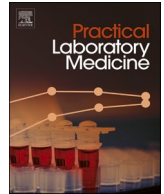




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## Development of a robust blood smear preparation procedure for external quality assessment

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### ABSTRACT

**Introduction:** The external quality assessment (EQA) scheme is particularly important for laboratory performance evaluation. Peripheral blood smears are necessary to identify morphological features, and the procedure for preparing such smears must be robust to meet the ISO 15189 standard. Although blood smear preparation is a routine activity in medical laboratories, an appropriate procedure for preparing a series of blood smears with high homogeneity and durable stability for EQA purposes has not yet been published elsewhere. For this reason, a robust procedure was developed and validated in this study.

**Methods:** Various factors affecting blood smear preparation, such as the amount of time collected blood samples are stored before fixation, suitable reagents, and specification parameters for each step, including fixation, staining, and timing of the staining steps, were studied. Each experiment was evaluated based on homogeneity and stability characteristics.

**Results:** Whole blood mixed with EDTA anticoagulant was used to make the blood smears. Samples were fixed with pure anhydrous alcohol and stained in Coplin jars using the Wright-Giemsa method.

**Conclusion:** The homogeneity and stability of two lots of smears suitable for EQA purposes was confirmed based on intact morphology of the smears for more than 8 months at room temperature.

### 1. Introduction

The goal of EQA is to evaluate participant performance against pre-established criteria through interlaboratory comparisons. Additionally, interlaboratory comparisons are required to obtain ISO 15189: 2012 accreditation and to fulfill local regulatory criteria [1].

Automatic hematology analyzers are commonly used in clinical laboratories to evaluate complete blood counts. In some cases,

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testing results need to be verified by checking blood films to provide necessary information for clinical hematologic diagnosis [2]. Furthermore, morphological analysis of blood smears is used as a reference method for WBC differential counts in quality control and verification of the automated instrument-based method according to CLSI H2O-A2 [3]. In short, it is important to confirm that a laboratory is competent in the morphological examination.

Published guidelines for peripheral blood smear EQA include methods for the process from preparing blood smears to staining with a suitable dye reagent; however specific details about achieving robust manipulation are lacking [4–6]. Therefore, the purpose of this article is to report a standard procedure for the preparation of homogeneous and stable blood films.

## 2. Materials and methods

### 2.1. Materials

Microscope slides (Sainty International Group Jiangsu Yangzhou Sumex Import & Export Co., LTD, China), cover slips (Hirschmann®, Deckglaser, German) were kept clean, dry conditions.

Reagents, including Wright's eosin methylene blue solution, Giemsa's azure-eosin-methylene blue solution, buffer tablets with pH 7.2 and pH 6.4, anhydrous methanol for analysis (max. 0.003% H<sub>2</sub>O), absolute ethanol for analysis (EMSURE® ACS), and Entellan® glue were supplied by Merck, Germany and used without further modification. Ethanol VP 90% was purchased from Vinh Phuc Health Equipment Trading Corporation, Vietnam.

### 2.2. Methods

#### 2.2.1. Blood smear preparation

Blood samples were supplied by the clinical laboratory at University Hospital Medical Center at Ho Chi Minh City, branch 2, Vietnam.

Blood was drawn as clinically relevant using BD Vacutainers® containing K2 EDTA 3.16 mg anticoagulant agent (BD Company, USA). Samples were checked for Hepatitis B and C and HIV. Blood was transported to the EQA center in a cooler box with freeze-dry ice packs, and blood smears were prepared within 4 h [5]. This research was approved by the Ethical Committee of the University of Medicine and Pharmacy at Ho Chi Minh City for EQA purposes.

The manual wedge method was used to make blood smears according to Ref. [5]; briefly: The blood tubes were gently inverted 8 times. Five microliters of blood were then withdrawn by micropipette (Mettler Toledo, Switzerland) and carefully dispensed approximately 1 cm from the frosted part of the slide. The edge of the spreader was placed at an angle of 30–45° in front of the drop and then drawn back into it. The spreader was gently pushed forward in a smooth, quick motion, resulting in a thin film of blood over the slide. By strictly following the steps described above, the tail of the blood film was made straight, and two-thirds of the glass slide was covered with a thin smear that ensures the red cells did not overlap.

As a first step, after smear creation, the optimal drying time before the fixing step and best fixative for the blood smears needed to be identified. The dried blood smears were kept at room temperature for 0, 2, 4, and 6 h to identify the effect of drying time. When the drying time was determined, three fixatives, including 90% ethanol, pure anhydrous ethanol, and pure anhydrous methanol, were considered. The samples prepared with different fixatives were then stained and observed. When the appropriate fixative was selected, the selected fixed samples were kept at room temperature from 0 to 18 h before staining to determine if there was any effect in terms of cell shape. At predetermined times (0, 6, 12, and 18 h), the fixed samples were stained and observed. In this first step of fixing procedure research, to simplify the procedure, the Wright method was used to stain the blood smear. The acceptable blood smear needed to meet the requirements described in Table 1.

When the fixing parameters were established, the Wright-Giemsa staining procedure was used. The smear was dipped completely into the mixture of Wright – Giemsa solution in 1:1 ratio (vol/vol). Then, the smear was washed by dipping in the pH 7.2 buffer for 12 min. In this step, the smear was dipped in Coplin jars versus on rack was studied. The stained smear was gently rinsed with tap water until the edges show faintly pinkish red [6]. The smear was then left to be dried at room temperature. For EQA purposes, the staining color should be homogeneous, and precipitate formation and scratches should be avoided. The critical features of the stained cells are shown in Table 1 [7].

The slides were then dried at room temperature for 2 h. The opposite surface was cleaned with 90% ethanol and soft tissue. One Entellan® drop (used to adhere the cover slip to a slide) was added as a rapid nonaqueous mounting medium in the middle of the smear. Cover slip (20 × 40 mm) was manually gently applied over the Entellan®, avoiding air bubbles. Covered slides were kept at room temperature for 5 days to be completely dry according to our experimental results. The covered slides were then used for further

**Table 1**  
Observed and recommended color for staining blood smear (16, 17).

	Nuclei	Cytoplasm				Granulocytes		
		Eryth-rocyte	Neutrophil	Lympho-cyte	Mono-cyte	Neutrophil	Eosinophil	Thrombocyte
Recommended color	violet	pink	pink	blue	gray blue	pink/violet	orange	violet
Wright – Giemsa method (Fig. 2)	violet	gray	pink	blue	gray blue	pink violet	brick red	violet

studies such as: evaluation of homogeneity and stability.

### 2.2.2. Evaluation of homogeneity and stability

For the homogeneity and stability study, two different lots were designed: Lot EH01 was prepared from a sample with a normal differential leukocyte count and Lot EH02 was prepared from a sample with normal blood cell morphology. The Lot number was given to show the differential purposes of EQA scheme. At the specified time intervals (2, 4, and 8 months), WBC differentials were counted and recorded for Lot EH01, and erythrocyte, leukocyte, platelet morphology was observed and recorded for Lot EH02. The interval time was selected as the multiples of EQA cycle (2 months).

For the homogeneity test, according to ISO 35: 2017 [8], from 70 samples, 10 samples (more than 10%) were randomly selected and analyzed (observation of each sample was repeated twice by two different researchers at the same time without discussion to ensure the fairness). All statistical analyses were performed using SPSS 16.0. The homogeneity and stability of random samples were evaluated using Microsoft Office Excel 2013 software. One-way ANOVA was used for homogeneity testing with statistically significant differences defined at p-value < 0.05.

For the stability test, according to ISO 35: 2017, 3 samples were randomly selected, and the analysis was repeated twice. The WBC differential in Lot EH01 was analyzed using Student's t-tests with the level of significance set at p-value < 0.05. Erythrocyte, leukocyte, and platelet morphology in Lot EH02 needed to be intact.

## 3. Results

### 3.1. Blood smear preparation procedure

The effect of various fixing parameters (blood smear drying time, fixatives, fixed smear storage time before staining) on the blood smear preparation is shown in Table 2 and Fig. 1. The morphology of the blood cells on the smears was acceptable, but blue-gray streaks were observed in the smear background 6 h after smear preparation. Thus, the blood smear drying time must be less than 6 h.

For the effect of fixatives, water artifacts were observed in erythrocytes when using 90% ethanol. Both pure anhydrous ethanol and methanol gave good results in all types of blood cells. The cell shape was remained intact up to 18 h since fixation. Considering the toxicity of methanol, ethanol was selected as a suitable fixative.

The blood smears prepared using the Wright-Giemsa methods are presented in Table 1 and Fig. 2. There was only a slight difference between the observed and recommended color in Table 1 in terms of erythrocytes. In terms of staining conditions, smears immersed in Coplin jars were stained the same color across the batch, without scratching or staining precipitate formation. Thus, in this step, blood smears should be stained using the Wright-Giemsa method and in Coplin jars.

According to these results, the blood smear preparation procedure should involve blood storage for less than 6 h, fixation with pure anhydrous ethanol, and staining using the Wright-Giemsa method in Coplin jars.

### 3.2. Evaluation of homogeneity and stability

#### 3.2.1. For the evaluation of homogeneity

The result of homogeneity of Lot EH01 was presented in Table 3. The neutrophils, lymphocytes, eosinophils, and monocytes of nine random slides were counted by two different technicians. The mean SD and F-statistic of each leukocyte were calculated. All the calculated F-statistic values were smaller than F-distribution showed the uniformity of this experiment.

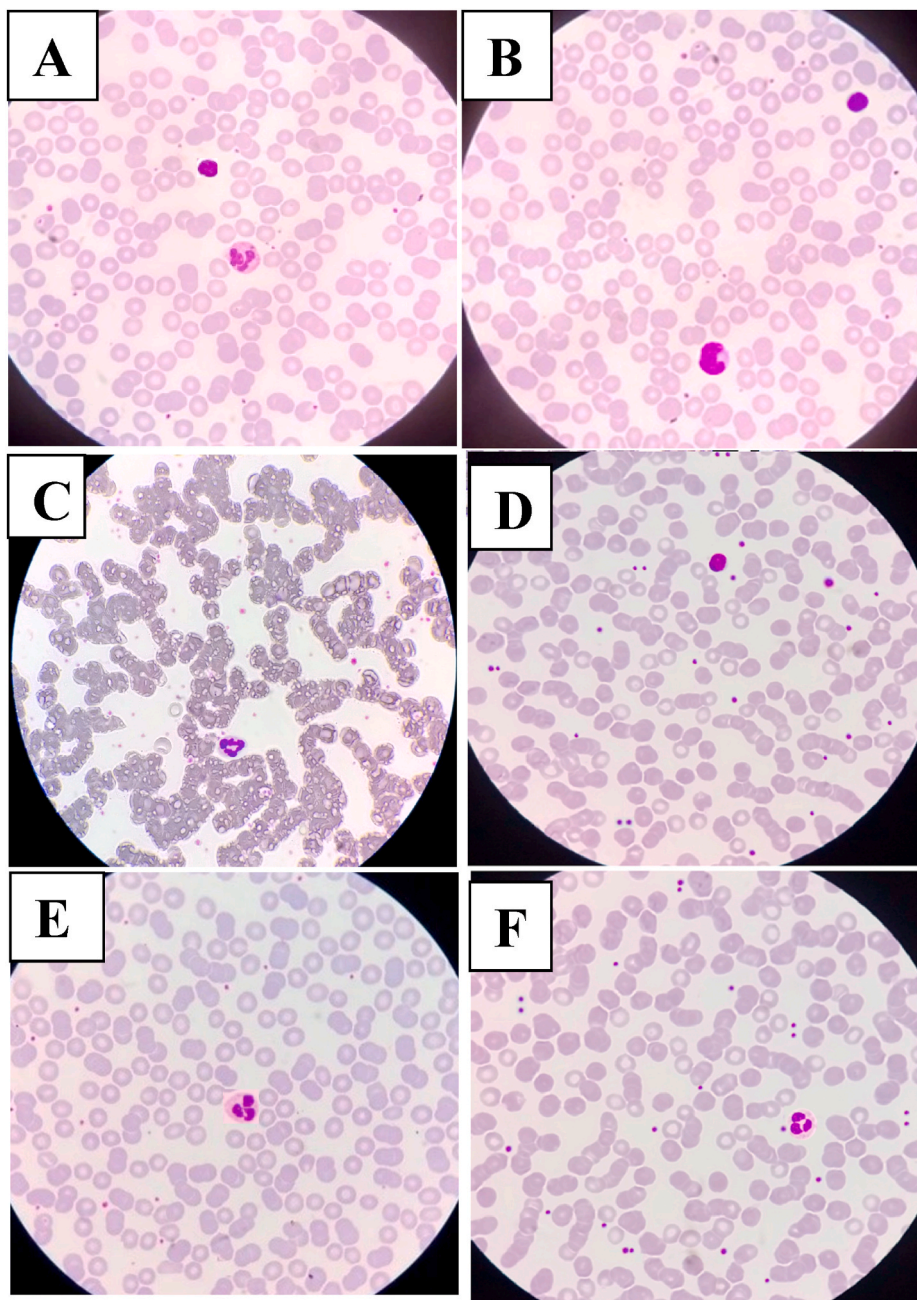
#### 3.2.2. For the evaluation of stability

Based on the optical microscope observation of Lot EH02, the morphology of blood cells remained intact after 2, 4, and 8 months (Fig. 3). Furthermore, as shown in Table 4, the P-values for all 4 leukemia parameters of Lot EH01 were greater than 0.05 at all their

**Table 2**  
Characterization of blood smears under different preparation conditions.

Variable		Erythrocytes	Leukocytes	Platelets	Smear Background
Time before fixation (hours)	0	x	x	x	x
	2	x	x	x	x
	4	x	x	x	x
	6	x	x	x	Blue gray streaks
Fixative	ethanol 90%	Water artifacts	x	x	x
	pure anhydrous ethanol	x	x	x	x
	pure anhydrous methanol	x	x	x	x
Time between fixation and staining (hours)	0	x	x	x	x
	6	x	x	x	x
	12	x	x	x	x
	18	x	x	x	x

x: acceptable.



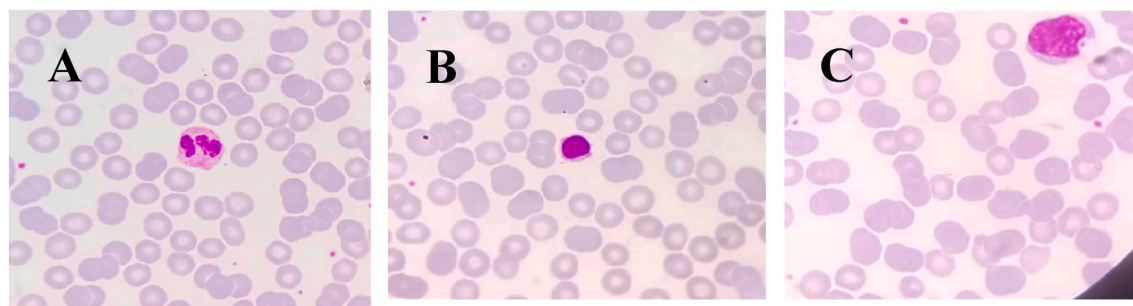
**Fig. 1.** Photomicrographs (100X) from (A) a slide with a 2-h interval before fixation; (B) a slide with a 6-h interval before fixation; (C) a slide fixed in 90% ethanol; (D) a slide fixed in pure anhydrous ethanol; (E) a slide fixed in pure anhydrous methanol; and (F) a slide stained after fixation by pure anhydrous ethanol for 18 h.

interval time points (2 months, 4 months, and 8 months). Basophils were detected at a rate of 0% in all trials. Thus, the samples stained by our procedure reached stability at least for 8 months in the term of blood cell morphology and leukocytes count (Table 4 and Fig. 3).

#### 4. Discussion

With the development of modern hematology analyzers, checking the morphology of blood cells is necessary as a reference method for clinicians to verify abnormal results and detect abnormal blasts that occur in the periphery. To obtain a clear interpretable morphology, the slide must be in good condition, including the correct morphology of erythrocytes, leukocytes, platelets, and have proper staining color. According to the International Council for Standardization in Hematology (ICSH), the difference in pH of EDTA





**Fig. 2.** Cropped and magnified 1.5x photomicrographs of blood smears prepared with Wright-Giemsa staining. (100X) (A) Neutrophil; (B) Lymphocyte; (C) Monocyte.

**Table 3**

Results from 10 slides in Lot EH01: leukocyte differential count for homogeneity testing.

N = 10 Slide #	Neutrophils		Lymphocytes		Eosinophils		Monocytes	
	Slide reviewer		1	2	1	2	1	2
	1	2						
EH01-002	58	54	37	39	1	2	4	5
EH01-050	56	55	37	39	2	2	5	4
EH01-011	54	56	38	37	2	2	6	5
EH01-042	52	54	40	39	2	2	6	5
EH01-020	54	52	38	40	2	3	6	5
EH01-061	54	51	39	40	2	3	5	6
EH01-035	52	52	39	40	3	2	6	6
EH01-030	56	55	38	37	2	2	4	6
EH01-029	54	54	37	38	3	2	6	6
Mean (SD)	54.1 (1.71)		38.45 (1.09)		2.1 (0.55)		5.35 (0.75)	
F-statistic	0.59*		2.289*		1.037*		1.020*	

(\*) The F-statistic smaller than F-distribution (F-distribution = 3.020).

The Basophils were not observed in this experiment.

salts ( $K_2$ ,  $K_3$ , and  $Na_2$ ) may affect the size of erythrocytes because of osmotically dependent shrinkage [13]. The laboratory of the University Medical Center at Ho Chi Minh City branch 2 has already verified the morphology of blood cells contained in  $K_2$  EDTA vacuum tubes remained intact. After withdrawal, the blood was kept at room temperature for approximately 1 h and a cool temperature for 3 h (including sample transportation in an icebox) [5,9]. This allowed for the necessary time to find morphological features suitable for the EQA program to make slides for EQA purposes [10]. Using this method, morphological changes were not detected in the slides compared with the fresh smears. Our result was compatible with other publications [9,11,12]. In a study by Aparna Narasimha, artifacts were observed in smears that were mounted on slides more than 2 h after they were collected in EDTA tubes, but the preservation temperature was not mentioned [13]. In some studies and guidelines, the blood volume dispersed on glass was approximately 2–7  $\mu$ L [5,14], which can cause variation in blood slide lots. To standardize the essential blood volume to make a perfect glass, in this research, 4  $\mu$ L of blood were chosen to ensure the robustness of smear creation.

As can be seen in Fig. 1(A) and (B), the drying time before fixation often influences the smear background; until 4 h after drying, the film is still in good condition, and after 6 h, the smear exhibits many blue-gray streaks because of plasma sticking on the glass [5]. In Table 2, among the fixing solutions, pure anhydrous methanol and ethanol maintained blood cell morphology in good condition. The blood film developed water artifacts that often occur when films are not completely dried at room temperature. With 90% ethanol, as can be seen in Fig. 1(C), the water in the solution was retained in the red blood cell wall, so the staining reagent had difficulty penetrating the cell wall, resulting in water artifacts in erythrocytes. As can be seen in Fig. 1(D) and (E), fixing the film with pure anhydrous ethanol and pure anhydrous methanol resulted in similar morphology of the blood cells in both slides. Although methanol has been suggested in many articles, it is poisonous, so we chose pure anhydrous ethanol, which is safer for clinician health. In this study, no difference in the blood cell morphology was observed as the time after fixation extended to 18 h.

In EQA, homogeneity ensures the slides that the laboratory uses have the same characteristics, including the smear, staining color, and absence of scratches. Therefore, we assessed slides that were stained on a staining rack and in a staining cell. Slides that were stained in a staining cell had similar staining colors across the batch. This characteristic is suitable for EQA purposes, which requires all samples supplied for participants have the same features. In the staining rack, the slides were stained manually, so using the same staining duration for every slide was unrealistic. Staining precipitates sometimes occur on slides using the staining rack method, especially when the staining or buffer solutions overflow the slides.

The final stage was to evaluate the homogeneity and stability of the slides according to ISO 13528 [15]. As can be seen in Table 3, for Lot EH01, we evaluated the uniformity of five white blood cell lineages. The results showed that all four white blood cell lineages

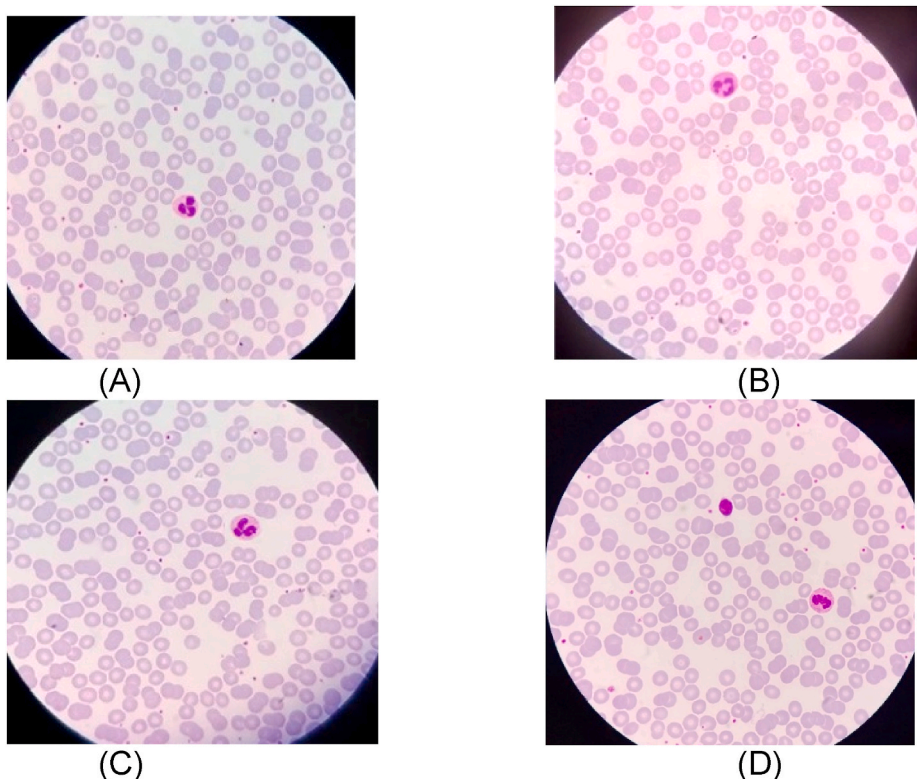


Fig. 3. Microscopy of newly prepared EH02 (A) and after 2 (B), 4 (C) and 8 (D) months.

Table 4

Results 3 slides in Lot EH01: leukocyte differential count for stability testing with three periods (2, 4, and 8 months).

	Sample#	Neutrophils			Lymphocytes			Eosinophils			Monocytes		
		1	2	P value	1	2	P value	1	2	P value	1	2	P value
After 2 months	EH01-059	56	55	0.102	37	38	1.000	3	2	1.000	6	5	0.093
	EH01-066	54	54		39	40		1	1		6	5	
	EH01-052	55	56		37	38		2	2		6	4	
After 4 months	EH01-009	55	56	0.490	39	38	1.000	2	2	1.000	6	4	0.328
	EH01-062	54	53		39	38		2	2		5	7	
	EH01-055	57	56		38	37		1	2		4	5	
After 8 months	EH01-019	53	54	0.341	39	38	0.611	2	2	0.465	6	6	0.328
	EH01-036	56	55		38	37		2	2		4	6	
	EH01-032	55	56		38	37		2	3		5	4	

The Basophils were not observed in this experiment.

had an experimental F value less than the theoretical F, which means the EH01 sample achieved uniformity for all four white blood cell lineages. Therefore, Lot EH01 was considered homogeneous and could be used for EQA purposes. In Lot EH02, the morphology of erythrocytes, leukocytes, and platelets was checked in 10 samples by 2 hematology technicians; the results were the same across replicates, confirming homogeneity.

The slide used for EQA purposes must be stable for at least 7 months because the EQA cycle involves sending a box containing 12 items twice every 6 months. The covered slides were kept in a dry box to avoid direct light permanently staining the color. Every month, a technician checks 2 slides that were coded and introduced in the packed insert. Many studies have reported that slides will be stable for at least 1 year [4], and possibly up to 3 years [13], but these studies were performed in regions with different weather conditions from Vietnam, which has a tropical climate. In our study, the staining color of neutrophils, lymphocytes, eosinophils, and monocytes was maintained for at least 8 months.

In conclusion, we have established the following relevant procedure to prepare slides for EQA purposes: using EDTA tubes for venous blood collection, preparing slides within 4 h after withdrawal, fixing them with pure anhydrous methanol, using the Wright-Giemsa staining method, and staining in a staining cell to prepare slides with the appropriate morphology, homogeneity, and stability.

### Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Practical Laboratory Medicine*.

### Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

#### Category 1.

Conception and design of study: Vu Quang Huy, Van Hy Triet.

Acquisition of data: Huynh Thi Diem Phuc.

Analysis and/or interpretation of data: Tran Van Thanh.

#### Category 2.

Drafting the manuscript: Van Hy Triet, Tran Van Thanh.

Revising the manuscript critically for important intellectual content: Nguyen Van Chinh, Le Dinh Thanh.

#### Category 3.

Approval of the version of the manuscript to be published (the names of all authors must be listed):

Vu Quang Huy, Van Hy Triet, Tran Van Thanh, Huynh Thi Diem Phuc, Nguyen Van Chinh, Le Dinh Thanh.

### Declaration of competing interest

The authors did not receive financial or any other support what so ever in the experimental work or in the preparation of their manuscript.

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