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ICSH guidelines for the evaluation of blood cell analysers including those used for differential leucocyte and reticulocyte counting

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SUMMARY

This revision is intended to update the 1994 ICSH guidelines. It is based on those guidelines but is updated to include new methods, such as digital image analysis for blood cells, a flow cytometric method intended to replace the reference manual 400 cell differential, and numerous new cell indices not identified morphologically are introduced. Haematology analysers are becoming increasingly complex and with technological advancements in instrumentation with more and more quantitative parameters are being reported in the complete blood count. It is imperative therefore that before an instrument is used for testing patient samples, it must undergo an evaluation by an organization or laboratory independent of the manufacturer. The evaluation should demonstrate the performance, advantages and limitations of instruments and methods. These evaluations may be performed by an accredited haematology laboratory where the results are published in a peer-reviewed journal and compared with the validations performed by the manufacturer. A less extensive validation/transference of the equipment or method should be performed by the local laboratory on instruments prior to reporting of results.

INTRODUCTION

Since the previously published ICSH guidelines in 1994, haematology analysers have evolved greatly. Automated analysers speed up the workflow in the laboratory and improve precision as more cells are counted and cell classifications are based on more measured objective properties (light scatter, fluorescence,

digital imagine, etc.). For leucocyte, differentials provide clinicians with more reliable data for patient treatment as compared to manual microscopic methods. Data obtained in one laboratory on one instrument should give comparable results to that provided from another laboratory using a different analyser. Several parameters have been introduced to the complete blood count (CBC), such as nucleated red blood cells (NRBC), immature granulocytes (IG), and cell indices such as immature reticulocyte fraction, immature platelet fraction and red cell fragments, as well as new red cell parameters for detection of functional iron deficiency. New parameters related to cell size such as mean platelet volume (MPV) and red cell distribution width (RDW) have also been established. Digital imaging analysis for all blood cells has also been shown to be equivalent to manual microscopy with improved traceability as all significant images can be re-reviewed and stored for future reference [1], but these too must be evaluated for both normal and pathological samples before introduction into clinical laboratory practice.

The proposed reference for an extended flow cytometric differential [2] to replace the CLSI manual counting method [3], while not intended for routine laboratory use, but for manufacturers testing prototype and preproduction models, more accurate value assignments to calibrator and control products and use at select sites performing a full regulatory evaluation . A defined immunophenotypic reference method for high-accuracy nucleated cell identification will further diminish subjectivity on cell classification and statistical imprecision, a problem for both microscope and digital imaging systems.

Advances in instrumentation have resulted in some parameters, previously only indicated by abnormal cell flags, are now being quantitated, such as NRBC, fragmented RBCs, IG and some new reticulocyte and platelet maturation parameters. Choosing a new analyser is an important decision; research should begin with government or peer-reviewed reports on comparative evaluations, as well as information from instrument manufacturers. A laboratory purchasing an evaluated instrument may perform an abbreviated assessment/validation appraising aspects of the equipment in its intended location. Verification/validation is a conformation of the evaluation performed by a manufacturer or other centre of excellence published in a peer-reviewed journal. Such a focused validation should provide evidence that the analyser can meet specific requirements within the test site. Certainly, a normal or reference range and other abbreviated validations must be performed by each laboratory before the analyser is used for patient testing. It should be noted that claims by the manufacturer about throughput may be overestimated due to testing in optimal conditions using relatively normal samples in nonclinical environments. Therefore, the performance attributes of the device should be obtained in the environment where the instrument will be sited and by the routine staff that will be operating the instrument. The scope of any validation should depend upon the availability of independent evaluation data, the range of CBC parameters reported by the laboratory, the range of samples available for the validation and regional laboratory regulations.

LEVELS OF EVALUATION

Manufacturers must conduct validation of their instruments in accordance with European and International Conference on Harmonization (ICH) Guidelines [4, 5], the Chinese State Food and Drug Administration [6], the American National Standards Institute [7, 8] and U.S. FDA guidelines [9], Health Canada, Ministry of Health and Welfare of Japan and any other regional/national requirements.

In the USA, the FDA clearance process, which encompasses all diagnostic devices, requires a description of the study design and the results of the studies. These should be conducted to demonstrate that the device shows an insignificant risk of yielding erroneous results in the hands of the intended user [10]. A laboratory in the USA buying an instrument cleared by the FDA should have confidence that the product meets FDA requirements; however, these instruments may have different requirements when sold outside the USA. Europe has its own *in vitro* diagnostic devices directive, which is currently under rapid transition. In the European community, a CE mark is required indicating that the manufacturer, its authorized representative or the person placing the product on the market or putting it into service asserts that the item meets all the essential requirements of the relevant European Directive(s) [11], but an independent evaluation is still necessary. The Australian

Therapeutic Goods Administration is seeking to harmonize its decision-making with that of overseas authorities, including the FDA. This may offer an opportunity to harmonize diagnostic device approval systems internationally. In Japan, for international standardization on reference measurement procedures of haematology, manufacturers are required to harmonize with the internationally recognized method shown by the Japanese Society for Laboratory Haematology (JSLH).

In some countries, a national evaluation may be carried out by an official organization at an approved centre and performed in accordance with the protocol for the evaluation of blood analysers produced by ICSH [12] and in accordance with this updated guideline; however, these organizations are increasingly being reduced. Where a national evaluation is not available, an evaluation published in a peer-reviewed journal should be sought. The local laboratory user may wish to perform a less extensive assessment/validation that appraises aspects of the equipment and user-dependent steps in its intended location or focused on any unique aspect of the medical practice at that institution. The recommendations for evaluation of coagulation analysers [13] provide some general advice relevant to other haematology analysers.

STAGES OF PERFORMING A FULL EVALUATION

Preliminary information required from the manufacturer and planning the technical evaluation

Instrument installation

Methodology and principles of operation should be assessed and validated with the support of existing literature.

Planning of the evaluation should include an estimate of the availability of internal resources of the evaluating organization. As a preliminary step, a selection should be carried out of adequate reference methods, the evaluation of staff, statistical tools and experienced morphologists: their predictable working time and availability should be verified in advance. Establish the range of instrument parameters that have to be evaluated and should be selected.

The instrument manufacturer should be responsible for instrument installation, set-up and initial calibration using the method specified by the manufacturer. A written report on the calibration and performance of the instrument using control material and/or patient samples should be provided to the evaluating or validating laboratory.

Training. The supplier should provide training for the intended evaluators. This should encompass the principles of the methods of measurement, operation and maintenance of the instrument, and troubleshooting. The instruction manual supplied by the manufacturer should cover these topics in more detail: the evaluation should include assessment of the instruction manual quality and completeness. It is vital that manufacturer's instructions are carefully followed.

Blood samples. Fresh, human whole blood samples used in the evaluation should be anticoagulated using K ethylene diamine tetraacetic acid (K_2EDTA or K_3EDTA as specified by the manufacturer), and its concentration recorded. Advice should be sought from the manufacturer on the minimum volume of blood needed for testing in both automatic and manual modes, including dead space. Blood samples should be processed within 4–8 h of venesection, with the exception of those samples used for the assessment of sample stability. Transport and storage of samples should satisfy the conditions for appropriate international or national codes of safe practice [14]. All blood specimens should be surplus after all other laboratory testing is complete and would otherwise have been discarded. No donor identity should be recorded other than a numerical specimen coding system that allows comparison between instruments and methods. Clinical information is permitted as long as it does not make the patient identifiable, and even necessary for the study of pathological samples. Planning is needed to ensure that samples are available with a wide variety of quantitative and qualitative abnormalities. The range of samples tested should cover the entire clinical reportable range and include the most severe abnormalities encountered by the laboratory. Samples should be included with possible interfering substances, such as lipid, high bilirubin concentration, haemolysis or the presence of

Table 1. Abnormal samples and potential interfering substances that should be included in the evaluation of the haematology analyser

WBC	RBC	Platelets	Interfering substances
Extreme leucocytosis	Sickle cells	Giant platelets	Haemolysis
Extreme leucopenia	Target cells	Platelet clumps	Cryoglobulins
Neutrophilia			
Lymphocytosis			
Monoctyosis			
Eosinophilia			
Basophilia			
Blast cells	Fragmented cells	Immature Platelets	Paraproteins
Atypical lymphocytes	Microcytic cells	CD61 labelled platelets	High bilirubin
Smear/smudge cells	Macrocytic cells		Lipaemia
Immature granulocytes	Spherocytes		
Left shift/band neutrophils	Extreme polycythaemia		
CD3/CD4/CD8 Lymphocytes	Extreme anaemia		
	Nucleated red blood cells		
	Reticulocytosis		
	IRF		
	Low Retic Hb Conc/Content		
	Howell–Jolly bodies		
	Heinz bodies		
	Pappenheimer bodies		
	Malarial parasites		

WBC, white blood cell count; RBC, red blood cell count; IRF, immature reticulocyte fraction; Hb, haemoglobin; Retic, reticulocytes; Conc, concentration.

cryoglobulins. Examples of diseases and interfering substances to be included are listed in Table 1. Not all parameters are available on all instruments. Samples with visible clots should be excluded, but those with platelet clumps and/or red cell agglutinates and detected microscopically should be included, as this will allow assessment of the platelet clump flag. One-third to half the total number of samples should be from normal or nondiseased individuals. Comparability results can be unreliable if the proportion of normal samples included in the evaluation is too high or too low.

Blood films. At least two blood films should be prepared for each sample. These should be made using the laboratory's standard protocol. For digital image analysis systems, the stain may need to be optimized according to the manufacturer's instruction.

Records. Accurate records of all results, including quality control data, should be kept in worksheets or computer spreadsheets. It is also mandatory to keep the original instrument printouts of results for future reference in some regions. Records should be kept of instrument downtime, with reasons for any breakdown, service response times and maintenance schedules. An operator log with the name and professional level of the operator should be used to record any instrument problems encountered, as well as reagent and control usage, batch numbers and expiry dates.

Preliminary assessment

Advice on the safety of the instrument should be sought from the manufacturer and is usually included within the instrument operator manual. Before any evaluation can proceed, an assessment of the safety of the instrument should be made. Health and safety, legal and insurance implications, data storage and security, efficiency and ease of operation should be assessed and recorded. The staff skill level intended to

use the instrument should be taken into consideration as well as cost-effectiveness. Failure of any of the following categories, electrical, mechanical or chemical, should result in the suspension of the evaluation.

Electrical. Electrical specifications should comply with appropriate national or international standards, such as the Geprüfte Sicherheit (GS) mark, American National Standards Institute (ANSI), Conformité Européene (CE) mark, Underwriters Laboratories (UL) listing, Japanese Industrial Standards (JSA) and Canadian Standards association (CSA) mark. In addition, the manufacturer may also cite various international standards with which the instrument complies.

Mechanical. Checks should be made for any hazards that may cause injury, such as exposed moving parts and sharp edges.

Chemical. Any reagents that may be corrosive, carcinogenic or toxic should be considered, for example cyanide reagents used in the measurement of haemoglobin. All reagents used must have undergone complete control of substances hazardous to health (COSHH) assessments and should be supplied with the material safety data sheet information. Different countries have different labelling requirements, but these should conform to local requirements. Any reagents that are premarketed should be labelled as for performance evaluation only or for research use only.

Microbiologic. Ideally specimen analysis should be by closed-vial sampling; however, all instruments should be tested for infective aerosols. The manufacturer should perform a microbiologic assessment by testing for aerosolization and surface contamination using a fluorescent bacterial tracer [15]. All procedures should conform to the appropriate legislation [16–18]. Protocols must also be available for the disinfection and decontamination of equipment, containment of spillages and disposal of waste and samples.

Sample identification and handling

Sample identification either achieved by barcode or by manual input should be assessed. The reliability of barcode readers should be monitored throughout the evaluation, so should the clarity of data and graphics, validation process, quality control programmes and data storage and retrieval.

Performance assessment

Sample mode. Where the instrument has multiple sampling modes utilizing separate sampling pathways (such as automatic from a closed tube and manual from an open tube or prediluted sample), performance should be tested for all methods. Precision, carry-over and linearity (or parameters as stated by the manufacturer) should be assessed in all modes and comparability between modes on a limited number of samples (at least 30).

Precision. Precision may be defined as the closeness of agreement between test results when a sample is run repeatedly. It depends on the distribution of random errors and is not a measure of accuracy. Imprecision rises with lower cell concentrations because fewer cells are counted. Imprecision can be reported as the standard deviation (SD) or the percentage coefficient of variation (CV%), which is the SD expressed as a percentage of the mean value of the replicate measurements on the same sample within a short period of time. An increasing SD or CV% indicates increasing imprecision. Where practical, precision should be established for the full reportable range of each measurand, this includes low counts especially for haemoglobin and platelets at the transfusion threshold.

Within-run precision. This is the repeatability and usually consists of a single run of 10 measurements on the same sample, with all reported parameters analysed. Normal, abnormal low and abnormal high samples for white blood cell count (WBC), haemoglobin concentration (Hb) and platelets should be sought. Low and high values should be outside the reference limits as low and high as samples seen in the laboratory, for example chemotherapy patients, polycythaemic patients and patients with untreated leukaemia. If the analyser reports reticulocytes or NRBC, samples with values around clinical decision points for these parameters should also be tested [19]. Data need to be collected for all parameters reported by the instrument not just those listed above. The

results for mean, SD and CV% should be calculated [12].

Between-batch precision. Between-batch precision may be affected by calibration or drift. A single measurement on the same sample repeated each day for a period 20–30 days is used to measure the total between day (batch) precision for all parameters. As for within-run precision, abnormal low and abnormal high samples for WBC, Hb, platelets, reticulocytes and NRBCs, if appropriate, should be included. Samples are required for a long period of time, and fixed blood may be required; it may be convenient to use quality control material supplied by the manufacturer for this purpose where all parameters available on the instrument will be assessed. For some parameters and from some manufacturers, this is not the case and laboratories should treat the reporting of these parameters with caution without specific internal quality control [19, 20]. As these samples are analysed at any time during the daily workload, the effect of carry-over from high to low specimens needs to be considered and should be assessed in separate studies see below.

Carry-over. Carry-over is defined as the contamination of a sample by the sample analysed immediately preceding it [21]. The clinical concern is that results from a high sample analysed routinely may elevate the results in a cytopaenic or anaemic sample analysed subsequently. Carry-over from a high sample to a low sample should be assessed by running sample A (high sample) three times, A1, A2, A3, followed by sample B (low sample) three times, B1, B2, B3. This should be performed at least three times for WBC, Hb, platelets, reticulocytes and NRBC.

Percentage carry-over is calculated by:

$$\frac{\mathrm{B1}-\mathrm{B3}}{\mathrm{A3}-\mathrm{B3}}\times 100$$

Linearity. Regulatory Affairs agencies require that a laboratory validates an instrument for the reportable range before patient testing. Assuming no constant bias, this is the ability to provide results that are directly proportional to the concentration of cells.

There should be a linear relationship for the parameter measured at various dilutions over as large a range as possible. Dilutions should be chosen to include the entire analytical measurement range, from the highest counts to the lowest. In laboratories where very low WBC and platelet are encountered, it is advisable to examine linearity in the low range separately. For example, a sample with a platelet count of 50×10^9/L could be serially diluted down to a count of 5×10^9/L or a WBC of 2.0×10^9/L to 0.2×10^9/L. Replicate tests should be performed to give results at evenly spaced concentrations, for example reducing in increments of 10% from 100% to 0%. Group AB serum or the diluent reagent for the analyser may be used as the diluent; however, certain haematological parameters, for example, the red cell indices or percentage reported results, will not be affected by dilution of the sample. Some commercial companies provide products that can be used for linearity checks for validation of reportable ranges if no patient samples are available, such as Streck, international@streck.com, R&D Systems, CustomerService@RnDSystems.com. As part of the evaluation, the analytical measurement interval (AMI) and the clinically reportable interval (CRI) should be assessed.

The AMI is the range of analyte values that a method can directly measure on a specimen without any dilution, concentration or other pretreatment not part of the usual assay process. In some instances, AMI may be considered synonymous with linearity. Verification of AMI may be accomplished by evaluation of known samples of abnormal high and abnormal low values or by the use of high and low calibration materials with known values. The CRI of patient test result is the range of analyte values that a method can measure, allowing for specimen dilution, concentration or other pretreatment used to extend the direct analytical measurement range. The CRI is based on diagnostic needs and is a clinical decision by the laboratory authorities, which is related to dilution and concentration protocols used in the laboratory [22–24]. The verification of reportable range may be accomplished by evaluation of known samples of abnormal high or abnormal low values or with low and high calibration materials with known values. Precision of results from the analyser will affect linearity results so this needs to be taken into consideration and may be best tested before linearity. Limit of blank (LoB) and limit of detection (LoD) are measurements determined from precision and linearity procedures and biases of the instrument. This may be more

important for very low cell counts of WBC, red blood cell (RBC) and platelets and for body fluid counting.

A regression graph should be plotted, with concentration on the *x*-axis and cell count or concentration on the *y*-axis. The regression line should pass through the origin and the *R*-value (correlation coefficient) should be as close to 1.0 as possible, but cells in low numbers will always show higher variability between methods due to counting statistics.

Sample stability. Sample stability may be defined as the ability of a sample to retain a consistent value for a measured quantity over a defined time period and within specific limits when stored under defined conditions [25]. A change in the measured quantity of various components of the CBC over time following venesection is a well-known phenomenon, and there may be a higher or lower change in the direction of results. To determine changes on different cell counts or CBC parameters, blood should be taken from five normal individuals and five patients with abnormalities of different cell lines. Analysis is performed at time zero (or as close as possible), the blood sample should then be divided into two sets of six aliquots, one set stored at room temperature (which should be recorded) and the other at 4 °C. Subsequent testing should then be performed after 4, 8, 12, 24, 48 and 72 h. Samples stored at 4 °C should be allowed to come to room temperature before analysis. The effect of storage time and temperature assessed by plotting differences from the initial, or time zero, results against times of testing. The effect of precision of results also needs to be considered and should be assessed in separate studies.

Reference intervals. To establish the clinical utility of an instrument for diagnosis, screening and monitoring of disease, it is necessary for the laboratory to establish a reference range. Reference ranges specific to the instrument, for all components of the CBC, should be calculated during the instrument evaluation [26, 27]. Ideally at least 120 samples, from apparently healthy individuals, all normal values for CBC and differential parameters, of each sex, 60 from each, are tested within 4 h of venesection. Sex and ethnic groups should be examined where possible. Special care should be used to ensure adequate venipuncture technique, time of tourniquet application and blood flow from the vein to avoid haemoconcentration and clotting. If appropriate, ranges for neonates and children of different ages should be tested. Mean, SD and 95% confidence ranges should be calculated for each group. The reference interval for each measurand having a normal distribution (not skewed) within a group is defined from the lower bound (mean − 2 SD) to the upper bound (mean + 2 SD). Mann–Whitney *U*-test for percentiles and confidence limits is applied, if the population results are not normally distributed. Residual samples from the laboratory may be used if all testing is complete; but if volunteers are bled, informed consent should be sought in line with regional ethical guidelines.

Accuracy. Accuracy is defined as the closeness of agreement between the result of a measurement and a known true value. The concept of a true value for many components of the CBC is often not applicable as the true value, which is obtained by a definitive reference method and may not be available. The only relevant parameters that can be estimated correctly are Hb [28], packed cell volume (PCV) [29, 30], RBC count and WBC [31], platelet count [32], reticulocyte count [33, 34] and differential leucocyte count [3, 19]. The haemiglobincyanide (cyanmethaemoglobin) method is the internationally recommended method for determining the haemoglobin concentration of blood. The basis of the method is dilution of blood in a solution containing potassium cyanide and potassium ferricyanide. Haemoglobin and HbCO, but not haemoglobin sulphate, are converted to haemoglobincyanide. The absorbance of the solution is then measured in a spectrometer at a wavelength of 540 nm or a photoelectric colorimeter with a yellow green filter [35]. Most automated counters measure haemoglobin by a modification of the manual method with cyanide reagent or with a nonhazardous chemical such as sodium lauryl sulphate, which avoids possible environmental hazards from disposal of large volumes of cyanide-containing waste. Whatever method is used for Hb measurement, the haemoglobincyanide method is the only accepted reference method.

For reticulocytes, a flow cytometric reference method has been proposed, which has shown to be more precise than the manual method using new methylene blue [36–38]; however, the manual

method, which is subjective and imprecise, currently remains as an accepted reference method. CLSI H26-A2 [19] states that the reference is the flow reticulocytes method with manual reticulocytes as an alternative as this not always available in routine laboratories.

In practice, many laboratories would compare the instrument under evaluation to results from the instrument in routine use, with the exception of the differential leucocyte count, where it is recommended that the results are compared with the reference 400-cell manual differential [3]. However, for a national evaluation, the available reference methods, listed above, may be used if results between systems are significantly different.

Comparability. A comparison of the results from the evaluation instrument with those obtained by the current routine procedures should be made for as many normal and abnormal samples as possible, together with samples with interfering substances; normal samples should be half to one-third of the total. Total number should be at least 250–300 for a full validation. Table 1 lists the samples that should be included in the evaluation. Samples should be measured over a period of time, at least a week or more, to assess variability of the instrument and daily differences in the patient population in the laboratory. Results should be presented graphically showing the difference between the result from the instrument under evaluation (*y*-axis) and the routine instrument (*x*-axis). Regression analysis, correlation and in particular Bland–Altman [39] plots should be used to assess agreement [40]. The correlation coefficient is a measure of how well the data fall on a straight line; the closer the correlation is to 1.0, then the more linear. Slope, intercept, correlation or bias assessments are all important information. It is important to know the medical diagnosis of outliers when comparing methods, as it is not possible for instruments to correctly identify all cells, for example leucemic blast cells. It is important to look at mean difference or Bland Altman plots in leukopenic, anaemic or thrombocytopenic samples, as differences in low values may be masked when analysing the whole data set. Total analytical error (based on analyses of differences) should be calculated [40, 41]. Paired results from the same sample should also be analysed using the paired *t*-test (when results are normally distributed) as linear regression analysis may show good correlation even when there is a bias between results from the two instruments. For non-Gaussian data, the Wilcoxon rank sum test or Mann–Whitney *U*-test should be used for paired data. A *P*-value of <0.05 is usually considered as statistically significant for all the listed tests. Any samples with extreme results where the reason can be explained should not be merged with other results; this would influence the statistical analysis. When discrepant results are found between the current instruments and those under evaluation, the samples should, where possible, be measured using the reference methods previously described. Blood films should be made on all samples analysed and examined microscopically.

Some parameters are only available on a single or limited number of instrument types, such as the percentage hypochromic red cells, reticulocyte haemoglobin content/concentration or the immature platelet fraction. In these cases, it may not be possible to compare the results to another method; in such circumstances, the results should be assessed to determine whether they are consistent and appropriate with the diagnosis and clinical condition of the patient. Where routine haematology analysers use monoclonal antibodies and flow cytometry methods for the measurement of some cells such as platelets labelled with anti-CD61 and lymphocyte subsets with anti-CD4 and anti-CD8, the results should be compared with the results from a validated flow cytometer using guidelines for performing $CD4^+$ T cell populations [42] or the current routine laboratory flow cytometric method for the measurement of $CD4^+$ and $CD8^+$ lymphocyte subsets.

Reference leucocyte differential including NRBC and IG. It is recommended that the reference 2×200-cell leucocyte differential [3], including NRBC and IG counts, is performed by two experienced examiners on all samples and a third person if the first two results disagree. Different automated counting techniques deal with abnormal cells differently; some analysers now enumerate NRBC and IG where previously their presence was indicated only by an abnormal cell flag. Blast cells are indicated by a flag and should be correlated with morphological findings. A similar

approach should be used for the red cell fragment flag or research count available on some instruments. For a full evaluation or for manufacturers, a flow cytometric differential will in future be recommended [2]. The use of digital imaging systems for leucocytes may be used instead of the microscope, but these will have to be evaluated/validated before introduction to the evaluating or routine laboratory. This should be performed using the 2 × 200-cell manual leucocyte differential or the flow cytometric method using both normal and abnormal samples and include morphological abnormalities of all cell lines.

Digital-image-based haematology systems. Digital imaging systems have a long history, starting in the late 1960s and the early 1970s [43, 44]. These systems performed a five-part WBC differential and morphologic examination on wedge smears. The cost of the systems, continued maintenance, system performance limitations (based on wedge smears, cell distribution on the slide and staining consistency) followed by the availability of three-part and then five-part automated differentials on routine haematology cell counting analysers contributed to the demise of these original systems.

With the improvement of computer systems, sophisticated processing and graphics software and the advent of artificial neural networks, image-based systems have returned to routine haematology laboratories. The systems available are two types:

- Cell locator systems with preclassification of cells for verification of cells by a skilled operator.
- Image-based haematology systems that perform a CBC, five-part normal differential and, in some instruments, a reticulocyte count. If any abnormal cells are detected, the sample is flagged by the instrument for further review by a skilled operator.

Digital cell locator/preclassify systems. Currently two digital cell locator/preclassify systems are cleared by the FDA as a 'Cell Locator with preclassification of cells for verification by skilled operator' with final classification of all cells (normal and abnormal) performed by the operator. Other systems are available outside of the United States.

The digital cell locator/preclassify systems are highly dependent on the quality of the blood films and the stain quality for accurate identification of cells. Manually made blood films must have an adequate feather edge to find cells. Automated blood films are usually more consistently made and stained. The systems are designed to work with different staining protocols, and the manufacturers may recommend specific settings or provide an instrument protocol to assess the individual laboratory's current slide making/staining protocol.

Quality control on these systems allows the laboratory to assess stain and smear quality within day or day-to-day using in-laboratory prepared blood films as IQC slides.

Evaluation of these digital cell locator/preclassify systems needs to encompass performance characteristics such as reproducibility/precision, accuracy, comparability and clinical sensitivity and specificity. The CLSI H20-A2 [3] is considered the reference method for the cell locator/preclassify system if a previously cleared cell locator/preclassify system is available, that can be used instead of a protocol based on H20-A2 [3].

Because blood samples are not aspirated by the cell locator/preclassify systems, carry-over, linearity and LoB/detection/quantification are not applicable to these systems. Sample stability also does not strictly apply in the cell locator/preclassify system even though sample stability is important to determine whether cells can be identified in older samples. Interferences with cell locator/preclassify systems are minimal (rouleaux, RBC agglutination and platelet clumps) as these typical interferences can be visually seen if present.

Individual laboratory reference ranges established for manual or automated differentials should be verified with the installation of any system.

Digital-image-based CBC/differential/reticulocyte haematology analysers. For the image-based systems that perform a CBC and identify normal WBCs and reticulocytes, a full evaluation of all performance characteristics must be performed as they are applicable to these instruments. (Currently no system is FDA cleared at this time). See Table 2 for a comparison of performance characteristic testing that should be examined for each type of digital imaging system.

Testing such as carry-over, linearity and LoD/quantitation may have to be modified from the more common methods to accommodate differences in the image-based systems *vs.* 'traditional' impedance or optical methods used my most haematology analysers.

Carry-over assessment could require the use of modified samples (such as a serum blank or manufactured sample with low RBC count and depleted of WBCs and platelets) as the 'low' sample analysed after 'high' samples to detect carryover in WBCs, RBCs, haemoglobin and platelets with some of these systems. Eliminating WBCs and platelets from a low sample could be difficult to achieve [45]. It is important that consideration is given to carryover of abnormal cells.

Interfering substances that impact impedance or optical-based haematology analysers (lipemia, bilirubinemia, paraproteins, etc., see Table 1) will not impact image-based determination of CBC/Diff/retic parameters. Extreme situations for rouleaux or cold agglutinins/RBC agglutination may affect CBC/Diff/Retic results requiring the preanalytical treatment of these types of samples. Morphologic abnormalities (as outlined in Table 1) should be included in the evaluation. Both the image-based systems' haematology analysers and cell/preclassify systems include recognition or counting of these abnormalities in the analyser software.

Flow cytometric immunophenotypic counting methods. An alternative method to the manual microscopic method of leucocyte counting, expected to improve precision and accuracy, is a flow cytometric method using specific monoclonal antibodies to different haematolymphoid lineage markers of leucocytes [2]. This improves precision as many more cells are counted, and additional cells not identified microscopically can be further classified. Any subjectivity in classifying cells microscopically is markedly decreased. A preliminary study has been performed [2] using three different six colour combinations of monoclonal antibodies and fluorochromes. This preliminary study performed under the auspices of ICSH indicated that an eight or more colour panel was required to achieve the desired specifications for a new leucocyte differential method. The specification for the immunophenotypic method is that with a single analysis clear identification can be afforded to lymphocytes (defining various subsets), monocytes (activated, resting and immature), granulocytes (mature and immature), plasma cells and account for 'blast' cells and nucleated red cells. The final proposed reference method for leucocyte counting, intended as a more robust replacement of the CLSI H20-A2 guideline, currently is under further evaluation and validation by an ICSH working group using the most recent ICSH/ICCS Guidelines for Validation of Cell Based Fluorescent Assays [46]. The antibody combinations used in the study are summarized in Table 3.

Abnormal cell flags. As well as cell counts, the morphology of the cells needs to be assessed to

Table 2. Performance characteristic testing comparison for image-based haematology systems

Performance validation testing	Digital image cell locator	Digital image CBC/Diff/Retic
Precision/repeatability	YES	YES
Within run	YES	YES
Between run (day to day)	YES	YES
Between techs	YES	YES
Accuracy	YES	YES (Reference H20-A2)
Comparability	YES	YES (compare to H20-A2 and predicate haematology analyser)
Sensitivity/Specificity	YES	YES
Sample stability	NO	YES
Carryover	NO	YES/NO (if cartridge system)
Linearity	NO	YES
Limit of blank (LoB)/Limit of detection (LoD)/Limit of quantitation (LoQ)	NO	YES – May be modified or adapted to analyser methodology
Reference intervals	NO	YES

CBC, complete blood count.

compare the efficiency of the suspect abnormal cell flags generated by the instrument. Most instruments will generate flags in the presence of abnormal white cells, red cells and platelets. In other circumstances, a flag may be generated to indicate that the instrument results are unreliable or corrupted, and should not be used. The ideal is that the instrument identifies abnormal cells for further study in the laboratory. The sensitivity, specificity, positive predictive value, negative predictive value and overall efficiency should be calculated for each individual flag that relates to the morphology or presence of abnormal cells according to Galen and Gambino [47]. Blood films need to be examined on all samples. Sensitivity is the ability to produce positive results in the presence of abnormality and specificity, the ability of the instrument to show negative results when an abnormality is not present. The method for the classification of results is shown in Table 4.

Quality assurance. It may be useful to register the instrument into an accredited external quality assurance (EQA) scheme that will give an indication of the instrument's performance in comparison with others. Partially or fully stabilized blood is usually used for EQA schemes (CAP, UK NEQAS or others) and may not perform in the same way as fresh blood. Results are often instrument specific, especially for leucocyte differentials, platelet counts and reticulocyte counts. Instrument groups from multiple laboratories are compared against each other rather than across all groups of instruments. Target values and acceptable limits are calculated and returned to the participating laboratory. All reportable results should have an assigned value for internal quality control (stabilized blood supplied by the manufacturer). This is currently not true for some parameters on some instruments. This brings into question whether these parameters should be used for clinical decision-making, at least in the minds of laboratory regulator. Ideally, this is true for EQA but this is not always possible for instrument-specific or new parameters. It should be noted that for some extended parameters and even some established parameters such as NRBC, IG, mean platelet volume, immature platelet fraction, immature reticulocyte fraction and red cell distribution width, there is no EQA scheme available in many countries [20, 45].

Local validation/transference of a haematology analyser. In all cases, the performance of a new system should be compared with the current in-house system using a range of samples designed to test for sensitivity, specificity, reproducibility and results robustness.

Table 3. Monoclonal antibody combination protocols, along with DNA dye Syto16, protocols under evaluation by ICSH study group as candidate method for immunophenotypic leucocyte differential reference method

Antibody specificity	Fluorochrome
CD16	Pacific Blue
CD45	Krome Orange
Syto16	FITC
CD7	PE
CD123	PerCP-Cy5.5
CD14	PE-Cy7
$CD3^+$	APC
CD11b	APC-A750

Table 4. Calculations for the assessment of efficiency of morphologic classification of cells (a) Definition of sensitivity, specificity, predictive value and overall efficiency and (b) Definition of true/false positive/negative

Efficiency measure	Calculation
(a)	
Sensitivity (positivity in abnormal samples)	$\frac{TP}{TP + FN} \times 100$
Specificity (negativity in normal samples)	$\frac{TN}{TN + FP} \times 100$
Predictive value of a positive result	$\frac{TP}{TP + FP} \times 100$
Predictive value of a negative result	$\frac{TN}{TN + FN} \times 100$
Overall efficiency	$\frac{TP + TN}{TP + FP + TN + FN} \times 100$

	Results of test method	
Reference method	Positive (abnormal)	Negative (normal)
(b)		
Positive (abnormal)	TP (true positive)	FN (false negative)
Negative (normal)	FP (false positive)	TN (true negative)

If a complete evaluation, as described in this guideline, has not been performed, a literature search should be undertaken to find independent peer-reviewed evaluations of the equipment. Validation or transference may be defined as establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce results meeting its predetermined specifications and quality attributes. Instrument precision, comparability to reference methods, linearity, carry-over and drift should have been assessed during any formal national evaluation, but still need a brief validation and reference range study. In general, the routine laboratory should perform an abbreviated validation based on the evaluation described above in this document. It is necessary for the laboratory to determine whether or not the reference ranges are the same as with existing instruments, at least 120. If the laboratory tests neonatal or paediatric samples, these should also be examined separately. If paediatric samples are tested in open mode, this should also be assessed. On many analysers, capillary blood can be analysed using diluted blood in the analyser-specific diluent. Dilution of blood to diluent will vary, but usually the instrument will calculate the whole blood results if run in capillary mode. If this is used in the routine laboratory, then this should be assessed with at least 30 samples compared with the whole blood mode.

Before the start of any evaluation, it is important to ensure that the instrument is compatible with the laboratory and the service for which it is intended. The evaluator should obtain the following information: – name, manufacturer and distributor of the instrument, list price including options for rental or leasing, reagent and consumable costs, and terms of service contracts. The overall dimensions of the instrument, power requirements, drainage, operational environment (temperature range) and heat produced by the instrument should be investigated. Information on consumables (reagents, controls and calibrators), formulation and shelf life of reagents, quantity and number of tests possible, and storage requirements would be part of any complete instrument assessment. The ability of the instrument to be interfaced with the laboratory/hospital information system should also be understood at this stage.

The repertoire of parameters available, measurement principles, minimum sample volume, data presentation and compatibility with other systems should also be considered. New instruments should generate results that are comparable both in the units reported and reference ranges to those already in place in the laboratory including other instruments in the laboratory and point-of-care (POC) instruments within the same institution. The instrument should be able to process those sample tubes most commonly received by the laboratory.

A plan, with a realistic time-scale should include the time to obtain the relevant specimens, maintain records, perform analysis of results and statistical process to be used, and write a final report. This is particularly important when the instrument is loaned or leased. The quantities of reagents and consumables required for the evaluation must be estimated. Arrangements must be in place for service and maintenance of the instrument during the evaluation period and, if training is available, this must take place before the evaluation begins.

A signed statement from the manufacturer or supplier that the instrument installed has undergone testing and calibration and are all within predetermined limits. Appropriate grades of staff should be selected to complete the evaluation and their availability ensured. Staff with skills similar to those of the potential users of the equipment should be used for the evaluation.

The purchaser should perform a local assessment of the instrument and validation of the results. Preliminary information regarding the instrument installation, training and suitable blood samples is required and sought from the manufacturer before planning the technical evaluation. A written protocol should be established that specifies how validation will be conducted. Firstly, the laboratory should establish that performance specifications are the same, or better, as those demonstrated by the manufacturer and on par with previous published reports. Comparability of results, reference ranges, reliability and acceptability of the instrument are also considerations in a local evaluation and should be analysed as for a national evaluation. Fewer samples are needed to validate compared with the full evaluation but at least 50 should be used for comparability and reference ranges.

In the future, more core/centralized laboratories will be implemented with samples travelling some distances and over time for testing, up to 48 h. The

results for these samples need to be validated to ensure that results given to clinicians are correct and clinically safe.

Some guideline documents, such as the Clinical Laboratory Standard Institute (CLSI) H20-A2: Reference Leukocyte (WBC) Differential, state that at least 200 samples should be included in the study (1/2 normal, 1/2 abnormal) [3, 19, 45]. As a minimum, good laboratory practice should dictate that at least 50–100 samples (1/3–1/2 normal and the remainder abnormal) should be compared between the manual differential and the automated differential.

Efficiency

Throughput. The throughput of samples per hour, including control material, should be recorded, taking into consideration any tests that require more time than the standard CBC and leucocyte differential, for example, reticulocyte counts, and on some analysers, there is a reflex option to another channel, extended counting or different method for abnormal samples The time needed for start-up, shutdown and routine maintenance of the instrument should also be determined. The number of samples that need repeating, for any reason, should also be documented.

Sample identification. Sample identification through the use of a barcode reader is now almost universal and offers significant advantages for laboratories, but the manual input of patient identification and tests should be available for flexibility. If barcodes are used for sample identification during the evaluation, barcode read errors by the instrument should also be assessed and documented as they will also impact instrument efficiency. Information should be sought regarding information technology (IT) options, availability of middleware (software communication from the analyser to the laboratory information system) and expert rule-based systems for autovalidation of results or automatic ordering of blood films [48].

IT, presentation and storage of results. The compatibility of the instrument with the laboratory and hospital information systems, particularly bidirectional interfacing, should be established. The quality, format of data presentation and graphics, including the display of quality control results, should be assessed, together with the process of result validation, data storage capacity, speed of retrieval of results and the quality control programs available.

Training. The quality of training given by the manufacturer should be described, as well as the ease of use and clarity of the operator's manual. Laboratories should be on the manufacturer's contact list for any laboratory upgrades, recall notifications or technical updates.

Reliability. The length of time that an instrument was unusable due to breakdown should be recorded as well as the response time for the manufacturer's repair.

Cost. The cost per test should be determined, including costs for controls and the staff time needed to maintain and operate the instrument and process samples to final interpretation.

Acceptability. Staff opinions and preferences should be taken into consideration. An assessment of the level of expertise required for the operation of the instrument should be examined, as well as the impact of the instrument on the workflow and organization of the laboratory. Any modifications to the laboratory design should be considered.

Record keeping of the evaluation should be performed as previously described in these guidelines. The manufacturer's claims for within- and between-batch precision should be confirmed. The suitability of equipment and comparability with current methods must be studied using samples including abnormal samples and samples with interfering substances as possible. The number of abnormal cell flags generated and any failure of the analyser to provide a result should be documented and compared with existing methods. Precision, comparability of results, reference ranges, reliability and acceptability of the instrument are also considerations in a local evaluation and should be analysed as for a national evaluation.

Once the equipment has been established within a few months, an audit should be undertaken to ensure that all manufacturers' claims are being met and that parameters being reported are those needed by the laboratory as well as any problems with middleware or the laboratory information system.

Special considerations for the evaluation of POC testing analysers. Point-of-care testing (POCT) can be defined as any analytical test performed for a patient by a healthcare worker outside the laboratory environment [49, 50]. POCT is designed to move laboratory testing closer to the patient providing a more rapid service than can be achieved from the central laboratory. POCT devices can vary from hand-held devices, designed to be used at the patient bedside, intensive care units or in operating theatres, to small bench top analysers, but all should generate results with reference ranges that are comparable with those of the main haematology analysers. Portable haemoglobinometers, some of which now measure WBC and platelets, which use capillary blood and blood gas analysers that test for haemoglobin alone, should also be evaluated in the same way.

Ideally, where several POCT instruments are required at different sites within a single institution, only one instrument type should be selected so that results and reference ranges are the same wherever a patient is tested. This also simplifies training, ordering and storage of reagents, as well as servicing and maintenance contracts.

Three extensive reviews of POCT validation have been previously published [49–51].

The performance of the device should be tested in the environment where the instrument will be sited and, importantly, by the staff that will be operating the instrument, so as to ensure that ease of operation by nonlaboratory staff can be confirmed. It has been previously demonstrated that experiences of skilled *vs.* unskilled users can be different, and usually, the quality of testing by the POCT user is poorer than that by experienced laboratory staff [52].

CONFLICT OF INTEREST

Carol Briggs: The department has received an unrestricted grant from Sysmex Europe. Naomi Culp: Employment by Trillium Diagnostics, LLC; consult to Beckman Coulter, Horiba Medical, and Abbott Diagnostics. Bruce Davis: Employment by Trillium Diagnostics, LLC; consult to Beckman Coulter, Horiba Medical, Abbott Diagnostics, and BD Biosciences. Giuseppe d'Onofrio, Gina Zina: None. Samual J Machin: The department has received an unrestricted grant from Sysmex Europe. *Contributors.* Sue Mead: Siemens; Nigel Llwelyn Smith: Abbott; Elena Sukhacheva: Beckman Coulter; Jolanta Kunickaj: Sysmex; Yutaka Nagai: Nihon Khoden.

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