Highest-order reference measurement procedure compliant with international protocol for the concentration of erythrocytes and leukocytes provided by provided by International Council for Standardization in Haematology

Revision 01-06e, 2025-5-20
Subcommittee on Standardization of Blood Cell Counting (SBCC)
The Japanese Society for Laboratory Hematology (JSLH)

Contents

1. Title

Highest-order reference measurement procedure compliant with international harmonization protocol for the concentration of erythrocytes and leukocytes provided by International Council for Standardization in Haematology.

2. Introduction

The quantities measured by this RMP are the number of erythrocytes and the concentration of leukocytes in the peripheral venous blood. The blood cells that are the object of this measurement quantity are not stable, such as latex particles. Therefore, measurands of concentration of erythrocytes and leucocytes are not traceable to SI units. The concentration has been designed for hematology analyzer manufacturers and reference laboratories to assure the metrological traceability in the end-user with harmonization/commutability to assure transferability the RMP results to end-users.

This document presents requirements for manufacturers of IVD Medical Devices (MDs) and Reference Laboratories located in Japan in documenting the calibration hierarchy for a measured quantity in human samples using a specified IVD MD. The document includes various model of calibration hierarchies offering potential technical solutions for different kinds of measurands in establishing metrological traceability of values assigned to human samples and calibrators based on The International Council for Standardization in Haematology (ICSH) documents.

The ICSH has defined a reference method for the concentration of erythrocytes and leukocytes in a published guideline for the assignment of values to fresh blood used for calibrating automated blood cell counters in 1988 [01]. Then, ICSH published a reference method that extended ICSH1988 to include more accurate coincidence corrections for the concentration of erythrocytes and leukocytes [02]. Since the previously published ICSH guidelines in 1994, hematology analyzers have evolved greatly. Almost all manufacturers of automated hematology analyzer use a higher order reference measurement procedure (RMP) according to ISO 17511 'In vitro diagnostic medical devices - Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples -' [03]. The ICSH guidelines should be complemented by some descriptions to comply with 'In vitro diagnostic medical devices - Measurement of quantities in samples of biological origin - Requirements for content and presentation of reference measurement procedures (RMP)' by ISO15193 [04]. This document specifies technical requirements and documentation necessary to establish metrological

traceability of values assigned to human samples and human calibrator for quantities measured by IVD MDs.

3. Scope

This document describes a higher order RMP based on the ICSH guidelines for conforming ISO15193/17511, concerning of the assignment of values to fresh blood for concentration of erythrocyte and leukocyte. The reference method provided is a reference measurement procedure that is ultimately metrologically traceable to measurement units and/or measurement standards and/or measurement procedures of the highest metrological level.

Higher order RMP can be used for the following:

a) assessing performance properties of measuring systems comprising measuring instruments, auxiliary equipment as well as reagents,
b) demonstrating if there is a functional interchangeability of different routine measurement procedures purporting to measure the same quantity,
c) assigning quantity values to fresh blood as reference materials (calibrators) that are then used for purposes of calibration or trueness control of routine measurement procedures by the manufacturer.

This document is applicable to:

a) Objectives of measurement:
   The assignment of values to blood used for calibrating automated hematology analyzer (automated blood cell counters) at reference laboratories of manufacturers.
b) Types of sample material and interfering components for fresh blood calibrators:
   Healthy human fresh blood without some interfering components to RBC counting and WBC counting including of lysing process.
c) Measurement interval:
   The analytical measurement interval covered by the reference interval and clinically relevant interval. The analytical interval of erythrocytes and leukocytes concentration of fresh whole blood should be from 3.0 to 6.5 (10^{12}/L) and from 3.0 to 10 (10^{9}/L), respectively.
d) Measured volume interval:
   The clinically relevant upper limit and lower limit of volume axis for determining erythrocytes and leukocytes
e) Calibration of volumetric devices:

Internationally standardized measurements of mass and traceable to certification or other equivalent valid standard of accuracy.

f) Nomenclature:
ICSH recommendations for the standardization of nomenclature and grading of peripheral blood cell morphological features. Int. J. Lab. Hematol. 2015, 37, 287–303 [05]

g) Unit:
ICSH standardization in haematology. Recommendation for standardization of haematology reporting units used in the extended blood count. Int J Lab Hematol. 2016 Oct;38(5):472-82 [06].

h) Different cell types:
All types of erythrocytes and leukocytes are counted volumetrically included as leukocyte count. Leukocyte count can be expanded to measure small nucleated cells other than leukocytes (for example, nucleated erythrocytes) if the lower limit volume for all smallest nucleated cells and lysing method are validated.

This document is not applicable to:

a) Stored blood calibrators and trueness control materials for IVD MDs
b) Control materials that are used only for internal quality control purposes to assess the imprecision of an IVD MD

This document does NOT propose an International Harmonization Protocol (IHP) different from the one published by ICSH but supplements IHP compliant with highest-order measurement procedure (i.e. no internationally agreed RMP) for value assignment to fresh blood calibrators as required by calibration hierarchy 5 in ISO 17511 and 15193 and described in Introduction section. Except for the supplements, the contents follow the reference method published by ICSH.

4. Warning and safety precautions

Warnings and safety precautions regarding blood sample handling, transportation of infections substances and determining the safety level depending on whether it contains bloodborne pathogens should be based on the policies described in the following documents for laboratory biosafety level.

a) Laboratory biosafety manual 4th ed. 2020.12 [07]
b) Guidance on regulations for the transport of infectious substances 2021-2022. applicable as from 1 January 2021 [08]

c) OSHA's Bloodborne Pathogens Standard (29 CFR 1910.1030) [09]

5. Measurement principle and method

The chamber counting is replaced by a single channel semi-automated electronic counter using the aperture-impedance principle [10] provided that a known volume of diluted blood is displaced through the orifice. Only semi-automated single channel aperture-impedance particle counters are available. The instrument should have an impedance cell counter with optimized orifice diameter and length, detector for displaced volume through the orifice, and coincidence correction function. Measurement principles and method are shown in the following [11]:

- **Impedance detection-based Cell counter**

  Cell counts should be performed using a semi-automated single channel counter meeting design specifications which permit counting, by electronic means, of all the cells in a known displaced volume of the diluted blood sample. Aperture-impedance counters could be used for this purpose. The design of the electronic cell counting instrument should be such that each cell is counted once and only once. The probability of re-circulation of particles through the sensing zone must be low and any impulses so created should be below the appropriate lower threshold. The detection electronics should have the ability to discriminate, with a low probability of error, and by pulse amplitude between the cells of interest, other cells, and electrical noise. The detection electronics should have sufficient signal to noise ratio to identify erythrocytes and leukocytes, and it should have a signal amplitude roughly proportional to cell volume. The lower signal threshold should be adjusted to an incremental accuracy equivalent to ±5 fL cell volume or less over the range 0-100 fl. The following considerations should be made to ensure that the effect on erythrocyte and leukocyte counts is less than or equal to 1.5% for erythrocyte count and 2.0% for leukocyte count.

  a) The volume measurement range should include the entire volume range of erythrocytes [12] and leukocytes [10] [13]. Note: The volume range of leukocytes may vary depending on the hemolytic agent used.
  b) The lower volume limit of erythrocytes should be such that if the upper volume limit of platelets overlaps, measures should be implemented to automatically control the lower threshold of erythrocytes to minimize the effect of this overlap.

c) Since the upper volume limit of erythrocytes slightly overlaps with the lower volume limit of leukocytes, measures should be implemented as necessary, such as setting the upper threshold of erythrocytes to minimize the effect of this overlap.

d) The lower volume limit of the leukocyte measurement should be set so that measures are taken as necessary to ensure that the lower volume limit is not affected by erythrocyte ghosts (debris) caused by hemolysis. For example, using a lower threshold for the large debris detection which is set at a volume smaller than the lower volume threshold for leukocytes, the difference in counts by both thresholds may be used to detect the effect of debris.

The design should be such that the probability of recirculation of cells inside the orifice tube is low. Cavitation (trapped air bubbles) and turbulence must be avoided. The sample flow lines must not change volume in response to changes in fluid pressure nor should their materials contribute any measurable cell loss due to adherence.

- **Coincidence correction:**

Coincidence correction may be provided automatically or by means of a coincidence correction chart. The counting error after coincidence must not exceed 2%. The total electronic dead time for a signal pulse must not affect the coincidence correction.

- **Displaced liquid volume through the orifice:**

The liquid volume displaced during the counting period must be known to within an uncertainty of 1% traceable to a national or international metrological standard. This displacement can be achieved by the motion of incompressible fluid column (mercury or distilled water other suitable means) such as "manometer" built-in reference counter. The differential displacement like thermal effects or other influence quantities must be considered.
A quantification equipment for volume of displaced liquid through the orifice connects a manometer in reference counter to the quantification equipment and has the capability to measure the weight of a moving liquid column in the manometer and convert it to a moving volume. The flow path between the manometer and the equipment should be filled with incompressible liquid and measures should be taken to prevent bubbles from entering the flow path The

calibration procedure and evidence for volume measurement shall be validated for the quantification equipment used. There is an example of using mercury as the liquid column. The calibration of mercury manometer volume is shown in Appendix A [14]. Volumetric quantification using the water column can also be implemented to solve environmental issues. For quantitative validation by water column weight, the following measures should be taken in consideration as the main sources of error.

a) Metering syringe: Use glass micro syringes or equivalent to ensure accuracy. The movement of the metering syringe should be automated so that it can aspirate or dispense the volume of the column of liquid to be measured at a constant speed. The speed of movement of the column of liquid should be set according to the speed to the speed set by the fluid control of the reference counter.
b) The amount of fluid remaining on the inner wall in the manometer of the reference counter: It is advisable to check whether this source of error is significant by checking the difference in the amount of water column movement when the moving speed of the liquid column is set as low as possible and when the moving speed of the reference counter is set.
c) Weighing equipment: Select equipment that can measure 4 to 5 significant digits (equivalent to 0.1 to 0.01 μL), which is necessary to ensure valid quantitative accuracy.
d) Weighing environment: Take measures to prevent the effects of static electricity, vibration, and draught.
e) Room temperature control: Set the temperature at 20°C, which is the standard temperature for glass weighing instruments, and take measures to correct the capacity according to the measured temperature.
f) Weighing bottle: To minimize the effect of evaporation of the metered amount of water, use a weighing bottle with a lid or take other measures.
g) Sampling outlet: To minimize the volume of dispensed droplets and to minimize the amount of a droplet remaining on the outside of the needle tip, measures such as using an LC tip with a 90° cut at the tip of the needle should be taken.
h) Sampling: Conduct sampling as close as possible to the weighing equipment.

6. Apparatus

The RMP requires the dedicated analyzer and some equipment.

6.1. Name of the apparatus

Single-channel semi-automated aperture-impedance cell counter without flow focusing

6.2. Essential performance properties

The apparatus for the RMP should fulfill the properties below as the essential performance properties. The basic performance characteristics described in “16. Analytical reliability” should be validated by manufactures.

- Probability of re-circulation of particles through the sensing zone must be low and any impulses so created should be below the appropriate lower threshold.
- Detection electronics should have the ability to discriminate, with a low probability of error, by pulse amplitude between the cells and interest, other cells and electrical noise.
- Instrument should have an orifice diameter 80-100 um and length 70-100 [02]. If the instrument has the other orifice diameter and length, it should be confirmed that the instrument has the same level of analytical performance defined in this document.
- Volume displaced during the counting period must be known to within an accuracy of 1% traceable to a national or international metrological standard.
- This displacement can be achieved by the motion of a mercury column or other suitable means.
- The differential displacement due to thermal effects should not be greater than 0.1% per K.
- Noise must be below the threshold level. Cell counts should be sufficiently low when measuring background. To avoid artificial signals, the threshold settings should ensure sufficient level so that cell counting is not affected noise. The cell counting number in blank measurement should be confirmed low enough not to affect the counting.

6.3. Other equipment

To prepare the appropriate equipment described below is essential to assure the results of the RMP with enough quality.

- **Pipettes**

Positive displacement pipettes should be used, calibrated to an accuracy of ± 0.5%, validated by a process traceable to a primary metrological standard.

- **Volumetric flasks**
  Grade A volumetric flasks made of borosilicate glass, should be used, each with a stated volume which has been tested by an appropriate national calibration service, or which has been checked by the individual user by means of a gravimetric method using certified weights and corrections for buoyance and temperature.
- **Counting vials**
  The counting vial should have a minimum volume of 10 ml. It should be of sufficient height that the entry office of the electronic cell counter is at approximately half the depth of the fluid before counting commences and there should be more than 1 cm of fluid above that orifice after the completion of counting. Before use, the vial should be cleaned free of chemical contaminants and adventitious particles. It is necessary to confirm that cells do not adhere to the counting vial and cause a progressive fall in the count. This is ascertained by testing a number of vials from each batch. Counts are made at measured intervals after the dilutions have been placed in the counting vials. The dilution should be mixed in the vial before counting.

7. Reagents

The reagents for the RMP should have the appropriate quality defined by manufactures and the operator should follow manufacturer specification: storage conditions, shelf life, and so on described on the labels or in the package inserts, etc.

7.1. Diluent for blood sample preparation

The diluent must be a sterile, non-toxic, buffered salt solution suitable for the measurement system. The diluent must not cause cell lysis or later changes of cell volume during diluting blood to the completion of measurement; hence it must be verified that there is no significant volume change that may affect cell count. Its tonicity and the range should be stated. The constituents and their concentrations must be given on the label of the container or on the package insert. When counted in the vial, it must contain less than 1×10^5 particles per liter measurable at the threshold setting for the cells which are to be counted. The operator should follow

the any description such as storage conditions, shelf life, and so on described on the labels or in the package inserts.

7.2. Lytic Agent for blood sample preparation of leukocyte counting

The lytic agent is necessary when white blood cells are to be counted in a whole blood sample and the red cells must first be lysed. The lytic agent must not affect the ability of the white cells to be counted whilst the red cell stroma must be reduced to a residue which does not contribute to the white cell count. For example, typical traditional hemolytic agents used since the early days include quaternary ammonium salts, anionic surfactants, cationic surfactants or and non-ionic surfactant. The different hemolytic agents may change the volumetric range of leukocytes. Regarding condition of lytic agent for sufficient performance, threshold (LD) to discriminate leukocytes from debris (remaining erythrocyte membranes cause by dissolved) and evaluate the “time-stability” after adding the lysis should be validated by impedance counter. The operator should follow the any description such as storage conditions, shelf life, and so on described on the labels or in the package inserts.

7.3. In-house reagents

If the RMP requires the procedure to prepare the reagents, the operator should prepare the dedicated SOP describing the procedure.

7.4. Expression of concentration

Manufactures should prepare the document describing the concentration expressed as amount-of-substance concentration of the necessary reagents for the RMP. The operator should prepare the document describing the concentration for the in-house reagents.

8. Sampling and sample

Fresh venous blood specimens should be obtained by syringe or evacuated container. Ideally the period between venous sampling and processing should not exceed 8h of venesection [15].

8.1. Tubes

Standard evacuated containers, 10 – 12×75mm, containing 5ml blood require an air bubble comprising at least 20% of the tube volume.

8.2. Blood collection

Specimens should be anticoagulated with K2 EDTA in a final concentration of 3.7 – 5.4 μmol (1.5 – 2.2mg/ml) and placed in containers with allow sufficient air remaining to enable proper mixing [16].

8.3. Storage of the specimen

Specimens should be maintained at 20 ± 2℃ until processing. Ideally the period between venous sampling and processing should not exceed 8h.

8.4. Sample handling

Before testing the specimen should be well mixed by gently inverting the container. The number of inversions of a container, to achieve adequate homogeneity, will depend on the type and dimension of the container. Blood samples should be processed within 4-8 h of venesection [15].

8.5. Special notes

- Liquid anticoagulants are not recommended because the cell count is affected due to the dilution by the anticoagulants.
- Vortex mixers should not be used.
- Non-standard tubes, particularly narrower tubes require more complete inversions.
- Specimens should be rejected if there is visible haemolysis or if micro-clots are present.

9. Control materials and blank materials

It is recommended for the manufactures of the RMP to provide the appropriate control materials so that the quality of the RMP in each laboratory can be assured by performing the internal quality control.

9.1. Control materials

It is recommended for the manufactures of the RMP to provide the appropriate control materials so that the quality of the RMP in each laboratory can be assured by performing the internal quality control. Commercial control materials are available to perform quality control for the measurement system. Pre-analytical procedure should be performed following the description of the package insert for the materials. Use diluent and operational control materials from manufacturer.

9.2. Blank materials

As described in 7.1., the diluent for the measurement system must contain less than 1×10^5 particles per liter measurable at the threshold setting for the cells which are to be counted; therefore, the diluent is suited as the blank material. Other solutions that can be assured to have the same quality as the diluent can also be used as blank materials. Use diluent and operational control materials from manufacturer.

10. Analytical solution

Sample preparation for validating of coincidence correction should be performed for every blood specimen which is to be counted. Make two primary dilutions (each of 0.1 ml blood plus 20 ml diluent). Take 0.02, 0.04, 0.06 and 0.08 ml of the primary dilutions and add these to 20 ml aliquots of diluent to make two sets of four secondary dilutions, the most concentrated of these, i.e., 0.08 ml + 20 ml, having a dilution factor of 1/50451*. Each secondary dilution is then counted as follows:

Secondary dilutions	0.02+20	0.04+20	0.06+20	0.08+20
No. of counts	12	6	4	3

The diluted sample must be transferred to counting vials and gently mixed for about 30 s to get rid of bubbles. The count must be completed within 5 min of performing the dilution.

* “Macro-dilution” is an alternative option:
A volumetric flask (1,000 mL) is filled with an isotonic electrolyte solution (diluent) up to the mark.
Aliquot of the blood is taken using a micro dispenser of a nominal 20 µL.
The blood is dispensed to the volumetric flask while the tip of the micro dispenser is placed in diluent in the volumetric flask.

11. Preparation of the apparatus

11.1. Requirement for the manufactures

For manufactures providing the measurement system, it is required to prepare appropriate instruction for the users which covers the items such as warning and safety precautions, assembly, checking that the tolerance limits of performance quantities are not exceeded, operating mode and user’s preventive maintenance.

11.2. Requirement for the laboratories

Operators should follow any guidance described in the instruction in laboratories using the apparatus for the RMP: Refer to manufacturer instructions: Warnings and safety, Assembly, Control of tolerance limits, Operating mode, Preventative maintenance.

12. Operation

Regarding the operation including the sample measurement, the operator should follow the defined measurement steps and should notice deviations which would happen during the operation. Exclude certain observations from data analysis if there is an assignable cause. Assignable causes are defined as events in the following categories:

a) Failure to adhere to study-specific protocol or procedures
b) Instrument failure
c) Operator-related (e.g., removal of sample before complete aspiration occurs)
d) Specimen-related (e.g., hemolyzed or clotted)
e) Any fresh blood numerical result that has data-invalidating flags as described in the operating instructions for each of the instruments

Exclude only the invalidated numerical results for a given specimen run. All other valid numerical results for other measurands within that run may be used for analysis, provided that such measurands are not potentially analytically impacted by other invalidated results, as defined by the manufacturer.

12.1. Sequence of measuring steps

1) Sample preparation should follow the description in section 8. "Sampling and sample" and section 10. "Analytical solution".
2) The apparatus should be prepared following the description in 11. Preparation of the apparatus
3) Measurements are made 3 times with the 100% sample concentration, 4 times with the 75% sample concentration, 6 times with the 50% sample concentration, and 12 times with the 25% sample concentration using the blood cell counter, and the counts of each sample concentration are summed up.
4) Draw the graph based on the measurement results and calculate the erythrocyte/leukocyte count following the description in 13. Data processing

12.2. Errors

The errors below should be cared during the operation and the error which happens during the operation should be recorded in the report.

- **Analysis of error**

  The maximum permissible bias is 2.0% for red cell counts and 4.0% for white cell counts. Potential sources of error are listed below and apply to both red and white cell counting.

- **Sampling error**

  This error arises if the measured sample is not representative of the original whole blood specimen. Factors to be considered include poor mixing, haemorheological effects in extracting the sample and the inaccuracy in dilution.

- **Transport error**

  Errors due to transport occur if each and every cell is not counted in a known volume of diluted blood. Such errors are caused by sedimentation, cell loss and/or recirculation, as well as the inaccuracy of the displaced volume.

- **Counting error**

  Errors can arise if the final count is distorted because of improper discrimination, spurious counts or inaccurate correction for coincidence loss. The imprecision of the reference cell count should be no greater than 1%. Potential sources of error include extracting the sample, dilution, the imprecision of the displaced volume and the statistical limitation of the number of cells counted.

13. Data Processing

The calculation of measurement results shall contain the following steps;

- Processing of initial data
- Construction of measuring function
- Minimum number of points to generate the measuring function
- Kind-of-quality & measurement unit
- Model for statistical treatment of measured quantity values
- Number of replicates measured quantity values necessary to calculate a measurement result, their allowable maximum difference and the equation used
- Number of significant figures in the measurement result and any rounding procedure
- Calculation of measurement uncertainty

13.1 Calculation of measurement results

a) Processing of initial data:

Initial data are “measured count of 50,000-fold dilution sample”.

Initial data shall be corrected by coincidence-correction study.

b) Construction of measuring function:

RBC count can be obtained by the following formula (1) and (2);

$$X = \frac{c_c}{v_M} \times \frac{v_D + v_F}{v_D} \quad (1), \qquad c_c = a + bc_m \quad (2)$$

X : Blood Cell Count in WB sample (/ L)

c_c : Corrected Count of primary dilution (50,000-fold) sample

v_M : Displaced volume by the manometer (L)

v_D : Displaced volume by the micro-dispenser (L)

v_F : Nominal volume of the volumetric flask (L)

a : y-axis of linear-regression formula for coincidence-correction

b : Slope of linear-regression formula for coincidence-correction

c_m : Observed count of primary dilution (50,000-fold) sample

c) Kind-of-quantity & measurement unit:

Kind-of-quantity is RBC concentration, erythrocyte count per 1 L of whole blood sample. Measurement unit for RBC and WBC is " x 10^{12} / L", and " x 10^{9} / L", respectively.

d) Model for statistical treatment of measured quantity values:

Statistical model shall be given in the following formula (1) and (3);

$$X = \frac{c_c}{v_M} \times \frac{v_D + v_F}{v_D} \quad (1), \qquad c_c = a + bc_m + \varepsilon_{LR} + \delta_{CA} \quad (3)$$

X : Blood Cell Count in WB sample (/ L)

c_c : Corrected Count of primary dilution (50,000-fold) sample

v_M : Displaced volume by the manometer (L)

v_D : Displaced volume by the micro-dispenser (L)

v_F : Nominal volume of the volumetric flask (L)

a : y-axis of linear-regression formula for coincidence-correction

b : Slope of linear-regression formula for coincidence-correction

c_m : Observed count of primary dilution (50,000-fold) sample

ε_{LR} : Deviation of corrected-count originated by linear regression

δ_{CA} : Deviation of corrected-count originated by volume equipment used for dilution-line preparation

e) Description of any algorithm used:

There are possibly 2 options for assigning the RBC count – ICSH original recommended procedure and its modified procedure, as follows;

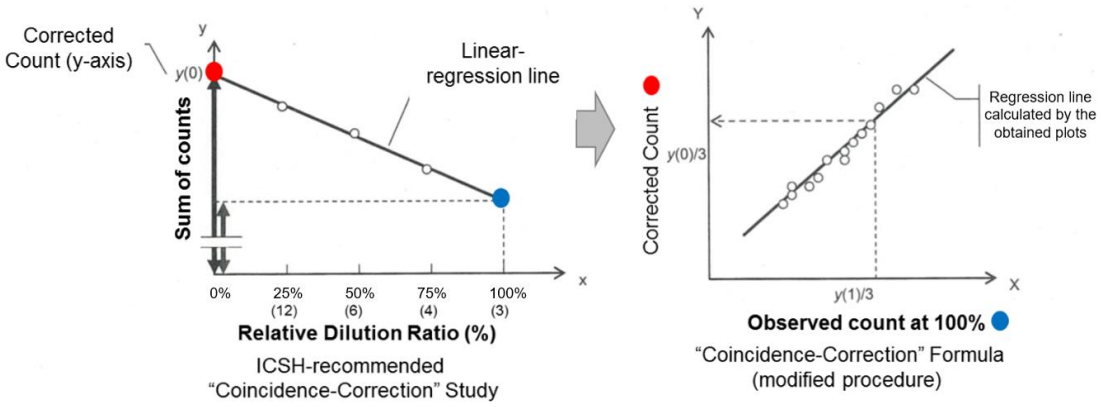
ICSH original RMP

Coincidence-correction study should be performed, sample-by-sample.

Modified Procedure

"Coincidence-Correction Formula" can be applied, which shall be created by the validation study based on multiple samples & analyzers.

* As the ICSH original RMP requires more time to do, the modified procedure can be used as an optional RMP.



f) Number of repeat measurements of quantity values necessary to calculate a measurement result, their allowable maximum difference and the equation used:

	25%	50%	75%	100%
# of measurement	12	6	4	3

g) Number of significant figures in the measurement result and any rounding procedure;

RBC count and WBC count assigned by RMP are rounded to two places of decimals; e.g. RBC: 4.965 -> 4.97 (x 10^{12} / L), WBC: 7.532 -> 7.53 (x 10^{9} / L).

h) Calculation of measurement uncertainty

See the budget sheet in Appendix C.

13.2 Comparison with measurement results obtained by other measurement procedures

If relevant for comparability, reproducibility data shall be given on measurement results on various types of sample to which the reference measurement procedures is claimed to apply with the procedure presented and with alternative measurement procedures differing in measurement principle, measurement method or details of measurement procedure, such as national metrology institute: PTB's reference method using blood cells, AIST's optical particle count method using monodisperse polystyrene latex (PSL) standard solution[17], etc.

PTB: Physikalisch-Technische Bundesanstalt

AIST: The National Institute of Advanced Industrial Science and Technology

14. Reporting

The report of the results should contain the information described below.

- Identification of source and type of sample
- Date of sampling and possibly date of measurement
- Results with measured quantity name, numerical value, and measurement unit
- Statement of measurement uncertainty
- Observations of unusual properties of sample
- Observations regarding unusual features of the measurement procedure or use of modifications
- Physiological and clinical information, if relevant

15. Validation of a reference measurement procedure

The reference laboratory shall validate the reference measurement procedure that it is fit for its intended use. The validation may include the following items;

- Comparison of measurement results achieved with other measurement procedures
- Inter-laboratory comparisons
- Performance validation using reference materials
- Systematic assessment of factors influencing the results
- Assessment of the measurement uncertainty of the results based on scientific understanding of the theoretical principles of the method and practical experience

15.1 Comparison of measurement results achieved with other measurement procedures

Using multiple fresh whole blood samples (at least 50 samples), the comparison study

as a validation for secondary standards (hematology analyzers) of RBC count and WBC count shall be performed. The samples used for this comparison study shall have – RBC: 3.0 – 6.5 (x 10^{12} / L) and WBC: 3.0 – 10.0 (x 10^{9} / L). Do correlation analysis between the both methods, and confirm if the R^2 (coefficient determination) is > 0.95 for RBC and WBC.

15.2 Inter-laboratory comparisons

Perform the inter-laboratory comparisons among the reference laboratories performing the reference measurement procedures for RBC and WBC, periodically. If it is not available, the alternative methods are participating in the External Quality Assessment or proficiency testing program, such as CAP, UK-NEQAS and INSTAND. The recommended frequency of inter-laboratory comparisons is more than twice per year, for monitoring the z-score (SDI) or %bias to the target value.

15.3 Performance validation using reference materials

Though there are no certified reference materials for blood cell counting, one possible way to validate the performance of reference counter is to use the calibration standards for number concentration of liquid-borne particles – e.g. PSL standard solution which is provided and value-assigned by the national metrological institute.

15.4 Systematic assessment of factors influencing the results

n/a

15.5 Assessment of the measurement uncertainty of the results

Combined standard measurement uncertainty of assigned values for these kinds of reference materials for hematology analyzers (that are not calibrators) shall be estimated by the manufacturer and provided to end users on request. The calibration hierarchy is able to be clarified by using measurands defined by international harmonization protocol (No CRM; not traceable to SI) in ISO17511-2020 (5.6 Cases with metrological traceability supported by an international harmonization), and more detailed explanations can be found at ISO21151-2020[18]. The model of calibration hierarchy for metrological traceability is shown in Appendix B. The assessment of the measurement uncertainty of RBC and WBC count performed by the reference measurement procedure shall be evaluated and documented in the reference laboratory according to the JCGM 200;2012[19].

16. Analytical reliability

The reference laboratory shall demonstrate the analytical reliability of the reference measurement procedure for RBC and WBC, for the following items;

- Linearity
- Analytical influence quantities
- Blank measurement
- Measurement uncertainty
- Measurement precision
- Repeatability
- Intermediated precision
- Reproducibility
- Detection limit
- Lower and higher measurement limits

16.1 Linearity

The linearity range of the reference measurement procedure for RBC and WBC shall be stated and demonstrated, which should cover the measuring range of the whole blood samples – RBC: 3.0 – 6.5 (x 10^{12} / L) and WBC: 3.0 – 10.0 (x 10^{9} / L).

16.2 Analytical influence quantities

The analytical influence quantities in the whole blood samples used for the reference measurement procedure for RBC and WBC shall be stated and demonstrated, such as visible hemolysis, micro-clots or turbidity.

16.3 Blank measurement

Before measuring the diluted whole blood samples, confirm if the blank measurement count is lower the criteria – e.g. criteria for blank measurement < 50; < 0.2% for RBC, < 0.5% for WBC.

16.4 Measurement uncertainty

The measurement uncertainty of the reference measurement procedures for RBC and WBC shall be evaluated and stated in the uncertainty budget sheet (see Appendix C).

There are the following steps to evaluate the measurement uncertainty [11];

- List up the major uncertainty sources such as listed in Appendix C.
- Estimate standard uncertainty for major uncertainty source (type A, type B) : u_i
- Calculate combined standard uncertainty: u_c

- Determine expanded standard uncertainty: $U = k\, u_c$ (coverage factor, k=2)
- State the measurement uncertainty : $y \pm U$

Prepare documentation for evaluating process of the measurement uncertainty to show the analytical reliability. The model equation for measurement and equation of evaluating uncertainty is shown in Appendix D which describes more detailed model than the model in Appendix B [14]. The manufactures shall evaluate the measurement uncertainty of the reference measurement procedures for RBC and WBC following these models.

16.5 Measurement precision

The measurement precision shall be evaluated for the following conditions;

- Repeatability conditions, i.e. the intra-run situation
- Intermediate precision conditions, i.e. defined between-run situation in a given laboratory
- Reproducibility conditions where several laboratories are involved

a) Repeatability

Prepare the diluted samples of whole blood or QC samples for RBC and WBC, and run 10 times consecutively. Calculate the mean, standard deviation (SD) and coefficient of variance (%CV) for RBC and WBC.

b) Intermediate precision (Internal quality control)

Perform the QC using the stabilized QC materials – e.g. 4-runs per day x 25 days (2-3 time / week). Calculate the mean, SD and %CV for RBC and WBC.

c) Reproducibility (Interlaboratory comparisons)

Perform the QC using the stabilized QC materials at multiple reference laboratories – e.g., 4-runs per day x 45 days (2-3 time / week) at 3 reference laboratories. Do ANOVA analysis and calculate the means, inter-lab and intra-lab SDs and %CVs for RBC and WBC.

16.6 Detection limit

n/a

16.7 Lower and higher measurement range

The lower and higher measurement range shall be demonstrated in the linearity study results for RBC and WBC.

17. Quality assurance

Laboratories should perform the internal quality control before the sample measurement and perform the interlaboratory comparisons to assure the quality of the RMP in each laboratory,

17.1. Internal quality control

- Internal quality control should be performed prior to the sample measurement.
- Appropriate control material which has the assay value for RBC and WBC should be selected. It is preferable to use 2 levels of control materials.
- QC chart with target and limit values should be prepared based on the appropriate statistical calculation in each laboratory.
- The operator should strictly follow the pre-analytical procedure described in the package inserts for the materials.
- Measure for each control material at least once before and after sample measurement and plot the result in the QC chart. Outlying measurement values should be checked for each control before sample measurement.
- An example of Internal quality control is shown in Appendix E.

17.2. Interlaboratory comparisons

- Interlaboratory comparisons should be planned and performed regularly (e.g. once a year, once per 3 years)
- Fresh human blood collected by the appropriate procedure (it is preferable to prepare the standard operation procedure about fresh human blood collection for interlaboratory comparison) or commercial control materials are available for interlaboratory comparison.
- Organizer of interlaboratory comparisons should prepare the instruction covering the pre-analytical and measurement procedure.
- Organizer should calculate the statistics based on the data submitted from laboratories and define the outlier based on the defined criteria of the comparison.
- For the outlier laboratory, the operator in the laboratory should look for the cause, perform the countermeasure, and record the measure.
- The example of interlaboratory comparison is shown in Appendix F.

18. Special cases

When a manufacturer's laboratory performs routine assays on fresh blood samples, the laboratory's own secondary assay procedures should ensure consistency with the results of coincidence loss measurements using dilution series methods that comply with the internationally harmonized protocols provided by ICSH.

18.1. Reduced coincidence correction method

Coincidence correction should be performed for every blood specimen which is to be counted, however in case to measure many specimens, it is available to perform the coincidence correction by applying the regression line obtained by the measurement for some samples.

References

[01] The assignment of values to fresh blood used for calibrating automated blood cell counters. Clin lab Haematol 1988; 10: 203-212

[02] Reference method for the enumeration of erythrocytes and leucocytes. Prepared by the Expert Panel on Cytometry. Clin Lab Haematol 1994; 16: 131–138.

[03] In vitro diagnostic medical devices – Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples –‘ by the International Organization for Standardization (ISO)17511

[04] In vitro diagnostic medical devices – Measurement of quantities in samples of biological origin – Requirements for content and presentation of reference measurement procedures (RMP)’ ISO15193 .

[05] ICSH recommendations for the standardization of nomenclature and grading of peripheral blood cell morphological features. Int. J. Lab. Hematol. 2015, 37, 287–303.

[06] ICSH standardization in haematology. Recommendation for standardization of haematology reporting units used in the extended blood count. Int J Lab Hematol. 2016 Oct;38(5):472-82

[07] Laboratory biosafety manual 4th ed. 2020.12

[08] Guidance on regulations for the transport of infectious substances 2021-2022. applicable as from 1 January 2021

[09] OSHA’s Bloodborne Pathogens Standard (29 CFR 1910.1030)

[10] Davis B, Barnes PW. Chapter 3, Automated Cell Analysis: Principles, In: Laboratory Hematology practice, 1st edn. International Society for Laboratory Hematology (Kottke-Marchant K, Davis B eds). New Jersey: John Wiley & Sons; 2012.

[11] Shinkai E, Shirakami A, Fujimoto K, The reference measurement procedure for erythrocyte enumeration. Kensa to Gijutsu, 2008; Vol. 36(6): 518-522
*English version: Sysmex Int 2009; 19:1-5 (This article was translated and republished with permission from IGALU-SHOIN Ltd.)

[12] Constantino BT. The Red Cell Histogram and The Dimorphic Red Cell Population. Laboratory Medicine. 2011 May; 42(5): 300-308.

[13] Niitani K. Advance on blood cell counting, especially on volumetric analysis and related problems. Jpn J Clin Hematol. 1984. 25(6). 768-775

[14] E.Shinkai, A.Shirakami, K.Fujimoto; Research Group for Case Studies on Uncertainty Evaluation, NMIJ (National Metrology Institute of Japan) Uncertainty Club, 183-195 (2009)

[15] International Council for Standardization in Haematology, Writing Group, Briggs C, Culp N, Davis B, d'Onofrio G, Zini G, Machin SJ; International Council for Standardization of Haematology. ICSH guidelines for the evaluation of blood cell analysers including those used for differential leucocyte and reticulocyte counting. Int J Lab Hematol. 2014 Dec;36(6):613-27. PMID: 24666725

[16] Recommendations of the ICSH for EDTA Anticoagulation of blood for blood counting and sizing, A.J.C.P October 1993

[17] Y. Kuruma et.al, A. Validation of number concentration measurement equivalent to ICSH reference method for impedance-type blood cell counter by comparison with a primary standard for number concentration of liquid-borne particles, XXXIII International Symposium on Technical Innovations in Laboratory Hematology, Melbourne, Australia - Melbourne Convention and Exhibition Centre, May 21-23, 2020

[18] ISO21151-2020 “In vitro diagnostic medical devices — Requirements for international harmonisation protocols establishing metrological traceability of values assigned to calibrators and human samples”

[19] JCGM 200:2012 International vocabulary of metrology – Basic and general concepts and associated terms (VIM) 3rd edition, 2008 version with minor corrections

Appendices

Appendix A. Example of calibration of mercury manometer volume

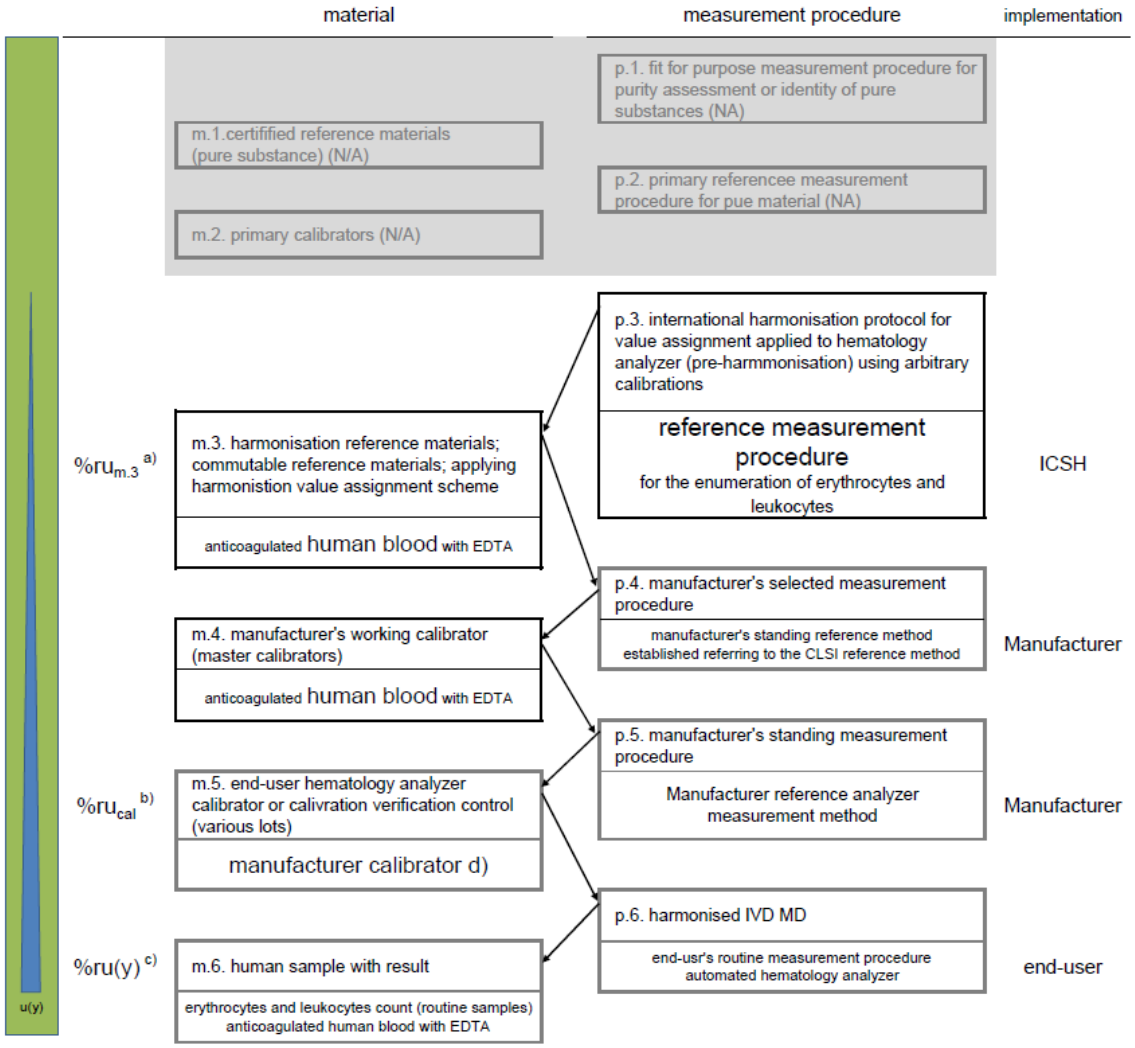
Before equipping a manometer with the reference counter, the manometer volume can be calibrated using a dedicated quantitative device consisting of quartz-glass passage and special syringe to quantify the mass of mercury column's displacement, which was precisely calibrated by the metrological institute, JCSS (Japan Calibration Service System) [14]. An example of calibrating the mercury manometer volume is shown below.

	Measured Volume (mL)		
1	1.0496		
2	1.0497		
3	1.0501		
4	1.0504		
5	1.0502		
Mean	1.05000		
SD	0.00034	Criteria	Judgment
CV(%)	0.032%	< 0.5%	**OK**
Expanded Uncertainty (*k=2*)	0.00221	< 1%	**OK**

Certified Volume (mL)	1.05000

Appendix B. Model of calibration hierarchy for metrological traceability

The model of the calibration hierarchy in the concentration of erythrocytes and leukocytes shall be selected cases with metrological traceability supported by an international harmonization protocol, as shown in the following figure. The hierarchy shall apply to cases in which the measurand in human samples is defined by an international harmonization protocol. There are no internationally agreed upon reference measurement procedures, no primary reference materials, no conventional reference measurement procedures or reference materials, and no metrological traceability to the SI.



Measurand defined by international harmonization protocol (No CRM; not traceable to SI).

Materials [m.1], [m.2], and measurement procedures [p.1] and [p.2] are not applicable (N/A)

(ISO 17511:2020, 5.6 Cases with metrological traceability supported by an international harmonization)

a) Relative percent combined value assignment uncertainty of the [m.3] reference material, calculated according to the following formula:

$$\%ru_{m.3} = (\%ru_c^2{}_{p.1} + \%ru^2_{Rw\text{-}p.3})^{(1/2)}$$

where

$\%ru_c^2{}_{p.1}$ is the relative percent combined standard measurement uncertainty for the [p.1] higher order

MPs with relation to e.g. thermometry, volumetry, time, length, etc.; N/A

$\%ru^2_{Rw\text{-}p.3}$ is the relative percent standard deviation (CV%) for MP [p.3] under repeatability conditions.

b) Relative percent combined value assignment uncertainty of hematology analyzer calibrator [m.5] calculated according to the following formula:

$$\%ru_{cal} = (\%ru^2_{m.3} + \%ru^2_{Rw\text{-}p.4} + \%ru^2_{Rw\text{-}p.5})^{(1/2)}$$

where $\%ru_{Rw\text{-}p.4}$, $\%ru_{Rw\text{-}p.5}$, represent the percent relative standard uncertainties for each applicable MP in the calibration hierarchy.

c) Relative percent combined standard measurement uncertainty for reported values of the measurand with the end-user hematology analyzer, calculated per the following formula:

$$\%ru(y) = (\%ru^2_{cal} + \%ru^2_{Rw\text{-}p.6})$$

where $\%ru^2_{Rw\text{-}p.6}$ is the relative percent standard uncertainty of hematology analyzer based on long-term precision (repeatability conditions of measurement).

d) The documentation of the status of the material’s commutability with human samples when used with any intended measurement procedures, including hematology analyzers: 123-456789 (manufacturer's report number)

Appendix C. Uncertainty Budget of RMP for RBC and WBC concentration

The measurement uncertainty of the reference measurement procedures for RBC and WBC shall be evaluated and stated in the uncertainty budget sheet. There are the following steps to evaluate the measurement uncertainty [11];

- List up the major sources of uncertainty such as listed below.
- Estimate standard uncertainty for major uncertainty source (type A, type B) : u_i
- Calculate combined standard uncertainty: u_c
- Determine expanded standard uncertainty: $U = k\, u_c$ (coverage factor, k=2*)
- State the measurement uncertainty : $y \pm U$

Prepare documentation for evaluating process of the measurement uncertainty to show the analytical reliability.

- Uncertainty of inter-laboratory standard deviation:

U_3 (RBC) and U_4 (WBC) indicated the expanded uncertainty $\%ru_{m.3}$ according to ISO 17511:2020 under repeatability conditions. The calibration hierarchy applied the highest measurement procedure called "International Harmonisation Protocol (IHP)" using fresh blood calibrators valid for up to 8 hours. Definitions of uncertainty in ISO 15193:2009 are also limited repeatability, intermediate and reproducibility standard deviation in single laboratory, and inter-laboratory standard deviation is excepted. It is reasonable that the IHP does not incorporate inter-laboratory variability as a factor in the uncertainty calculation because fresh blood calibrators are not transportable for global use.

Conversely, in use of transportable CRMs should additionally be considered the measurement uncertainty factor for inter-laboratory standard deviation (U_{IL}). For the purpose of calibration verification of global laboratories. According to the policy of uncertainty budget (ISO/IEC Guide 98-3), it is recommended to perform inter-laboratory comparison using stabilized blood for implementation of calibration verification. $U3_{-IL}$ (RBC) and $U4_{-IL}$ (WBC) indicate the uncertainty including the interlaboratory variation factor in calibration verification. $U3_{-IL}$ (RBC) and $U4_{-IL}$ (WBC) are different from the combined uncertainty required by CRM because no fresh blood calibrators are used.

- Measurement uncertainty calculation for RBC

code	Component of Uncertainty	Uncertainty, U(xi)	Type	Distribution	Divisor	Std Unc. u(xi)	sensitivity coefficients	Std. Unc.u(xi)	Remarks
u_{R3}	Repeatability of measurement by RMP	0.014 ($\times10^{12}$/L)	A	Normal distribution	2	0.0068 ($\times10^{12}$/L)	1.00	0.0068 ($\times10^{12}$/L)	Mean of RBC result by RMP 5.051 ($\times10^{12}$/L)
u_V	Calibration of manometer volume	2.5 μL	B	Normal distribution	2	1.26 μL	0.0049 ($\times10^{18}$/L^2)	0.0061 ($\times10^{12}$/L)	Calibration volume of manometer (No.M10-015) 1038.9 μL
u_{DC}	Calibration of microdispenser volume	0.031 μL	B	Normal distribution	2	0.0154 μL	0.25 ($\times10^{18}$/L^2)	0.0039 ($\times10^{12}$/L)	Mean of microdispenser measurement (No.73483) 19.85 μL
u_F	Tolerance of flask volume	0.40 mL	B	Rectangular distribution	$\sqrt{3}$	0.23 mL	0.0051 ($\times10^{15}$/L^2)	0.0012 ($\times10^{12}$/L)	Nominal value 1000 mL
u_{CC}	Coincidence correction	246 count	B	Normal distribution	2	123 count	0.000049 ($\times10^{12}$/(L*count))	0.0060 ($\times10^{12}$/L)	Corrected count of coincidence 104114 count
u_{C3}	combined standard uncertainty			Normal distribution				0.012 ($\times10^{12}$/L)	
U_3	Expanded uncertainty			Normal distribution (k = 2)				0.023 ($\times10^{12}$/L)	

RBC result by reference method procedure = **5.051** ($\times10^{12}$/L) ± **0.023** ($\times10^{12}$/L) , (k = 2) 0.46%

The result in the table above was calculated by the calibration laboratory accredited by ISO/IEC 17025:2017. This method has been reported by the National Institute of Advanced Industrial Science and Technology (AIST) as examples of uncertainty calculation in Japan (Journal of the Japan Society for Precision Engineering Vol.87, No.11, 2021; 849-852) and the result in the table below was calculated including the interlaboratory comparison uncertainty.

Component of Uncertainty	Uncertainty, U(xi)	Type	Distribution	Divisor	Std Unc. u(xi)	sensitivity coefficients	Std. Unc.u(xi)	Remarks
RBC result by reference method procedure	0.023 ($\times10^{12}$/L)	A	Normal distribution	2	0.012 ($\times10^{12}$/L)	1.00	0.012 ($\times10^{12}$/L)	Mean of RBC result by RMP 5.051 ($\times10^{12}$/L)
Inter-Lab variation	0.063 ($\times10^{12}$/L)	A	Normal distribution	2	0.032 ($\times10^{12}$/L)	1.13	0.036 ($\times10^{12}$/L)	Mean of RBC result of Inter-Lab results 4.479 ($\times10^{12}$/L)
combined standard uncertainty			Normal distribution				0.038 ($\times10^{12}$/L)	
Expanded uncertainty			Normal distribution (k = 2)				0.075 ($\times10^{12}$/L)	

RBC result by reference method procedure = **5.051** ($\times10^{12}$/L) ± **0.075** ($\times10^{12}$/L) , (k = 2) 1.5%

* Coverage factor, k=2 (95% confidence interval, assumed Normal distribution)

- Measurement uncertainty calculation for WBC

code	Component of Uncertainty	Uncertainty, U(xi)	Type	Distribution	Divisor	Std Unc. u(xi)	sensitivity coefficients	Std. Unc. u(xi)	Remarks
u_{R4}	Repeatability of measurement by RMP	0.034 ($\times10^9$/L)	A	Normal distribution	2	0.017 ($\times10^9$/L)	1.0	0.017 ($\times10^9$/L)	Mean of WBC result by RMP 5.433 ($\times10^9$/L)
u_V	Calibration of manometer volume	2.5 uL	B	Normal distribution	2	1.3 uL	0.005 ($\times10^{15}$/L^2)	0.0066 ($\times10^9$/L)	Calibration volume of manometer (No.M10-015) 1038.9 uL
u_{DC}	Calibration of microdispenser volume	0.6 uL	B	Normal distribution	2	0.3 uL	0.027 ($\times10^{15}$/L^2)	0.008 ($\times10^9$/L)	No.73175 Latest calibration value 198.56 uL
u_{PC}	Calibration of micropipett volume	2.0 uL	B	Normal distribution	2	1.0 uL	0.0054 ($\times10^{15}$/L^2)	0.005 ($\times10^9$/L)	No.358537 Latest calibration value 999.2 uL
u_{CC}	Coincidence correction	58 count	B	-----	2	29 count	0.000488 ($\times10^{12}$/(L*count))	0.014 ($\times10^{12}$/L)	Corrected count of coincidence 11123 count
u_F	Tolerance of 100mL flask volume	0.10 mL	B	Rectangular distribution	$\sqrt{3}$	0.058 mL	0.054 ($\times10^{12}$/L^2)	0.0031 ($\times10^9$/L)	Nominal value 100.0 mL
u_{C4}	combined standard uncertainty			Normal distribution				0.025 ($\times10^9$/L)	
U_4	Expanded uncertainty			Normal distribution (k = 2)				0.051 ($\times10^9$/L)	

WBC result by reference method procedure = **5.433** ($\times10^9$/L) ± **0.051** ($\times10^9$/L) , (k= 2) **0.93%**

The result in the table above was calculated by the calibration laboratory accredited by ISO/IEC 17025:2017. This method has been reported by the National Institute of Advanced Industrial Science and Technology (AIST) as examples of uncertainty calculation in Japan (Journal of the Japan Society for Precision Engineering Vol.87, No.11, 2021; 849-852) and the result in the table below was calculated including the interlaboratory comparison uncertainty.

code	Component of Uncertainty	Uncertainty, U(xi)	Type	Distribution	Divisor	Std Unc. u(xi)	sensitivity coefficients	Std. Unc. u(xi)	Remarks
u_{C3}	WBC result by reference method procedure	0.051 ($\times10^9$/L)	A	Normal distribution	2	0.025 ($\times10^9$/L)	1.0	0.025 ($\times10^9$/L)	Mean of WBC result by RMP 5.433 ($\times10^9$/L)
u_{IL}	Inter-Lab variation	0.157 ($\times10^9$/L)	A	Normal distribution	2	0.078 ($\times10^9$/L)	0.731	0.057 ($\times10^9$/L)	Mean of WBC result of Inter-Lab results 7.435 ($\times10^9$/L)
u_{C4}	combined standard uncertainty			Normal distribution				0.063 ($\times10^9$/L)	
U_4	Expanded uncertainty			Normal distribution (k = 2)				0.125 ($\times10^9$/L)	

WBC result by reference method procedure = **5.433** ($\times10^9$/L) ± **0.125** ($\times10^9$/L) , (*k*= 2) **2.3%**

* Coverage factor, *k*=2 (95% confidence interval, assumed Normal distribution)

Appendix D. Model equation for measurement and equation of evaluating uncertainty

Equations (1) and (2) in order to calculate RBC concentration are as follows ((1)' for WBC):

$X = \frac{C_c}{V_M} \times \frac{V_D+V_F}{V_D}$ (1) $X = \frac{C_c}{V_M} \times \frac{V_D+V_F+V_L}{V_D}$ (1)'

where

$C_c = a + bC_m C_c = a + bC_m$ (2)

X: Erythrocyte concentration (/L)
C_C: Corrected count in the diluted sample (measurement unit one)
V_M: Volume aspirated by the manometer (L)
V_D: Volume dispensed by the micro dispenser (L)
V_F: Volume of the volumetric flask (L)
V_L: Volume of the lysing solution(L)
a: Intercept of the linear regression equation obtained in advance for correcting for coincidence-loss (measurement unit one)
b: Slope of the linear regression equation obtained in advance for correcting for coincidence-loss (measurement unit one)
C_m: Measured count in the diluted sample (measurement unit one)

By applying the law of propagation of uncertainty, the model equations (1) yield equation (3) shown below.

$$u_c^2(X) = \left(\frac{1}{V_M} \times \frac{V_D+V_F}{V_D}\right)^2 u^2(C_c) + \left(-\frac{C_c}{V_M^2} \times \frac{V_D+V_F}{V_D}\right)^2 u^2(V_M) + \left(\frac{C_c}{V_M} \times \frac{1}{V_D}\right)^2 u^2(V_F) + \left(-\frac{C_c}{V_M} \times \frac{V_F}{V_D^2}\right)^2 u^2(V_D) \quad (3)$$

where

$u_c(X)$: Combined standard uncertainty of RBC concentration by the reference measurement procedure (/L)

$u(V_M)$: Standard uncertainty of the volume aspirated into the manometer (L)

$u(V_D)$: Standard uncertainty of the volume dispensed by the micro dispenser (L)

$u(V_F)$: Standard uncertainty of the volume of the volumetric flask (L)

$u(V_{PC})$: Standard uncertainty of the volume of the micro pipette (L)

$u(C_c)$: Standard uncertainty of the corrected count in the diluted sample (measurement unit one)

* Combined standard uncertainty of WBC concentration by the reference measurement procedure (/L) which adds $u(V_{PC})$ on equation (3)

Appendix E. Example of internal quality control

These data represent internal quality control (IQC) measurements and the judgement from a single site. The commercial IQC material (IQC-M) was measured four runs per day, twice per week. The table below shows 96 runs of IQC data collected for 24 days of operation. Twelve vials of the IQC-M for the same lot were used within the two-week valid period after opening the vials. The allowance range was defined using the CLSI H26-A2 guidance. The allowance range was determined based on data from 16 runs, with four runs per day for four days, and all IQC results were within the allowance range.

Day No.	Week No.	Time	RBC	RBC (Mean)	Pass Fail
1	1-1	14:41	4.46	4.455	---
		14:42	4.46		
		14:43	4.46		
		14:44	4.44		
2	1-2	10:28	4.46	4.443	---
		10:29	4.44		
		10:30	4.43		
		10:31	4.44		
3	2-1	17:19	4.48	4.465	---
		17:20	4.47		
		17:21	4.46		
		17:22	4.45		
4	2-2	11:36	4.44	4.453	---
		11:37	4.45		
		11:38	4.47		
		11:39	4.45		
5	3-1	14:18	4.42	4.423	Pass
		14:19	4.44		
		14:20	4.41		
		14:22	4.42		
6	3-2	10:11	4.45	4.448	Pass
		10:12	4.44		
		10:13	4.45		
		10:27	4.45		
7	4-1	11:57	4.47	4.460	Pass
		11:58	4.48		
		11:59	4.47		
		12:09	4.42		
8	4-2	9:32	4.49	4.473	Pass
		9:34	4.46		
		9:35	4.49		
		9:36	4.45		
9	5-1	11:46	4.39	4.398	Pass
		11:51	4.39		
		11:51	4.40		
		11:52	4.41		
10	5-2	10:06	4.49	4.488	Pass
		10:08	4.50		
		10:09	4.48		
		10:10	4.48		
11	6-1	13:35	4.42	4.413	Pass
		13:36	4.41		
		13:37	4.40		
		13:39	4.42		
12	6-2	14:46	4.48	4.485	Pass
		14:49	4.50		
		14:50	4.49		
		14:51	4.47		
13	7-1	13:26	4.44	4.448	Pass
		13:27	4.47		
		13:28	4.45		
		13:29	4.43		
14	7-2	15:43	4.51	4.500	Pass
		15:45	4.52		
		15:50	4.50		
		15:51	4.47		
15	8-1	11:29	4.44	4.473	Pass
		11:30	4.49		
		11:31	4.48		
		11:32	4.48		
16	8-2	14:21	4.43	4.435	Pass
		14:21	4.45		
		14:22	4.44		
		14:23	4.42		
17	9-1	16:48	4.47	4.465	Pass
		16:49	4.48		
		16:50	4.48		
		16:51	4.43		
18	9-2	15:25	4.46	4.443	Pass
		15:26	4.44		
		15:27	4.42		
		15:28	4.45		
19	10-1	11:06	4.47	4.470	Pass
		11:07	4.50		
		11:08	4.47		
		11:09	4.44		
20	10-2	16:18	4.45	4.433	Pass
		16:19	4.43		
		16:20	4.42		
		16:20	4.43		
21	11-1	15:18	4.43	4.455	Pass
		15:19	4.44		
		15:20	4.48		
		15:21	4.47		
22	11-2	14:19	4.47	4.480	Pass
		14:20	4.50		
		14:21	4.50		
		14:22	4.45		
23	12-1	14:10	4.45	4.455	Pass
		14:11	4.43		
		14:12	4.48		
		14:22	4.46		
24	12-2	13:28	4.50	4.475	Pass
		13:29	4.49		
		13:30	4.48		
		13:31	4.43		

Target Assay Data from Day No 1 - 4	RBC
Taget value (Median)	4.454
Allowance range	±1.5%
Lower limit	4.461
Upper limit	4.893

All data (1 - 24)	RBC (Mean)	Pass Fail
Mean	4.455	---
SD	0.024	---
CV	0.55%	---
Minimum (Min)	4.398	Pass
(Min - Target) / Target	-1.27%	
Maximum (Max)	4.500	Pass
(Max - Target) / Target	1.03%	
Range (Max - Min)	0.10	---

Appendix F. Example of interlaboratory comparison

There are several considerations for interlaboratory comparison:

a) The calibration hierarchy shown in Appendix B serves as the highest-order refence measurement procedure for assigning values to fresh blood due to the inability to use certified reference materials with long-term stability such as clinical chemistry testing. It must be used the mean of multiple fresh blood calibrators to account for specimen variability.

b) The usable period for a fresh blood calibrator is within four hours, exceeding this period imposes a restriction where only stabilized human blood control materials or calibration verification materials must be used and distributed to participants of reference laboratories.

c) If stabilized blood is distributed as blind test samples, the assay values should not be disclosed to prevent cheating. The use of accuracy assurance reference materials, such as calibrators or calibration verification materials with previously published values, is not desirable.

d) It is possible to assign values to stabilized blood, such as commercially available internal quality control materials (IQC-M), and use them as blind samples. This is because the expected range of the IQC-M in the package insert is not intended to assure accuracy but rather serves as a criterion for assessing its usability as a stable material.

e) The primary laboratory assigns an allowance range with target value for blind test samples, and other participating reference laboratories distribute stabilized blood from the same lot as the blind test sample.

f) The primary laboratory operates a quality assurance scheme in which it determines whether the measurement values from the participating facilities fall within the assigned allowance range. If the values fall outside the acceptable range, a review is conducted to investigate the cause and implement corrective measures.

As an example of resolving the previously mentioned concerns, the following presents an example of interlaboratory comparison using commercial control materials:

These data represent an inter-laboratory comparison between the primary test laboratory (Lab. A) and the other five reference test laboratories (Lab. B to F) and the judgements. The table below presents a comparison between the IQC data from 98 runs conducted over 24 days at the primary laboratory and the IQC data collected during the same period at other reference test laboratories. Several vials of the IQC-M for the same lot were used within the two-week valid period after opening the vials. The allowance range for percentage biases to primary laboratory was defined using ICSH guidance and all results from the other five reference laboratories were within the acceptable range of 2%.

Day No.	Week No.	Lab. A (Primary)	Lab. B	Lab. C	Lab. D	Lab. E	Lab. F
1	1-1	4.455	4.510	4.424	4.443	4.430	
2	1-2	4.443		4.428	4.453	4.435	
3	2-1	4.465	4.445	4.416	4.410	4.408	4.403
4	2-2	4.453	4.420	4.404	4.423	4.385	
5	3-1	4.423	4.358	4.380	4.420	4.403	4.393
6	3-2	4.448	4.378	4.392	4.433	4.398	4.380
7	4-1	4.460	4.453	4.394	4.445	4.430	
8	4-2	4.473	4.450	4.429	4.425	4.420	
9	5-1	4.398	4.403	4.389	4.418	4.410	4.450
10	5-2	4.488	4.460		4.410	4.458	4.450
11	6-1	4.413	4.385	4.508		4.485	4.365
12	6-2	4.485	4.430			4.480	4.410
13	7-1	4.448	4.428	4.504		4.413	4.385
14	7-2	4.500	4.448			4.430	4.393
15	8-1	4.473	4.415	4.571		4.465	
16	8-2	4.435	4.425			4.418	4.393
17	9-1	4.465	4.450	4.508		4.488	4.295
18	9-2	4.443	4.423			4.340	4.413
19	10-1	4.470	4.455	4.417			
20	10-2	4.433	4.560	4.395			
21	11-1	4.455	4.540	4.369		4.443	4.465
22	11-2	4.480	4.490	4.414		4.455	4.425
23	12-1	4.455		4.392		4.455	4.398
24	12-2	4.475		4.402			4.448
Mean		4.455	4.444	4.428	4.428	4.431	4.404
SD		0.024	0.050	0.054	0.015	0.036	0.040
Maximum (Max)		4.500	4.560	4.571	4.453	4.488	4.465
Minimum (Min)		4.398	4.358	4.369	4.410	4.340	4.295
Range (Max - Min)		0.103	0.203	0.202	0.042	0.148	0.170
%Bias from Primary Lab.		0.00%	-0.26%	-0.61%	-0.62%	-0.55%	-1.16%
Allowance range		±2.0%	±2.0%	±2.0%	±2.0%	±2.0%	±2.0%
Pass / Fail		Pass	Pass	Pass	Pass	Pass	Pass