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Mathematical Modeling of DNA Yield Using Box-Behnken Design for Key Extraction Parameters

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ABSTRACT

The current research was initiated to model an enzyme-assisted inorganic salt-based DNA extraction protocol to obtain maximum yield from peripheral whole blood samples. **Objectives:** DNA Key extraction parameters were considered for model optimization such as incubation temperature (54,56,58°C), time (8,10,12hrs.), and proteinase K enzyme concentrations (8,10,12µL). **Methods:** Box-Behnken Design (BBD) was employed to model and simulate the extraction process with three factors/variables and three levels each, which is one of the widely used functional approach of response surface methodology (RSM). With this selected design, RSM entails a mathematical model with experimental data to optimize DNA yield. **Results:** The best fit simulation settings yielded the maximum DNA at an incubation temperature of 56°C for 10hours with a proteinase K enzyme volume of 10µL. Utilizing these parameters, a yield of 300ng/µL was obtained. A confirmation run under these settings validated the prediction, with observed yields closely matching the model's estimates ($p<0.050$), indicating strong agreement between the model and experimental outcomes. **Conclusions:** Regression modeling identified the role of three key factors for optimal DNA yield. Additional extraction parameters can be integrated to develop a more robust and controlled model, ensuring efficient use of limited crime scene samples and reagents in forensic investigations.

INTRODUCTION

The DNA isolation of the specimens is a very important process in modern biomedical studies and diagnosis, and has wide-ranging applications that include genetic studies, as well as in identifying diseases. With the growing need to achieve efficiency and reliability in extraction methodologies, researchers are considering new methods of extraction using materials like magnetic nanoparticles and silica matrices as an overcoming of limitations inherent in traditional methods. This paper is concerned with the mathematical modeling of the response surface modeling (RSM) of Box-Behnken Design (BBD) of the entire whole-blood by inorganic salt-based DNA extraction, which is a basic traditional technique, to comprehend and optimize the parameters that can in turn enhance its economic viability in terms of time, reagents and cost saving [1-3]. DNA extraction procedures often use organic solvents, including phenol-chloroform-isoamyl alcohol (PCI), which is renowned due to its efficiency in getting high yields of DNA, but is very expensive. On the contrary, inorganic salt-based techniques make use of cheaper substances such as sodium chloride (NaCl). The optimization of extraction methods based on inorganic is, however, still a research topic. Regression, ANOVA, and the R2 values are mathematical models that are critical in explaining the complex interplay between inorganic compounds and biological elements and offer a logical structure of forecasting and controlling the results. This can be a groundbreaking strategy of DNA extraction,

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especially in low-resource environments, to facilitate the study of genetic medicine, forensics, and molecular diagnostics research in the third world developing nations [2, 4]. The use of inorganic DNA extraction techniques has a wide range of reagents and protocol parameters, such as incubation temperature, time period, and the most important enzyme concentration or proteinase K, which is the most important in increasing the yield of DNA in whole blood samples. Other statistical methods, like RSM, are used in this study to perform the optimization of DNA extraction parameters rigorously. The key goal is to identify conditions that will maximize the yield of DNA with the use of BBD surface design. This research plays an important role in the establishment of the optimum values of the minimum required incubation temperature, duration of time, and concentration of proteinase K enzyme to maximize the yield of DNA before experimentation [5, 6].

Despite the widespread use of DNA extraction methods in biomedical, forensic, and genetic research, traditional techniques often face challenges such as high cost, time consumption, and variability in yield. While enzyme-assisted inorganic salt-based extraction provides a low-cost alternative, the optimal combination of key parameters—incubation temperature, time, and proteinase K concentration—remains underexplored. Existing studies lack a systematic mathematical modeling approach to predict DNA yield under varying conditions, creating a gap in cost-effective, reproducible, and high-yield extraction strategies. This study aims to develop an integrated model that synthesizes principles from chemistry, biology, statistics, and mathematics to optimize key parameters.

METHODS

This study used Box-Behnken Design (BBD) to model and simulate the extraction process with three factors and three levels each. The study was conducted from August 2023 to July 2024 at Decode Genomics, Lahore, Pakistan and Ethical approval was obtained from the institutional review board (Ref. No. DG-adm-127). For our experiment, DNA was extracted from 200 µL of whole blood using a salt-based inorganic method. After lysis with TE buffer, SDS, and Proteinase K, 6M NaCl was added to pellet protein debris, incubating further on ice as well as centrifuging. DNA was precipitated in the supernatant using chilled isopropanol, which was monitored via gentle inversion. DNA pellets were washed sequentially three times with ethanol before a final harvest to remove impurities. Pure DNA was assayed using gel electrophoresis for intactness, as well as its concentration was determined [7, 8]. The following process variables and their ranges were used regarding the key DNA extraction reagents and chemicals (Table 1).

Table 1: Process variable labels and levels

Labels	Variables (units)	Levels		
		-1	0	1
A	Incubation Temperature (°C)	54	56	58
B	Incubation Time (Hour)	8	10	12
C	Proteinase-K Enzyme (µL)	8	10	12

These variables include levels, variables (incubation temperature (°C), incubation time (hours), and proteinase K enzyme (µL). Each variable is categorized into three levels: -1, 0, and 1. For temperature, the range spans from 54°C, 56°C, 58°C. Time varies from 8, 10, and 12 hours, while proteinase K enzyme volume ranges from 8, 10 and 12 µL. This table serves as a reference for understanding the experimental conditions and their corresponding parameter ranges, crucial for ensuring consistency and repeatability in the experiment's outcomes. BBD was used with Design Expert Software to design a DNA extraction optimization protocol. The software-designed experimental conditions that were simulated to run practical extraction trials as well as to simulate response surface to get a regression line (Table 2).

Table 2: Box-Behnken Experimental Design

Sr. No.	A	B	C	Yield
1	56.00	10.00	10.00	350
2	56.00	8.00	8.00	90
3	56.00	10.00	10.00	350
4	54.00	10.00	8.00	110
5	56.00	10.00	10.00	350
6	58.00	10.00	12.00	310
7	56.00	8.00	12.00	270
8	56.00	10.00	10.00	350
9	56.00	10.00	10.00	350
10	54.00	10.00	12.0	100
11	58.00	10.00	8.00	180
12	58.00	12.00	10.00	300
13	56.00	12.00	8.00	150
14	54.00	12.00	10.00	220
15	54.00	8.00	10.00	200
16	56.00	12.00	12.00	400
17	58.00	8.00	10.00	180

Through systematic exploration of these combinations, we conducted 17 experiments aimed at DNA yield from whole-blood samples followed by DNA quantification. Response Surface Methodology (RSM) is a statistical technique used in modeling and multi-variable process optimization. In our research work, we applied the RSM to optimize the DNA extraction protocol, considering the interactive effects of DNA concentration, incubation time, and incubation temperature on the yield. The technique allows the establishment of predictive models to identify the optimum conditions for efficient and reproducible DNA recovery [9, 10]. The BBD embedded into the RSM was

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applied to optimize DNA extraction conditions by the exploration of the linear and the quadratic effect of three variables. Three levels per parameter (–1, 0, and +1) decrease the number of run experiments economically in comparison with full-factorial experiments. In combination with the RSM, it enabled the building of a predictive model for the identification of the condition providing the optimum DNA recovery [11]. The second-order polynomial model used in response surface methodology (RSM) can be expressed as:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=0}^{k} \sum_{j=2}^{k} \beta_{ij} X_i X_j + e_j \quad (1).$$

After putting all the values in equation (1) and simplifying we get:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + 2\beta_{22} X_2^2 + 2\beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + 2\beta_{23} X_2 X_3 + e \quad (2)$$

Now, by substituting the actual experimental factors: X_1=A (Incubation Temperature), X_2 =B (Incubation Time), X_3 =C (Proteinase K Volume) The model becomes:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + 2\beta_{22} B^2 + 2\beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + 2\beta_{23} BC + e \quad (3)$$

Where Y is the predicted DNA yield $\left(\frac{ng}{\mu L}\right)$, β_0 is the intercept, and β_j , β_{jj} , β_{ij} are the linear, quadratic, and interaction coefficients, respectively and ε is the residual error term. A second order polynomial regression model was applied to correlate the response variable (DNA yield) and the independent variables: proteinase K enzyme concentration, time of incubation, and incubation temperature. The model applied in the Response Surface Methodology (RSM) was as follows: proteinase K enzyme concentration, time of incubation, and incubation temperature. The model applied in the Response Surface Methodology (RSM) was as follows: $Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$ Analysis of variance (ANOVA) was performed to test the significance and adequacy of the model. The model performance was tested based on the values of R^2, adjusted R^2, and predicted R^2 and also from lack-of-fit tests. All statistical analysis and model fitting were performed by Design Expert software (Stat-Ease Inc., USA). To authenticate the validity and predictive capacity of the constructed RSM model, a further experimental run was performed according to the optimal settings indicated by the model. The optimal levels of the concentration of the enzyme proteinase K, the time of incubation, and the temperature of incubation used for this purpose were derived from the numerical optimization function of the Design Expert software. The DNA extraction was performed according to the above-mentioned optimized parameters and the extracted DNA yield was validated against the corresponding value anticipated from the model to check the model's accuracy [12]. Model selection applied sequential sum of squares and model summary statistics. The quadratic model was the preferred model of fit because its p-value was significant, its R^2 and it's adjusted R^2 The values were reasonable, and it was able to capture linear and interaction effects between the variables that influenced DNA yield.

RESULTS

This equation represents the response function used for the estimation of DNA extraction yield from whole blood. The equation contains various independent variables, i.e., incubation temperature (A), incubation time (B), and Proteinase K Enzyme Concentration (C), with corresponding coefficients estimated from the experimental data. The equation follows the following form: $Y= 350.00 + 32.50A + 51.25B + 68.75C + 45.00AB +35.00AC +17.50BC - 98.75A^2 - 46.25B^2 - 76.25C^2$. Here, (Y) represents the DNA extraction yield in a ng/uL. The independent variables and the interaction effects between the variables show the influence of the variables on the extraction. The linear and nonlinear relationships between the variables and the extraction yield represented by the quadratic terms (such as A^2, B^2, and C^2) give insight about the potential nonlinear relationships. The above equation serves as the predictive model to predict DNA extraction yield from the specified experimental condition. The adequacy of the Box-Behnken model constructed was statistically validated, showing a very good fit with a high R^2 value of 0.975, an adjusted R^2 of 0.958, and a predicted R^2 of 0.922, while the lack-of-fit test value of 0.231 indicated an insignificant lack-of-fit, confirming that the model can be applied for the estimation of DNA yield under different extraction conditions. The relationship between incubation temperature, incubation time, and Proteinase K enzyme concentration and DNA yield was captured by the model, with linear, quadratic, and interaction terms indicating that the effects were not necessarily linear and allowing for the determination of optimal extraction conditions. Regression analysis showed that incubation temperature had a significant effect on DNA yield, with the response surface being concave and the optimal yield achieved at approximately 56°C; deviations to 54°C or 58°C resulted in decreased yield, reflecting the heat sensitivity of the enzymatic lysis reaction. Incubation time also significantly affected DNA yield, with a parabolic response surface and optimal yield at 10 hours, while shorter or longer durations reduced efficacy. Proteinase K volume exerted a significant influence, with the highest yield at 10 µL and lower or higher volumes leading to reduced yield due to incomplete lysis or over-digestion. Sequential sum of squares analysis and model summary statistics indicated that the quadratic model was the most appropriate for predicting DNA yield, as it was statistically significant, captured the relationships between variables effectively, and demonstrated a suitable fit with R^2=0.8751 and adjusted R^2=0.7146, while linear, 2FI, and cubic models were less suitable due to lack of explanatory power, aliasing, or inconsistencies between predicted and observed responses (Table 3).

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Table 3: Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob>F	Remarks
Sequential Model-Sum of Squares for Total Yield						
Mean	1.028×10^{006}	1.00	1.028×10^{006}	–	–	–
Linear	67275.00	3.00	22425.00	2.43	0.1118	Suggested
2F1	14225.00	3.00	4741.67	0.45	0.7238	–
Quadratic	82336.76	3.00	27445.59	8.22	0.0108	Suggested
Cubic	23375.00	3.00	7791.67	6.366 x10007	<0.0001	Aliased
Residual	0.000	4.00	0.000	–	–	–
Total	1.21×10^{006}	17.00	71470.59	–	–	–
Model Summary Statistic						
Source	Std. Deviation	R^2	Adjusted R^2	Predicted R^2	PRESS	Remarks
Linear	96.05	0.3594	0.2115	-0.0618	1.988×10^{005}	–
2F1	102.82	0.4353	0.0965	-0.7405	3.258×10^{005}	–
Quadratic	57.79	0.8751	0.7146	-0.9977	3.740×10^{005}	Suggested
Cubic	0.000	1.0000	1.00000	–	–	Aliased

ANOVA outcomes indicated the statistically significant fitted model ($p = 0.0180$), so the variation in DNA yield could be well explained using the selected factors. Incubation time (B) and Proteinase K volume (C) indicated significant effects ($p=0.0405$ and $p=0.0120$), respectively, while temperature (A) indicated a moderate effect ($p=0.1557$). Of particular interest was the statistical significance of the second-order terms A^2 and C^2, indicating non-linear behavior of the parameters. The interaction terms (AB, AC, BC) indicated statistical non-significance. The model indicated a large coefficient of determination ($R^2=0.8751$), showing significant agreement between the predictive and the observed responses. An adequate precision (AP=5.895) confirmed the signal-to-noise ratio of the model being satisfactory for the exploration of the design space (Table 4).

Table 4: ANOVA for Response

Source	Sum of Squares	DF	Mean Square	F-Value	p-Value
Model	1.63×10^{005}	9	18204.08	5.45	0.0180
A-Temperature	8450.00	1	8450.00	2.53	0.1557
B-Time	21012.50	1	21012.50	6.29	0.0405
C-Proteinase k	37812.50	1	37812.50	11.32	0.0120
AB	8100.00	1	8100.00	2.43	0.1633
AC	4900.00	1	4900.00	1.47	0.2651
BC	1225.00	1	1225.00	0.37	0.5638
A^2	41059.21	1	41059.21	12.30	0.0099
B^2	9006.58	1	9006.58	2.70	0.1445
C^2	24480.26	1	24480.26	7.33	0.0303
CV	23.50				
AP	5.895				
PRESS	3.740+005				
Mean	245.88				
R^2	0.8751				
Adj-R^2	0.7146				

The next graphs provide the combined diagnostic plots employed to check for the fitness of the fitted regression model. The normal probability plot of studentized residuals indicates that the residuals are normally distributed since they lie along a straight line. The predicted vs. actual plot shows a good correlation between observed and model-predicted values, with data points tightly scattered around the diagonal line, indicating the accuracy of the model. The residuals vs. predicted plot shows a random distribution of residuals, indicating homoscedasticity and the lack of systematic error. Both plots confirm that the regression model is statistically valid and accurate for prediction. (A) Normal probability plot. Blue circles closely follow the reference line, indicating normally distributed residuals. (B) Predicted vs. actual values. Green triangles lie near the diagonal, showing strong agreement between observed and predicted DNA yield. (C) Residuals vs. predicted values – Red squares are randomly scattered around zero, confirming homoscedasticity and model validity. Axes represent expected vs. actual residuals (A), actual vs. predicted yield (B), and predicted yield vs. residuals (C) (Figure 1).

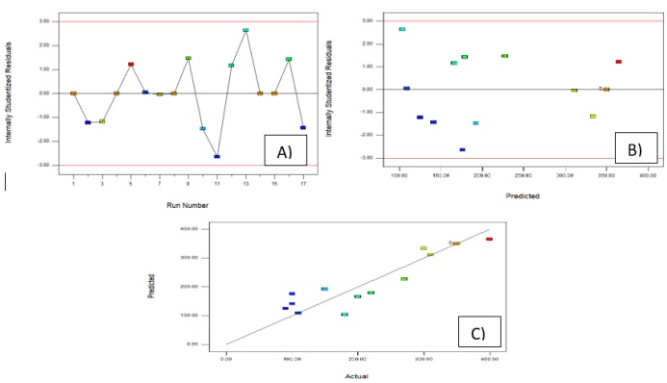

Figure 1: (A) Normal Probability Plot. (B) Predicted Vs. Actual Values. (C) Residuals Vs. Predicted. Axes Represent Expected Vs. Actual Residuals (A), Actual Vs. Predicted Yield (B), and Predicted Yield Vs. Residuals (C)

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A three-dimensional scatter plot was created to demonstrate the interaction effects of incubation temperature, incubation time, and Proteinase K enzyme concentration on DNA yield using the Box-Behnken experimental design. The plot visually shows a nonlinear response surface with the highest DNA yield (~350–400 ng/µL) at points closest to the center values of 56°C, 10 hours, and 10 µL, validating the regression model's prediction. Deviation from these optimal conditions resulted in a drop in yield, validating the occurrence of quadratic effects and that there is a very small range within which optimal extraction takes place. The visualization serves to reinforce that incubation time and enzyme concentration, specifically, need to be precisely balanced to preclude under- or over-digestion, both of which have a detrimental impact on yield. The even gradient of DNA concentration through the plotted surface gives a clear, intuitive view of the sensitivity of the extraction protocol to changes in each parameter. Generally, the plot reinforces the statistical results and verifies the robustness of the optimized conditions that were identified by the model (Figure 2).

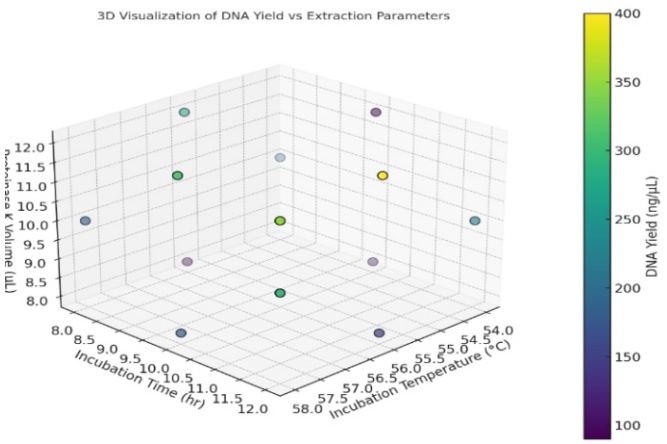

Figure 2: Three-Dimensional Scatter Plot

DISCUSSION

The optimization of the DNA extraction process by Box-Behnken design (BBD) showed that incubation temperature, incubation time, and Proteinase K enzyme concentration had a great nonlinear influence on the generation of the DNA, as had been previously reported [13]. The high-predictive quadratic regression model (R2=0.8751) illustrated that even minor alterations to the optimal conditions (56°C, 10 hours, 10 µL) had a major impact on the DNA recovery, which shows the sensitivity of enzymatic activity [14, 15]. The results are in line with the earlier optimization experiments with the use of factorial design and response surface methodology, which found the same quadratic behavior in the course of enzymatic extraction [16-18]. The concentration of proteinase K is critically important in determining extraction yield, due to the existence of two opposing conditions between total lysis and possible over-digestion [14, 16]. Comprehensively, the paper highlights the usefulness of response surface methodology (RSM) and Box-Behnken design in the effective determination of optimal conditions using a limited number of experimental programs to increase reproducibility and cost-efficiency [18, 19]. The improved salt-based extraction process was found to be appropriate in extracting high-quality DNA using a small amount of whole blood, and it is possible that it can be used in clinical diagnostics, forensic cases, and genetic studies [7, 20]. Even though the model system was chicken blood, the principles realized in this case can be applied in other species after validation. The model can be further improved in future research by adding some parameters to it, like buffer composition, storage conditions, and sample type.

Although the current model effectively identifies optimal extraction conditions for whole blood, it is limited to specific sample types and does not consider additional variables such as buffer composition, storage conditions, or variations across species. Future research could integrate these factors into an expanded model, enabling broader applicability, improved reproducibility, and the development of standardized protocols for clinical, forensic, and molecular biology applications.

CONCLUSION

An enzyme-based DNA extraction of whole blood was optimized by box-Behnken design and response surface methodology. The quadratic equation (R 2=0.8751) established optimal conditions (56 0 C, 10 hours, 10 0 -1) to give maximum yield. Reliability was also experimentally validated, and provides a low-cost, fast, and repeatable alternative to commercial kits with low sample volumes.

Authors' Contribution

Conceptualization: SZ, RS
Methodology: SZ, FA, RS
Formal analysis: SZ, FA, RS
Writing and Drafting: SZ, FA, RS
Review and Editing: SZ, FA, RS

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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