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**WORLD HEALTH ORGANIZATION**

**CALIBRATION AND CONTROL OF BASIC**

**BLOOD CELL COUNTERS**

Prepared on behalf of the World Health Organization by the Expert Panel on Cytometry  
of the International Council for Standardization in Haematology<sup>1</sup>

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## CALIBRATION AND CONTROL OF BASIC BLOOD CELL COUNTERS

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of the International Council for Standardization in Haematology<sup>1</sup>

This document deals with methods for calibrating single channel blood cell counters for red cell and white cell counting and for the production of quality control preparations. The use of control charts with this material and also control charts with patient's data are described. There is a short section on identifying and correcting faults in a counter.

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## CONTENTS

	Page
<b>1. INTRODUCTION</b> .....	3
<b>2. RED BLOOD CELL COUNT (RCC)</b> .....	3
2.1 Blood sample .....	3
2.2 Diluent .....	3
2.3 Calibration .....	4
2.4 Method for establishing threshold .....	4
2.5 Calibrator .....	4
2.6 Quality control .....	4
2.7 Patients' data .....	6
<b>3. TOTAL WHITE BLOOD CELL (LEUCOCYTE) COUNT (WCC)</b> .....	7
3.1 Reagent .....	7
3.3 Calibration .....	8
3.3 Quality control .....	8
<b>4. APPENDIX</b> .....	9
4.1 Red blood cell count (RCC) by visual (counting chamber) method .....	9
4.2 Total white blood cell count (WCC) by visual (chamber counting) method .....	10
4.3 Blood cell counter faults .....	10
<b>Reference</b> .....	11
<b>Figures</b> .....	12

## 1. INTRODUCTION

The commonly used blood cell counters function on the principle of "aperture impedance" as originally described in 1956 by W.H. Coulter. There are now many types of electronic counters which use this process; they are of varying degrees of complexity and cost:

- (a) fully automated systems which measure all the components of a full blood count, including the differential leucocyte count;
- (b) semi-automated systems which are preset to count the cells and also to measure Packed Cell Volume (PCV) and Mean Cell Volume (MCV) on whole blood or on prediluted samples;
- (c) simple single-channel instruments which require the operator to set the instrument for counting red blood cells and leucocytes in separate procedures in prediluted solutions of a blood sample. This type of instrument was described in a previous WHO document.<sup>1</sup> It is relatively cheap and is generally adequate for the needs of a small laboratory. Only this category of instrument will be considered here.

## 2. RED BLOOD CELL COUNT (RCC)

### 2.1 Blood sample

Blood should be collected under standard conditions into an anticoagulant. A suitable anticoagulant is dipotassium ethylenediamine-tetraacetic acid ( $K_2$ -EDTA) in a concentration of 1.5 - 2.2 mg/mL ( $4.55 \pm 0.85 \mu\text{mol/mL}$ ) of blood.<sup>2</sup> Other anticoagulants may result in significant shrinking or swelling of red cells.<sup>2,3</sup> The count should be carried out within 8 hours of collection, but the count can generally be delayed for up to 24 hours without affecting the results if the blood is stored at 4°C.

### 2.2 Diluent

A buffered particle-free isotonic salt solution is required. As a rule the manufacturer will recommend a specifically produced reagent. This will have standardized specifications of pH and tonicity which are important with complex multi test instruments, especially for correct measurement of MCV. For red cell counting by simple single-channel counters physiological saline (9g/L) or phosphate buffered saline, pH 7.3-7.5, is adequate provided that the counts are performed immediately after dilution in order to avoid errors due to sphering of the cells. Commercial solutions of saline ("for intravenous use") are usually particle-free; other solutions may require filtration as follows.<sup>4</sup>

Filter through 0.45  $\mu\text{m}$  micropore filter to remove particle so as to ensure that there is a background of less than 50 particles per mL when counted at the RCC setting. If necessary, repeat the filtration through an 0.22  $\mu\text{m}$  micropore filter.

For counting, the blood sample is diluted in accordance with the manufacturer's instructions. As a rule a dilution of 1:50 000 is required; to obtain this a primary dilution of 20  $\mu\text{L}$  blood in 10 mL of the diluent is followed by diluting this suspension 1:100.

### 2.3 Calibration

Most modern counters are preset and cannot be calibrated by the user. In others it is necessary to establish the threshold setting at which all red cells, but a minimum of other cells, will be included in the count. Some counters have a lower but no upper threshold, so that white cells are included in the RCC. Since the white cell count is usually very low (<0.2% in blood from healthy subjects) in relation to the red cell count this is not usually of practical importance; however, an appreciable error can be introduced if the white cell count is greatly elevated, particularly if the patient is also anaemic. The setting of the lower threshold is very important since it is necessary to ensure that microcytic red cells, but not platelets, are included in the count.

### 2.4 Method for establishing threshold

A fresh blood sample is diluted as described above and successive counts are made of the suspension with the lower threshold control being moved incrementally from maximum to minimum position. At the maximum setting the count should be zero or close to zero, and the counts will increase as the amplitude is reduced. The counts at each setting are plotted on arithmetic graph paper (Figure 1). The correct threshold setting is at the far left of the horizontal part of the graph before the line becomes vertical. It is important to carry out the procedure with an abnormal microcytic blood as well as with normal blood. The threshold must be checked weekly, and if necessary readjusted. If the threshold is preset it must be checked with a calibrator (See # 2.5).

### 2.5 Calibrator

A calibrator is usually obtained from a commercial source with a stated assigned value. In some cases it may be provided by a national reference centre as a reference preparation. Home-made preserved blood (control preparation, see # 2.6.1) can be used as a calibrator only if the RCC is first obtained by 5-10 carefully made dilutions using precalibrated pipettes, followed by careful counting by haemocytometer of sufficient numbers of cells to reduce the distribution error to 2% (See Appendix # 4.1). If the instrument count differs by 5% or more from the assigned value it may require to be recalibrated. This procedure for assigning a value to the calibrator is laborious and needs to be done only if a discrepancy is found in results with *control* preparations.

### 2.6 Quality control

#### 2.6.1 Preserved blood control preparation

Collect a unit of normal blood into a blood transfusion donor bag containing acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) anticoagulant. Run the blood through a transfusion giving set into a sterile 2-litre round-bottomed flask. Add antibiotic (e.g. 25-50 mg of penicillin and 25-50 mg of gentamycin per 500 mL). With continuous mixing, dispense aliquots in 2-mL volumes into sterile containers, cap tightly and seal with plastic tape. Unopened bottles should keep in good condition for about 3 weeks at 4°C. Sodium azide (0.1 g/L) can be added as a fungicide in a preparation used for red cell counting; however, it should not be used if the PCV or MCV is to be measured.

A new batch should be prepared every two weeks, so that two separate preparations will be available at all times. If the control preparations are being used as a calibrator check (see above), the validity of the value assigned to the new batch must be checked by measuring it in parallel with the current batch before the end of its stability. Samples of each of the preparations in use should be checked at intervals for signs of deterioration, e.g. discolouration of the blood, turbidity of the supernatant plasma, lysis, leakage at the cap; if any of these features are present, the individual samples or the whole batch must be discarded.

### 2.6.2 Stabilized whole-blood control

The preserved blood may begin to deteriorate after three weeks. Blood treated by fixation will have a longer stability. This is suitable for control of red cell, leucocyte and platelet counting as well as for measuring haemoglobin; its disadvantage is that for cell sizing the cells do not behave like fresh blood, and the packed cell volume (PCV) by centrifugation will be about 10% lower when measured by an automated counter.

#### Method<sup>4,5</sup>

Collect a unit of blood into a blood transfusion donor bag containing CPD anticoagulant. Carry out the subsequent processing no later than 24 hours after collection. Filter the blood through a blood transfusion recipient set into a 500-mL glass bottle.

Prepare the following reagent:

40% formaldehyde	6.75 mL
50% glutaraldehyde	0.75 mL
Trisodium citrate	26 g
Distilled water	to 100 mL

Add 1 mL of the reagent to every 50 mL of the blood. Add antibiotic (e.g. 25-50 mg of penicillin and 25-50 mg of gentamycin per 500 mL). Mix well by inverting and then leave on a roller mixer for one hour. At the end of this period, with continuous mixing dispense in 2 ml volumes into sterile containers. Cap these tightly and seal with plastic tape. Keep refrigerated at 4°C. For use equilibrate to ambient temperature and then mix gently on a roller mixture or by hand for five minutes before opening. Unopened samples should keep in good condition for at least two months.

As an alternative the use of glutaraldehyde-fixed avian blood provides pseudo-leucocytes of longer stability.<sup>5</sup> The procedure for preparation is the same as for stabilized human red cells. Such a pseudo-leucocyte cell concentrate may be unsuitable for direct use in a fully automated blood cell counter, in this event it should be diluted in preserved blood, or added to a lysate for the simultaneous control of leucocyte count and haemoglobin or after it has been prediluted manually in 9 g/L NaCl.

The cell count on the control is established by five replicate counts on each of two vials from the batch either by visual counting or by an electronic cell counter. The dispensing procedure is checked by repeated counts on 5-10 tubes selected at random from each batch.

### 2.6.3 Control charts

Control charts are widely used in haematology. Measurement of the control preparation should be included with each batch of patients' specimens and the result checked on the control chart. This provides a check of precision and is a way to evaluate technical competence and instrument stability. When a batch of material has been prepared and distributed into a number of containers as described above, the homogeneity must be checked by measuring the haemoglobin on four or five subsamples; these should vary by not more than 2%. The mean of the red cell count (RBC) and its Standard Deviation (SD) calculated from ten replicate measurements<sup>2</sup> must then be determined and a control chart drawn, showing the range of mean  $\pm$  2SD (Figure 2). Thereafter, one subsample is included with each successive batch of routine blood counts and the results are plotted on the chart.

If the sample for this procedure has been kept at 4°C it must be allowed to equilibrate to ambient temperature and then mixed on a roller mixer or continuously by hand for about five minutes before opening. As the control is intended to simulate routine practice on random samples, it must be treated exactly like routine specimens.

**Interpretation:** If the test is satisfactory results will oscillate about the mean value and less than 5% of the results will be outside the 2 SD limits. The following indicate a fault in technique, instrument or reagent:

- A result outside the 3 SD limit (+ or -)
- Two results on or just outside the 2 SD limits
- Four consecutive results outside 1 SD limit
- Six consecutive values on one side of the mean
- Difference between two consecutive results greater than 4 SD (e.g. from +2 SD to -2 SD)

When an unsatisfactory result occurs, repeat the test with the same sample of the control preparation. If still unsatisfactory repeat with a fresh sample of the same preparation and then with a sample of the second control preparation which is in stock (see # 2.6.1). If the test continues to be unsatisfactory there is likely to be a fault in the instrument which requires recalibration (see # 2.3-2.5).

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$$SD = \sqrt{\sum (x - \bar{x})^2 \div n - 1},$$

where x = individual measurement

$\bar{x}$  = mean

n = number of measurements

## 2.7 Patients' data

In large hospitals where at least 100 blood counts are performed each day, there should be no significant daily variability in the averages of red cell indices (MCV, Mean Cell Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC)). Demonstrating this is an important method for quality control of automated cell counters, as any substantial deviation may indicate a change in instrument calibration or a fault in its function. With sophisticated systems there is often an inbuilt facility which makes it possible to monitor the blood count results continuously. In smaller laboratories using simple counters results can be analysed at least on a daily basis, using a programmable calculator or personal computer.

To set up the procedure it is first necessary to establish the SDs of the daily averages on several consecutive working days:

$$SD = \sqrt{\Sigma (x - x^d)^2 \div [n - 1]},$$

where n = number of days, x = daily average,  $x^d$  = average of daily averages.

If the test is performed satisfactorily the average on any subsequent day will not vary by more than 2 SD. However, this interpretation is valid only if the population from which the routine specimens come does not vary significantly and the tests are not selectively biased, e.g. by tests carried out only on specific days on patients with iron deficiency or other conditions which affect MCV, MCH and/or MCHC.

It is helpful and has educational value to plot the daily results on a graph as illustrated in Figure 3. It must be remembered that the basic measurements (Haemoglobin, RBC and PCV) are controlled only indirectly by this procedure.

## 3. TOTAL WHITE BLOOD CELL (LEUCOCYTE) COUNT (WCC)

### 3.1 Reagent

The total white cell count is measured in whole blood after the red blood cells are lysed. The lytic agent should reduce the stroma from the lysed cells to a residue which will cause no detectable response in the counting system but will not affect the counting of leucocytes. Manufacturers recommend specific reagents and it is essential to use these for multi-channel instruments which also perform automated differential leucocyte counts. However, for simple single-channel counters the following reagent is satisfactory:

Cetrimide	20 g
10% formaldehyde (in 9g/L NaCl)	2 mL
Glacial acetic acid	16 mL
NaCl	6 g
Water to	1 litre

For the count dilute 20  $\mu$ L of blood in 10 mL of diluent (# 2.1) and add two drops of the lytic reagent. Let stand for at least two minutes but for no longer than 20 minutes. Then mix gently by inverting ten times and introduce into the counter.

### **3.2 Calibration**

In most modern counters the threshold setting is preset by the manufacturer. In this case it should be checked with a reference preparation in the same way as for the red cell count (see # 2.4). A control preparation can also be used for this purpose provided that its WCC has been measured as accurately as possible by counting-chamber. In other instruments it is necessary to establish the threshold setting by preparing a threshold curve from a diluted lysed fresh blood sample as is described in # 2.2.

### **3.3 Quality control**

The stabilized whole-blood control preparation described in # 2.6.2 is suitable.



From the count calculate the RCC per litre as:

$[\text{Number of cells counted} \times 200 \text{ (i.e. dilution)} \times 10^6] \div 0.1 \text{ (i.e. vol counted in } \mu\text{L)}$

#### 4.2 Total white blood cell count (WCC) by visual (chamber counting) method

The counting chamber is as described above (#4.1).

Method: Blood is diluted 1:20 in a lysing solution of 2% (20 ml/L) acetic acid, coloured pale violet with gentian violet. To make the dilution add 20  $\mu\text{L}$  of blood to 0.38 ml of the diluting fluid in a 75 x 10 mm glass or plastic tube. After tightly corking the tube, mix the suspension for at least one minute in a rotating mechanical mixer and then fill the counting chamber as for red blood cells. Count the cells at a magnification of x40. To obtain a CV of 5% it is necessary to count about 400 cells; to minimize distribution errors, count the cells in the entire ruled area (i.e. 0.9  $\mu\text{L}$ ).

Calculate WCC per litre as:

$[\text{Number of cells counted} \times 20 \text{ (i.e. dilution)} \times 10^6] \div 0.9 \text{ (i.e. vol counted in } \mu\text{L)}$

#### 4.3 Blood cell counter faults

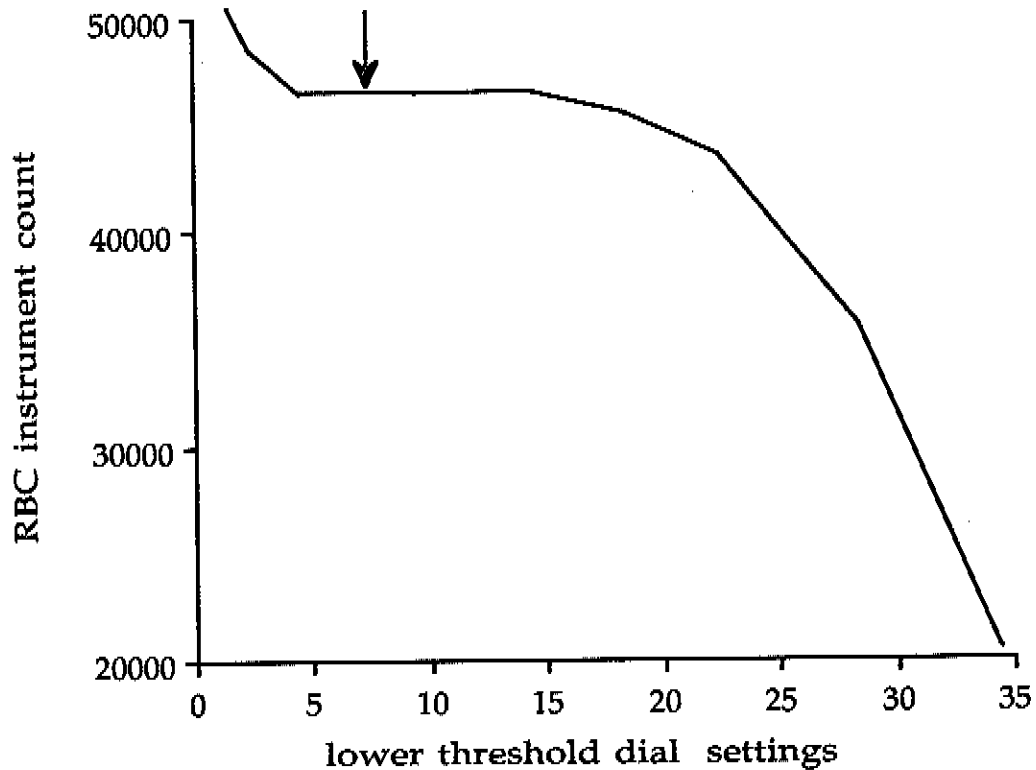
Various technical errors and instrument defects will result in an incorrect count. Most of these causes, as listed below, can be overcome by careful standardized technique.

1. Unrepresentative sampling. If the specimen has been stored in a refrigerator it must first be allowed to equilibrate to ambient temperature on the bench. Then, to ensure homogeneous sampling, the specimen must be carefully mixed by inverting the tube repeatedly, but without shaking, as this will cause foaming, making accurate pipetting impossible.
2. Wrong diluent and/or lysing agent, and diluent contaminated with particulate matter.
3. Inaccurate dilution.
4. Inappropriate dilution. If the suspension is too concentrated there will be coincidence error due to pairs of cells passing through the aperture together, producing a single pulse which is counted as one cell.
5. Instrument drift and unstable current, especially when there are voltage surges during measurement. Electronic noise due to other electric equipment being used in close proximity.
6. Calibration error due to incorrect threshold setting and insensitive discrimination.
7. Carry-over of cells from one sample to the next.

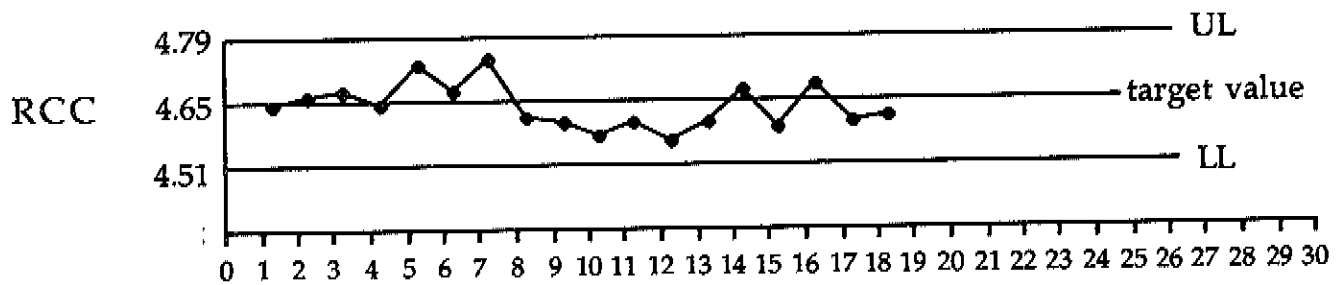
8. Loss of homogeneity of cell suspension after dilution. The diluted sample must be transferred to a counting vial and counted within 5 minutes after completing the dilution.
9. Aperture blockage. The system must be washed thoroughly with diluent at the start and end of each working day.
10. Daily instrument maintenance. Failure to keep the instrument clean and dust free is a major cause of dysfunction. During the course of the day the aperture must be kept in a vial filled with diluent. Overnight, it should be left in a vial of distilled or deionized water. If the counter is not used every day it should be checked regularly to ensure that the aperture has not been allowed to dry and that no crust forms. When not in use, it should be kept covered to avoid dust contamination.

### References

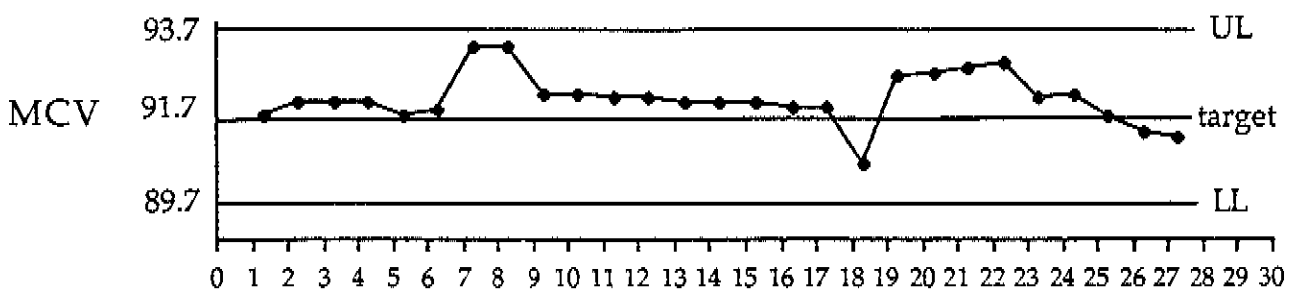
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**Figure 1.** Method to establish threshold setting of cell counters. The position shown by the arrow is selected to exclude noise pulses without missing signal pulses produced by the red cells in the sample.



**Figure 2.** Example of quality control chart for RCC. The upper level (UL) and lower level (LL) indicate the limits of satisfactory control.



**Figure 3.** Use of patients' data as a control. The graph shows the daily averages obtained for MCV in one laboratory over a month. The upper level (UL) and lower level (LL) indicate the limits of satisfactory performance.