

# Experience of Reticulocytes Measurement at 720 nm Using Spectrophotometer

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## 분광광도계를 이용한 720 nm에서 망상적혈구 측정 경험

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Currently, reticulocyte experimental calculation technology used in clinical laboratories are divided two types: manual and automated. Manual reticulocyte counting using a microscopy lacks accuracy due particularly to its low reproducibility, affecting the accuracy of manual reticulocyte count. Moreover, Automatic blood corpuscle analyzer flow cytometry is difficult to be used in underdeveloped countries and small scale laboratories due to relatively high cost. Therefore, this study tried to find a new method to complement these drawbacks. The aim of this study was to compare the stained reticulocytes count by spectrophotometer and also to analyze the statistics of spectrophotometer and flow cytometer. The same 8 EDTA samples were repeated 36 times to compare the agreement between spectrophotometer and flow cytometer. This study measured the specimen diluted 600 times at 700~780 nm by 10 differences. Wavelength between 710 to 730 by absorbance showed a positive correlation between standard data and test data ( $r=0.967$ ,  $p<0.01$ ), presenting a correlation between variables. Statistical analyses of regression for test and standard parametric data, the optimal dilution factor was 600 times. Therefore, this study tried to technical utilizes such as contributing economical for the reticulocyte absorbance apply from the auto spectrophotometer, a monitoring system for the reticulocyte relation anemia, etc. Therefore, more extensive studies, including an auto chemical analyzer application, will be needed.

**Key words:** Flow cytometry, Reticulocyte, Spectrophotometer

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## INTRODUCTION

Reticulocytes are immature erythrocyte, usually composing about 1% of the erythrocyte in the peripheral blood at human body. In the process of erythropoiesis, reticulocytes develop and mature in the bone marrow and then

circulate for about one to two days in the blood stream, penultimate phase of maturation [1]. As process which produces erythrocytes, becomes a basophilic or early normoblast, then a polychromatophilic or intermediate normoblast, then an orthochromatic or late normoblast. At this stage the nucleus is expelled before the cell

becomes a reticulocyte. However, some extranuclear RNA remains. This residual RNA generally is lost progressively during the 24 hours after the cell enters the circulation.

However, some extranuclear RNA remains in reticulocytes. Reticulocytes differ from other red cells in that they have a more convoluted shape, and are about 8% larger than the more erythrocytes (10~15  $\mu\text{m}$  versus 6~8  $\mu\text{m}$ ). The normal value of reticulocytes in the blood depends on the clinical situation but is usually 0.5% to 2.5% in adults and 2% to 6% in infants [2]. The number of reticulocytes is a good diagnosis factor of bone marrow activity because it represents recent production and allows for the determination of reticulocyte count and the reticulocyte production index. These values can be used to determine anemia and can also be used to monitor the progress of treatment for anemia.

A reticulocyte count learns if your bone marrow is producing enough red blood cells. If your red blood cell count is too low or too high, your body will try to achieve a better balance by producing and releasing more or fewer reticulocytes. The reticulocyte experimental calculation technology at clinical laboratories are currently divided into manual and automated. The manual reticulocyte counting by blood slide smear microscopy became traditional and has been considered the standard method, for its simplicity and low cost since 1940 [3]. Reticulocytes are present in the peripheral blood although it is immature red blood cell so it can be used for verification of hematopoietic status and classification of anemia. The most common supravital stain is performed on reticulocytes using new methylene blue or brilliant cresyl blue, which makes it possible to see the reticulo filamentous pattern of ribosomes characteristically precipitated in these live immature red blood cells by the supravital stains [4]. However, the observer's visual difference, the technician's experience to distinguish reticulated cells from other cells with inclusions that also stain with the dye, besides the quality and the resolution power of the microscope are other important factors and that presents some inconvenience and limitations, such as lack of accuracy, low reproducibility, time spent in the laboratory routine, lack

of quality of the used stains, inappropriate blood and films affect the accuracy of the manual reticulocyte count [5,6]. Reticulocyte was stained by RNA/DNA-specific fluorochrome and fluorescence method was first reported by Kozenow and Mai in the early 1950s [7]. This principle was applied to flow cytometry analyzers. It is predominantly used to measure fluorescence intensity produced by fluorescent-labeled antibodies detecting proteins, or ligands that bind to specific cell-associated molecules such as propidium iodide binding to DNA/RNA. Also, automatic blood corpuscle analyzer flow cytometry is difficult to use in underdeveloped countries and small scale laboratories because of the cost. Therefore, this study tried to find a new method to complement these drawbacks. Spectrophotometer which can utilize the stain ability of RNA component and easy to use at relatively is applied. In this background, the objectives of this study were to compare stained reticulocytes count by spectrophotometer and also to analyze the statistic of spectrophotometer and flow cytometer.

## MATERIALS AND METHODS

### 1. Sample and setting

This study was part of a new optical technique for measuring reticulocyte with the Spectrophotometer, for new experiments blood was collected by median cubital vein into 3 mL ethylene diamine tetra acetic acid K3 (EDTA-K3) from university students at Suwon-si, Gyeonggi-do, Korea. In this paper were completed from July 2017 to September. The participants in this study were 8 female university students without the other disease and written informed consent of the participants was obtained from all individuals.

### 2. Confirm the quality of dye

To place 3 or 4 drops of new methylene blue and 3 or 4 drops of blood (venous or capillary) in a small test tube. Mix the tube contents and allow standing for a minimum of 15 minutes. This allows the reticulocytes adequate time to take up the stain. At the end of 15 minutes, mix the

contents of the tube well. Place a small drop (10  $\mu\text{L}$ ) of the mixture on a clean glass slide and prepare a thin smear. Allow smear to air-dry. Place the slide on the microscope stage and, using the low power objective, locate the thin portion of the smear in which the red cells are evenly distributed and are not touching each other. Then let the mixture stand for 10 minutes check the degree of dyeing using the oil lens ( $\times 1000$ ).

### 3. Preparation of diluted samples

To determine the proper dilution, use the different quantity of 0.9% saline and fixed amount of whole blood and dye by 10  $\mu\text{L}$ . It is used for simple dilutions, one in which a unit volume of a liquid material of interest is combined with an appropriate volume of a solvent liquid to achieve the desired concentration. The diluted material must be thoroughly mixed to achieve the true dilution. When dilute under 200 times, the absorbance exceeded 1.000 multiple. And so the dilution had to increase more and more. The check proper dilution rate results were presented in Table 1.

### 4. Reticulocyte count

Reticulocyte count was measured venous blood samples collected in ethylene diamine tetra acetic acid K3 (EDTA-K3) as an anticoagulant of 3 mL and were analyzed

in a Sysmex XN- 2000 analyzer (Sysmex, Kobe, Japan) equipment. Spectrophotometric reticulocyte counting was performed using Spectro SC (Labomed, LA, USA). The same sample to read at another wavelength, reset the wavelength, insert the reference cuvette, adjust to 100% transmittance with the light control knob, then the sample inserted and has been read absorbance. Another sample to read at the same wavelength, replace the first sample cuvette with the next one, the instrument reseted with the standard for each wavelength.

### 5. Statistical analysis

All statistical analyses were performed using SPSS version 21.0 (SPSS, Armonk, NY, USA) program for windows. Descriptive statistics including mean and standard deviation for quantitative variable were used. We used the Kolmogorov-Smirnov test to evaluate normal distribution. Differences in baseline results between reticulocytes variables were tested for significance using the t-test, ANOVA or chi-square test, Mann-Whitney. In addition, the Spearman correlation analysis or Pearson correlation coefficient was utilized for determining the strength of linear association between various results for flow cytometry and spectrophotometry separately. In addition, the multiple regression analysis and simple regression analysis were performed according to the correlation. All statistical significance level was set as  $p < 0.05$ .

**Table 1.** Preparation of diluted samples

Multiple	Whole blood ( $\mu\text{L}$ )	0.9% Saline ( $\mu\text{L}$ )	Brome cresyl blue ( $\mu\text{L}$ )
$\times 400$	10	3,990	10
$\times 450$	10	4,490	10
$\times 500$	10	4,990	10
$\times 550$	10	5,490	10
$\times 600$	10	5,990	10

**Table 2.** Reticulocyte results by Flow cytometer

Parameter	A	B	C	D	R	F	G	H	M $\pm$ SD
Hemoglobin (g/dL)	8.50	10.091	13.26	9.73	14.75	6.68	14.75	8.85	10.83 $\pm$ 3.04
Reticulocyte (%)	2.57	2.61	2.65	2.74	2.75	2.61	2.74	3.54	2.77 $\pm$ 0.31
Reticulocyte ( $10^6/\mu\text{L}$ )	0.0828	0.0829	0.1156	0.0834	0.1323	0.0520	0.1378	0.0853	0.0965 $\pm$ 0.03
Mean reticulocyte volume (fL)	119.8	114.0	109.6	113.0	111.1	116.8	101.0	142.1	115.9 $\pm$ 11.95
Immature reticulocyte fraction	0.46	0.50	0.45	0.42	0.48	0.41	0.46	0.61	0.47 $\pm$ 0.062

A, B, C, D, E, F, G, H; low hemoglobin vale 8 healthy subjects.

## RESULTS

### 1. Reticulocyte results by flow cytometer

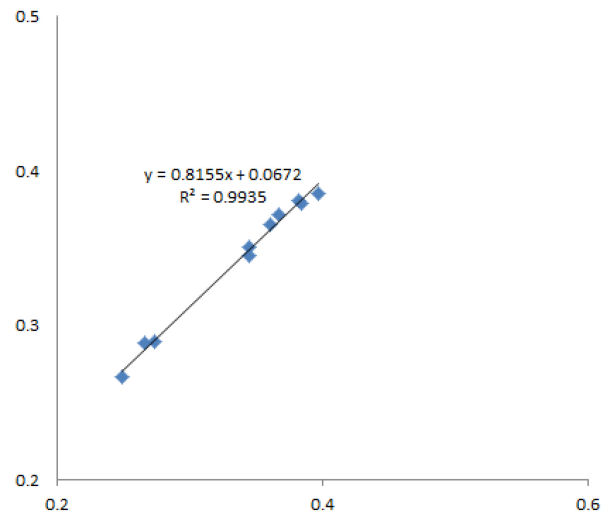
During study selected low hemoglobin vale 8 healthy subjects were taken to establish the reference values for reticulocyte profile in this study. Hemoglobin mean of

Healthy subjects were  $10.83 \pm 3.04$  g/dL. The average Reticulocyte of the subject were  $2.77 \pm 0.31\%$  and the average Reticulocyte quantification of the subjects was  $56.54 \pm 10.56$  106/ $\mu$ L. The average MRF (mean reticulocyte volume) of the subjects was  $115.9 \pm 11.95$  fL and the IRF was  $0.47 \pm 0.062$  (Table 2).

**2. Result of dilution rate**

The samples were made a diluted for 400, 450, 500, 550, 600 multiples and 650~780 nm spectrometry methods involve development of each colored products in the presence of reticulocyte. When samples do not contain substances that interfere with the specific reagent or dye binding interaction, the intensity of the colored product is proportional to the amount of reticulocyte in the sample. The normal distribution was the Kolmogorov-Smirno test, but there was no statistical difference. Fisher's test was statistically significant ( $p < 0.01$ ). Data were analyzed using ANOVA, Dunnett T3 post hoc test. There was no statistical difference. As shown in Table 4, the 600 multiply by absorbance was positive correlated with standard and test ( $r = 0.997$ ,  $p < 0.01$ ), presents the Pearson parametric cor-

relations for variables. The statistical analyses for agreement of regression analysis for test and standard parametric data, was illustrated in Table 4 and Figure 1. The optimal dilution factor was 600 times. The results of dilution rate were presented in Table 3.



**Figure 1.** Spectrophotometer absorbance; the Pearson correlation  $r$  was 0.997. Line-estimated regression line ( $y = 0.8155x$ ); dotted line-95% limit of confidence for residues. Lines in cross-averages values for  $x$  and  $y$ .

**Table 3.** Result of absorbance by dilution rate in 650~780 nm

	Multiple	M $\pm$ SD (N=10)	SE	Z	F	Dunnett T3
Standard	$\times 400$ (a)	$0.772 \pm 0.117$	0.012	0.548	119.320**	d,e,f,g,h,i,j < b,c < a
	$\times 450$ (b)	$0.529 \pm 0.053$	0.529	0.941		
	$\times 500$ (c)	$0.352 \pm 0.033$	0.352	0.884		
	$\times 550$ (d)	$0.313 \pm 0.043$	0.313	0.697		
	$\times 600$ (e)	$0.336 \pm 0.054$	0.336	0.800		
Test	$\times 400$ (f)	$0.558 \pm 0.023$	0.558	0.834		
	$\times 450$ (g)	$0.385 \pm 0.042$	0.385	0.647		
	$\times 500$ (h)	$0.389 \pm 0.046$	0.389	0.929		
	$\times 550$ (i)	$0.363 \pm 0.045$	0.363	0.709		
	$\times 600$ (j)	$0.341 \pm 0.044$	0.341	0.724		

\*\* $p < 0.01$

No statistically significant difference by Kolmogorov-Smirnov.  $p$ -values were calculated by Kolmogorov-Smirnov, one-way analysis of variance. Post hoc test by Dunnett T3.

**Table 4.** Regression analysis result of absorbance by 600 multiply in 650~780 nm

Independent variable	Dependent variable	SE	$\beta$	t	r/R2/F
Standard	Constant	0.008	0.997	8.459**	0.997/0.994/1224.722**
	Test	0.023		34.996**	

\*\* $p < 0.01$ .

$p$ -values were calculated by regression analysis.

### 3. Result of appropriate wavelength

The spectral absorbance density curves of reticulocyte in the spectral range between approximately 650 and 780 nm shows that the absorptions showed result of the same concentrations associated with 600 times dilution solution. To research appropriate wavelength used ten wavelengths within the wavelength range 700 to 780 nm corresponding to measurement that be used for the purpose of establishing a set of photometric scale standards. This study measured the specimen diluted 600 times at 700~780 nm by 10 differences. The normal distribution was the Kolmogorov-Smirno but there was no statistical difference. Data were analyzed using ANOVA, Sheffe post hoc test. Fisher's test was statistically no significant. As shown in

Table 7, the 710 to 730 wavelength by absorbance was positive correlated with standard and test ( $r=0.967$ ,  $p<0.01$ ), presents the Pearson parametric correlations for variables. The statistical analyses for agreement of regression analysis for test and standard parametric data was illustrated in Table 6, 7 and Figure 3, 4.

### DISCUSSION

Reticulocyte counts make available for use clinically useful information, not only in the diagnosis and classification of anemia, but also in monitoring therapeutic replication. The reticulocyte parameters are available for realtime assessment of marrow erythropoietic activity and clinically they will help in the diagnosis and treatment of

**Table 5.** Result of absorbance by wavelength in 600 times dilution

Wavelength		M±SD (N=6)	SE	Z	F	Scheffe
Standard	700 nm (a)	0.293±0.010	0.004	0.974	1.593	a,b,c,d,e,f,g,h,i,j,k,l
	710 nm (b)	0.290±0.011	0.004	0.994		
	720 nm (c)	0.286±0.011	0.010	0.955		
	730 nm (d)	0.284±0.010	0.010	0.976		
	740 nm (e)	0.282±0.015	0.015	0.980		
	750 nm (f)	0.281±0.011	0.011	1.000		
Test	700 nm (g)	0.350±0.082	0.082	0.974		
	710 nm (h)	0.348±0.080	0.080	0.969		
	720 nm (i)	0.342±0.082	0.081	0.841		
	730 nm (j)	0.344±0.082	0.082	0.811		
	740 nm (k)	0.337±0.079	0.080	0.875		
	750 nm (l)	0.334±0.044	0.078	0.893		

No statistically significant difference by Kolmogorov-Smirnov.

$p$ -values were calculated by Kolmogorov-Smirnov, one-way analysis of variance.

Post hoc test by Scheffe.

**Table 6.** Regression analysis result of standard absorbance by 710 nm and 720 nm in 600 times dilution

Independent variable	Dependent variable	SE	$\beta$	t	r/R2/F
720 nm	Constant	0.050	0.937	0.373	0.937/0.877/28.638**
	710 nm	0.172		5.351**	

\*\* $p<0.01$ .

$p$ -values were calculated by regression analysis.

**Table 7.** Regression analysis result of test absorbance by 720 nm and 730 nm in 600 times dilution

Independent variable	Dependent variable	SE	$\beta$	t	r/R2/F
720 nm	Constant	0.007	0.999	0.156	0.999/0.998/2299.181**
	730 nm	0.021		47.950**	

\*\* $p<0.01$ .

$p$ -values were calculated by regression analysis

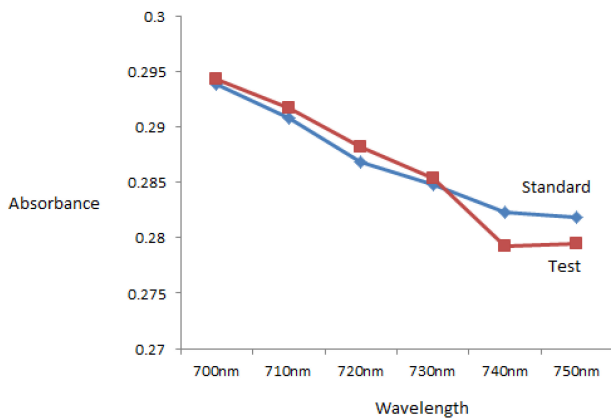


Figure 2. Absorbance by wavelength in 600 times dilution.

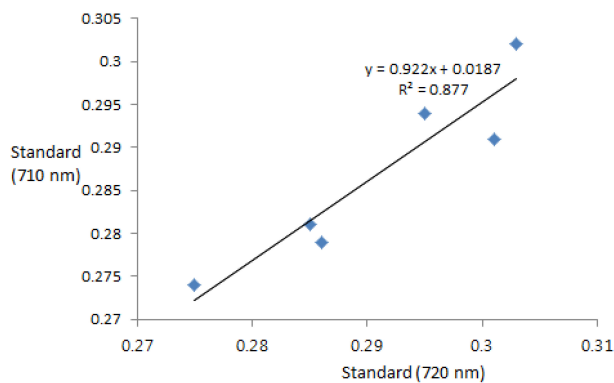


Figure 3. Spectrophotometer absorbance; the Pearson correlation r was 0.877. Line—estimated regression line ( $y=0.922x$ ); dotted line—95% limit of confidence for residues. Lines in cross—averages values for x and y.

various anemias [8]. The study of reticulocyte production index (RPI) could be used as a potential surrogate marker to indicate sufficient bone marrow regenerative capacity [9]. New red blood cell and reticulocyte parameters and reference values for healthy individuals and in chronic kidney disease in the development of new techniques and hematological parameters have revealed important information about functional integrity of bone marrow, diagnosis of anemia and recombinant human erythropoietin monitoring therapy used in hemodialysis [10]. Higher reticulocyte counts were associated with higher mortality. We speculate that this result reflects tissue hypoxia, which results in a higher erythropoietin level, or a compensatory erythropoietic response due to the accelerated clearance of erythrocytes [11]. Reticulocytes are immature eryth-

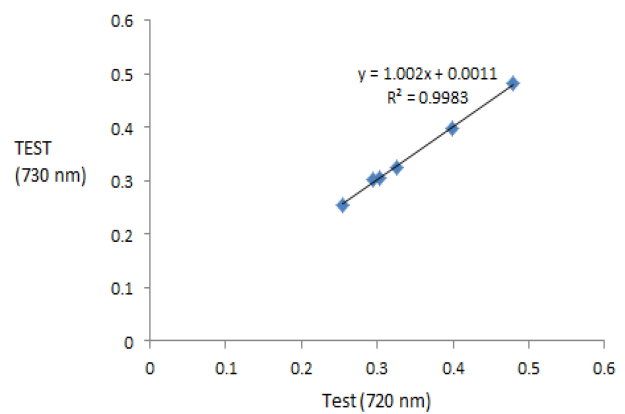


Figure 4. Spectrophotometer absorbance; the Pearson correlation r was 0.182. Line—estimated regression line ( $y=-3.308x$ ); dotted line—95% limit of confidence for residues. Lines in cross—averages values for x and y.

rocytes that have immediately left the bone marrow but still include intracellular RNA. The clinical availability of the production of erythrocytes can be measured by staining blood with a supravital dye and determining the number of erythrocytes that contain this residual RNA.

The most universally used method for counting reticulocytes was a manual microscopic procedure. Although it is relatively cheap and simple to perform, this method takes a long TAT (turnaround time), expert skill required and lack of relatively objectivity. In most laboratories which is still conducted microscopically and the standard method for counting reticulocytes has not changed for many years and remains imprecise and unreliable [12]. In this background, automated reticulocyte methods, using flow cytometry and fluorescent dyes that bind RNA, have been developed and used. The development of practical techniques using flow cytometry and fluorescent dyes could be possible many clinical laboratories to automate reticulocyte counting and bring it to an acceptable level of reliability. However, most of used fluorescent dyes combine polynucleotides nonspecific, and thus stain DNA as well as RNA [13,14]. The quantity of circulating reticulocytes is an important indicator of erythropoietic activity in response to a wide range of haematological pathologies. While most modern laboratories use flow cytometry to quantify reticulocytes, most field laboratories still rely on ‘supravital’ staining [15]. Spectrophotometry is the quan-

titative measurement of the reflection or transmission properties of a material as a function of wavelength [16]. Spectrophotometry is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. A flow cytometry is a popular laser-based technology to analyze the characteristics of cells or particles. Flow cytometry is a widely used method for analyzing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations and analyzing cell size and volume [17].

In this background, this study has attempted to study by the spectroscopic method of reticulocyte count to complement the disadvantage of the blood smear microscope method and the relatively expensive flow cytometry method. This study performed the reticulocyte based on flow cytometer and compared results with spectrophotometer absorbance. First, a dilution step was required after BCB staining with the conventional method. Brilliant cresyl blue gave apparent absorbance differences with the 600 nm, standard staining an intense absorbance and test sample a pronounced absorbance. As a result of confirming proper dilution rate, it was suitable at 600 times (Table 4). Typical spectrophotometer can be set to measure at these optimal wavelengths in the visible spectrum, but reticulocytes aren't equipped with filter sets for specific wavelengths that differ from the absorbance maxima required by the reticulocyte assay. In such situations, it is important to know the range of wavelengths that can be used and still obtain acceptable results. Data are plotted in two different ways in the Table 6: by wavelength thereby produced a ANOVA analysis for each reticulocyte concentration and by reticulocyte concentration thereby produced a Statistical regression for each of several selected wavelengths. Therefore, this study the action of Develop new optical technique and these results can arrange a reticulocyte count methods and a method that has for

rapid and effective diagnostic results offer and counting of reticulocytes. This study can indicate that using spectrophotometer measurement based on supravital stained reticulocytes are an efficient, economical, and rapid technique, yielding predictable results.

The limitation of this study was that there were no previous studies and it was difficult to obtain various samples. However, automatic blood corpuscle analyzer flow cytometry is difficult to use in underdeveloped countries and small scale laboratories because of the cost. Therefore, this study tried to find a new method to complement these drawbacks. Technical utilizes such as contributing economical for the reticulocyte absorbance apply from the auto spectrophotometer, a monitoring system for the reticulocyte relation anemia, etc. can be contributed.

## 요 약

임상 실험실에서의 망상 적혈구 계산은 현재 수동 및 자동으로 구분된다. 현미경에 의한 망상 적혈구 수 검사는 정확성의 결여, 낮은 재현성 등이 망상 적혈구 결과의 정확성에 문제가 있다. 또한, 자동 혈액 분석기의 유동 세포 계측법은 개발 도상국 및 소규모 실험실에서 비용 때문에 사용하기가 어렵다. 따라서 본 연구는 이러한 단점을 보완 할 수 있는 새로운 방법을 찾기 위해 노력했다. 이 연구의 목적은 분광 광도계로 염색 된 망상 적혈구 수를 비교하고 또한 분광 광도계 및 유동 세포 계측기의 통계를 분석하는 것이다. 동일한 8 개의 EDTA 샘플을 36 회 반복하여 분광 광도계와 유동 세포 계측기 사이의 일치도를 비교 하였다. 이 연구는 700~780 nm에서 600 배 희석 한 표본을 10 개의 차이로 측정하였다. 흡광도에 의한 710~730 파장은 표준 및 검사와 양의 상관 관계가 있으며( $r=0.967$ ,  $p<0.01$ ), 변수에 대한 상관 관계를 나타내었다. 결과에 대한 회귀 분석결과 최적 희석 계수는 600 배였다. 따라서 본 연구는 자동 분광 광도계를 이용한 망상 적혈구 흡광도 측정에 대한 경제적인 기여 그리고 망상 적혈구 관련 빈혈에 대한 모니터링 시스템 등에 기여 할 수 있는 기술적 활용을 시도한 것이다. 따라서 더욱 광범위한 연구가 필요할 것으로 사료된다.

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Conflict of interest: None

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