-Ruiz O., Aleman-Castillo S. E., Esparza-Araiza, M. J., & Leon-Felix, J. (2021). Optimization of PCR-based TYLCV molecular markers by response surface methodology, *Gene*, *785*, 145606

Myers, R. H., & Montgomery, D. C. (2002). *Response surface methodology process and product optimization using designed experiments*, John Wiley & Sons, Inc., ABD.

Nabi, N., Zellama, M. S., Hafsa, A. B., & Chaouachi, M. (2016). A new Multiplex PCR-based strategy to detect GM maize events with 2k fractional factorial plan in processed food. *Journal of Research in Biological Sciences*, 35-43.

Niens, M., Spijker, G. T., Diepstra, A., & Meerman, G. J. (2005). A factorial experiment for optimizing the PCR conditions in routine genotyping. *Biotechnol. Appl. Biochem.*, 157-162.

Souza, H. J., Moyses, C. B., Pontes, F. J., Duarte, R. N., Silva, C. E., Alberto, F. L., & Silva, M. B. (2011). Molecular assay optimized by Taguchi experimental design method for venous thromboembolism investigation. *Molecular and Cellular Probes*, 231-237.

Persing H. D. (1991). Polymerase Chain Reaction: Trends to benches, *J. Clin. Microbiol.*, *29*, 1281-1285.

Pryor, R. J., & Wittwer C. T. (2006). Real time polymerase chain reaction and melting curve analysis, *Methods Mol Biol*, *336*(19), 19-32.

Siqueira J. F., & Roças I. N. (2003). PCR methodology as avaluable tool for ıdentification of endodontic pathogens, *J. Dent.*, *31*(5), 2003, 333-339.

Thanakiatkrai, P., & Welch, L. (2011). Using the Taguchi method for rapid quantitative PCR optimization with SYBR Green I. *Int J Legal Med*.

Wadle, S., Lehnert, M., Rubenwolf, S., Zengerle, R., & von Stetten F. (2016). Real-time PCR probe optimization using design of experiments approach, *Biomolecular Detection and Quantification, 7*, 1-8.

Zhang, Q. Y., Zhou W. W., Zhou Y., Wang X. F., & Xu, J. F. (2012). Response surface methodology to design a selective co enrichment broth of Escherichia coli, Salmonella spp. and Staphylococcus aureus for simultaneous detection by multiplex PCR, *Microbiological Research*, *167*, 405-412.