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Original Article

Optimizing peripheral blood chromosome analysis: effects of refrigeration time and blood volume

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Abstract: Objective: This study aims to investigate the impact of refrigeration time and blood volume on the success rate of peripheral blood chromosomal analysis using response surface methodology (RSM). Methods: Peripheral blood samples from 30 volunteers were subjected to chromosomal analysis under different refrigeration duration periods (≤7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days) along with different blood volumes (0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, and 0.8 mL). The effects of refrigeration time and blood volume on the success rate of peripheral blood chromosomal analysis were determined using the Chi-square test for trend, followed with Spearman's rank correlation coefficient, and RSM analysis to identify the optimal combination of refrigeration time and blood volume. Results: The refrigeration time within 10 days had a minor impact on the success rate, while refrigeration time more than 11 days significantly decreased the success rate. An increase in blood volume slightly improved the success rate. The success rate showed both linear and nonlinear changes with refrigeration time, while the effect of blood volume was primarily linear. The highest success rate was observed at a refrigeration time of ≤7 days and a blood volume of 0.8 mL. The interaction between refrigeration time and blood volume had a significant impact on the success rate. Conclusion: It is recommended to keep the refrigeration time of blood samples within 7 days and control the blood volume at 0.8 mL to maximize the success rate of chromosomal analysis.

Keywords: Refrigeration time, blood volume, chromosome analysis, response surface methodology, success rate

Introduction

Chromosomes and chromatin, as carriers of genetic information, play a crucial role in the accurate transmission of genetic information through their morphological changes during the cell cycle [1]. Peripheral blood chromosomal karyotype analysis is a key technique for revealing genetic diseases and guiding clinical diagnosis and treatment [2-4]. Since Hungerford published the techniques for culturing human peripheral blood leukocytes and preparing chromosomes in 1965, lymphocyte culture, cell harvesting, slide preparation, and chromosome banding have played vital roles in the process of chromosome preparation [5, 6]. However, due to the lengthy experimental cycle, environmental factors, differences in technician proficiency, or occasional operational errors, it is challenging to obtain metaphase cells that meet the requirements for karyotype analysis every time. Thus, the test subjects have to undergo repeated blood draw for remedial testing, thereby increasing the time, cost, and testing cycle [7, 8]. Currently, there is no unified standard for karyotype preparation, and there are significant differences in laboratory operations [9, 10]. The chromosome preparation process involves numerous steps, and any improper handling can affect the quality of

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specimens. The European Guidelines for Cytogenetic Analysis encompass sample preparation, analysis, reporting, and the application of specific techniques, emphasizing the necessity of professional knowledge and skills among staff, as well as the quality control and assurance measures that laboratories should follow [11]. Bulla et al. [6] investigated the effects of different culture establishment times (0 hour, 24 hours, and 48 hours) on cell viability and band resolution, suggesting that cell culture should be initiated within 24 hours after blood collection to ensure high-quality results in karyotype analysis. Although these studies mention some details about sample handling and preservation, they do not address specific refrigeration duration or the blood volume.

Furthermore, we must confront a practical issue, which is in primary healthcare facilities, due to limitations in technology and equipment, samples are often sent to higher-level hospitals or specialized laboratories for analysis. The quality and preservation status of the samples during this process directly affect the final examination results [12]. Investigating the relationship between blood sample volume, refrigeration time, and the success rate of examinations can help us understand the changes in sample quality under different conditions. This, in turn, provides specific operational guidelines for primary healthcare institutions, ensuring that even with limited resources, the integrity and viability of samples can be maximized through proper sample handling and preservation methods.

To this end, this study aims to achieve high-quality peripheral blood chromosomal karyotypes by employing response surface methodology (RSM) to investigate the optimal combination of blood sample refrigeration time and blood volume. RSM, a method that combines mathematics and statistics, is used for developing, enhancing, and optimizing procedures [13, 14]. As a classic multifactorial analysis method, RSM can effectively address the optimization of peripheral blood chromosomal examination methods under conditions of multifactorial interactions. RSM will help establish a model between blood sample volume, refrigeration time, and examination success rate, thereby providing a scientific basis for improving the quality control level of peripheral blood chromosomal examinations. In this study, we conducted a retrospective analysis to investigate the impact of refrigeration time and injected blood volume on the success rate of peripheral blood chromosomal analysis. The study aimed to optimize the conditions for chromosomal karyotype examination by examining historical data from July 2021 to June 2022.

Subjects and methods

Subjects

The retrospective analysis was conducted using data and peripheral blood samples collected from volunteers between July 2021 and June 2022. The study involved reviewing the previously collected samples, their processing, and the results of chromosomal analysis performed during that period. Thirty volunteers from the health examination outpatient clinic of Liuzhou Maternity and Child Healthcare Hospital were included in the study.

Inclusion criteria: age between 20 and 50 years, with normal routine blood indicators and blood sugar levels, and informed consent from the participants. Exclusion criteria: history of medication use within one week prior to blood collection. The study was approved by the Ethics Committee of Liuzhou Maternity and Child Healthcare Hospital (approval number: Quick review - scientific research-2020-022), and volunteers signed informed consent forms for clinical research.

Blood sample collection and processing

Peripheral blood samples were collected from the study subjects via venipuncture at the elbow, with a total volume of 30 ml, divided into 6 vacutainers (5 ml in each) containing sodium heparin anticoagulant. After collection, the blood samples were gently mixed to ensure uniform distribution of the anticoagulant, labeled with group identifiers, and then stored in a medical refrigerator at 4°C. The refrigeration time groups were: ≤7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, and 14 days. The injection blood volume groups were: 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, and 0.8 mL.

Cell culture and chromosome harvesting

Under a biological safety cabinet, cell injection was performed for each study subject based on

Table 1. Impact of different refrigeration durations on chromosome examination results [n (%)]

Refrigeration time (days)	+	-	±	Total
≤7	152 (72.38)	37 (17.62)	21 (10.00)	210
8	85 (40.48)	73 (34.76)	52 (24.76)	210
9	52 (24.76)	98 (46.67)	60 (28.57)	210
10	39 (18.57)	113 (53.81)	58 (27.62)	210
11	3 (1.43)	180 (85.71)	27 (12.86)	210
12	0 (0.00)	205 (97.62)	5 (2.38)	210
13	0 (0.00)	208 (99.05)	2 (0.95)	210
14	0 (0.00)	210 (100.00)	0 (0.00)	210

"+" indicates a positive result, meaning that cell culture and karyotype analysis were successful; "-" indicates a negative result, meaning no analyzable chromosome karyotype was available; "±" indicates the presence of a karyotype, but with substandard banding or insufficient numbers for analysis.

the combination of refrigeration time and blood volume of the blood samples using a sterilized pipette. After injection, the samples were placed in a CO_2 incubator for cell culture for 67 hours. Thirty minutes before the end of the culture, colchicine was added to terminate the culture, and the cells were harvested using an automated chromosome harvester.

Chromosome slide preparation and G-banding

The volume of the harvested cell suspension was adjusted to 2 mL, and slide preparation was performed using a chromosome spreader under conditions of 25.0°C and 50.0% humidity. Two slides were prepared from each sample tube, which were then placed in a drying oven and baked at 80.0°C for 4 hours. The slides were treated in a 0.25% EDTA pancreatic enzyme solution for 12 seconds, with the duration adjusted according to the clarity of the banding. Subsequently, the enzymatic action was terminated in a phosphate buffer solution at pH 6.8, and the slides were stained with Giemsa stain, washed, and air-dried for subsequent analysis.

Karyotype analysis and result interpretation

Suitable karyotypes were scanned using a Zeiss automated scanning analyzer (sensitivity coefficient set at 7.0, scanning the entire slide), and karyotype analysis was performed using the iKaryoS software. Samples that successfully completed karyotype analysis were recorded as "+", samples with no analyzable karyotype were recorded as "-", and samples with karyotypes present but with substandard banding or insufficient numbers for analysis were recorded as "±".

Data collection and outcome measures

The primary outcome of this study was the success rate of peripheral blood chromosomal analysis, defined as the proportion of samples that yielded analyzable karyotypes suitable for chromosomal examination. The secondary outcome measures included the evaluation of the impact of different refrigeration durations and injected blood volumes on the success rate. Additionally, the identification of the optimal combination of these factors was achieved through RSM analysis.

Statistical analysis

Data analysis was conducted using IBM SPSS 27.0. Initially, the number of positive and negative cases were counted under different refrigeration time and injected blood volumes, and the positive and negative ratios for each group were calculated. Comparisons of successful chromosome detection rates among various refrigeration durations and injected blood volumes were analyzed using the chi-square test for trend. If significant differences were indicated by the chi-square test for trend, Spearman's rank correlation coefficient between refrigeration time, injected blood volume, and the success rate of chromosomal examination was calculated, and online plotting was conducted using the website (http://www.bioinformatics.com.cn/) [15]. Locally weighted regression (Lowess) was used to plot the scatter plots of correlation coefficients. Response surface analysis was employed to determine the optimal experimental conditions, and R Studio 4.3.0 software was utilized for conducting the response surface analysis and plotting the response surface graphs. The significance level for the test was set at α=0.05.

Results

Descriptive statistics of chromosome examination

The results of cell culture and karyotype analysis under different refrigeration durations are shown in **Table 1** and **Figure 1**. The percentage

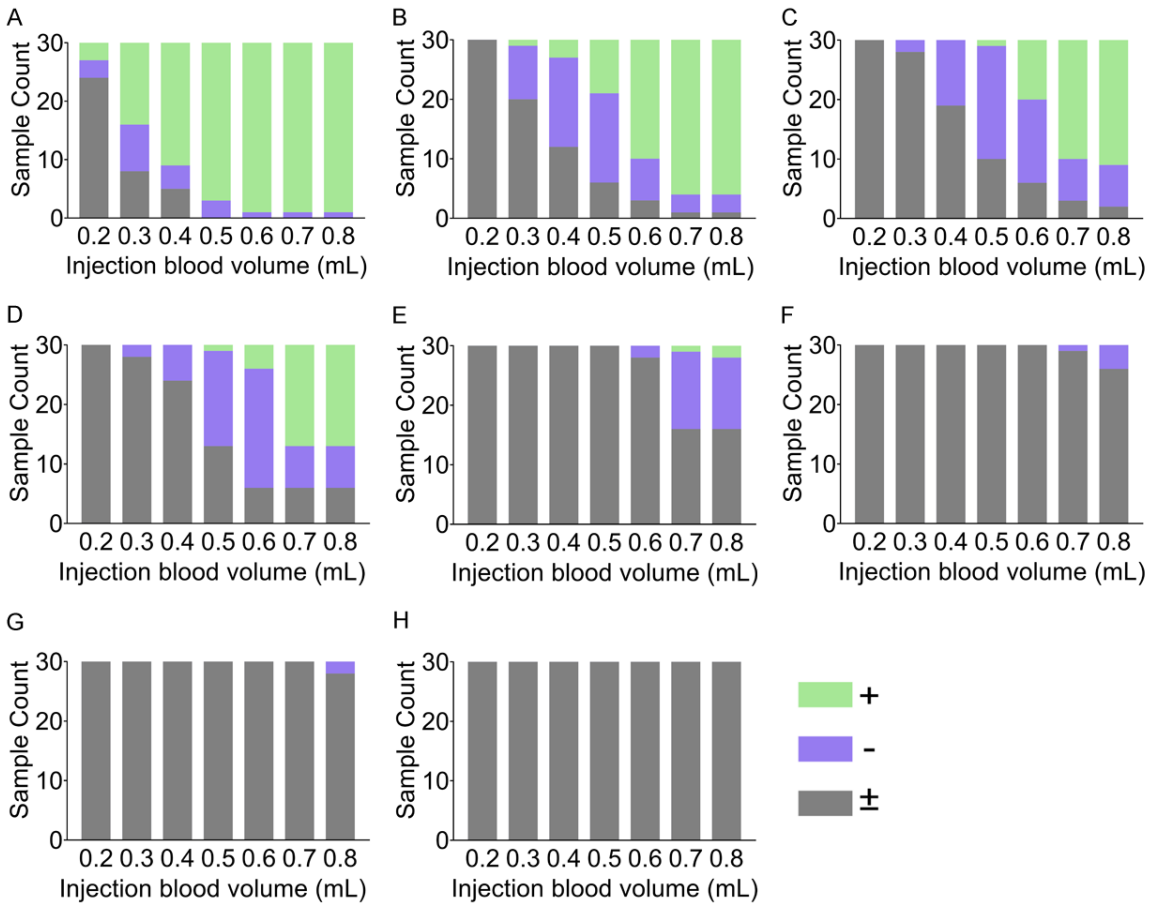

Figure 1. Frequency distribution of chromosome examination results under different refrigeration durations and blood volumes. The frequency distribution chart illustrates the changes in chromosome examination results under different refrigeration durations (A-H representing ≤7 days to 14 days) and blood volumes. The horizontal axis represents the blood volume, and the vertical axis represents the number of chromosome examination results. Green represents positive results, purple represents negative results, and gray represents cases with a karyotype present but with substandard banding or insufficient numbers for analysis.

of positive cases gradually decreased with the increase in refrigeration duration, especially after exceeding 10 days, while the proportion of "±" cases (karyotype present but not meeting the standard) showed little fluctuation.

Table 2 presents the results of chromosome examination under different blood volumes. As the injected blood volume increased, the proportion of positive cases gradually rose and peaked at 0.7 mL and 0.8 mL, with the overall positive ratio exceeding 30%. Meanwhile, the proportion of "±" (karyotype present but not meeting the standard) showed some fluctuations, but the overall trend was not significant. When the refrigeration time exceeded ≥11 days, the positive case ratio did not increase with the increase in blood volume (**Figure 1**).

Comparison of differences in examination outcomes among different refrigeration durations and blood volumes

In the analysis of the impact of different refrigeration durations on the success rate of chromosome detection, we utilized the chi-square test for trend. **Table 3** shows that the refrigeration time significantly influenced the success rate of chromosome detection ($P<0.001$). Further multiple comparisons showed no significant difference between ≤7 days and 8, 9 and 10 days. However, significant differences were identified between ≤7 days and all subsequent groups (11 days and beyond). No significant differences were noted among the groups from 8 days onwards.

Table 2. Chromosome examination results by blood volume [n (%)]

Blood volume (mL)	+	-	±	Total
0.2	3 (1.25)	234 (97.50)	3 (1.25)	240
0.3	15 (6.25)	204 (85.00)	21 (8.75)	240
0.4	24 (10.00)	180 (75.00)	36 (15.00)	240
0.5	38 (15.83)	149 (62.08)	53 (22.08)	240
0.6	63 (26.25)	133 (55.42)	44 (18.33)	240
0.7	93 (38.75)	115 (47.92)	32 (13.33)	240
0.8	95 (39.58)	109 (45.42)	36 (15.00)	240

Table 3. Descriptive statistics and chi-square test for trend for success rate of chromosome detection at different refrigeration durations (≤7 days to 14 days)

Refrigeration time (days)	M (P25, P75)	Chi-square test for trend	
		χ^2	P
≤7	90.00 (46.70, 96.70)	505.972	<0.001
8	30.00 (3.30, 86.70)		
9	3.30 (0.00, 66.70)		
10	3.30 (0.00, 56.70)		
11	0.00 (0.00, 3.30)		
12	0.00 (0.00, 0.00)		
13	0.00 (0.00, 0.00)		
14	0.00 (0.00, 0.00)		

It includes the median (M) and interquartile range (P25, P75) for chromosome detection success rates, along with the chi-square and corresponding *P*-values from the chi-square test for trend.

Table 4. Descriptive statistics of success chromosome detection rate at different blood volume levels with chi-square test for trend

Blood volume (mL)	M (P25, P75)	Chi-square test for trend	
		χ^2	P
0.2	0.00 (0.00, 0.00)	208.542	<0.001
0.3	0.00 (0.00, 2.48)		
0.4	0.00 (0.00, 7.50)		
0.5	1.65 (0.00, 23.33)		
0.6	6.65 (0.00, 58.35)		
0.7	30.00 (0.00, 81.70)		
0.8	31.70 (0.00, 82.53)		

It includes the median (M) and interquartile range (P25, P75) for success chromosome detection rates at blood volumes ranging from 0.2 to 0.8 mL, as well as the chi-square and corresponding *P*-values for the chi-square test for trend.

The median success rate of chromosome detection exhibited an overall upward trend with increasing blood volume (**Table 4**). The chi-square test for trend showed that there was significant difference in the success rate of chromosome detection across varying injected blood volumes (P<0.001). The results indicated a positive trend in the effect of increased blood volume on the success rate of chromosome detection.

Correlation analysis

The Spearman rank correlation coefficients between refrigeration time, injected blood volume, and the success rate of chromosome examination are depicted in **Figure 2**. The Spearman rank correlation coefficient between refrigeration time and success rate was -0.766 (P<0.001), which signifies a strong negative correlation between the two variables. Conversely, the Spearman rank correlation coefficient between injected blood volume and success rate was 0.414 (P=0.002), indicating a moderate positive correlation.

Response surface analysis

We conducted a response surface analysis to evaluate the impact of refrigeration time and blood volume on the success rate of chromosome examination. The response surface model accounted for approximately 88.9% of the response variance, suggesting a good fit (**Table 5**). The adjusted R-squared value of 87.8% indicated that the model maintained high explanatory power even after accounting for its complexity. The F-statistic value was 80.38 with P<0.001, indicating that the model was overall significant. The model coefficients revealed that both the primary and interaction terms of refrigeration time and injected blood volume significantly influenced the response variable. Specifically, an increase in refrigeration time was associated with a decrease in success rate, whereas an increase in injected

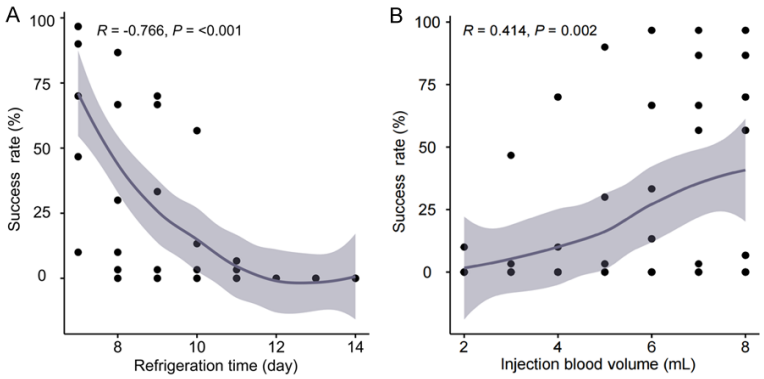

Figure 2. Spearman rank correlation analysis of refrigeration time, blood volume and chromosome examination success rate. A: The negative correlation between refrigeration time and success rate is evident, with a coefficient of -0.766 (P<0.001). B: The positive correlation between blood volume and success rate is evident, with a coefficient of 0.414 (P=0.002).

Table 5. Statistical coefficients for response surface analysis of the effect of refrigeration time and blood volume on the success of chromosome examination

Coefficient	Estimate	Standard error	t	P
(Intercept)	1.803	0.411	4.387	<0.001
Refrigeration time	-0.423	0.072	-5.843	<0.001
Blood volume	3.231	0.569	5.679	<0.001
Refrigeration time × Blood volume	-0.278	0.033	-8.311	<0.001
Refrigeration time^2	0.022	0.003	6.651	<0.001
Blood volume^2	0.383	0.442	0.868	0.390
R-squared	0.889			
Adjusted R-squared	0.878			
F	80.38			
P	<0.001			

This table details the estimates, standard errors, t-statistics, and corresponding *P*-values for both the linear and quadratic effects of refrigeration time and blood volume on the examination success rate. The R-squared and Adjusted R-squared values represent the model's goodness-of-fit and adjusted goodness-of-fit, respectively, while the F-statistic and its corresponding *P*-value are employed to assess the overall significance of the model.

blood volume tended to improve the success rate. Furthermore, a significant interaction effect was observed between refrigeration time and injected blood volume. The quadratic term reflects the nonlinear impact of the independent variables on the dependent variable. Notably, the coefficient of the quadratic term for refrigeration time was significant (P<0.001), while the coefficient for the quadratic term of injected blood volume was not (P=0.390), suggesting that the effect of refrigeration time on the success rate exhibited both linear and nonlinear variations, whereas the effect of injection blood volume on the success rate was primarily linear.

The response surface plots delineate the interplay between refrigeration time and injected blood volume on the success rate of chromosome examination, highlighting the optimal combination points. As depicted in **Figure 3**, the most favorable combination for achieving the highest success rate was identified at a refrigeration time of ≤7 days and a blood volume of 0.8 mL. Conversely, the lowest success rate was observed with a refrigeration time of 13 days and an injection volume of 0.6 mL.

Discussion

To the best of our knowledge, based on a review of the literature, there is no work that correlates the refrigeration time of human peripheral venous blood specimens, the injection blood volume, and the success rate of their karyotype analysis. Although the operation of peripheral blood chromosome examination is intricate, it is possible to analyze the effectiveness of chromosome karyotype analysis by examining the impact of the cellular state at the time of injection (with different preservation durations and blood volume gradients) without altering the conditions of culture, harvesting, and banding preparation. This approach can provide insights into the optimal blood volume and effective preservation time for peripheral blood chromosome examination.

From the perspective of different refrigeration durations, within the first 10 days of refrigeration (from ≤7 days to 10 days), although there were changes in the median, these changes were not statistically significant. This indicates

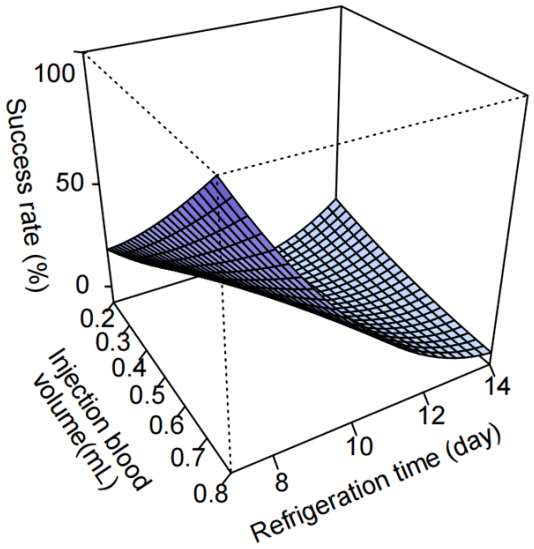

Figure 3. Response surface analysis (RSM) of refrigeration time and blood volume on success rate of chromosome examination. It illustrates the predicted success rate across varying conditions of refrigeration time and blood volume. The optimal condition, characterized by a refrigeration time of ≤7 days and a blood volume of 0.8 mL, is situated at the apex of the response surface, signifying that the highest success rate for chromosome examination is achieved under these parameters.

that the impact of refrigeration time on the samples is relatively minor within this time frame. However, significant differences were observed between the groups from ≤7 days to 11 days and beyond. This suggests that after exceeding a certain threshold (in this experimental design, 11 days), the samples underwent significant changes. In recent years, it has been reported in the field of cytogenetic techniques that circulating nucleic acids can remain stable under appropriate storage conditions for up to 7 days [16]. Doeleman et al. [17] suggested that although lymphocyte counts remained stable within 7 days after blood collection, culturing and chromosome preparation beyond 24 hours post-collection might lead to a decrease in the acceptability of analysis results. The negative correlation between refrigeration time and success rate indicates that the success rate of chromosome examination gradually decreases with the extension of sample refrigeration time. This is because the reduction in cell count or cell viability over time may result in an insufficient number of metaphase cells, thereby affecting the outcome of chromosome analysis [18, 19]. Therefore, to ensure the accuracy and reliability of chromosome preparation, it is generally recommended to perform cell culture and chromosome preparation as soon as possible after blood sample collection [20]. However, in clinical practice, blood samples may need to be stored for 24 hours or longer, or even transported to external laboratories, which can take several days [16, 21]. To ensure an adequate number and vitality of cells for chromosome preparation and analysis, considering a higher injection blood volume is a viable solution.

Our study results indicate a positive trend in the impact of increased injection blood volume on the success rate of chromosome detection, although this effect is not statistically significant. The positive correlation between blood volume and success rate also suggests that, within a certain range, increasing the blood volume can contribute to a higher success rate in chromosome examination. Insufficient blood volume may lead to a lack of cell numbers, which can affect the preparation and examination of chromosomes. In cell culture, cell density is a critical factor influencing cell proliferation and division [22, 23]. For instance, pre-B acute lymphoblastic leukemia cells (ALL3) exhibit poor or no growth under low-density conditions, yet thrive better under high initial cell density conditions. ALL3 cells secrete soluble factors when grown at high density, which can stimulate the growth of low-density ALL3 cells. A high-density environment is conducive to mimicking the function of the stromal layer, facilitating the generation of human B lymphocytes [24, 25]. Similarly, in the context of chromosome examination, increasing the blood volume (i.e., cell density) can, to some extent, enhance the success rate of chromosome detection. However, further research is needed to validate the impact of increased blood volume on the success rate of chromosome detection, the specific mechanisms by which cell density affects cell proliferation and division, and the generalizability of these findings across different cell types and conditions. Additionally, it is important to investigate other factors that may influence the success rate of chromosome detection.

The response surface analysis revealed that both the linear and nonlinear coefficients of refrigeration time were significant, indicating

that within the range of ≤7 days to 14 days, the success rate may gradually increase or remain stable with the extension of refrigeration time. However, beyond a certain threshold, the success rate may begin to decline. The effect of blood volume on the success rate was primarily linear, suggesting that the success rate may gradually increase or remain stable as the blood volume increases. Notably, there is a significant interaction between refrigeration time and blood volume. The optimal blood volume may vary under different refrigeration durations. For instance, with shorter refrigeration time where cell viability is higher, less blood volume may be needed to achieve a high success rate. Conversely, with longer refrigeration time and reduced cell viability, more blood volume might be required to provide sufficient nutrients and growth factors to support cell survival and division. However, this does not imply that simply increasing the blood volume can resolve all issues [9, 26]. In practical applications, increasing the blood volume may introduce other issues, such as weakened cell-to-cell interactions and intensified competition for nutrients [27]. Therefore, the optimal strategy is to consider multiple factors and determine the most suitable culture conditions through experimental optimization. In our study, the highest point on the response surface revealed that the highest success rate was achieved with a refrigeration time of ≤7 days and blood volume of 0.8 mL. By identifying the optimal combination point, we can provide specific guidance for experimental design to maximize the success rate of chromosome examination.

In summary, this study analyzed the impact of refrigeration time and blood volume on the success rate of chromosome examination using a response surface model. The model not only has a good fit but also provides valuable information about the optimal operating conditions and the shape of the response surface. These results are significant for understanding the mechanisms by which refrigeration time and blood volume affect the examination outcomes and for guiding practical operations. However, the study's sample source is limited and may not fully represent all possible scenarios, which could affect the generalizability of the results. Furthermore, while the response surface model can provide information for optimizing operating conditions, the accuracy of the model depends on the quality of the data and the choice of analysis methods. A small sample size may impact the model's predictive power. Additionally, in clinical settings, there may be more variables at play, such as individual patient differences, sample transportation, and processing conditions. Therefore, future research could consider expanding the sample size, incorporating additional influencing factors, or employing alternative models to further validate and extend the conclusions of this study.

Conclusion

To maximize the success rate of chromosome examinations, we recommend adhering to strict refrigeration time of no more than 7 days for blood samples and controlling the blood volume at 0.8 mL in practical operations. This approach can provide better cell growth conditions for chromosome preparation, enhancing the reliability and success rate of the experiments.

Acknowledgements

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Disclosure of conflict of interest

None.

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