



THE EFFECT OF FREE SPACE VARIATION IN 3 ML K₂EDTA VACUUM TUBE ON THE ACCURACY OF HEMATOCRIT AND RED BLOOD CELL COUNT TESTS

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Abstract

The accuracy of hematology test results is influenced by various factors, including the quality of the pre-analytical phase. One condition that is still encountered in laboratory practice is the inconsistency of blood filling volume in EDTA vacuum tubes, which creates headspace and can alter the ratio of blood to anticoagulant. This condition has the potential to affect hematocrit and erythrocyte count test results. The aim of this study was to analyze the effect of free space variation in 3 mL K₂EDTA vacuum tubes on the accuracy of hematocrit and red blood cell count results. This study was a laboratory experiment with a quantitative approach and a paired design (within subjects). The study subjects consisted of 37 healthy young adults aged 18 to 23 years. Venous blood was collected from each subject and placed in two 3 mL K₂EDTA vacuum tubes with filling volumes of 2.2 mL and 3.0 mL. Hematocrit and erythrocyte counts were examined using a validated automatic hematology analyzer. Data analysis included descriptive statistics, Shapiro-Wilk normality test, and paired samples t-test with a significance level of $p < 0.05$. The mean erythrocyte count at a filling volume of 2.2 mL was 4.93 ± 0.40 million cells/ μ L and at a filling volume of 3.0 mL was 4.92 ± 0.39 million cells/ μ L. The mean hematocrit values were $41.32 \pm 3.27\%$ and $41.14 \pm 3.18\%$, respectively. The results of the paired samples t-test showed no statistically significant difference in red blood cell count ($p = 0.633$) or hematocrit ($p = 0.051$) between the two filling volume variations. Variations in free space in 3 mL K₂EDTA vacuum tubes with 2.2 mL and 3.0 mL blood filling did not show statistically significant differences in hematocrit and erythrocyte count results. Under well-controlled pre-analytical conditions, blood filling up to 2.2 mL still produced hematological values comparable to full filling.

Keywords: Free Space ; K₂EDTA ; Hematocrit ; Erythrocytes ; Pre-Analytical Phase

1. Introduction

Hematology testing is one of the most commonly used laboratory tests in healthcare because it plays an important role in assessing the physiological and pathological conditions of patients. Basic hematology parameters, such as hematocrit and erythrocyte count, are widely used to evaluate anemia, hydration disorders, and various clinical conditions related to circulation and tissue oxygenation. The accuracy of hematology test results is highly dependent on the quality of the blood sample being analyzed, so quality control at every stage of the test, especially the pre-analytical phase, is crucial in ensuring the reliability of laboratory results. (Lippi et al., 2019)

The pre-analytical phase covers all procedures prior to sample analysis, ranging from patient preparation, venipuncture techniques, tube and anticoagulant selection, to blood sample handling and storage. Various reports indicate that most laboratory errors occur at this stage, and these errors have the potential to cause biased test results even when the analysis is performed using validated automated hematology instruments. (Alshaghдали et al., 2022; Lima-Oliveira et al., 2017).

In this context, identifying and controlling pre-analytical factors that

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could potentially affect hematology test results is an important focus in modern clinical laboratory practice.

One of the pre-analytical factors often encountered in practice is the inconsistency of blood filling volume in EDTA vacuum tubes. EDTA tubes are manufactured with a specific nominal volume that has been adjusted to the amount of anticoagulant inside to maintain the optimal ratio of blood and anticoagulant. Filling the tubes below the recommended volume will create free space in the tubes, which could potentially alter the blood-anticoagulant ratio and affect blood cell stability during storage and analysis. (Biljak et al., 2016; Lippi et al, 2023)

The changes in blood ratio and anticoagulants due to underfilling of EDTA tubes have been reported to affect the microenvironment of red blood cells, including osmotic balance and erythrocyte membrane integrity. This condition has the potential to cause changes in the size and characteristics of red blood cells, which in turn can affect hematocrit and erythrocyte count results. Several studies have reported that significant underfilling of EDTA tubes can cause meaningful changes in certain hematological parameters, particularly erythrocyte indices and hematocrit, while mild underfilling tends to have minimal effects and remains within acceptable analytical variation limits. (Biljak et al., 2016; KA et al., 2014; Lippi et al., 2019)

In addition to blood volume, the type of EDTA used and the design of the vacuum tube have also been reported to affect hematology test results. Differences between K₂EDTA and K₃EDTA, as well as variations in tube design between manufacturers, can cause minor differences in some hematology parameters, although clinically these are often still within acceptable limits if pre-analytical procedures are performed correctly. (Ahn et al., 2016; Mehmood et al., 2018). These results confirm that controlling the filling volume of EDTA tubes remains an important aspect of maintaining blood sample quality.

Several studies have shown that filling EDTA tubes with a lower than standard volume of blood can still produce acceptable test results. However, data on the safe filling tolerance limits for hematocrit and erythrocyte count parameters are still limited and show varying results between studies. These differences are likely influenced by variations in tube design, anticoagulant type, hematology analyzer platform, and research subject characteristics (Lippi et al, 2023; Unal, 2019). Further studies are needed to evaluate the impact of free space variation in EDTA tubes on hematology test results under controlled pre-analytical conditions.

Within the framework of these pre-analytical issues, this study was conducted to analyze the effect of free space variation in 3 mL K₂EDTA vacuum tubes on the accuracy of hematocrit and erythrocyte count test results. The results of this study are expected to provide additional scientific evidence regarding the tolerance of EDTA tube filling volume and support the strengthening of pre-analytical phase quality control in clinical laboratory practice, especially in conditions where blood collection is limited in volume.

2. Method

This research is a laboratory experiment with a quantitative approach, where each respondent acts as an internal control to compare examination results under two tube filling volume conditions. Blood sampling was conducted at the Laboratory of the Faculty of Health Sciences, Musi Charitas Catholic University, while hematology testing was carried out at the Balai Besar Laboratorium Kesehatan (BBLK) Palembang on April 22, 2025.

The research subjects consisted of 37 respondents aged 18-23 years who met the inclusion and exclusion criteria. Inclusion criteria included being in a healthy condition based on anamnesis, willing to sign informed consent, and not experiencing dehydration or infection at the time of blood sampling. Exclusion criteria included a history of hematological abnormalities or chronic diseases, use of medications or therapies affecting hematology test results, unwillingness to follow all research procedures, as well as blood samples that did not meet laboratory examination requirements. The subject selection technique used total sampling. Venous blood was collected by medical laboratory technical personnel. For each respondent, blood was placed into two 3 mL K₂EDTA vacuum tubes with a filling volume of 2.2 mL and 3.0 mL using a syringe, then homogenized using the inversion technique 8-10 times. Hemolyzed samples were excluded from the analysis. All examinations were conducted on the same day to minimize the effects of storage.

Hematocrit values and erythrocyte counts were examined using a validated automatic hematology analyzer. Internal quality control was performed according to laboratory procedures prior to sample analysis to ensure that the instrument performance was within acceptable limits.

The independent variable was the variation in free space in the 3 mL K₂EDTA vacuum tube, which was determined by the blood filling volume, namely 2.2 mL and 3.0 mL. The dependent variables included hematocrit values and erythrocyte counts. Data were presented descriptively as means and standard deviations. Normality tests were performed using Shapiro Wilk. Differences in results between the two filling volume conditions were analyzed using a paired t-test because the data were paired, with a significance level of p less than 0.05. The analysis was performed using validated statistical software.

All respondents received an explanation of the purpose and procedure of the study and gave their voluntary consent by signing an informed consent form before blood sampling. The confidentiality of the respondents' identities was maintained during data processing and reporting.

3. Results and Discussion

Subject Characteristics

This study involved 37 young adult respondents aged 18 to 23 years who met the inclusion criteria and did not meet the exclusion criteria. All respondents were in good general health at the time of blood sampling. No samples showed hemolysis, so all specimens were deemed suitable for analysis. Each respondent underwent venous blood collection with two variations in filling volume in 3 mL K₂EDTA vacuum tubes, allowing for direct comparison of hematocrit and erythrocyte count results under different free space conditions.

Results of Hematocrit and Red Blood Cell Count Examinations

The mean red blood cell count at a blood volume of 2.2 mL was 4.93 ± 0.40 million cells per microliter, while at a blood volume of 3.0 mL it was 4.92 ± 0.39 million cells per microliter. The mean hematocrit values were 41.32 ± 3.27 percent at a blood volume of 2.2 mL and 41.14 ± 3.18 percent at a blood volume of 3.0 mL. All hematocrit and red blood cell count values were within the physiological range.

Data Normality Test

The results of the normality test using the Shapiro Wilk test show that the data on erythrocyte count and hematocrit in both filling volume variations have a normal distribution with a significance value greater than 0.05. Thus, the data meets the assumptions for parametric statistical analysis.

Comparison of Examination Results Based on Variations in Blood Volume

The results of the paired t-test showed that there was no statistically significant difference in the number of erythrocytes between 2.2 mL and 3.0 mL blood filling, with a p-value of 0.633. In the hematocrit parameter, the difference in mean between the two filling volume variations showed a p-value of 0.051, which was still above the statistical significance threshold. These findings indicate that variations in free space in the 3 mL K₂EDTA vacuum tube within the range of filling volumes studied did not produce significant differences in hematocrit or red blood cell count test results. The hematocrit and red blood cell count test results and statistical tests are presented in the following table:

Table 1. Mean Hematocrit Values and Red Blood Cell Counts Based on Variations in the Filling Volume of 3 mL K₂EDTA Tubes

Parameter	Filling volume	n	Mean $\pm$ SD
Red Blood Cell Count (million cells/ μ L)	2.2 mL	37	4.93 ± 0.40
Red Blood Cell Count (million cells/ μ L)	3.0 mL	37	4.92 ± 0.39
Hematocrit (%)	2.2 mL	37	41.32 ± 3.27

Hematocrit (%)	3.0 mL	37	41.14 ± 3.18
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Table 2. Results of the Shapiro Wilk Normality Test

Parameter	Filling volume	Statistics	p
Red Blood Cell Count	2.2 mL	0.966	0.311
Red Blood Cell Count	3.0 mL	0.967	0.322
Hematocrit	2.2 mL	0.970	0.417
Hematocrit	3.0 mL	0.964	0.278

Table 3. Results of the Paired t-test on Red Blood Cell Count and Hematocrit

Parameter	Difference average	SD difference	p
Red Blood Cell count (2.2 mL vs 3.0 mL)	0.01	0.12	0.633
Hematocrit (2.2 mL vs 3.0 mL)	0.18	0.55	0.051

This study assessed the effect of free space variation in 3 mL K₂EDTA vacuum tubes manipulated through differences in blood filling volumes of 2.2 mL and 3.0 mL on two hematological parameters, namely hematocrit and erythrocyte count. The results show that the difference in filling volume in this range did not produce statistically significant differences in red blood cell count ($p = 0.633$) or hematocrit ($p = 0.051$). The mean difference between the two parameters was also relatively small and still within the analytical variation commonly used in routine hematology tests. These findings reinforce the understanding that mild underfilling of K₂EDTA tubes does not always result in meaningful bias in results, provided that pre-analytical conditions are well controlled and analysis is performed consistently.

The Relationship Between Findings and The Concept of Pre-Analytical Quality

The quality of blood samples is a key prerequisite for accurate hematology test results. The literature confirms that the pre-analytical phase plays a major role in laboratory result variation because it involves a series of processes that are prone to procedural deviations, from venipuncture to sample handling before analysis. (Lima-Oliveira et al., 2017; Lippi et al., 2019). In this study, the use of EDTA anticoagulants must ensure the appropriate ratio of blood and anticoagulant so that the microenvironment of blood cells remains stable. Inconsistent filling volumes have the potential to increase the relative concentration of EDTA in relation to blood, which can alter the characteristics of erythrocytes through changes in osmotic balance and membrane surface interactions. (Lippi et al., 2019)

In this study, a volume of 2.2 mL represents approximately seventy-three percent of the nominal capacity of a 3.0 mL tube, so conceptually it is still within the range of mild underfilling, which is unlikely to be significant enough to trigger measurable cellular changes affecting hematocrit and erythrocyte count.

The consistency of the pre-analytical process in this study, such as standard venipuncture by Medical Laboratory Technicians, sample homogenization according to procedure, and same-day examination, provides a strong control framework for assessing the effect of filling volume more specifically. These conditions are in line with the principles of pre-analytical quality management, which emphasize standardization of procedures to minimize unwanted variations. (Alshaghдали et al., 2022; Ialongo & Bernardini, 2016)

In line with this concept, the influence of underfilling K₂EDTA tubes is not only limited to hematology parameters but also to molecular-based examinations, indicating that inappropriate tube fill volume can be a source of cross-method pre-analytical variation. Inadequate fill volume of K₂EDTA tubes can affect nucleic acid extraction results and gene expression, including changes in RNA concentration and cycle threshold values in GAPDH gene analysis. Although these effects are moderate, these findings suggest that variation in fill volume can be a source of pre-analytical bias that not only affects hematology parameters but also molecular-based laboratory tests (Benati et al., 2024).

Stability of Erythrocyte Count in Mild Underfilling

There is no significant difference in erythrocyte count between the two filling volume variations, which can be understood from the characteristics of this parameter, which tends to be stable as long as the structure and function of red blood cells are maintained and the sample is well homogenized. Previous studies have shown that filling K₂EDTA tubes with a volume lower than the standard can still provide acceptable complete blood count results for many parameters, especially if the degree of underfilling is not extreme. (KA et al., 2014). Conversely, more severe underfilling in EDTA anticoagulant tubes has been reported to cause changes in some hematology parameters, including erythrocyte count under certain conditions, due to an increase in relative EDTA concentration and changes in cell characteristics. (Biljak et al., 2016). Differences in findings between studies are often determined by the degree of underfilling, tube design, and the hematology analyzer platform used.

The results of this study can also be considered as evidence that at a filling range of about two-thirds to near full, the measurement system on automatic hematology devices tends to produce consistent erythrocyte counts. Verification and quality control of the analyzer are important factors in maintaining measurement performance under varying sample conditions (Vis & Huisman, 2016). The resistance of erythrocyte counts to variations in filling volume in this study is likely a combination of relatively mild underfilling and standardized pre-analytical processes.

Underfilling K₂EDTA tubes does not always cause significant changes in hematology parameters. Filling up to 1 mL ($\approx 67\%$ below the recommended volume) did not result in significant differences in any complete blood count parameters using the Beckman Coulter LH 780 analyzer, indicating that the analytic system is still able to maintain the stability of measurement results, even with a considerable volume deviation (Dayalan et al., 2020).

Other studies evaluating blood volume variations in K₃EDTA tubes showed no significant differences in erythrocyte parameters, hemoglobin, hematocrit, MCV, MCH, and MCHC for volume variations of 1–3 mL. There was a significant effect on platelet count, indicating that the sensitivity of each hematology parameter to volume variation can differ (La Ode Marsudi et al., 2024).

Interpretation of Hematocrit with p-Values Approaching the Significance Threshold

The p-value for hematocrit of 0.051 is very close to the commonly used statistical significance threshold. Inferentially, this value is still classified as insignificant at a p-value threshold of less than 0.05. Proper interpretation requires placing these results in two contexts: effect size and analytical significance. First, the difference in mean hematocrit between 2.2 mL and 3.0 mL is 0.18 percent, which is a small difference in absolute terms. In practice, hematocrit is influenced by the analyzer measurement method and mathematical relationships with erythrocyte indices, particularly mean corpuscular volume and erythrocyte count. Under conditions of relatively increased EDTA ratio, cell shrinkage may occur and potentially shift hematocrit estimates through changes in cell size or measurement signal (Lippi et al., 2019). Small shifts like those found in this study are still plausible within the range of analytical and biological variation commonly used in routine testing.

Second, p-values close to the threshold may reflect small effects that are difficult to distinguish from random variation in a given sample size. A study on the Sysmex XN 10 reported that filling spray-dried K₂EDTA tubes to less than about two-thirds of their nominal volume can cause more noticeable variations in several hematological parameters, including hematocrit and erythrocyte indices (Lippi et al, 2023). These findings are consistent with the understanding that the greater the degree of underfilling, the higher the likelihood that changes in the blood-to-anticoagulant ratio will affect sensitive parameters. In this study, the 2.2 mL volume was still above the two-thirds threshold, so the observed effect on hematocrit was minimal and did not reach statistical significance.

Another study comparing small-volume tubes and standard vacuum tubes also highlighted that differences in results may arise under certain conditions, but are not always significant for all parameters, depending on tube design and pre-analytical procedures (Unal, 2019). This variability supports the need for laboratories to establish internal quality control

policies that take into account the local context, including the type of tubes and equipment used.

The Role of EDTA Type and Tube Design in Result Variation

EDTA type and tube characteristics are factors that can modify the impact of underfilling. A comparison between EDTA tubes from different manufacturers shows that differences in design and composition can cause small variations in some hematology parameters, including result stability during storage (Ahn et al., 2016). Another study assessed the differences between K₂EDTA and K₃EDTA and reported that differences in certain parameters, particularly those related to cell volume, may arise even though they are often clinically acceptable if the filling and handling procedures are appropriate. (Mehmood et al., 2018). his study used a single type of K₂EDTA tube with uniform specifications, thereby minimizing variations arising from differences in tube design. These conditions helped position the study results as a more focused evaluation of the impact of filling volume on a relatively homogeneous tube and analyzer system.

In the literature, the impact of underfilling can also differ between tubes with spray-dried EDTA and tubes with other forms of EDTA. Studies on incompletely filled K₂EDTA tubes emphasize that the proportion of anticoagulant to blood volume is the main determinant of changes in results, especially when the filling volume falls below a certain limit (Biljak et al., 2016; Lippi et al, 2023). Operationally, this is important because laboratory practices can result in varying degrees of underfilling, so the determination of internal tolerance limits needs to take into account the characteristics of the tubes used.

Practical Implications for Clinical Laboratories

The results of this study have practical relevance, especially in situations where blood sampling is limited in volume, such as difficult venous access or the need for multiparameter testing with limited blood volume. The results show that under controlled pre-analytical conditions, filling a 3 mL K₂EDTA tube with 2.2 mL of blood produces hematocrit and erythrocyte counts comparable to those obtained with full filling. This evidence supports a realistic quality approach, namely understanding that minor deviations from ideal conditions do not always produce unreliable results, as long as these deviations remain within analytical tolerance and are compensated for by good pre-analytical process control (Lippi et al., 2019) .

These results are not intended to replace manufacturer recommendations or standard tube filling guidelines. Standardization of filling remains the primary goal to minimize potential errors and ensure consistency across time and operators. Practices that may be considered include establishing internal tolerance limits for the minimum acceptable filling volume for specific tests, accompanied by documentation and education of blood collection operators. Quality indicator systems can also be used to monitor the frequency of underfilling and assess its impact on laboratory performance (Alshaghdali et al., 2022)

Limitations and Directions for Further Research

Several important limitations need to be considered. First, this study evaluated two filling volume points, namely 2.2 mL and 3.0 mL, so it did not describe the response of hematocrit and erythrocyte count parameters in a broader spectrum of underfilling. Second, the respondents were relatively homogeneous healthy young adults, so generalizing the results to populations with hematological disorders, changes in osmolarity, or severe clinical conditions requires caution. Third, this study focused on hematocrit and red blood cell count. Other parameters that are more sensitive to preanalytical variations, such as mean corpuscular volume, mean corpuscular hemoglobin concentration, and red cell distribution width, were not analyzed. Previous studies have shown that underfilling and preanalytical conditions can affect erythrocyte indices and cell distribution parameters, which need to be evaluated in further studies (Biljak et al., 2016; Lippi et al, 2023).

The next study could test several additional filling volumes, for example, approaching two-thirds of the nominal volume and below, to map the analytically and clinically relevant threshold points. Variations in EDTA tube brands and the use of different analyzer platforms are also important for testing the consistency of results between systems, as shown in

comparative studies of tubes and hematology testing devices (Ahn et al., 2016; Vis & Huisman, 2016). In this study, quality improvement research can also integrate pre-analytical quality indicators and service outcomes to strengthen the relevance of findings to patient safety.

Overall, the results of this study indicate that variations in free space in 3 mL K₂EDTA tubes within the filling range of 2.2 mL to 3.0 mL do not cause statistically significant differences in red blood cell count and hematocrit. These findings are consistent with the concept that the impact of underfilling depends on the degree of deviation from the nominal volume, tube characteristics, and the quality of pre-analytical control. This study adds operational evidence for clinical laboratories to understand the safe filling tolerance limits under certain conditions, while maintaining filling standardization.

4. Conclusion and Suggestion

This research shows that variations in free space in 3 mL K₂EDTA vacuum tubes resulting from differences in blood filling volumes of 2.2 mL and 3.0 mL do not cause statistically significant differences in erythrocyte count and hematocrit test results. The difference in the values of both parameters is relatively small and falls within the acceptable range of analytical variation in routine hematology tests. These results confirm that, under well-controlled pre-analytical conditions, filling the K₂EDTA 3 mL vacuum tube with up to 2.2 mL of blood still produces hematology values comparable to those obtained with full filling. Control of the blood collection procedure, sample homogenization, and the use of validated automated hematology instruments are important factors in maintaining the consistency of test results.

Based on the results of this study, clinical laboratories are advised to maintain the standard filling of EDTA tubes in accordance with manufacturer recommendations and international guidelines. In certain situations with limited blood volume, the results of this study can be used as a basis for consideration that filling tubes to approximately two-thirds of their nominal volume is still acceptable for hematocrit and erythrocyte count tests, as long as pre-analytical factors are strictly controlled. Further research is recommended to evaluate other hematology parameters known to be more sensitive to pre-analytical variations, particularly erythrocyte indices such as mean corpuscular volume and mean corpuscular hemoglobin concentration, as well as erythrocyte distribution parameters such as red cell distribution width. Analysis of these parameters is expected to provide a more comprehensive picture of the impact of free space and blood-to-EDTA anticoagulant ratio variations on the stability of hematology test results. Future studies may involve a wider range of blood filling volumes, the use of various brands of EDTA tubes, and different hematology analyzer platforms to assess the consistency of findings across testing systems. This approach is expected to strengthen the scientific evidence base and support the development of more standardized specimen quality policies in laboratory practice.

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