

International Council for Standardization in Haematology (ICSH) Recommendations for “Surrogate Reference” Method for the Packed Cell Volume

B. S. Bull, K. Fujimoto, B. Houwen, G. Klee, L. van Hove, O. W. van Assendelft
on behalf of the ICSH Expert Panel on Cytometry*

*ICSH Expert Panel on Cytometry

Chair: G. G. Klee, MD, PhD (USA)

Members:	O. W. van Assendelft, MD, PhD (USA)	L. van Hove, MD, PhD (USA)
	B. S. Bull, MD (USA)	J. A. Koepke, MD (USA)
	A. Bunyaratvej, MD (Thailand)	S. M. Lewis, MD (UK)
	M. Buttarello, MD (Italy)	S. J. Machin, MD (UK)
	B. Davis, MD (USA)	G. d’Onofrio, MD (Italy)
	K. Fujimoto, MEng (Japan)	R. M. Rowan, MD (UK)
	B. Houwen, MD, PhD (USA)	N. Tatsumi, MD (Japan)

ABSTRACT

The spun packed cell volume (PCV, hematocrit) is a key measurement on which are based hematology instrument calibration, reference range determination, and assignment of values to calibrators/controls. In 2001, the International Council for Standardization in Haematology (ICSH) recommended a Reference PCV method, which is fully traceable to the ICSH reference hemoglobin method. Because of its complexity, however, this method is impractical for occasional use in routine laboratories and is therefore intended primarily for use by manufacturers of capillary microhematocrit tubes, liquid calibrators, and multi-channel analyzers. In response to the need for a simpler method—accessible to all routine laboratories—the ICSH offers this “Surrogate Reference” PCV procedure. It is traceable to the original ICSH Reference PCV method and is based on spun PCVs obtained using borosilicate capillary tubes with an already-known relationship to this reference procedure. This ICSH “Surrogate Reference” PCV method is substantially simpler, thus putting it within the reach of most routine hematology laboratories.

KEY WORDS: Hematocrit · Packed cell volume (PCV) · Reference method

TABLE OF CONTENTS

1.	INTRODUCTION.....	3
1.1	Background.....	3
1.2	Review of the Original ICSH Reference PCV Method.....	3
2.	SPECIMEN COLLECTION.....	4
3.	MATERIALS AND APPARATUS.....	4
3.1	Supplies.....	4
3.2	Equipment.....	4
4.	METHOD.....	4
4.1	Overview of the ICSH “Surrogate Reference” PCV Procedure.....	4
4.2	Sample Relationship between “Reference PCV Capillary Tubes” and the ICSH Reference PCV Method.....	5
4.3	Assigning ICSH “Surrogate Reference” PCV Values to Blood Samples.....	5
4.4	Using Assigned ICSH “Surrogate Reference” PCV to Validate Other Equipment/Methods.....	6
5.	SUMMARY.....	7
6.	REFERENCES.....	9

1. INTRODUCTION

1.1 Background

The manual, spun packed cell volume (PCV, hematocrit) is a key measurement, underpinning much of hematology. The calibration of virtually all hematology autoanalyzers can be traced in some way back to the PCV. Reference ranges for the hematocrit and red cell indices depend on the validity of this calibration, as do the assignment of expected values to calibrators and controls, and the assignment of target values¹ for statistical population-based quality control programs. Any errors in PCV assignment have far-reaching implications.

Although the manual, spun PCV is simple and inexpensive to perform, it is affected by several variables including:

- trapped plasma^{2,3,4}
- white blood cell and platelet contamination of the red cell layer⁵
- indistinct margin between red and white cell layers⁵
- non-flat tube seals⁵
- red cell dehydration⁶
- oxygenation state of the red cells^{7,8}

Fortunately, these biases tend to counterbalance each other, so the net PCV error is typically small—less than 1 PCV unit.⁵ Although of little clinical consequence for *individual* patients, hematocrit errors such as these can have wide-ranging implications when applied globally. For example, biases as small as 1 PCV unit can result in the inappropriate deferral of up to 3.5% of potential blood donors.⁹ PCV errors of this order of magnitude are totally unacceptable in terms of autoanalyzer calibration/control and reference range determination. Even slight autoanalyzer miscalibration based on erroneous PCV values can lead to the incorrect categorization of patients being investigated for anemia.¹⁰

In 2001, the International Council for Standardization in Haematology (ICSH) Expert Panel on Cytometry recommended an ICSH Reference PCV method,¹¹ which was to be used to validate working methods for the PCV. This hemoglobin/mean corpuscular hemoglobin concentration (MCHC)-based reference method eliminates errors due to all of the above 6 factors and requires only standard laboratory glassware, calibrated micropipettes, microhematocrit centrifuge, and spectrophotometer. It does, however, require significant expertise and time if it is to be performed correctly. Although this requirement should not pose a problem for instrument and reagent manufacturers who regularly validate the performance of their products, it does make the procedure impractical for occasional use in routine laboratories. The ICSH therefore offers this alternate ICSH “Surrogate Reference” PCV procedure,¹² which is traceable to the original ICSH Reference PCV method¹¹ and which can be easily performed by most laboratories.

1.2 Review of the Original ICSH Reference PCV Method¹¹

The ICSH Reference PCV is the ratio of whole blood hemoglobin to packed red cell hemoglobin. Collect venous blood samples by syringe or evacuated tubes and anticoagulate with di- or tri-potassium ethylenediaminetetraacetic acid (K₂EDTA, K₃EDTA), 3.7 to 5.4 μmol per mL blood. Complete testing within 3 hours if K₃EDTA is used and within 6 hours if K₂EDTA is used. Determine whole blood hemoglobin concentration by dispensing three 6.66-μL aliquots (positive displacement pipette with glass capillary tips, accuracy ±1%) into each of two 13 × 100-mm glass tubes containing 5.0 mL (transfer pipette 5.0 ± 0.025 mL) of hemoglobin-cyanide (HiCN; methemoglobin cyanide) reagent.^a Cap the tubes and mix by inversion. Determine the packed red cell hemoglobin concentration as follows. Draw well-mixed blood into 2 Reference PCV Capillary Tubes (borosilicate glass; 1.55 ± 0.085-mm inner diameter, 1.9 ± 0.085-mm outer diameter, 75 ± 0.5-mm length). Seal the tubes and centrifuge at 10,000g to 15,000g in a “microhematocrit” centrifuge for approximately 5 minutes. Determine and mark (diamond-tip etching tool) the center point of the red cell column in each tube. Scribe and break the tubes at 1.75 mm above this center point and discard the plasma-containing portion of

^aICSH-recommended HiCN reagent contains 50 mg KCN, 200 mg K₃Fe(CN)₆, 140 mg KH₂PO₄ (anhydrous), 0.5-1.0 mL nonionic detergent (eg, Nonidet P40, Triton X-100), and clinical laboratory reagent water, Type 1, to 1000 mL.

each tube. Carefully aspirate the uppermost 6.66 μL from the packed red cell column in the retained halves of the capillary tubes. Wipe the exterior of the pipette and dispense the contents into two 13 \times 100-mm glass tubes, each containing 5.0 mL HiCN reagent. Cap the tubes and mix by inversion. Allow tubes to stand at room temperature for at least 15 minutes to allow for complete red cell lysis.

Filter the dilute HiCN solutions (5-mL syringe, 0.2-0.25- μm mean pore diameter low-binding/low-release membrane filter) into clean labeled test tubes. Measure the absorbance of the filtered solutions with a calibrated spectrophotometer at 504, 540, and 750 nm in 1.000-cm cuvettes against an HiCN reagent blank. The absorbance values are within acceptable limits if $A^{750} \leq 0.003$ and if $1.59 \leq (A^{540}/A^{504}) \leq 1.63$. The replicates of A^{540} for whole blood (A^{540} *w.bl.*) and for the packed red cells (A^{540} *p.r.c.*) must be within 0.009 absorbance units. The ICSH Reference PCV is calculated as follows:

Equation 1

$$\text{ICSH Reference PCV} = (A^{540} \text{ w.bl.} \times 367.7) / (A^{540} \text{ p.r.c.} \times 1100.2)$$

$$= \text{whole blood hemoglobin} / \text{packed red cell hemoglobin}$$

2. SPECIMEN COLLECTION

All human blood specimens are to be treated as potentially infectious and handled according to Standard Precautions.¹³ Venous blood samples should be collected by syringe or evacuated tube and immediately anticoagulated. The preferred anticoagulant (used primarily in Europe and Japan) is K_2EDTA (dipotassium ethylenediaminetetraacetic acid); an acceptable alternative (used in North America) is K_3EDTA (tripotassium ethylenediaminetetraacetic acid). Care should be taken to avoid red cell hemolysis.¹⁴ To avoid potential errors due to swelling of the erythrocytes over time, testing should be completed within 3 hours if K_3EDTA is used and 6 hours if K_2EDTA is the anticoagulant.

3. MATERIALS AND APPARATUS

3.1 Supplies

- 3.1.1 Reference PCV Capillary Tubes: Wide-bore^b borosilicate^c glass capillary tubes previously validated against the ICSH Reference PCV method.¹¹ This primary validation procedure must be repeated any time tube specifications are altered in any way (dimensions, tube composition, mold release agents, etc). An example of an acceptable tube and its relationship to the ICSH Reference PCV method is given in section 4.2 below.
- 3.1.2 Capillary tube clay sealant.
- 3.1.3 Capillary tube holder (a simple holder to position the PCV tube on the microscope stage can be made by mounting a standard 25 \times 75-mm glass slide on a larger 50 \times 75-mm slide. This holder can then be placed on the microscope stage and the PCV tube positioned against the edge of the 25-mm slide).

3.2 Equipment

- 3.2.1 Microhematocrit centrifuge meeting NCCLS specifications.¹⁵
- 3.2.2 Light microscope with vernier scale.

4. METHOD

4.1 Overview of the ICSH "Surrogate Reference" PCV Procedure

To obtain ICSH "Surrogate Reference" PCV values, spun PCVs are first obtained using so-called Reference PCV Capillary Tubes (wide-bore capillary tubes with a known relationship to the original ICSH Reference PCV

^bTube specifications: 1.55 \pm 0.085 mm (inner diameter) \times 1.9 \pm 0.085 mm (outer diameter) \times 75 \pm 0.5 mm (length).

^cSoda lime tubes are not acceptable because they may induce shrinkage of the red cells in contact with the glass walls.

method). These spun PCV values are then mathematically converted back to what *would have been* obtained had the original ICSH Reference PCV procedure been used instead.

By definition, the slope and intercept values of the ICSH Reference PCV method are 1.0 and 0.0. Theoretically, the relationship (Deming slope, intercept) of a particular PCV capillary tube to the ICSH Reference PCV method can be determined after a little practice by anyone with laboratory expertise. For Reference PCV Capillary Tubes, however, it is recommended that manufacturers determine the relationship and assign appropriate Deming^d slope and intercept values to their tubes. For this purpose, a minimum of 25 blood samples spanning the entire range of clinical PCVs should be used. The slope thus determined should fall between 0.96 and 0.98 and the intercept between 0.012 and 0.015.¹⁶

4.2 Sample Relationship between “Reference PCV Capillary Tubes” and the ICSH Reference PCV Method

One example of an acceptable “Reference PCV Capillary Tube” is the “Special Collection Tube” from Drummond Scientific.^c These tubes have been compared to the ICSH Reference PCV method in 4 different laboratories using a total of 50 blood samples spanning the PCV range of approximately 0.2 to 0.7 l/l, giving the following relationship (Deming^d regression).

Equation 2
$$\text{Spun PCV} = (0.9736 \times \text{ICSH Reference PCV}) + 0.0119$$

And so:

Equation 3
$$\text{ICSH “Surrogate Reference” PCV} = \frac{\text{Spun PCV} - 0.0119}{0.9736}$$

This relationship is depicted graphically in Figure 1 and was used to create Table 1, which provides, in tabular form, a conversion of spun PCVs to ICSH “Surrogate Reference” PCVs across a hematocrit range of 0.1 to 0.7 l/l. (Note that this table applies only to the specific Drummond tubes used in this example.)

4.3 Assigning ICSH “Surrogate Reference” PCV Values to Blood Samples

- 4.3.1 Obtain blood samples as per section 2 above and mix well.
- 4.3.2 Perform **duplicate spun PCV** determinations on each specimen using Reference PCV Capillary Tubes^b that have been previously certified by the original ICSH Reference PCV method¹¹ (see sections 4.1 and 4.2 above).
- 4.3.3 Promptly remove tubes from the centrifuge and maintain in vertical position until they can be read. For best results, read as soon as possible after centrifugation, because the red cells begin to swell and the red blood cell/white blood cell (RBC/WBC) interface becomes progressively more indistinct. By 60 minutes, most samples will have progressed beyond the point at which accurate readings can be made.
- 4.3.4 Examine each tube microscopically to determine appropriate interfaces for PCV calculations:
 - 4.3.4.1 Use a light microscope equipped with ocular cross hairs and a stage vernier scale.
 - 4.3.4.2 Place capillary tube holder (see section 3.1.3 above) on microscope stage and lay capillary tube down against the straight edge of the smaller slide. Make sure that the holder and tube are aligned in a true horizontal position relative to the field of view so that no vertical adjustment is necessary as the stage is moved horizontally.

^dDeming regression tests for agreement between 2 methods or is used to predict values of a response variable (Y) based on a predictor variable (X). Deming regression has been widely adopted in clinical medicine because it allows for imprecision in both variables—unlike least squares linear regression, which allows for imprecision only in the response variable (Y).

^cDrummond Cat# 1-000-7510 (Drummond Scientific Co, Broomall, PA 19008).

4.3.4.3 Examine each Reference PCV Capillary Tube under low power, using the vernier scale to note the following interfaces:

- 4.3.4.3.1 RBC/Clay.
- 4.3.4.3.2 RBC/WBC.
- 4.3.4.3.3 Plasma/Air.

4.3.5 Calculate the spun PCV value for each sample from these interface readings (Equation 4).

Equation 4

$$\text{Spun PCV} = \frac{\text{Interface}_{\text{RBC/WBC}} - \text{Interface}_{\text{RBC/Clay}}}{\text{Interface}_{\text{Plasma/Air}} - \text{Interface}_{\text{RBC/Clay}}}$$

- 4.3.5.1 Determine acceptability of paired spun PCV data. Duplicates must agree within 0.007 PCV units, or the pair must be repeated.
- 4.3.5.2 Calculate average spun PCV value for each acceptable pair.

4.3.6 Convert average spun PCV value to ICSH “Surrogate Reference” PCV value for each sample:

- 4.3.6.1 Look up the Deming^d slope and intercept, which describe the relationship of the certified capillary tubes to the original ICSH Reference PCV method.¹¹
- 4.3.6.2 Use these values in Equation 5 to calculate the ICSH “Surrogate Reference” PCV value.

Equation 5

$$\text{ICSH “Surrogate Reference” PCV} = \frac{\text{Spun PCV} - \text{Deming Intercept}}{\text{Deming Slope}}$$

4.3.6.3 If using the tubes specified in section 4.2 above, this calculation becomes:

Equation 3

$$\text{ICSH “Surrogate Reference” PCV} = \frac{\text{Spun PCV} - 0.0119}{0.9736}$$

4.4 Using Assigned ICSH “Surrogate Reference” PCV to Validate Other Equipment/Methods

4.4.1 Select a **minimum of 6** different blood samples for analysis. (If necessary, the PCV of normal samples may be adjusted by the appropriate addition/removal of autologous plasma.^f)

- At least 2 with PCV \cong 0.20-0.25
- At least 2 with PCV \cong 0.40-0.45
- At least 2 with PCV \cong 0.60-0.65

4.4.2 Obtain **duplicate ICSH “Surrogate Reference” PCV** values on each specimen as described in section 4.3 above.

^fFormula to calculate the volume of plasma to add or remove to achieve the desired PCV:

$$\text{Volume of Plasma to ADD or REMOVE} = \left[\frac{(\text{Current Total Blood Volume} \times \text{Current PCV})}{\text{Desired PCV}} \right] - (\text{Current Total Blood Volume})$$

Note: If result is NEGATIVE, remove plasma
If result is POSITIVE, add plasma.

- 4.4.3 Obtain **duplicate PCV values on the alternate equipment or method being validated** (microhematocrit capillary tubes, multichannel hematology analyzer, blood gas instrument with hematocrit channel, etc) using the same blood samples.
- 4.4.4 Test for agreement between the 2 methods (the ICSH “Surrogate Reference” PCV and the alternate PCV equipment/method) using Deming^d regression¹⁷ (X-axis = ICSH “Surrogate Reference” PCV; Y-axis = alternate equipment/method). Note the Deming slope and intercept for use in Equation 6.
- 4.4.5 Determine the bias of the regression line at both extremes of the measurement range (ie, at PCV values of 0.2 and 0.6) as in Equation 6. The method/equipment is within specification if, for both extreme PCVs, the bias is less than ±0.01 PCV units:

Equation 6

$\text{Bias @ PCV}_{0.2} = [(\text{slope} \times 0.2) + \text{intercept}] - 0.2$ <p style="text-align: center;">AND</p> $\text{Bias @ PCV}_{0.6} = [(\text{slope} \times 0.6) + \text{intercept}] - 0.6$
--

4.4.6 The method/equipment is deemed acceptable if (for PCV_{0.2} and PCV_{0.6}) the following is true:

$$-0.01 < (\text{Equation 6}) < +0.01$$

Example:

	Capillary Tube/Analyzer A	Capillary Tube/Analyzer B
Deming slope	0.9464	0.9688
Deming intercept	0.0123	0.0143
Bias at PCV _{0.2}	0.0016	0.0081
Bias at PCV _{0.6}	-0.0199 (unacceptable)	-0.0044
Interpretation	Out of limits	Within specifications

5. SUMMARY

The PCV plays a central role in the hematology laboratory and is a linchpin for autoanalyzer calibration. It is therefore essential to define a “reference” PCV method with which the accuracy for any given working PCV method can be determined over a wide range of measurement. Just such a reference method—intended primarily for use by manufacturers of reagents, analyzers, and equipment—was recommended by the ICSH in 2001.¹¹ However, the complexity of this method rendered it impractical for occasional use in routine laboratories. The ICSH therefore recommends this ICSH “Surrogate Reference” PCV procedure (which uses capillary tubes already certified by the original 2001 ICSH Reference PCV procedure) as a simpler alternative appropriate for more general use in routine clinical laboratories for calibration checks and quality control. Ideally, manufacturers of capillary tubes will undertake to provide the end user with already-certified borosilicate tubes of the appropriate dimensions. Under such circumstances, in which a manufacturer is certifying an entire tube lot, 25 to 50 blood samples should be used rather than the minimum of 6 recommended in the original 2001 ICSH Reference PCV procedure.

Test Deming method comparison

n | 50

	Precision	
Reference PCV	0.0039	(SD of duplicates)
Spun PCV	0.0020	(SD of duplicates)

	Coefficient	SE	95% Confidence Interval	
Intercept	0.0119	0.0028	0.0062 to 0.0176	(constant bias detected)
Slope	0.9736	0.0064	0.9608 to 0.9864	(proportional bias detected)
r ²	0.9979			

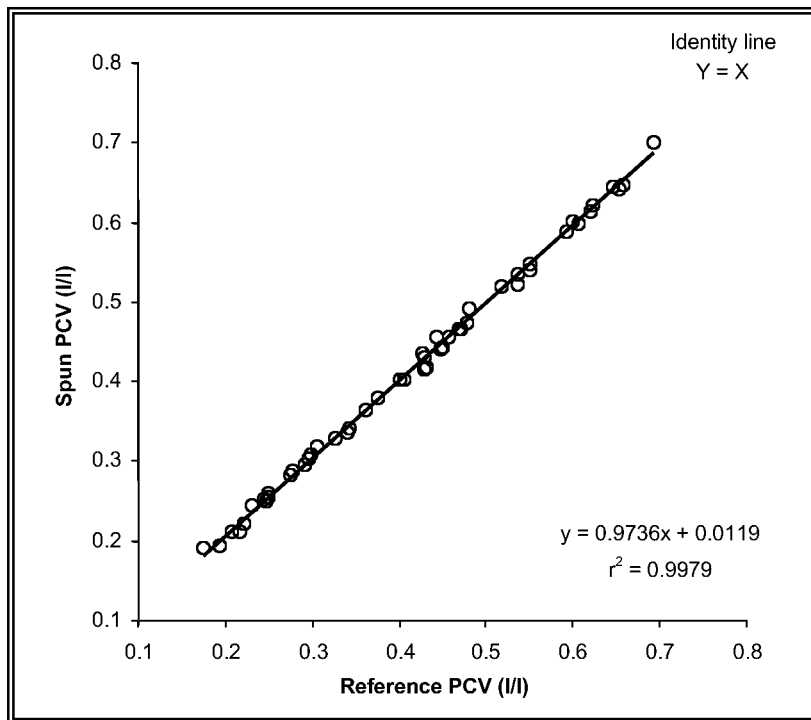


FIGURE 1. Deming^d method comparison of spun packed cell volumes (PCVs) obtained using so-called “Reference PCV Capillary Tubes” [Borosilicate tube, 1.55 mm (inner diameter) × 75 mm (length)] versus Reference PCV values obtained by the ICSH-recommended method.

TABLE 1. Sample Conversion of Spun PCV Values (Using Reference Borosilicate Capillary Tube Described in Footnotes b and e) to ICSH "Surrogate Reference" PCV Values*

Spun PCV	ICSH "Surrogate Reference" PCV	Spun PCV	ICSH "Surrogate Reference" PCV	Spun PCV	ICSH "Surrogate Reference" PCV
0.1000	0.0905	0.3000	0.2959	0.5000	0.5013
0.1100	0.1007	0.3100	0.3062	0.5100	0.5116
0.1200	0.1110	0.3200	0.3164	0.5200	0.5219
0.1300	0.1213	0.3300	0.3267	0.5300	0.5321
0.1400	0.1316	0.3400	0.3370	0.5400	0.5424
0.1500	0.1418	0.3500	0.3472	0.5500	0.5527
0.1600	0.1521	0.3600	0.3575	0.5600	0.5629
0.1700	0.1624	0.3700	0.3678	0.5700	0.5732
0.1800	0.1726	0.3800	0.3781	0.5800	0.5835
0.1900	0.1829	0.3900	0.3883	0.5900	0.5938
0.2000	0.1932	0.4000	0.3986	0.6000	0.6040
0.2100	0.2035	0.4100	0.4089	0.6100	0.6143
0.2200	0.2137	0.4200	0.4191	0.6200	0.6246
0.2300	0.2240	0.4300	0.4294	0.6300	0.6348
0.2400	0.2343	0.4400	0.4397	0.6400	0.6451
0.2500	0.2445	0.4500	0.4500	0.6500	0.6554
0.2600	0.2548	0.4600	0.4602	0.6600	0.6657
0.2700	0.2651	0.4700	0.4705	0.6700	0.6759
0.2800	0.2753	0.4800	0.4808	0.6800	0.6862
0.2900	0.2856	0.4900	0.4910	0.6900	0.6965

*Values from this table should not be assumed to apply to other makes of tubes. The conversion is based on the relationship: $Spun\ PCV = (0.9736 \times Reference\ PCV) + 0.0119$ (Deming^d regression). PVC indicates packed cell volume; ICSH, International Council for Standardization in Haematology.

6. REFERENCES

1. Bull BS, Hay KL. Are red cell indices international? *Arch Pathol Lab Med.* 1985;109:604-606.
2. Garby L, Vuille JC. The amount of trapped plasma in a high speed micro-capillary haematocrit centrifuge. *Scand J Clin Lab Invest.* 1961;13:642-645.
3. Rustad H. Correction for trapped plasma in micro-hematocrit determinations. *Scand J Clin Lab Invest.* 1964;16:677-679.
4. England JM, Walford DM, Waters DAW. Re-assessment of the reliability of the haematocrit. *Brit J Haematol.* 1972;23:247-256.
5. Bull BS, Rittenbach JD. A proposed reference haematocrit derived from multiple MCHC determinations via haemoglobin measurements. *Clin Lab Haematol.* 1990;12:43-53.
6. Karlow MA, Westengard JC, Bull BS. Does tube diameter influence the packed cell volume? *Clin Lab Haematol.* 1989;11:375-383.
7. Stewart JW. A comparison of different methods of determining the haematocrit. *Bibl Haematol.* 1966;24:101-108.
8. Bryner MA, Houwen B, Westengard J, Klein O. The spun micro-haematocrit and mean red cell volume are affected by changes in the oxygenation state of red blood cells. *Clin Lab Haematol.* 1997;19:99-103.
9. Solomon HM, Grindon AJ. The effect of capillary tube diameter on microhematocrit value. *Transfusion.* 1986;26:199-202.
10. Bull BS, Westengard J. Control of analyzer slope and intercept in the measurement of packed red cell volume (PCV), part II. *Blood Cells Mol Dis.* 2002;28:116-120.
11. Bull BS, van Assendelft OW, Fujimoto K, et al (International Council for Standardization in Haematology Expert Panel on Cytometry). Recommendations for reference method for the packed cell volume (ICSH Standard 2001). *Lab Hematol.* 2001;7:148-170.
12. Bull BS, Cacho VPR, Hay KL. Control of analyzer slope and intercept in the measurement of packed red cell volume (PCV), part I. *Blood Cells Mol Dis.* 2002;28:108-115.
13. Sewell DL, Callihan DR, Denys GA, et al. Protection of laboratory workers from occupationally acquired infections; approved guideline—second edition. NCCLS document M29-A2. 2001;21(23).
14. Standardization of blood specimen collection procedure for reference values: International Committee for Standardization in Haematology (ICSH). *Clin Lab Haematol.* 1982;4:83-86.
15. Bull BS, Koepke JA, Simson E, van Assendelft OW. Procedure for determining packed cell volume by the microhematocrit method; approved standard—third edition. NCCLS Document H7-A3. 2000;20(18).
16. Bull BS, Hay KL. Is the packed cell volume (PCV) reliable? *Lab Hematol.* 2001;7:191-196.
17. Cornbleet PJ, Gochman N. Incorrect least-squares regression coefficients in method comparison analysis. *Clin Chem.* 1979;25:432-438.