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QUALITY ASSURANCE IN HAEMATOLOGY

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Quality assurance is essential in haematology to ensure that laboratory tests are carried out reliably. The various aspects of quality assurance which are considered in this document are (1) internal quality control, (2) external quality assessment, (3) standardization of methods and (4) proficiency surveillance. The statistical procedures which are necessary to analyse the data are described in simple terms. Practical exercises are provided in which the blood count by electronic counters as well as by visual haemocytometry, and other fundamental diagnostic tests, are used to illustrate the application of quality control and external quality assessment procedures, and to explain their principles.

There are also exercises on selection of methods, evaluation of instruments, use of standards and how to check the reliability of routine methods in practice.

There is an introduction to standards of laboratory practice and a final section on methods for production of controls.

The document is intended as a training manual for all laboratory workers in the field of haematology, as well as for laboratory managers and teachers who are planning practical courses and workshops on quality assurance.

CONTENTS

PART 3: STANDARDS OF LABORATORY PRACTICE

PART 4: PREPARATION OF CONTROL MATERIALS

PREFACE

For a clinical laboratory to serve any real purpose (1) the results of tests from that laboratory must be correct, (2) the tests themselves must be relevant for diagnosis and clinical care of patients and for health screening and epidemiological studies, and (3) the laboratory must be efficient, effective and as economical as possible without sacrificing its standards. To achieve these objectives of good laboratory practice requires skilled management with critical supervision of the work of the laboratory which must include a quality assurance programme.

Quality assurance is concerned with all aspects of laboratory practice. Specific activities include internal quality control, external quality assessment, proficiency surveillance and standardization. These are defined and their principles described in Part 1.

Internal quality control (IQC) is concerned essentially with precision or reproducibility of results on a daily basis whilst external quality assessment (EQA) is concerned with interlaboratory and intermethod or interinstrument harmony, i.e. the comparability of results wheresoever the test is performed. EQA can be used to obtain a correct value for an analyte. Whilst quality assurance is concerned with all steps in the process from specimen collection to transmission of the report to the clinician, IQC and EQA test only the analytic procedure itself. But these are essential to ensure that the tests are performed correctly and that their results are reliable.

This document provides an introduction to the principles and methods of quality assurance in haematology together with a series of exercises which illustrate these principles based on the common tests which are performed in the haematology laboratory. The exercises will give the trainees an opportunity to become familiar with the recommended techniques, to achieve precision and accuracy in carrying out the tests and to appreciate the causes of error and how to avoid them. The course of exercises is intended for senior laboratory technicians and scientists, medical officers with some laboratory experience, directors of haematology laboratories and the staff of the central laboratories and institutes who are involved with the development of national quality assessment programmes. All laboratory staff at senior technician level or above should be able to apply the general principles of internal quality control in their work, interpret quality control charts and carry out tests on material received in an External Quality Assessment Scheme. In every laboratory at least one senior technician (technologist) should be able to prepare various quality control materials. Staff of laboratories directly involved in organizing EQA schemes also need to know how to prepare the quality control materials as well as how to dispatch specimens, process data statistically and report results in such schemes. The directors of central laboratories, responsible for the organization of national quality assurance programmes, should be familiar with all aspects of the subject.

Over the years there has been good collaboration between the World Health Organization and the International Council for Standardization in Haematology for developing standards which appertain to haematology. These include standardization of methods and the use of reference material standards. The role and the use of standards in the laboratory is illustrated in the training course exercises which are included in this manual.

The manual is divided into four parts. Part 1 deals with the principles and methods of Quality Assurance; it contains an explanation of the basic statistical procedures that are required for IQC and EQA, together with simple exercises which demonstrate their use. Part 2 consists of the training course manual and practical workbook, Part 3 is a brief introduction to standards of laboratory practice including a protocol for standard operating procedures and Part 4 provides detailed instructions for the preparation and calibration of control materials.

This document is a revision of WHO/LBS/92.4 in the light of practical experience gained from WHO training courses, and expanded to take account of development in laboratory practice, especially the increasing use of automated blood cell counters. But it is recognized that manual methods continue to be used in many laboratories and the principles of quality assurance apply equally to all laboratories.

Test procedures are not described as they are to be found in various practical textbooks. Selection and maintenance of instruments, safety standards for staff and equipment and other aspects of laboratory organization which are encompassed by proficiency surveillance as described in Part 1 are dealt with in a number of WHO documents. Details of publications to which readers are referred are given in Appendix 1.

PART 1: PRINCIPLES AND METHODS OF QUALITY ASSURANCE

1. INTRODUCTION

It is the responsibility of the person in charge of the haematology laboratory to ensure that the tests which are performed are relevant and that the results are reliable, reproducible and as accurate as possible according to the present state of the art. The results must be presented, without undue delay, to the clinician or public health worker who has requested the tests, in a report which is legible and readily understood. The aim is to ensure that they serve their intended purpose effectively, whether it be to provide help for a clinical diagnosis and for management of a patient, or for health care of a population at large.

It is obvious that the tests should be carried out as soon as possible after the specimens have reached the laboratory, and performed in accordance with a standard operating procedure, including the use of appropriate controls and reference materials to be assured of their technical reliability. But it must be remembered that there may be variables which are not always under control of the laboratory but which might significantly influence the test results. These include the effects on the blood count and on other blood components of exercise and mental stress just before blood is collected, the position of the patient (standing, sitting, lying in bed), a tourniquet left on the arm for too long before venepuncture, collection of capillary blood as compared to venous blood. The blood count, coagulation tests and certain chemical tests will be affected by incorrect anticoagulant, or by excess or deficient concentration of anticoagulant, by inadequate mixing of the specimens with the anticoagulant, by delays in sending the specimens to the laboratory and leaving them in an unfavourable environment such as room temperature in a hot climate, and, even worse, in direct sunlight. It is also important to ensure that the specimen containers do not leak, not only because contamination of the laboratory (and ward) staff is a potential health hazard, but also because a container without a secure cap is likely to disturb the constituents and their relationship, by evaporation of plasma and by leakage. The laboratory director must maintain close contact with the wards and staff who undertake specimen collection and also with the suppliers to ensure that these many factors are taken into account before a specimen actually arrives at the laboratory.

To achieve the necessary level of good laboratory practice and to be reassured that this level is constantly maintained (i.e. quality assurance), it is necessary to undertake a programme of quality management.

There are four separate aspects of such a programme, namely, internal quality control, external quality assessment, pre-analytic and post-analytic control and standardization.

The following glossary defines and explains these concepts. It is based on the definitions recommended by the International Standards Organization (ISO), and endorsed by WHO and the International Council for Standardization Haematology (ICSH):

Standards

Reference material

A material or substance with values of measurable quantities sufficiently homogeneous and well established to be used for calibration of an apparatus, the assessment of measurement procedures or for assigning values to materials. (VIM 1994)

International Biological Standards

Reference standards established by the World Health Organization which cannot be determined by physical or chemical measurement but are expressed as units of activity. These materials are not intended to be used in the laboratory working procedures but serve as the means by which national and commerical reference materials and calibrators can be controlled.

Certified reference material

Reference material, accompanied by a certificate one or more of its property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which the certified value is accompanied by an uncertainty at a stated level of confidence. (VIM 1994)

Materials and methods

Reference method

Thoroughly investigated method, clearly and exactly describing the necessary conditions and procedures, for the measurement of one or more property values that has been shown to have accuracy and precision commensurate with its intended use, and that can therefore be used to assess the accuracy of other methods for the same measurement, particularly in permitting the characterization of a reference method. (ISO 1992)

Calibrator

A reference material for calibration. (EN 375)

Accuracy of measurement

The closeness of agreement between a test result of a measurement and a true value of the measurement. (VIM 1994)

Bias

Systematic error of the indicator of a measuring instrument. (VIM 1994)

Precision

The closeness of agreement between independent test results obtained under prescribed conditions. (ISO 1993)

True value (of quantity)

Value consistent with the definition of a particular given quantity. (VIM 1994)

Assigned value (conventional true value)

The value attributed to a particular quantity and accepted, sometimes by conversion, as having an uncertainty appropriate for a given purpose. (VIM 1994)

Quality control material

A substance used in routine practice for checking the concurrent performance of an analytical process (or instrument). It must be similar in properties to and be analyzed along with patient specimens. It may have an assigned value, but this is intended only as an approximation and not for purpose of calibration.

Specimen

Material available for analysis. A representative part of a specimen which is used in the analysis is a sample. A portion of the sample on which the analysis is actually carried out (e.g. after dilution) is a subsample.

Specificity (analytical)

The ability of an analytical method to determine solely the component(s) it purports to measure.

Sensitivity (analytical)

Change in the response of a measuring instrument divided by the corresponding change in the structure. (VIM 1994)

Quality management

Quality assurance programme

The sum total of a laboratory's activities aimed at achieving the required standard of analysis. While ICQ and proficiency testing are very important components, a quality assurance programme must also include staff training, administrative procedures, management structure, etc. Accreditation bodies judge laboratories on the basis of their quality assurance programme.

Internal quality control

Internal quality control comprises all steps of activity from assessing clinical needs, via collection of sample and measurement of a measurable quantity to reporting of results of measurement. (preEN 1992)

External quality assessment

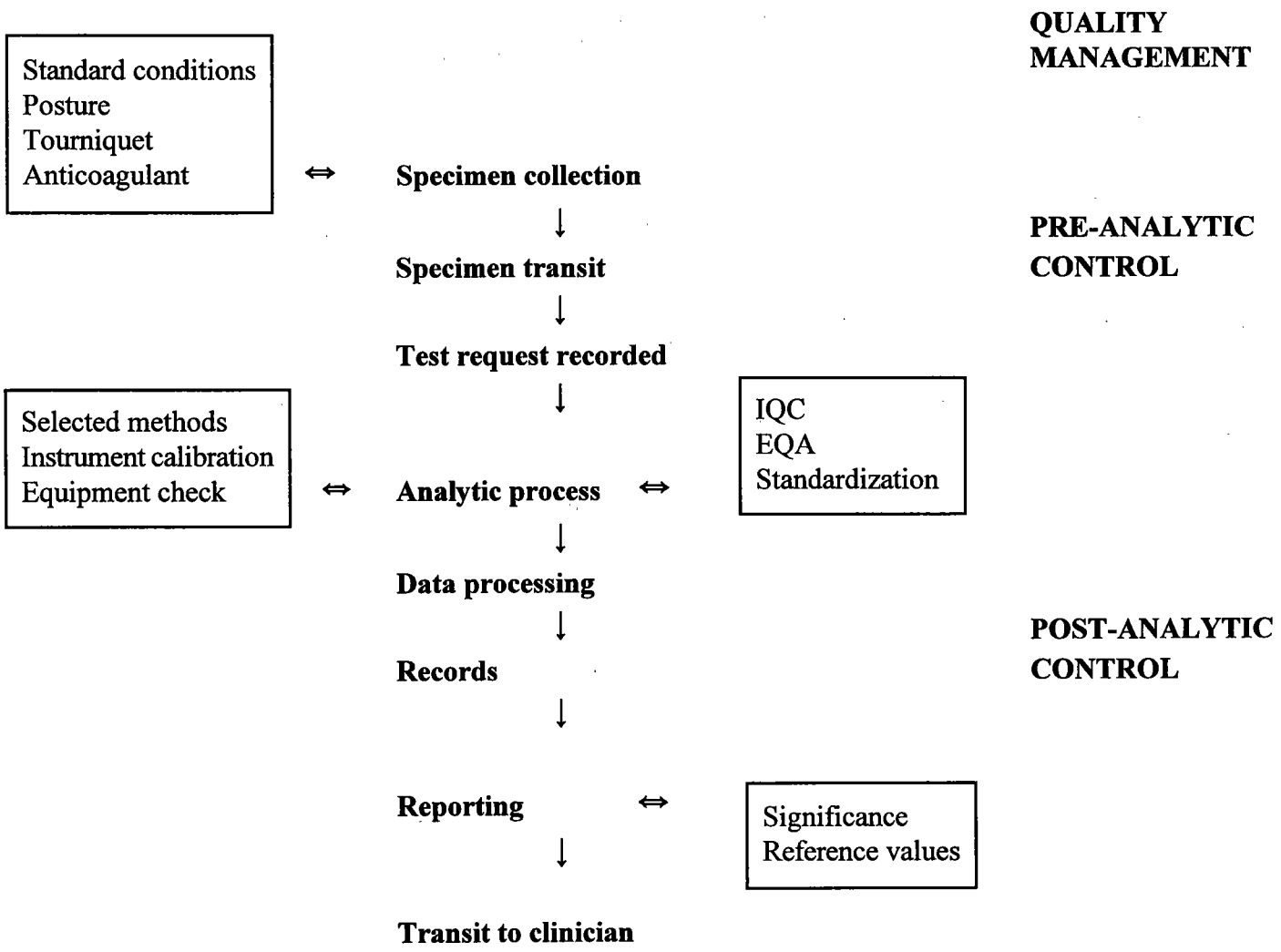
The assessment of results from measurements produced at a certain site by comparing the results obtained by other sites or the same material distributed by an external agency which also analyses the data statistically. (draft preEn 1992)

Audit

A systematic study to determine if the laboratory maintains a satisfactory level of good practice with effective application of quality-related issues in accordance with any regulatory requirements.

The inter-relationship of the various components of quality management as defined above, is illustrated in Fig. 1.

Fig. 1


QUALITY MANAGEMENT
Standard conditions
Posture
Tourniquet
Anticoagulant
Specimen collection
PRE-ANALYTIC CONTROL
Specimen transit
Test request recorded
Selected methods
Instrument calibration
Equipment check
Analytic process
IQC
EQA
Standardization
Data processing
POST-ANALYTIC CONTROL
Records
Reporting
Significance
Reference values
Transit to clinician

2. QUALITY ASSURANCE PROGRAMME

Every laboratory **MUST** undertake a programme of quality assurance. Every type of test undertaken by the laboratory must be subjected to some form of quality assurance, including internal quality control and participation in EQA surveys when available.

Internal quality control

The procedures which should be included in a programme in any individual laboratory will vary with which tests are undertaken, the equipment (especially if this includes a fully automated counting system), the size of the laboratory and level of training of its staff and the number of specimens handled each day. At least some of the following programme must be carried out.

At all times

Correlation system:

Cumulative report forms
Correlation of blood film appearances with blood count
Correlation of blood count changes with clinical events

Daily

Tests on control specimens:

Control sample with each batch of specimens
Control chart from results on the control samples
Duplicate measurements on a few of the patients' specimens - usually 3-4 from a batch.
Check test on a few patients' specimens from a previous batch (usually 3-4).
Check for significance of differences between results when tests are repeated (at intervals) on a patient. This is known as "delta check".

Calculation of daily mean value for selected parameters

If by automated counters - means of MCV, MCH, MCHC
If by manual methods - mean of MCHC

At intervals (daily or weekly)

Calibration check of blood cell counters, and haemoglobinometers

HAEMOGLOBIN	- haemiglobincyanide reference preparation
	- lysate
RBC	- preserved blood
	- stabilized blood

WBC	- stabilized human blood
	- stabilized avian blood (as surrogate leukocytes)
PLATELETS	- stabilized human platelet preparation

At intervals (initially and again when indicated)

Calibration of pipettes and automatic dispensers.

At intervals (initially, repeated if indicated and at least six-monthly)

Calibration graphs of photometers and spectrophotometers for specific analyses, e.g. haemoglobin by haemiglobincyanide method.

Laboratories vary in the way in which they are organized, the facilities which are available, the range of tests which they are able to undertake and their workload; they also vary in the number of staff and in their levels of training. It is thus not possible to give a rigid protocol for a quality assurance programme with procedures which must be carried out, nor even to indicate which procedures are more important than others. Each laboratory should establish a programme, as determined by local circumstances, with procedures selected from the above list. In addition there must be critical supervision of all aspects of the laboratory service, including specimen collection, labelling, delivery and storage before the tests are performed, efficiency of recording and reporting of results, maintenance and control of equipment and apparatus, staff training, protection of laboratory staff against health risks and hazards when handling specimens and equipment. This requires an understanding of the principles of good management as well as technical knowledge of the test procedures and an understanding of the likely causes of inaccuracy and imprecision in the test results. Laboratories must use reference values which are valid for their test methods and their local population, so that it can be appreciated when a result on an individual is significantly abnormal.

External quality assessment (EQA)

This is defined (see p.11) as the objective and retrospective evaluation by an external authority of performance by a number of laboratories on material which is supplied specially for this purpose. The objective is to achieve comparability; possibly also accuracy if the material supplied for the tests has been assayed by one or more reference laboratories, using methods of known precision, alongside a reference preparation of known value.

Most laboratories would expect to be participants in a scheme that is organized nationally by a central laboratory. However, staff working in rural or district hospital laboratories may also be required to organize and supervise a limited scheme for external quality assessment of any tests performed at health centres. Similarly, the staff of regional laboratories might be expected to provide a service for rural hospital laboratories. Accordingly, instructions for providing an external quality assessment scheme on a limited scale are also included.

It must be remembered that although IQC and EQA are very important, they relate only to one aspect of laboratory function. As illustrated in Fig. 1, surveillance of the pre-analytic and post-analytic phases is equally important. The supervisory role of the laboratory director in this is indicated in the next section.

3. ROLE OF LABORATORY DIRECTOR OR SUPERVISOR

Some of these functions may be delegated to other members of the laboratory staff, but the Director must assume ultimate responsibility.

3.1 General

Prepare standard operating procedures (SOP) for all aspects of laboratory function
Ensure that test procedures are up to date with detailed written instructions for bench-staff
Organize training programme for staff
Staff development and performance appraisal
Monitor workload
Establish clear lines of accountability and communication within the laboratory
Ensure adequate communication with clinicians and educate clinical staff
on how to make best use of the services of the laboratory
Inspect quality control and external quality assessment data
Check efficiency and effectiveness in specimen processing and reporting
Maintain inventory
Control budget and costs
Establish normal reference values for various tests
Laboratory safety with regard to choice of technique, handling of material and equipment, and waste disposal
Ensure adequate record keeping
Ensure compliance with professional and government regulations
Undertake regular audit of laboratory functions and performance

3.2 Checks for elucidation of causes of error

Patient preparation and specimen collection
Specimen container and anticoagulant
Transit time and environment at temperature
Patient or specimen mix-up
Information on request form
Prompt separation of plasma/sera (when required) and storage conditions before test
Unrepresentative sampling
Faulty pipettes and pipetting errors
Instrument faults
Incorrect instrument setting
Inadequate use of reference and control preparation
Lack of quality control system
Errors of calculation
Reporting and recording errors
Inaccurate or inappropriate dilution
Incorrect method (e.g. wrong diluent, inadequate reaction)
Wrong interpretation of observation

3.3 Equipment and test selection

Equipment maintenance
Policy on equipment replacement
Evaluation of kits
Evaluation of a test method
Evaluation of instruments and check of manufacturer's claims
Selection of appropriate tests and instruments
Check of colorimeters, cell-counters and other apparatus in use
Calibration of pipettes

4. CONTROL MATERIALS

In part 3 technical details are given for the production of materials which are suitable for use in quality control of blood counts. As the blood count constitutes the main work of most "routine" haematology laboratories, it is of considerable importance to have these preparations available as they can be used for a number of different purposes. Human or equine (i.e. donkey or horse) blood is used for some of the procedures for checking precision in internal quality control. The same material is suitable for use in external quality assessment and also as a calibrator.

When used as a control to check the precision of a test, it is not necessary to know the true concentration of the substance to be measured. But when the material is intended for use as a calibrator, it must have an assigned value; to obtain this value the test should be carried out by a reference method, and checked with an International Biological Standard, if one has been established. The measurements are made only after the preparation has been dispensed as aliquots in small (e.g. 2-5 mL) volumes in vials. The measurement must be made on at least 15 vials taken at random from the batch, and the results recorded as mean ($\bar{x}$) and standard deviation (SD). Intrabatch variation is expressed as CV%,

i.e. $\left(\frac{SD}{\bar{x}}\right) \times 100$. It should be within the limits stated in the methods for measurement described below.

As measurement of haemoglobin (Hb) and blood cell counting are the most common tests in routine haematology, it is in these areas that quality control is especially required, and will be described in this document. These procedures apply equally to manual methods and to cell counters and also to other quantitative tests on whole blood, e.g. Hb A_2; and to tests on plasma or serum (e.g. iron, iron binding capacity, vitamin B_{12}, folate). The descriptions given in a later section can be easily adapted for these tests. Obviously, this approach does not apply to qualitative tests such as identification of abnormal haemoglobins and blood film morphology.

5. STATISTICAL PROCEDURES

First of all it is necessary to calculate the most likely average result. There are three ways of doing this: Mean ($\bar{x}$), Median (m) and Mode.

Mean is the sum of all measurements divided by the number of measurements.

Median is the point on the scale at which there is an equal number of observations that are above and below.

Mode is the most frequently occurring result in the set.

Which of these is the most appropriate depends on how the individual measurements have been distributed, as described below.

5.1 Distribution graphs

All measurements obtained in laboratory tests show variation, so that there will always be a scatter of results. The type of scatter can be demonstrated by plotting the measurements against their frequency.

(a) Normal Gaussian curve

In this pattern, the data are distributed symmetrically, typically in a bell-shaped curve (Fig 2a) in which the mean is the midpoint value and this coincides with both median and mode.

(b) Skewed distribution

In this pattern, results are asymmetric (i.e. skewed), with a larger number towards one end, either to the left (positively skewed) or to the right (negatively skewed) as illustrated in Fig. 2b.

(c) Linear distribution

When there is a flat distribution of results with no clear predominance, identifying the central tendency and calculating the median is usually more reliable than the mean. An extreme example of this is illustrated in Fig. 2c which displays the results of haemocytometer chamber counts by a group of inexperienced students. A less obvious situation is that in Fig. 2d, showing the distribution of haemoglobin in a particular population. An attempt to draw a normal (Gaussian) distribution curve demonstrates the irregular distribution, indicating that this is not the correct way to interpret the data.

(d) Log-normal distribution

This occurs when the skewed distribution on an arithmetic graph will be transformed to a Gaussian distribution by plotting the data on a logarithmic scale (i.e. on semi-log graph paper). This is illustrated in Fig. 2e in which the log scale is shown at the top and the broken-line curve has been draw on this scale. The mid-point value then becomes the geometric mean.

Fig. 2a

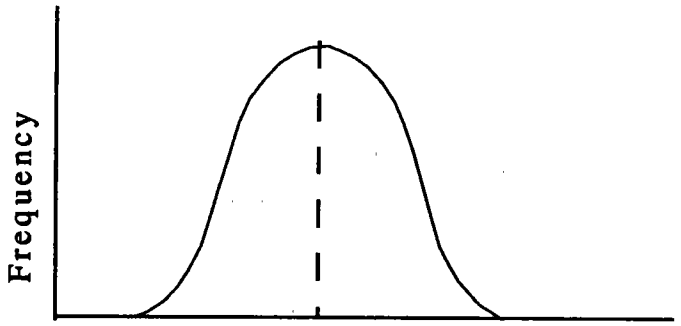


Fig. 2b

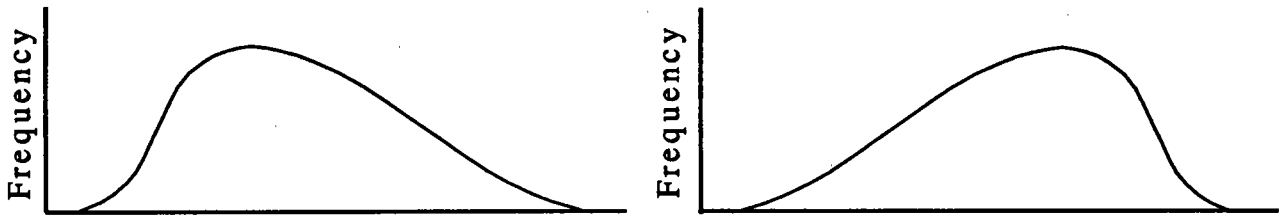


Fig. 2c

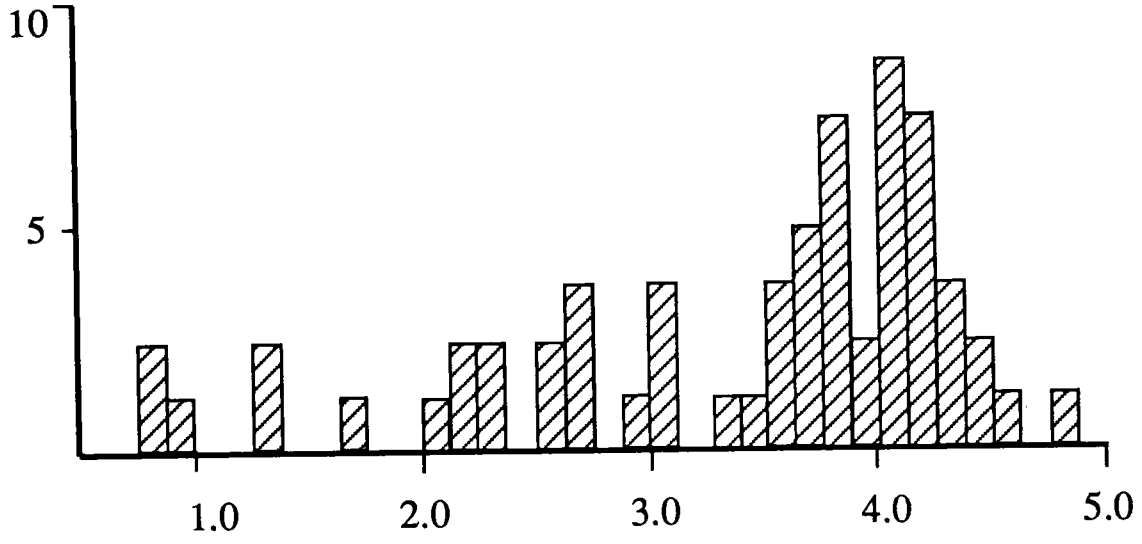


Fig. 2d

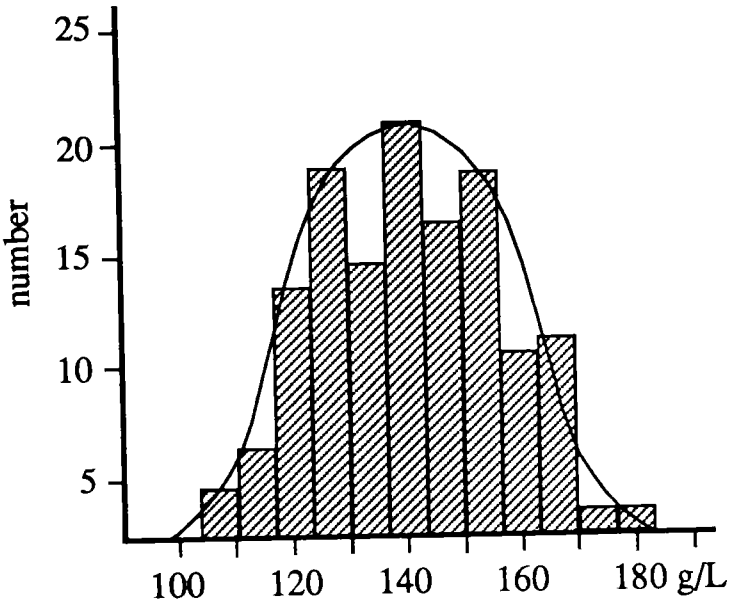
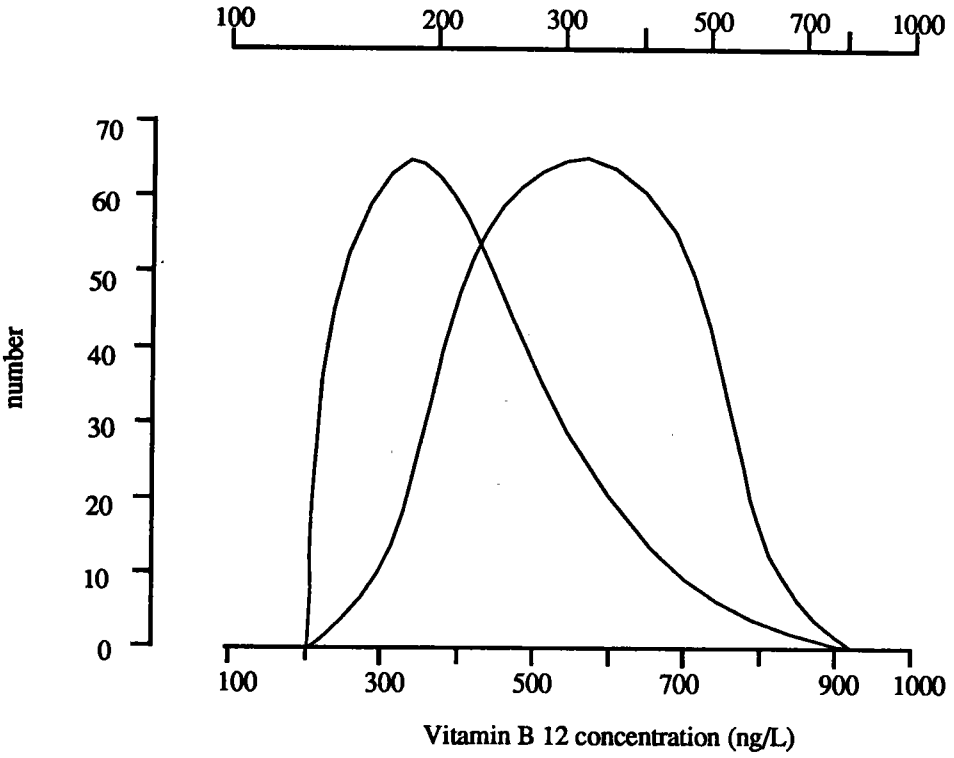


Fig. 2e



5.2 Mean ($\bar{x}$)

If the distribution of results is a normal Gaussian curve the mean is simply the total score of all the measurements divided by the number of measurements. Thus, for example, if the haemoglobin is measured on a specimen 11 times, with the following results:

126 120 118 124 115 110 112 115 122 120 115 (g/L)

then

$126 + 120 + 118 + 124 + 115 + 110 + 112 + 115 + 122 + 120 + 115 = 1297,$
$\bar{x} = 1297 \div 11 = 117.909$

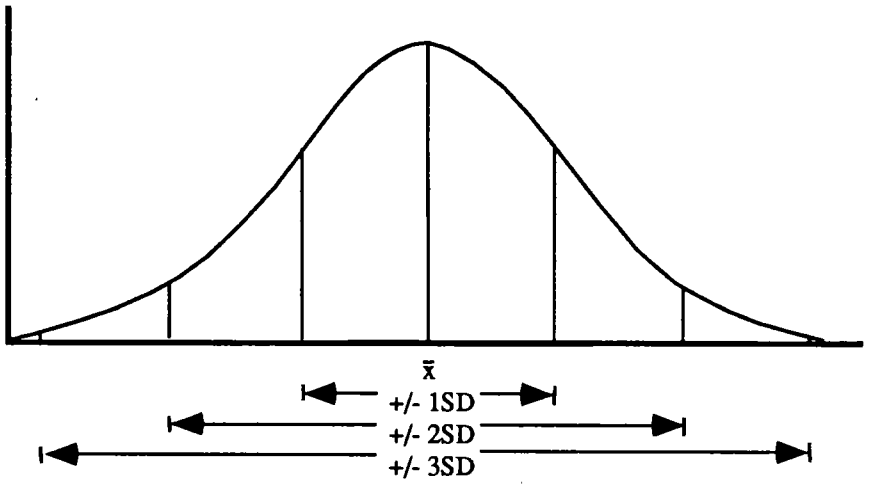
For subsequent statistical calculations retain one figure after the decimal point, e.g. 117.9.

For practical purposes, round up (or down). In this example, record as 118 g/L

5.3 Standard deviation (SD)

To understand the significance of SD in the context of quality control, the way in which the test results are likely to lie above and below the mean must be appreciated. When the results all belong to the same "population" (set) their distribution will be symmetrical and thus expressed as a Gaussian curve (Fig. 3).

Fig 3. Normal Gaussian Distribution Curve illustrating (see below) spread which includes 1SD, 2SD and 3SD



The measure of spread is conveniently expressed by the SD. The area under the centre of the curve is ± 1SD and this equals 69% of the whole area; ± 2SD equals 95%; ± 3SD equals 99.7%.

Calculation of the SD is useful in quality control procedures. If the limits are set at ± 2SD, when 100 measurements are performed, say of haemoglobin, then 95 of the 100 will fall within ± 2SD. This also means that by chance alone 5 out of 100 determinations will be outside the 95% range.

Calculation of standard deviation

This is the square root of variance, where variance (s^2) is calculated from the formula:

$\frac{\Sigma(x - \bar{x})^2}{n - 1}$ where "x" = individual measurement and "$\bar{x}$" = mean, and n = the number of measurements

Thus SD = $\sqrt{s^2}$ or $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$. It may also be expressed as $\sqrt{\frac{\Sigma d^2}{n - 1}}$ as d is sometimes used instead of $(x - \bar{x})$ to describe the differences of each individual result from the mean.

The calculation is readily obtainable by using a calculator which has this function, by means of which the SD may be obtained directly merely by entering the test results. It can, however, also be obtained by direct calculation. The method for this is shown in the following example with Hb:

(a) Carry out a number of consecutive measurements of Hb on a specimen. The usual number of measurements may be 10-20.

(b) Tabulate the measurements in a column (headed "x"). Calculate mean (= $\bar{x}$). In the example given below $\sum x = 3020$; $\bar{x} = \frac{3020}{20} = 151$.

(c) For each x, calculate $x - \bar{x}$ and enter in a second column.

(d) Then calculate $(x - \bar{x})(x - \bar{x})$, i.e. $(x - \bar{x})^2$ and enter this in a third column.

(e) To obtain SD calculate $\sqrt{\frac{(x - \bar{x})^2}{(n - 1)}}$. In the example given below

$\sum(x - \bar{x})^2 = 292$; $n - 1 = 19$.

Thus SD = $\sqrt{\frac{292}{19}} = \sqrt{15.36} = 3.9192$.

Thus, SD = 3.9, or practically 4 g/L.

Test No.	x	$(x - \bar{x})$	$(x - \bar{x})^2$
1	155	+ 4	16
2	148	- 3	9
3	152	+ 1	1
4	147	- 4	16
5	150	- 1	1
6	156	+ 5	25
7	156	+ 5	25
8	157	+ 6	36
9	153	+ 2	4
10	150	- 1	1
11	150	- 1	1
12	147	- 4	16
13	144	- 7	49
14	152	+ 1	1
15	157	+ 6	36
16	152	+ 1	1
17	147	- 4	16
18	152	+ 1	1
19	145	- 6	36
20	150	- 1	1
Totals 20	3020		292

5.4 Coefficient of variation (CV)

This relates the SD to the actual measurement so that measurements at different levels can be compared:

CV (as a percentage) is calculated by $\frac{SD}{mean} \times 100$.

Thus, using the example given above, $CV = \frac{3.9}{151} \times 100 = 2.58\%$.

In another situation, for example, if the mean Hb = 60 g/L and SD = 3.9, then

$CV = \frac{3.9}{60} \times 100 = 6.5\%$; thus, at this level, the test is being performed with a much lower precision.

When the mean Hb is 60 g/L, to obtain a CV of 2.5%, the SD must not exceed 1.5 g/L.

5.5 Median (m)

As described above the median is the point on the scale that has an equal number of observations above and below. It is an alternative to mean for non-parametric data when the distribution is skewed or flat, as the mean may not then give a true picture.

When the number of measurements (n) is even, median will be the measurement that is midway between $\frac{(n)}{2}$ and $\frac{(n+1)}{2}$.

When n is an odd number m will be at the position of $\frac{n+2}{2}$.

Example

The results of measurements are listed in increasing order to obtain the median as follows:

	g/L
1.	110
2.	112
3.	115
4.	115
5.	115
6.	118
7.	120
8.	120
9.	122
10.	124
11.	126

$n = 11$

$$\frac{n + 1}{2} = 6\text{th position}$$

Median (m) = 118 g/L

SD of Median is calculated approximately from the range about the median (central tendency) in which 50% of the results occur (i.e. 25% on either side):

$$SD = \frac{50\% \text{ spread}}{1.35}$$

5.6 Standard error of mean (SEM)

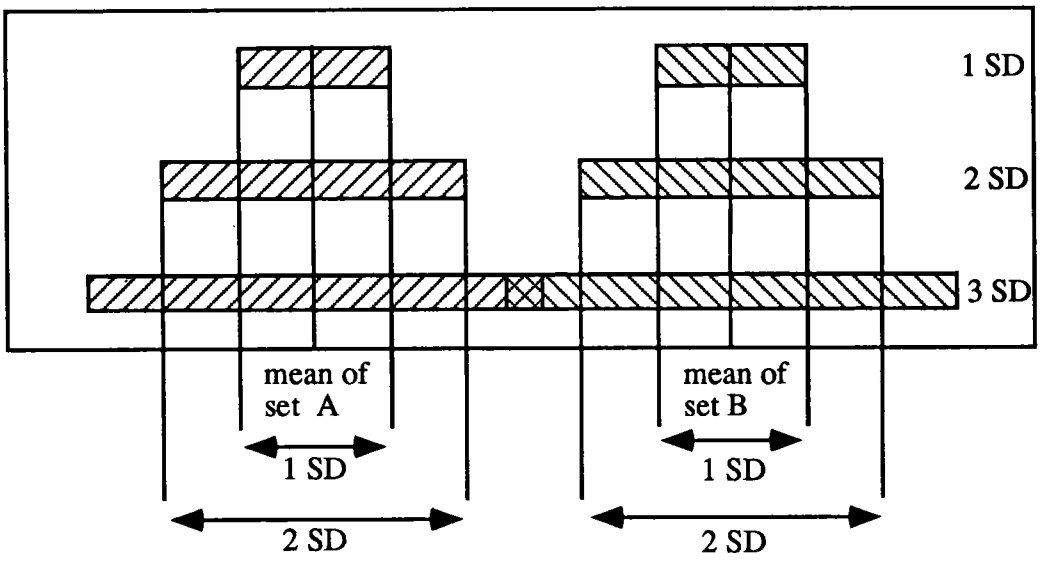
Although the mean is usually calculated and presented as a single measurement, there will be some dispersion around that figure. The extent of this variation is indicated by the SEM which is the SD of the mean. This is calculated by $\frac{SD}{\sqrt{n}}$. Thus, using the above data $SEM = \frac{3.9}{\sqrt{20}} = 0.87$ (i.e. 0.9).

5.7 Differences between means

The simplest method for assessing whether two sets of data are similar to or different from each other is to calculate their means and SDs. If the ranges, i.e. means ± SDs, do not overlap, the sets are significantly different. Thus, for example if the mean and SD are 40 ± 3 and 50 ± 3 respectively, their 1 SD range would be 37-43 and 47-53; clearly separate sets. Conversely, if their values were 40 ± 10 and 50 ± 10 their 1 SD range would be 30-50 and 40-60: with this overlapping the sets cannot be regarded as being separate entities.

If there are differences between the two sets, the extent of difference depends on whether they can be separated at 3 SD when there is a 99% probability, or at 2 SD when there is a 95% probability, or at 1 SD when there is only a 66% probability, that the difference is really significant. In other words when the sets appear separate at 1 SD range, this does not necessarily mean that they will remain separated at the 3 SD range, as illustrated in Fig. 4.

Fig. 4 Standard deviations of two sets of data, showing overlap at 3SD



This method assumes that the mean is a constant figure, and it does not take account of dispersion around that figure. This dispersion is indicated by standard error of mean (see section 5.6 above).

A more reliable method is to analyse the standard error of the difference between the means (SE diff). In this procedure the difference between two sets of data is regarded as significant only if their means differ from each other by an amount greater than the SE diff. This is obtained by the following calculation:

$$\text{Standard error of difference in means (SE diff)} = \sqrt{\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2}}$$

where SD_1 and SD_2 are the SDs of sets 1 and 2 respectively, n_1 and n_2 are the number of data items in the sets.

5.7.1 t-test

This is a procedure to assess the comparability of two sets of data. It can be used, for example, to determine the accuracy of a new method when compared with a reference method.

The t-test indicates the level of probability that there is no significant difference between the sets of data that are being compared ("null hypothesis"). It can be applied to differences in the means or to differences in paired results.

(1) Differences in means

$$t = \frac{\text{Differences in means (i.e. } x_A - x_B)}{\text{SE diff}}$$

(2) Differences in paired results

First determine the difference (d) for each pair of results. Then determine: sum of differences = $\sum d$

$$\text{Mean of differences} = \frac{\sum d}{n} = \bar{d}; \quad \text{Variance } (s^2) = \frac{\sum (d - \bar{d})^2}{n - 1}$$

$$\text{SE of the means of differences} = \sqrt{\frac{s^2}{n}}$$

$$t = \bar{d} \div \sqrt{\frac{s^2}{n}}$$

Enter t values on chart (Table 1) at n - 1 degrees of freedom.

Read level of probability (p) that there is no significant difference between the means (calculation 1) or the paired results (calculation 2).

When t = o, p = 1, i.e. results are identical.

When t = ∞, p = 0, i.e. results are completely different.

As a rough guide, when t >4 there is a significant difference.

Example:

(1) Differences in means

	COUNTER I	COUNTER II
1	12.0	11.5
2	12.5	12.0
3	13.0	12.0
4	13.0	11.8
5	11.5	11.0
6	12.0	11.4
7	12.8	12.0
8	11.6	10.6
9	10.0	11.5
10	12.0	11.0
Mean ($\bar{x}$)	12.04	11.48
Variance (s^2)	0.81	0.24
SD (= $\sqrt{s^2}$)	0.9	0.49

Difference between means $(\bar{x}_I - \bar{x}_{II}) = 12.04 - 11.48 = 0.56$

$$\text{SE diff} = \sqrt{\frac{(SD_I)^2}{n} + \frac{(SD_{II})^2}{n}}$$

Where SD_I = SD of data for counter I and SD_{II} = SD of data for counter II:

$$= \sqrt{\frac{(0.9)^2}{10} + \frac{(0.49)^2}{10}} = \sqrt{0.081 + 0.024} = 0.324$$

$$\text{Therefore } t = \frac{x_{II} - x_{II}}{\text{SE diff}} = \frac{0.56}{0.324} = 1.728$$

From Table 1, at 9 d.f. (degrees of freedom), when t = 1.728 there is 10% to 20% probability that there is **NO** significant difference between the means.

(2) Differences in paired results:

	d	$d - \bar{d}$	$(d - \bar{d})^2$
1	0.5	-.36	.1296
2	0.5	-.36	.1296
3	1.0	.14	.0196
4	1.2	.34	.1156
5	0.5	-.36	.1296
6	0.6	-.26	.0676
7	0.8	-.06	.0036
8	1.0	.14	.0196
9	1.5	.64	.4096
10	1.0	.14	.0196
$\bar{d}$	0.86	$\sum(d - \bar{d})^2$	1.044

Where d = the difference between each pair of results on counters I and II and d is the mean of the individual d results.

$$\text{Variance } s^2 = \frac{1.044}{9} = 0.116$$

$$\text{SE diff} = \sqrt{\frac{s^2}{n}} = 0.1077$$

$$t = \frac{\bar{d}}{\text{SE diff}} = \frac{0.86}{0.1077} = 7.985$$

From Table 1, at 9 d.f. there is <1% probability that there is **NO** significant difference when the paired results are compared individually.

Table 1: Critical values of t-test at various probability levels (rows) for selected degrees of freedom (columns).

	Percent Probability Level						
df	50	40	30	20	.10	5	1
1	1.000	1.376	1.963	3.078	6.314	12.706	63.657
2	0.816	1.061	1.386	1.886	2.920	4.303	9.925
3	0.765	0.978	1.250	1.638	2.353	3.182	5.841
4	0.741	0.941	1.190	1.533	2.132	2.776	4.604
5	0.727	0.920	1.156	1.476	2.015	2.571	4.032
6	0.718	0.906	1.134	1.440	1.943	2.447	3.707
7	0.711	0.896	1.119	1.415	1.895	2.365	3.499
8	0.706	0.889	1.108	1.397	1.860	2.306	3.355
9	0.703	0.883	1.100	1.383	1.833	2.262	3.250
10	0.700	0.879	1.093	1.372	1.812	2.228	3.169
11	0.697	0.876	1.088	1.363	1.796	2.201	3.106
12	0.695	0.873	1.083	1.356	1.782	2.179	3.055
13	0.694	0.870	1.079	1.350	1.771	2.160	3.012
14	0.692	0.868	1.076	1.345	1.761	2.145	2.977
15	0.691	0.866	1.074	1.341	1.753	2.131	2.947
16	0.690	0.865	1.071	1.337	1.746	2.120	2.921
17	0.689	0.863	1.069	1.333	1.740	2.110	2.989
18	0.688	0.862	1.067	1.330	1.734	2.101	2.878
19	0.688	0.861	1.066	1.328	1.729	2.093	2.861
20	0.687	0.860	1.064	1.325	1.725	2.086	2.845
21	0.686	0.859	1.063	1.323	1.721	2.080	2.831
22	0.686	0.858	1.061	1.321	1.717	2.074	2.819
23	0.685	0.858	1.061	1.321	1.717	2.074	2.819
24	0.685	0.857	1.059	1.318	1.711	2.064	2.797
25	0.684	0.856	1.058	1.316	1.708	2.060	2.787
26	0.684	0.856	1.058	1.315	1.706	2.056	2.779
27	0.684	0.855	1.057	1.314	1.703	2.052	2.771
28	0.683	0.855	1.056	1.313	1.701	2.048	2.763
29	0.683	0.854	1.055	1.311	1.699	2.045	2.756
30	0.683	0.854	1.055	1.310	1.697	2.042	2.750
40	0.681	0.851	1.050	1.303	1.684	2.021	2.704
50	0.680	0.849	1.048	1.299	1.676	2.008	2.678
60	0.679	0.848	1.046	1.296	1.671	2.000	2.660
120	0.677	0.845	1.041	1.289	1.658	1.980	2.617
∞	0.674	0.842	1.036	1.282	1.645	1.960	2.576

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5.8 Analysis of variance

There are several ways in which the SDs of two sets of data can be compared statistically to determine whether there are significant differences between them. The F-ratio and the t-test are described here.

5.8.1 F-ratio

The F-ratio compares the variances (s^2) of two sets of measurements. The F-ratio is calculated to estimate the significance in the precision (reproducibility) of two sets of measurement. Variations due to random factors will be demonstrated by the extent of the variance in each set.

$$\text{F-ratio} = \frac{s^2 \text{ of set A}}{s^2 \text{ of set B}}$$

The ratio must not be less than 1; accordingly, select the set with the greater variance as the numerator (i.e. set A). To determine the significance of the F-ratio as calculated, compare the result of this calculation with the figures in Table 2A (p.30) for 95% probability ($p = 0.05$) or Table 2B (p.31) for 99% probability ($p = 0.01$) at the appropriate degrees of freedom for the two sets of data (degree of freedom = number of measurements - 1). If the calculated ratio is greater than the figure shown in the Tables there is a 95% or 99% probability respectively that the differences in variance is significant.

Example:

When the leukocyte count (WBC) was measured 10 times on each of two counters, the results were as follows (expressed as 10^9/L):

Mean ($\bar{x}$)	12.04	11.48
$\sum(x - \bar{x})^2$	7.28	2.156
Variance (s^2)		
$= \frac{\sum(x - \bar{x})^2}{n - 1}$	0.81	0.24
F-ratio =		
$\left\lfloor \frac{s^2 \text{ Set A}}{s^2 \text{ Set B}} \right\rfloor$ =	3.37	

Comment

In this example the variance of the data for counter I (0.81) is used as the numerator ("s^2" of Set A in the F ratio equation) because it is greater than the variance of the data for counter II (0.24). Because the number of measurements (n) on each counter is the same (10) the degrees of freedom (n - 1 = 9) are also the same.

Table 2A indicates that when F >3.18 (corresponding to degrees of freedom of 9 for numerator and denominator) there is a 95% probability that there are significant differences between the two sets of data. Table 2B indicates that when F >5.35 there is a 99% probability that there are significant differences between the two sets of data. Since F = 3.37 in this example, there is a 95% probability that there are significant differences between the two methods.

5.8.2 Chi-squared (χ^2) test

This is another way of testing the "null hypothesis" (see 5.7.1) between two or more different variables by examining if an observed set of measurements differ significantly from the expected results. It can be used, for example, to check the goodness of fit of a distribution curve by comparing observed measurements against expected results in different parts of the curve. It can also be used to test the accuracy of a particular method by comparing results obtained on a set of specimens against a reference method.

It is calculated from the following formula:

$$\chi^2 = \Sigma\frac{(O - E)}{E}$$ where O is the observed result and E is the expected result.

If this derived value is greater than the χ value at the appropriate degree of freedom (n-1) and required probability level as shown in Table 3, the null hypothesis is rejected (i.e. there is a significant difference).

Table 2A: F-ratio values at a probability level of P=0.05 for selected degrees of freedom

d.f. Numerator ν_1

d.f. Denominator ν_2

ν_2	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	161.4	199.5	215.7	224.6	230.2	234.0	236.8	238.9	240.5	241.9	243.9	245.9	248.0	249.1	250.1	251.1	252.2	253.3	254.3
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40	19.41	19.43	19.45	19.45	19.46	19.47	19.48	19.49	19.50
3	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81	8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53
4	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00	5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63
5	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77	4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36
6	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10	4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67
7	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68	3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23
8	5.32	4.46	4.07	3.84	3.69	3.58	3.60	3.44	3.39	3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93
9	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18	3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71
10	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02	2.98	2.91	2.85	2.77	2.74	2.70	2.66	2.62	2.58	2.64
11	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90	2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40
12	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80	2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30
13	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71	2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21
14	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65	2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13
15	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59	2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07
16	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54	2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01
17	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49	2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.00	2.01	1.96
18	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46	2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92
19	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42	2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88
20	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39	2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84
21	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37	2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81
22	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34	2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78
23	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32	2.27	2.20	2.13	2.05	2.01	1.96	1.91	1.86	1.81	1.76
24	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30	2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73
25	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28	2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71
26	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27	2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69
27	4.21	3.35	2.96	2.73	2.57	2.40	2.37	2.31	2.25	2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67
28	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24	2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65
29	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22	2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64
30	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21	2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62
40	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12	2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51
60	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04	1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39
120	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.96	1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25
∞	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88	1.83	1.75	1.67	1.57	1.52	1.45	1.39	1.32	1.22	1.00

Table 2B: F-ratio values at a probability level of P=0.01 for selected degrees of freedom

d.f. Denominator (ν_2) \ d.f. Numerator (ν_1)	1	2	3	4	5	6	7	8	9	10	12	15	20	24	30	40	60	120	∞
1	4052	4999.5	5403	5625	5764	5859	5928	5981	6022	6056	6106	6157	6209	6235	6261	6287	6313	6339	6366
2	98.50	99.00	99.17	99.25	99.30	99.33	99.36	99.37	99.39	99.40	99.42	99.43	99.45	99.46	99.47	99.47	99.48	99.49	99.50
3	34.12	30.82	29.46	28.71	28.24	27.91	27.67	27.49	27.35	27.23	27.05	26.87	26.69	26.60	26.50	26.41	26.32	26.22	26.13
4	21.20	18.00	16.69	15.98	15.52	15.21	14.98	14.80	14.66	14.55	14.37	14.20	14.02	13.93	13.84	13.75	13.65	13.56	13.46
5	16.26	13.27	12.06	11.39	10.97	10.67	10.46	10.29	10.16	10.05	9.89	9.72	9.55	9.47	9.38	9.29	9.20	9.11	9.02
6	13.75	10.92	9.78	9.15	8.75	8.47	8.26	8.10	7.98	7.87	7.72	7.56	7.40	7.31	7.23	7.14	7.06	6.97	6.88
7	12.25	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.72	6.62	6.47	6.31	6.16	6.07	5.99	5.91	5.82	5.74	5.65
8	11.26	8.65	7.59	7.01	6.63	6.37	6.18	6.03	5.91	5.81	5.67	5.52	5.36	5.28	5.20	5.12	5.03	4.95	4.86
9	10.56	8.02	6.99	6.42	6.06	5.80	5.61	5.47	5.35	5.26	5.11	4.96	4.81	4.73	4.65	4.57	4.48	4.40	4.31
10	10.04	7.56	6.55	5.99	5.64	5.39	5.20	5.06	4.94	4.85	4.71	4.56	4.41	4.33	4.25	4.17	4.08	4.00	3.91
11	9.65	7.21	6.22	5.67	5.32	5.07	4.89	4.74	4.63	4.54	4.40	4.25	4.10	4.02	3.94	3.86	3.78	3.69	3.60
12	9.33	6.93	5.95	5.41	5.06	4.82	4.64	4.50	4.39	4.30	4.16	4.01	3.86	3.78	3.70	3.62	3.54	3.45	3.36
13	9.07	6.70	5.74	5.21	4.86	4.62	4.44	4.30	4.19	4.10	3.96	3.82	3.66	3.59	3.51	3.43	3.34	3.25	3.17
14	8.86	6.51	5.56	5.04	4.69	4.46	4.28	4.14	4.03	3.94	3.80	3.66	3.51	3.43	3.35	3.27	3.18	3.09	3.00
15	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.89	3.80	3.67	3.52	3.37	3.29	3.21	3.13	3.05	2.96	2.87
16	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.78	3.69	3.55	3.41	3.26	3.18	3.10	3.02	2.93	2.84	2.75
17	8.40	6.11	5.18	4.67	4.34	4.10	3.93	3.79	3.68	3.59	3.40	3.31	3.16	3.08	3.00	2.92	2.83	2.75	2.65
18	8.29	6.01	5.09	4.58	4.25	4.01	3.84	3.71	3.60	3.51	3.37	3.23	3.08	3.00	2.92	2.84	2.75	2.66	2.57
19	8.18	5.93	5.01	4.50	4.17	3.94	3.77	3.63	3.52	3.43	3.30	3.15	3.00	2.92	2.84	2.76	2.67	2.58	2.49
20	8.10	5.85	4.94	4.43	4.10	3.87	3.70	3.56	3.46	3.37	3.23	3.09	2.94	2.86	2.78	2.69	2.61	2.52	2.42
21	8.02	5.78	4.87	4.37	4.04	3.81	3.64	3.51	3.40	3.31	3.17	3.03	2.88	2.80	2.72	2.64	2.55	2.46	2.36
22	7.95	5.72	4.82	4.31	3.99	3.76	3.59	3.45	3.35	3.26	3.12	2.98	2.83	2.75	2.67	2.58	2.50	2.40	2.31
23	7.88	5.66	4.76	4.26	3.94	3.71	3.54	3.41	3.30	3.21	3.07	2.93	2.78	2.70	2.62	2.54	2.45	2.35	2.26
24	7.82	5.61	4.72	4.22	3.90	3.67	3.50	3.36	3.26	3.17	3.03	2.89	2.74	2.66	2.58	2.49	2.40	2.31	2.21
25	7.77	5.57	4.68	4.18	3.85	3.63	3.46	3.32	3.22	3.13	2.99	2.85	2.70	2.62	2.54	2.45	2.36	2.27	2.17
26	7.72	5.53	4.64	4.14	3.82	3.59	3.42	3.29	3.18	3.09	2.96	2.81	2.66	2.58	2.50	2.42	2.33	2.23	2.13
27	7.68	5.49	4.60	4.11	3.78	3.56	3.39	3.26	3.15	3.06	2.93	2.78	2.63	2.55	2.47	2.38	2.29	2.20	2.10
28	7.64	5.45	4.57	4.07	3.75	3.53	3.36	3.23	3.12	3.03	2.90	2.75	2.60	2.52	2.44	2.35	2.26	2.17	2.06
29	7.60	5.42	4.54	4.04	3.73	3.50	3.33	3.20	3.09	3.00	2.87	2.73	2.57	2.49	2.41	2.33	2.23	2.14	2.03
30	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	3.07	2.98	2.84	2.70	2.55	2.47	2.39	2.30	2.21	2.11	2.01
40	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.80	2.80	2.66	2.52	2.37	2.29	2.20	2.11	2.02	1.92	1.80
60	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.72	2.63	2.50	2.35	2.20	2.12	2.03	1.94	1.84	1.73	1.60
120	6.85	4.79	3.95	3.48	3.17	2.96	2.79	2.66	2.56	2.47	2.34	2.19	2.03	1.95	1.86	1.76	1.66	1.53	1.38
∞	6.63	4.61	3.78	3.32	3.02	2.80	2.64	2.51	2.41	2.32	2.18	2.04	1.88	1.79	1.70	1.59	1.47	1.32	1.00

Example

Reticulocyte counts were carried out by a proposed new method and compared with an automated reference method; the measurements are shown as absolute counts (x 10^9/L).

	Reference: Expected	New method: Observed	E-O	$(E-O)^2$	$\div E$
1	80	95	15	225	2.812
2	115	105	10	100	0.870
3	145	135	10	100	0.690
4	95	100	5	25	0.263
5	105	110	5	25	0.238
6	340	315	25	625	1.838

$\Sigma = 6.711$; N = 6; degrees of freedom = 5

From Table 3, at 5 degrees of freedom null hypothesis applies at $p<0.25$, i.e. a probability of less than 25% that the new method is comparable to the reference method.

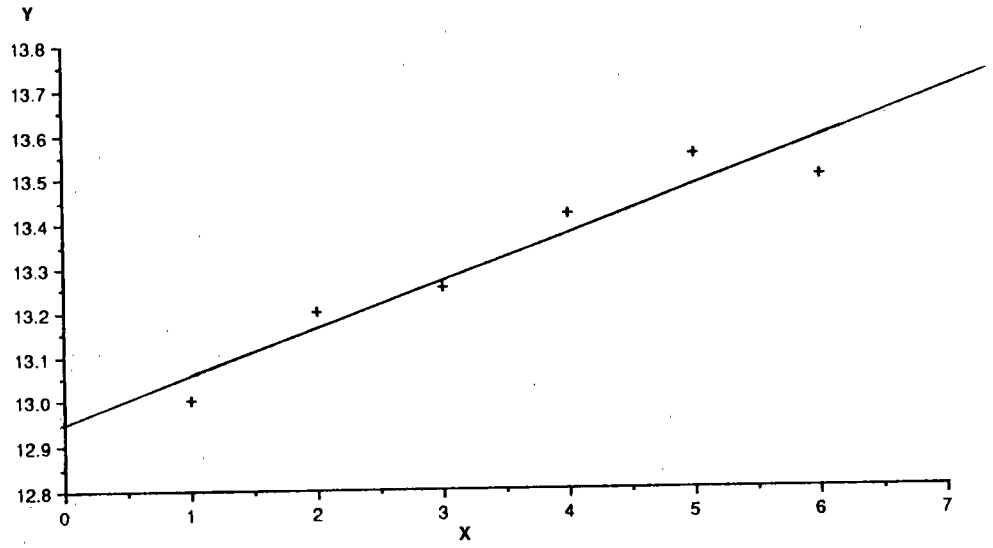
Table 3: Critical values of χ^2 at various probability levels (rows) for selected degrees of freedom (columns).

DF =	$P = 0.99$	0.95	0.10	0.05	0.01
1	0.000157	0.00393	2.706	3.841	6.635
2	0.0201	0.103	4.605	5.991	9.210
3	0.115	0.352	6.251	7.815	11.345
4	0.297	0.711	7.779	9.488	13.277
5	0.554	1.145	9.236	11.070	15.086
6	0.872	1.635	10.645	12.592	16.812
7	1.239	2.167	12.017	14.067	18.475
8	1.646	2.733	13.362	15.507	20.090
9	2.088	3.325	14.684	16.919	21.666
10	2.558	3.940	15.987	18.307	23.209
11	3.053	4.575	17.275	19.675	24.725
12	3.571	5.226	18.549	21.026	26.217
13	4.107	5.892	19.812	22.362	27.688
14	4.660	6.571	21.064	23.685	29.141
15	5.229	7.261	22.307	24.996	30.578
16	5.812	7.962	23.542	26.296	32.000
17	6.408	8.672	24.769	27.587	33.409
18	7.015	9.390	25.989	28.869	34.805
19	7.633	10.117	27.204	30.144	36.191
20	8.260	10.851	28.412	31.410	37.566
21	8.897	11.591	29.615	32.671	38.932
22	9.542	12.338	30.813	33.924	40.289
23	10.196	13.091	32.007	35.172	41.638
24	10.856	13.848	33.196	36.415	42.980
25	11.524	14.611	34.382	37.652	44.314
26	12.198	15.379	35.563	38.885	45.642
27	12.879	16.151	36.741	40.113	46.963
28	13.565	16.928	37.916	41.337	48.278
29	14.256	17.708	39.087	42.557	49.588
30	14.953	18.493	40.256	43.773	50.892
40	22.164	26.509	51.805	55.759	63.691
50	29.707	34.764	63.167	67.505	76.154
60	37.485	43.188	74.397	79.082	88.379

5.9 Assessment of linearity

The presence of linearity of measurement of an analytic variable at a certain concentration range is the basis of many analytic tests. For example, a material standard at one concentration can be used to measure that material at any concentration if the reaction has a linear relationship to concentration. This applies equally to spectrophotmetric readings of colour reaction or measurement of red cells in diluted suspension in a blood cell counter. Assessment of linearity is thus needed to check the range of dilutions which can be used reliably in a test or for comparing a new method with a reference method. A regression line which describes the relationship is obtained by plotting the two variables on arithmetic graph paper. The independent variable (x) is usually plotted on the horizontal axis and the dependent variable (y) on the vertical axis (see Fig. 5).

Fig. 5 Assessment of linearity



Drawing the regression line

When the data have been plotted on arithmetic graph paper a straight line must be drawn, connecting the points. If the line does not pass through all the points, it is necessary to draw a line which is the closest fit. There are several suitable procedures which give a reasonably close approximation.

(1) Best fit by eye

The regression line which is drawn should run midway between the various points. Some of the points will be above the line and an approximately equal number will be below the line. However, this method provides only a rough approximation which may be misleading.

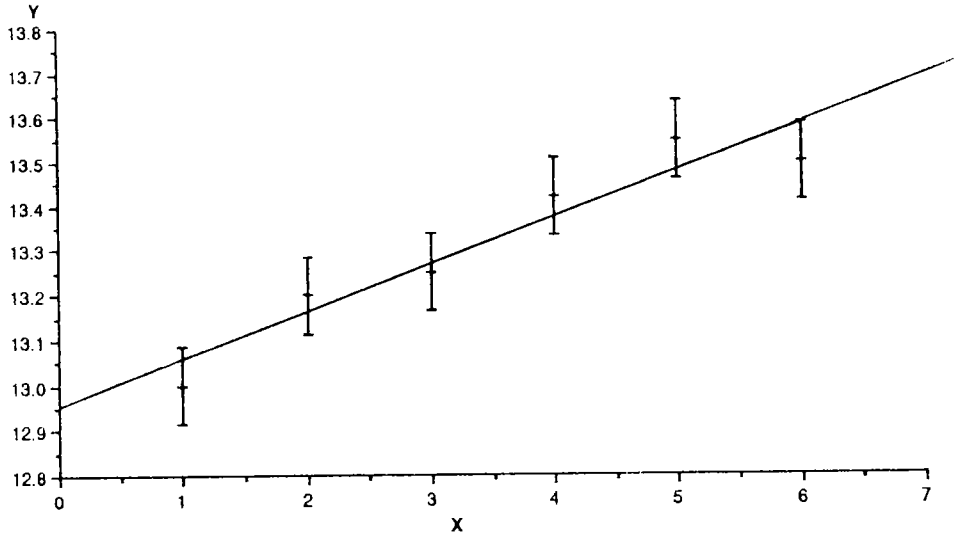
For a more reliable plot the sum of the squares of the perpendicular distance of the points to the line should be as small as possible, taking account of the sign, i.e. positive for the points above the line and negative for the points under the line. Any grossly outlying point may be ignored.

This is also the basis of the "least squares" method used for plotting the line by computer.

(2) Fit of regression line through SD range

Several measurements are obtained at each of the values. Instead of plotting the mean as a single point the 2SD range of the variable (y) is drawn at each value and the best fit obtained, as described above, provided that the line passes through any part of the 2SD range for each point (see Fig. 6).

Fig. 6 Fitting regression line through SD of plots

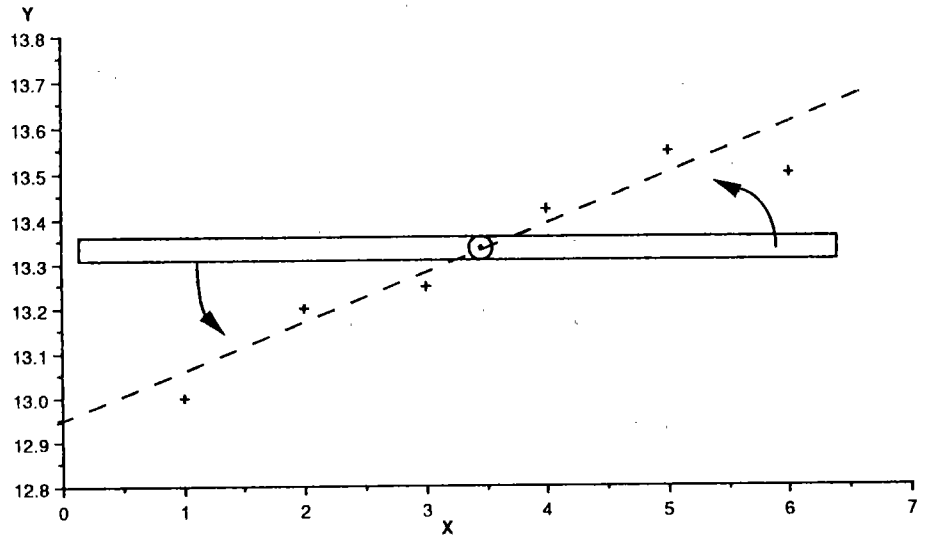


The vertical lines in the graph represent ± 2SD of the mean at each concentration.

(3) Average ratio

Plot the x/y values on arithmetic graph paper. Calculate the mean values for x and for y. Plot this point on the graph. Then, with a rule fixed at this point, rotate it so that it passes as closely as possible through as many of the points as is possible to obtain the best fit of the regression line (see Fig. 7).

Fig.7 Best fit of regression line from average ratio



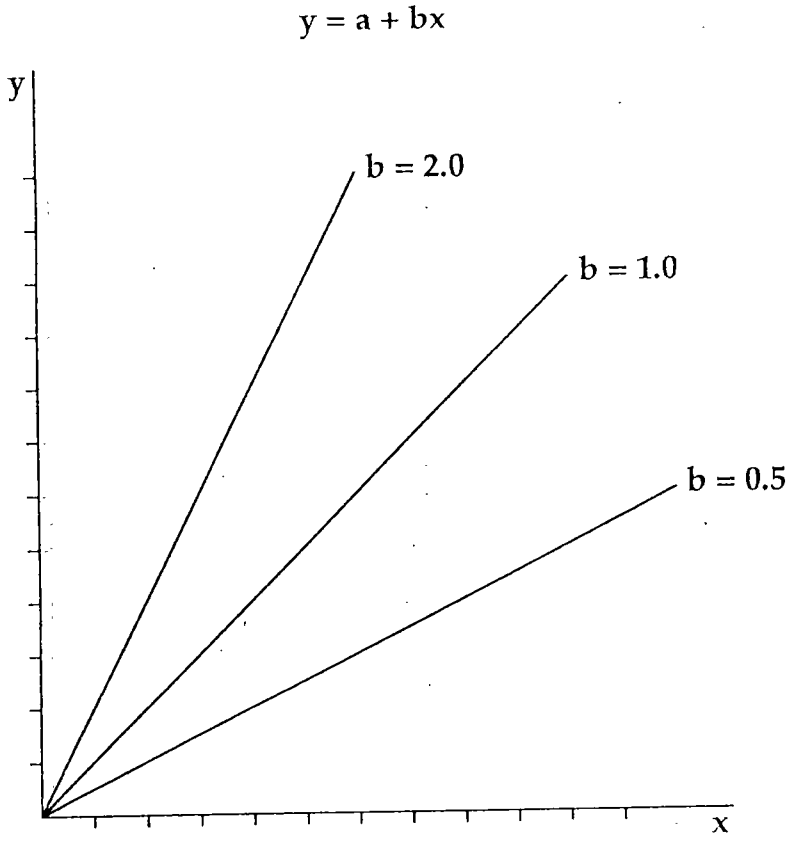
(4) The correlation coefficient (r) indicates the magnitude of the relationship between x and y. If this is perfect r = 1; if no relationship exists r = 0. For intermediate values the extent of the relationship can be estimated as (100 r^2)%: thus when r = 0.9 it is 81%; when r = 0.5 it is 25%. Calculation of r:

$$r = \frac{\Sigma xy - (\Sigma x)(\Sigma y)}{\sqrt{\left[\Sigma x^2 - (\Sigma x)^2\right]\left[\Sigma y^2 - (\Sigma y)^2\right]}}$$

This laborious calculation is readily obtained with the help of a suitably programmed calculator or computer.

(5) The slope (b) indicates the ratio of x to y at any point on the scale. Thus, when b = 1, x and y are identical, when b = 0.5, each value of y will = 0.5 x and when b = 2.0 each value of y will = 2.0 x (see Fig. 8)

Fig. 8 Slope of graph (b) illustrating ratio of x to y



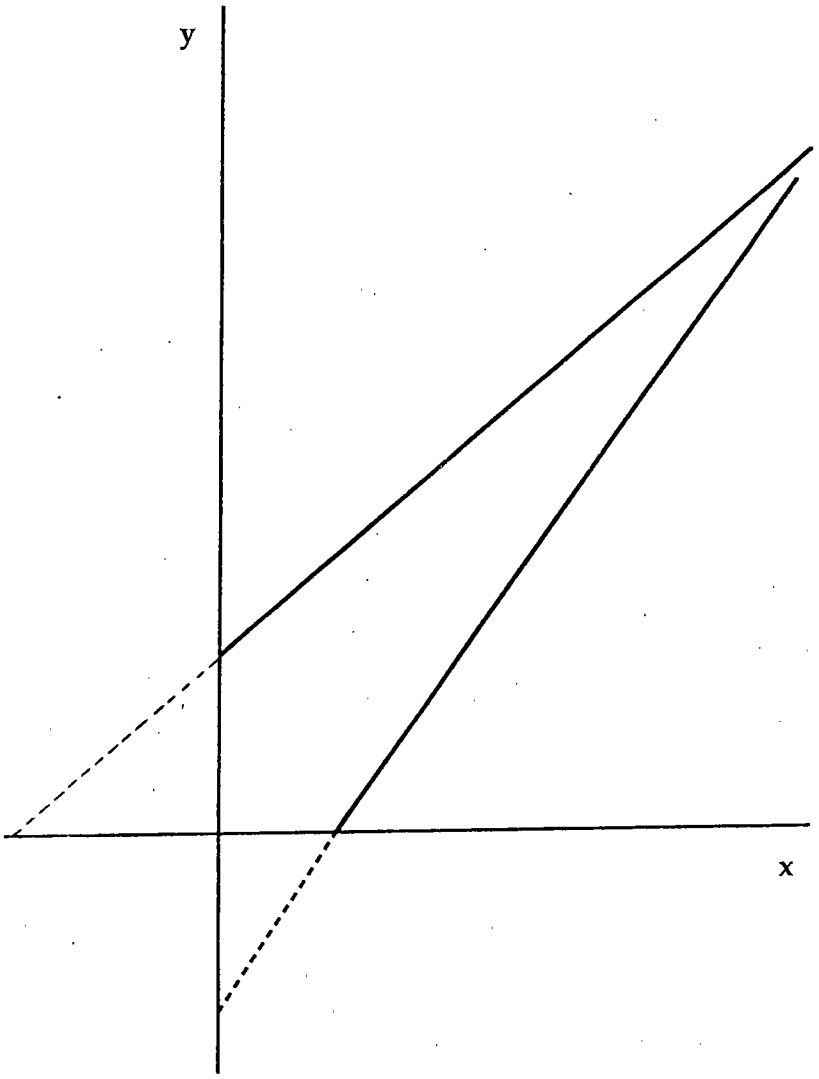
(6) The intercept (a) shows whether there is a consistent bias resulting in the regression line not passing through zero. As illustrated in Fig. 9, the intercept of x on the y axis or intercept of y on the x axis may be positive or negative; the negative intercepts are shown by the interrupted-line extensions of the regression lines.

The relationship of slope and intercept is:

$y = a + bx$, where x and y are paired results or their mean over a range, and a is the intercept of x on the y axis.

$$\text{Thus slope } (b) = \frac{y - a}{x}$$

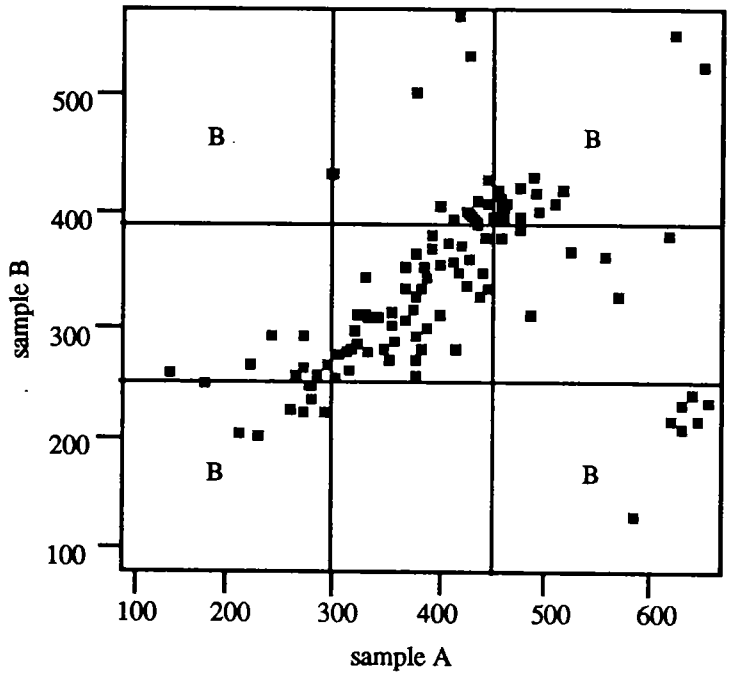
Fig. 9 Intercepts of x on y axis and y on x axis



5.10 X Y ratio plots (Youden plot)

When comparing two sets of data (e.g results on two samples in an EQA survey), the extent of random and systematic errors can be easily appreciated by plotting the individual results of x against y on arithmetic graph paper as shown in Fig.10.

Fig. 10 Youden plot to compare consistency of results of measurement



From the measurements obtained the means of the two sets are calculated. The ± 2SD limits or a clinically relevant reference interval for each are drawn on the graph. Results in this central area (A) for both samples are generally satisfactory. Those which fall in the areas marked by (B) are likely to have a systematic bias whilst the errors in results which fall in the other areas are more likely to have been random.

6. INTERNAL QUALITY CONTROL (IQC)

6.1 Duplicate tests on patients' specimens

This is the easiest procedure to carry out; if the day's work allows it, every specimen should be tested in duplicate; if there are too many test requests for this to be practical, test a few (e.g. 4 - 5) consecutive specimens in duplicate from time to time.

Calculate the standard deviation: $SD = \sqrt{\frac{\Sigma \text{ of } d^2}{2n}}$

where d^2 = difference between duplicates squared, n = number of specimens tested in duplicate.

Interpretation

None of the duplicate tests should differ from each other by more than 2SD as calculated. This is a method to identify random errors. If the test is always badly done the SD will be wide and will not be sensitive to individual errors.

Example

WBC (x 10^9/L)

Specimen	1st count	2nd count	d	d^2
1	5.4	5.8	0.4	0.16
2	8.3	10.5	2.2	4.84
3	17.2	18.0	0.8	0.64
4	5.4	5.4	0	0
5	12.2	11.8	0.4	0.16
6	14.3	13.8	0.5	0.25
7	6.2	6.4	0.2	0.04
8	8.2	8.6	0.4	0.16
9	7.3	7.5	0.2	0.04
10	5.4	5.9	0.5	0.25

$\sum d^2 = 6.54$

$\frac{d^2}{2n} = \frac{6.54}{20} = 0.327$

$\sqrt{\frac{\Sigma d^2}{2n}} = 0.5718$

Therefore SD = 0.57 2SD = 1.14

Conclusion

Test on specimen 2 is unsatisfactory and must be repeated.

6.2 Check tests

These are similar to duplicate tests but they use specimens which have been measured originally in an earlier batch.

Interpretation

The tests should agree with each other within ± 2SD. This procedure will detect deterioration of apparatus and reagents which may have developed between tests if it is certain that there has been no deterioration in the specimens on storage. Thus, this test is suitable for Hb and RBC, less so for WBC and platelets, and it is unsuitable for PCV if there is a delay of six hours or longer between the two tests.

It is useful to use the same specimens for check tests and duplicate tests. The SD can be established on the basis of technical competence from duplicate tests; if the SD for the check tests is greater this is a clear indication of deteriorating apparatus or reagents **PROVIDED THAT THE SPECIMENS HAVE NOT ALTERED.**

6.3 Delta test

This is a comparison of the current result with a recent previous result on the same patient. It is not appropriate when patients are being actively treated or when the clinical circumstances may result in a change in the blood count. As account must also be taken of diurnal variations, only relatively large differences in the count should be considered to indicate the likelihood of a fault:

Hb	2 g/dL
PCV	.05
MCV	>6 fL;
MCH	>5 pg
WBC	Normal to abnormal
Platelets	Reduced or increased by more than 50%

6.4 Replicate tests on control specimens

Repeated measurements on a single specimen will define the error of reproducibility (precision) and it is a method for evaluating technical excellence and/or an instrument which is unstable. As all the measurements are carried out with the same pipettes and reagents, it will not detect faults in these.

This test can be performed on any suitable blood sample but it is useful to use control material. When the SD of the repeat measurements has been obtained, as described below, this can be used for the control chart which is described in the next section. For Hb, use the lysate or preserved blood.

For replicate testing do eleven identical tests on one sample.

Calculate mean.

Calculate the difference from mean for each measurement.

$$\text{Calculate SD} = \sqrt{\frac{\Sigma d^2}{n - 1}}$$ where Σd^2 = sum of square differences.

$$\text{Calculate CV} = \frac{100\ SD}{mean}\%$$

Interpretation

The SD and CV give an index of precision. The desirable level of precision should be such that errors caused by the measurement procedure do not significantly affect clinical interpretation of the measurement. Thus, for example, if a clinician usually diagnoses that a haemorrhage has occurred when the haemoglobin falls by 10% of its previous level, it is necessary to be confident that the CV of the test is less than 5%. This means that the SD of measurement should not be greater than 4 g in 80 g/L or 8 g in 160 g/L.

With some tests, such as RBC by haemocytometry, it is not possible to achieve the desirable level of precision by the usual technique in the routine laboratory, as the CV is usually in the order of 10%. On the other hand, with automated cell counters one expects a CV of 2% for the RBC, as these instruments have a high level of precision.

Example

Consecutive measurements of Hb (g/L):

142 141 146 144 143 140 146 150 150 143 146

$$\text{Mean } (\bar{x}) = \frac{1591}{11} = 144.6$$

Set out columns:

No.	Measurement	$d = (x - \bar{x})$	$d^2 = (x - \bar{x})^2$
1	142	2.6	6.76
2	141	3.6	12.96
3	146	1.4	1.96
4	144	0.6	0.36
5	143	1.6	2.56
6	140	4.6	21.16
7	146	1.4	1.96
8	150	5.4	29.16
9	150	5.4	29.16
10	143	1.6	2.56
11	146	1.4	1.96

$$\sum d^2 = 110.56$$

$$\frac{\sum d^2}{n - 1} = 11.05$$

$$\sqrt{\frac{\sum d^2}{n - 1}} = \sqrt{11.05} = 3.32$$

$$SD = 3.3$$

$$CV = \frac{3.32 \times 100}{144.6} = 2.30, \text{ i.e. } 2.3\%$$

Conclusion

Although there was a 10 g/L difference between highest and lowest levels, overall haemoglobin measurement is being performed with an acceptable level of precision for clinical purposes.

6.5 Control chart

This uses the mean and SD obtained on the control material (lysate or preserved blood) as its basis. Originally devised for industrial purposes by W.A. Shewart, it was applied to clinical laboratories by Levey and Jennings.

Using arithmetic graph paper, calibrate the vertical scale in appropriate units, e.g. Hb in g/L, and the horizontal scale in days or batches of tests. Draw a horizontal line to represent the mean Hb. Draw two other lines above and two below the mean, respectively, to represent + 2 and 3SD and -2 and 3SD. In the example given, the following figures were obtained:

Mean: 144.6 g/L SD = 3.3 g 2SD = 6.6 g

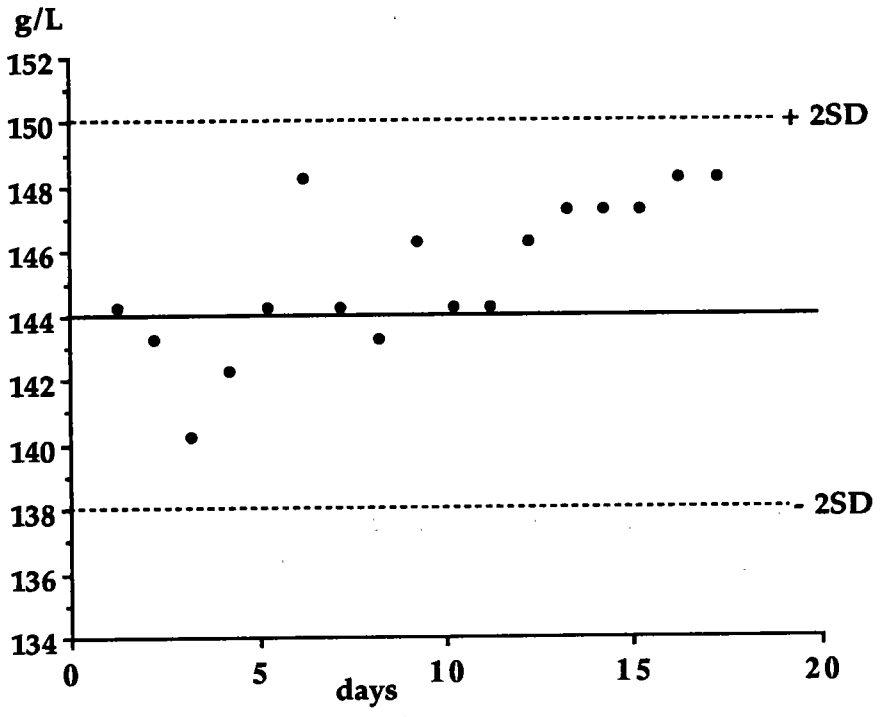
In rounding the figures it is convenient to plot the mean as:

144 g/L; +2SD as 150 g/L; and -2SD as 138 g/L.

With each batch of routine specimens include a sample of the control material and plot the result on the graph paper. This will give at least one value per working day. In the example shown in Fig. 11, the control material gave the following daily sequential results for Hb (g/L):

144 - 143 - 140 - 142 - 144 - 148 - 144 - 143 - 146 - 144 - 144 - 146 - 147 - 147 - 147 - 148 - 148.

Fig. 11 Control chart for haemoglobinometry



Loss of control had occured due to a systematic error (see below). This was found to have been caused by a new faulty reagent. When this was replaced, control was re-established as shown in the last part of Fig. 11

Interpretation

When the test is in control, all the measurements on successive samples will approximate to the established mean, with only minor deviations which will oscillate above and below the line of the mean. The chart will suggest that there is a fault in technique, instrument, pipette or reagents if one of the following occurs:

(1)	One control value outside the mean ± 2SD	Warning
(2)	One control value outside the mean ± 3SD	Reject: SE or RE
(3)	2 consecutive controls exceed mean ± 2SD	Reject: SE
(4)	4 consecutive controls exceed mean + 1 SD or mean - 1SD	Reject: SE
(5)	6 consecutive controls on one side of the mean	Warning: SE

SE = Systematic error
RE = Random error

But first check that the material itself has not become infected or in other ways has begun to deteriorate.

An example of good control followed by a systematic error which was then corrected is shown in the control chart illustrated in Fig. 11.

6.6 Cumulative sum (CUSUM) control

This is another way to display the data obtained in the test for precision. The CUSUM is the running total of the differences between successive measurements and the mean which was established initially. The plus and minus signs must be taken into account.

This is thought to be a more sensitive indicator of faulty technique or equipment. It is especially useful for detecting a consistent change in performance due to drift, as there will be a progressing increase in the deviation (plus or minus). By contrast, when there are only random differences from the mean some will be positive and some will be negative, i.e. plus and minus, so that the CUSUM will oscillate around zero.

An advantage of CUSUM is that it can be incorporated into a computer programme so that the user is alerted when a problem occurs without the need to plot the data as long as control results are within prescribed limits. The method is as follows:

1. Decide on minimum significant change to be detected; = d (usually 2SD).
2. Calculate "k"; = d/2
3. Calculate Upper reference value(URV); = mean + k
   Lower reference value(LRV); = mean - k
4. Calculate the Decision interval; = 2.0 k
5. Method of CUSUM result recording:

   (a) Set CUSUM at zero.
   (b) Do not start the CUSUM if the control value lies between URV and LRV.

(c) Start CUSUM if control value (x) lies outside URV or LRV. The CUSUM figure is the difference between the control value and the appropriate RV, i.e. either x - URV or LRV - x.

(d) To this difference add the next CUSUM difference and continue to add sequentially the difference between the control value and the same reference value, even when the control value falls within the reference values or outside the opposite reference value.

(e) If the CUSUM sign changes but the value is outside the opposite reference value, this indicates a possible abrupt shift in calibration.

(f) If the CUSUM equals or exceeds the decision interval, this suggests a significant change in accuracy: check calibration, correct as necessary, set CUSUM to zero and start a new CUSUM.

6.7 Patients' data

In a large hospital where at least 100 blood counts are performed each day, there should be no significant day-to-day or week-to-week variability in the means of red cell indices (MCV, MCH, MCHC). This is a useful method for quality control in laboratories with automated cell counters, as any significant change may indicate a change in instrument calibration or a fault in its function provided that the specimens are evenly distributed (see below). The more sophisticated automated cell counting systems have a computer programme incorporated in the system which makes it possible to analyse the data continuously. With other counters results can also be analysed using a programmable calculator or personal computer. By this means results are analysed in successive batches of 20 patients' specimens. Readers who require further information on the way in which this procedure can be used with an automated system should refer to one of the text books listed in Appendix 1.

Results are valid only if the population from whom the routine specimens are received does not vary significantly from day to day and the tests are not selectively biased. Such a bias might occur if, for example, as a result of specific outpatient clinics, tests are carried out on only certain days of the week on patients with iron deficiency or with some other condition which affects the MCHC, MCH and/or MCV. To overcome this problem results from these clinics should be excluded or the median should be used instead of mean.

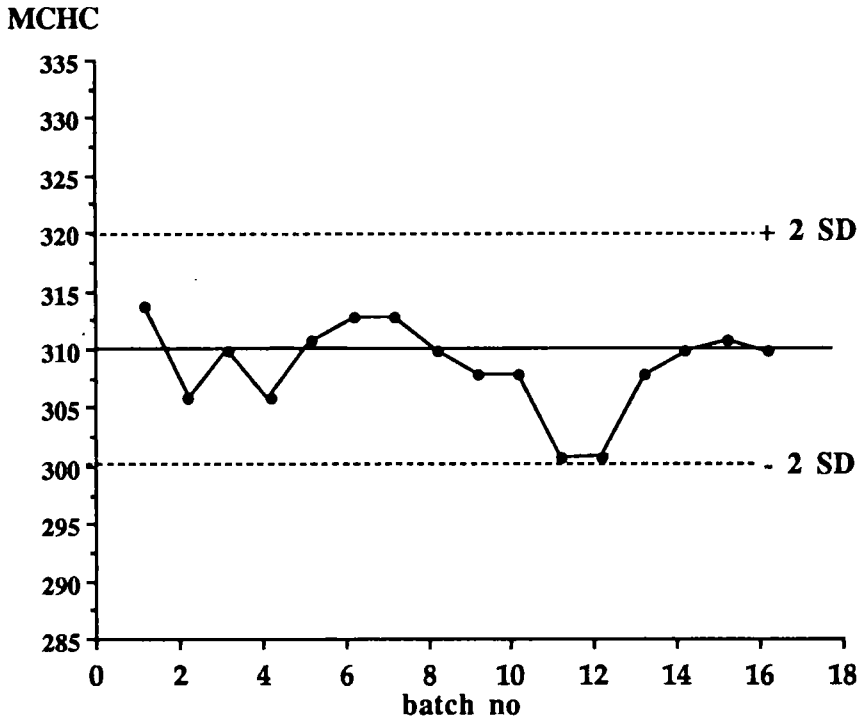
A simple adaptation of the same principle can be applied in laboratories using manual methods and which do not have computer facilities. In this situation the procedure is confined to the MCHC. The mean MCHC is calculated at the end of each day on all the measurements obtained during the course of the day. If the test is being performed satisfactorily, the mean will not vary by more than 2SD on any day. Before setting up this procedure it is necessary to establish the SD. This is done by calculating the mean on 11 consecutive working days and then calculating the SD.

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$$ where x = daily mean, $\bar{x}$ = mean of daily means and

n = number of days. In this case, therefore, n - 1 = 10.

It is convenient and educative to plot the data on a graph which should be updated each day (Fig.12). This method applies primarily to the control of absolute values. The component indices (Hb, RBC count and PCV) are controlled only indirectly by this procedure.

Fig. 12 Use of daily means of MCHC results as a control check



6.8 Correlation check

This implies that any unexpected result of a test must be checked to see whether it can be explained on clinical grounds or whether it correlates with other tests. Thus, for example, an unexpectedly higher or lower haemoglobin might be explained by a blood transfusion or a haemorrhage, respectively. A low MCHC should be confirmed by demonstrating hypochromic red cells on a Romanowsky-stained blood film; a high MCV must correlate with macrocytosis; similarly the blood film should be examined to confirm a leucocytosis or leucopenia, a thrombocytosis or thrombocytopenia - but be careful as the blood film itself may be misleading if not correctly made and stained.

Recording blood count data on cumulative report forms is good clinical practice as well as providing an inbuilt quality control system by making it easy to detect an aberrant result when compared with a previously determined baseline. This is especially useful in detecting the occasional wild errors caused by incorrect labelling, inadequate mixing or partial clotting of a blood sample. An example of a hand written record card is illustrated in Fig. 13. Similar presentations of sequential results is also possible by computerized processing

A formal way of testing for aberrant results is known as "Delta check" as described on p.42.

Fig. 13 Blood count cumulative report card

			CASE No.		DATE OF BIRTH	
Haematology Blood Count			SURNAME		SEX	
CONSULTANT			FIRST NAMES		WARD	
DATE						
WBC $x10^9/L$						
RBC $x10^{12}/L$						
Hb g/dL						
PCV						
MCV fL						
MCH pg						
MCHC g/dL						
Retics $x10^9/L$ %	$x10^9/L$ %	$x10^9/L$ %	$x10^9/L$ %	$x10^9/L$ %	$x10^9/L$ %	
Platelets $x10^9/L$						
	% $x10^9/L$	% $x10^9/L$		% $x10^9/L$	% $x10^9/L$	
Blasts			% $x10^9/L$			
Promyelocytes						
Myelocytes						
Metamyel						
Neutrophils						
Eosinphils						
Basophils						
Lymphocytes						
Monocytes						
Film morphology						
Signature						

For Lab. use only

7. EXTERNAL QUALITY ASSESSMENT

Even when all possible precautions are taken to achieve reliable results by internal quality control, errors will arise which are detectable only by external assessment; moreover, this is an essential process for ensuring harmonization or "transferability" of results between laboratories, and to recognize systematic errors. In haematology there are few tests which can be performed with such certainty that the result can be guaranteed as being accurate in the sense of absolute truth. Harmonization enables all laboratories to achieve results which are in agreement with each other and which will thus be clinically meaningful; external quality assessment identifies any laboratory which does not achieve this. The principle is that samples of the same material are sent from one centre (national or regional) to a large number of laboratories where the requested tests are performed. The materials described in Part 4 are suitable for this purpose. All the laboratories send their results back to the centre where they are analysed and interpreted in one of the ways described below.

7.1 Consensus method

From the results obtained by the participants, the centre calculates a mean or median and SD. If the mean is used its SD is calculated in the usual way by the formula:

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

where x = individual results
$\bar{x}$ = mean
n = total number of results.

The data are then adjusted by excluding any which are >2SD from the mean. The mean and SD (now termed "weighted") are then recalculated. If the median is used its SD is calculated as central 50% ÷ 1.35. By this procedure outlying results are automatically excluded in the calculation.

For these calculations of SD to be meaningful, there must be at least 15 participants in a set, and at least half the participants should have sufficiently good performance in comparison with each other to avoid having a (weighted) SD which is unhelpfully wide.

The SD may also be adjusted by one of the following methods:

(a) Restrict analysis to a selected group of good performance
(b) Use a predetermined constant CV from which the SD of the specific sample is calculated.
(c) Use a predetermined SD based on clinical significance.
(d) Calculate a mean SD from pooled SDs for all samples in the previous six months, including the present survey.

If different methods are known to react in different ways, giving different measurements on the same analytes, it may be necessary to treat the participant results in each group separately (Figs. 14 and 15).

A "deviation index" (DI) is then calculated for each individual participant. This is also known as "z-score". It indicates the difference between the individual laboratory results and the weighted mean of median and can be used to compare the performance of a laboratory with that of other laboratories as well as with its own performance in previous surveys.

The formula for calculating DI is:

$$\frac{\text{Actual result - weighted mean or median test}}{\text{Adjusted SD}}$$

The median should be used rather than the mean when there is a non-Gaussian distribution with a wide scatter of results (see page 23)

The deviation index score for any test may be interpreted as follows:

<1.0 = satisfactory performance
1.0-2.0 = still acceptable but borderline
2.0-3.0 = requires review of techniques and check on calibration
>3.0 = defect requiring urgent investigation

7.2 Assigned value method

As described in Part 4, when calibration and control materials are made it is possible to assign values by using reliable methods, as far as possible internationally recommended reference methods together with certified reference materials. It is also possible to establish the reproducibility for each test as performed by one or more skilled worker in the national or regional centre. Thus, results from participants can be judged by the extent or the deviation from the assigned mean in terms of clinical decision-making significance and taking account of unavoidable imprecision of the method used, as well as normal diurnal variations. The following limits are adequate to meet these requirements:

Hb, RBC (by electronic counter) and PCV: 4%
MCV MCH MCHC: 5%
WBC: 10%
Platelet count: 15%
Reticulocyte count by microscopy as a routine procedure: 30%

This method is valid no matter how few participants there are. Its limitation is that there is no assurance that the methods used in assigning values to the preparations are themselves free of bias. It is advisable for the centre to use several different techniques for each test, and preferably for the tests to be performed in three different laboratories which have been designated as reference centres because of their expertise.

Typical results in a United Kingdom NEQAS trial are given in Figs 14 and 15.

Fig. 14 Examples of results with partially fixed blood in an EQAS survey for all methods and for one specified group

	All methods combined n= 968		Specified group	
	Median	CV%	Median	CV%
Hb g/L	116.9	1.5	116.7	1.7
RCC x 10^{12}/L	3.70	1.7	3.68	1.8
PCV	0.349	4.0	0.350	2.2
MCV fL	94.4	3.6	95.3	1.5
MCH pg	31.6	2.0	31.8	2.1
MCHC %	33.4	3.9	33.3	2.4
WCC x 10^9/L	5.6	6.4	5.7	3.8
Platelets x 10^9/L	229	7.4	217	6.1

Fig. 15 Ilustration of part of report to an individual participant in an EQAS survey

Results for Survey No. Participant Refernce No.

Sample 1: Partially Fixed Human Whole Blood Sample Quality: Satisfactory
Sample 2: Partially Fixed Human Whole Blood Sample Quality: Satisfactory
Haemoglobin by reference method - Sample 1: 135 g/L
Sample 2: 126 g/L

Instrument	Test	Result	DI	Median	SD	CV%	N	
System A	Hb	[g/L]						
	1	139	1.35	136	2.22	1.6	950	All
			0.90	137	2.22	1.6	67	Group
	2	131	2.03	128	1.48	1.2	948	All
			0.90	129	2.22	1.7	67	Group
Sytem A	RCC	[x1012/L]						
	1	4.44	-0.14	4.45	0.074	1.7	907	All
			0.00	4.44	0.078	1.8	67	Group
	2	4.12	0.00	4.12	0.067	1.6	906	All
			-0.15	4.13	0.067	1.6	67	Group
System A	PCV							
	1	0.401	-0.16	0.403	0.0126	3.1	914	All
			0.56	0.396	0.0089	2.2	67	Group
	2	0.388	0.54	0.382	0.0111	2.9	912	All
			1.49	0.377	0.0074	2.0	67	Group
System A	WCC	[x109/L]						
	1	2.2	-1.36	2.5	0.22	8.8	916	All
			-1.43	2.3	0.07	3.0	67	Group
	2	3.6	-1.33	4.0	0.30	7.5	914	All
			-1.33	3.8	0.15	3.9	67	Group

8. CALIBRATION

8.1 Calibration of volumetric pipettes

The pipette is filled to the calibration mark with distilled water, which is then transferred to a weighed beaker in accordance with the normal usage of the pipette. The beaker is reweighed. The ambient temperature is noted. The volume of the pipette (in mL) is calculated by dividing the weight of the water (in mg) by one of the following factors depending on temperature:

Temperature (°C)	Factor
18	0.9986
19	0.9984
20	0.9982
21	0.9980
22	0.9978
23	0.9976
24	0.9973
25	0.9971
26	0.9968
27	0.9965
28	0.9963
29	0.9960
30	0.9957

The calibration must be performed in duplicate for each pipette.

8.2 Calibration of micropipettes

The micropipettes commonly used in haematology have a capacity of 20 μL (0.02 mL). The procedure described in 8.1 can be applied. However, as the weight of 20 μL of water is approximately 20 mg, to ensure a measurement error less than 2% it is essential to use a precision balance with a sensitivity of 0.1 mg (.0001 g). As this type of balance is not commonly available, the following modified procedure is recommended.

As a first step calibrate an 0.2 mL pipette, a 5 mL pipette and a 50 mL volumetric flask by measuring the weight of water on an analytic balance as described in 8.1 noting the ambient temperature. The true volume (in mL) is obtained by dividing the weight of water (in g) by the appropriate temperature-dependent factor given above.

Subsequently, for calibration of the micropipette a 2-3 mL specimen of fresh whole blood in EDTA is well mixed, lysed (e.g. by adding a few drops of saponin solution), and then diluted 1/251 in haemiglobincyanide reagent (see section 9) using the previously calibrated 0.2 mL pipette and the 50 ml flask. At the same time the blood is diluted (in duplicate) in haemiglobincyanide reagent using the 20 μL micropipette and the previously calibrated 5 mL pipette. The dilutions are carried out in duplicate and the absorbance (A) read at 540 nm on a spectrophotometer.

The dilution obtained by the micropipette is:

$$\frac{A_1}{A_2} \times 251$$

A_1 - Absorbance using the previously calibrated (0.2 mL) pipette
A_2 - Absorbance using the micropipette (20 μL)

This figure should be 251; if not, a correction factor must be applied to obtain the true dilution whenever the micropipette is used.

8.3 Calibration of autodilutors

A lysed sample of whole blood is diluted manually (in duplicate) in haemiglobincyanide reagent, by means of calibrated pipettes as described in 8.2.

Absorbance is read at 540 nm. Duplicate samples are then diluted in haemoglobincyanide reagent by means of the autodiluter and absorbance at 540 nm is read.

If Absorbance using manual dilution (A_1) differs from Absorbance using the autodilutor (A_2), a correction factor (f) must be applied:

$$f = \frac{A_1}{A_2}$$

Corrected A_{540} HiCN at 1/251 dilution = A_2 x f.

8.4 Control of calibration of photometer

To ensure that a photometer is functioning correctly a calibration graph should be prepared when it is first put into use in the laboratory, and thereafter at intervals - usually every six months, but every one to two weeks if there is any doubt about its performance. The following example illustrates the preparation of a calibration graph for use in haemoglobinometry, but the same principles also apply to other tests.

In preparing a calibration graph, a series of five cuvettes or tubes is set up. Into these tubes the following amounts of haemiglobincyanide (HiCN) reference preparation are pipetted by using a 10 mL graduated pipette.

Tube 1: 6 mL, approximately
Tube 2: 4.5 mL, accurately measured
Tube 3: 3.0 mL, accurately measured
Tube 4: 1.5 mL, accurately measured
Tube 5: none

After the reference solution has been pipetted, the pipette is rinsed through with haemiglobincyanide reagent. The rinsings are discarded and, with the same pipette, reagent is added to the five tubes as follows:

Tube 1: none
Tube 2: 1.5 mL, accurately measured
Tube 3: 3.0 mL, accurately measured
Tube 4: 4.5 mL, accurately measured
Tube 5: 6 mL, approximately

The contents of each tube are well mixed.

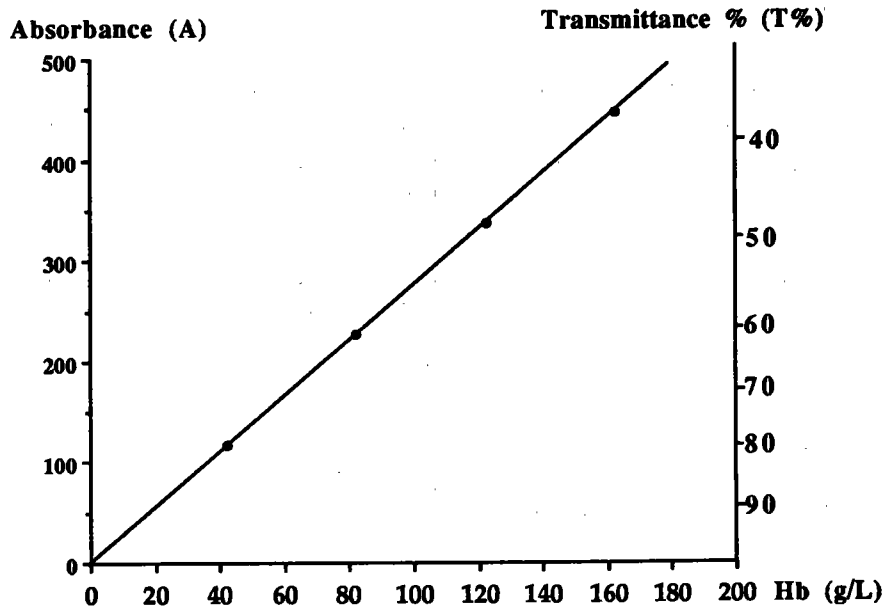
The haemoglobin concentration in the five tubes will be, respectively: labelled value (full strength); 3/4 times labelled value; 1/2 times labelled value; 1/4 times labelled value; zero strength (blank).

Using a spectrophotometer at A_{540} or a photometer with a filter at 540 nm, a cuvette containing distilled water as a blank is placed in the holder the holder and the instrument is set at zero absorbance (100% transmittance). The contents of tubes 1-5 are transferred to clean, dry matched cuvettes.

The outsides of the cuvettes are wiped carefully free of moisture, fingerprints, or lint. The cuvettes are placed successively into the instrument, and the readings are taken.

If the readings are in absorbance (A), they are plotted on the vertical scale and haemoglobin concentration on the horizontal scale of metric graph paper. If readings are in percentage transmittance, semilogarithmic paper must be used, with the transmittance plotted on the vertical (log) scale. The points should fall on a line passing through zero (see Fig. 16, p.59).

Fig. 16 Calibration graph for haemoglobin



In practice, the concentration of haemoglobin in blood which is diluted appropriately can be read from the calibration graph or, preferably, from a table which can be constructed from the values which were obtained. If the line of the calibration graph is not linear throughout its length, only the linear portion can be used for deriving measurements. If there is no linearity at all, the instrument requires attention by the manufacturer.

PART 2: PRACTICAL EXERCISES FOR TRAINING COURSE

INTRODUCTION

In the exercises which follow, haemoglobinometry and other fundamental haematological tests have been used as the model by means of which the principles and practical application of quality assurance are demonstrated. For the blood count it is assumed that all laboratories will have a general purpose photometer/spectrometer or a dedicated haemoglobinometer. The majority of laboratories will have a microhaematocrit centrifuge for packed cell volume and many laboratories now have an electronic cell counter, or an automated blood count system. Counting chamber method is not recommended for routine red cell counts because of its large clinically misleading errors, but as it is sufficiently reliable for total leukocyte counts and platelet counts, it has been included in those exercises; it is also included in Exercise 14 to demonstrate the principle and to provide an understanding of the statistics of random events (Poisson distribution).

This section should be used in conjunction with the CDC-WHO manuals on Fundamental Diagnostic Hematology "Anemia" and "Bleeding and Clotting Disorders". A by-product of the course is that the participants will have received training in techniques which are used for the detection and classification of anaemia; futhermore, they will have been introduced to the use of standards and methods for standardization.

It is recommended that the data obtained in the practical exercises be analysed in two ways: firstly, every participant should be regarded as an "individual laboratory" and secondly, his or her result should be incorporated in a communal databank and used for External Quality Assessment. When the principles have been mastered with fundamental haematological tests the practice of quality assurance can be extended into other tests, especially those which have a particular importance in diagnosis or health screening surveys in the area where the participants work.

Exercise 1: Calculation of standard deviation, variance and coefficient of variation.

The following data were obtained in 10 consecutive haemoglobin measurements on two colorimeters A and B:

	A	B
Test	**Hb g/L**	**Hb g/L**
1	155	150
2	148	135
3	152	145
4	147	137
5	150	153
6	156	163
7	157	155
8	153	149
9	150	144
10	150	137

Calculate for each series of readings:

(a) Mean ($\bar{x}$)

(b) $\sum(x - \bar{x})^2$

(c) Variance (s^2)

$$= \frac{\Sigma(x - \bar{x})^2}{n - 1}$$

(d) Standard deviation (SD) = $\sqrt{s^2}$

(e) Coefficient of variation (CV) which is a percentage $= \frac{100\ SD}{\bar{x}}$

(f) Standard error of mean (SEM) $= \frac{SD}{\sqrt{n}}$

Exercise 2: Differences between means

Assess whether the two sets of data in Exercise 1 are different or similar in the extent to which their SDs overlap.

	A	B
1 SD Range		
2SD Range		
3SD Range		

From this assess the 99%, 95% or 66% probability that the differences are significant (see p. 27).

Exercise 3: Differences between means cont.

Repeat Exercise 2, calculating standard error of differences in means (see p. 28).

$SD_A =$ $x_A =$

$SD_B =$ $x_B =$

SEdiff =

Is there any systematic difference (bias) between the two sets of measurements?

Exercice 4: Analysis of variance

Using the same data (Exercise 1) calculate the F-ratio of the two sets (see p. 32)

s^2 of set A =

s^2 of set B =

F-ratio =

Is there a significant difference?

Exercise 5: Analysis by t-test

Using the same data (Exercise 1) carry out a t-test by both methods (described on p. 27).

	Means	**Paired results**
t-value		
n-1 degrees of freedom		
Level of probability (p) that there is no significant difference		

Exercise 6: Comparability of methods

From the data provided in Exercise 1 assume that A gives accurate measurements (Reference method). On arithmetic graph paper plot the paired differences obtained with B (+ or -) on the vertical axis and the true results (i.e. A) or the horizontal axis.

Comment on the graph, noting whether there is consistent bias or random error or both.

Carry out a t-test on the data to determine the significance of differences.

Exercise 7: Evaluation of new method

The data below were obtained when haemoglobin was measured on a set of specimens (A) with the ICSH reference method and (B) with a new method using undiluted blood. Evaluate the new method by t-test on paired results and by demonstrating the extent of correlation or lack thereof by plotting the data on arithmetic graph paper.

	(A) Reference method (g/L)	(B) New method (g/L)	d	$(d - \bar{d})$	$(d - \bar{d})^2$
1)	70	65			
2)	76	75			
3)	110	120			
4)	90	75			
5)	116	110			
6)	113	105			
7)	97	80			
8)	95	105			
9)	85	65			
10)	64	50			
11)	120	115			
12)	117	115			
13)	101	100			
14)	98	90			
15)	73	60			

$\sum d =$ $\bar{d} =$ $\sum (d - \bar{d})^2 =$

$$s^2 = \frac{\sum (d - \bar{d})^2}{n - 1}$$

$$\text{SE diff} = \sqrt{\frac{s^2}{n}}$$

$t = d \div \text{SE diff} =$ ☐

Exercise 8: Evaluation of a new method (cont.)

In a trial of a new method for serum-iron, the results given below were obtained using five control sera with iron concentration determined with a reference method. Results are given in μg/dL.

Present the figures on graph paper. Calculate standard deviation and coefficient of variation (in %) at the five levels.

Control serum	Reference method	New method
A	40	51 - 58 - 67 - 61 - 63 - 59 60 - 58 - 61 - 64 - 59 - 59
B	100	126 - 118 - 126 - 121 - 123 - 117 114 - 120 - 122 - 125 - 118 - 120
C	160	184 - 180 - 177 - 185 - 181 - 174 179 - 178 - 180 - 182 - 183 - 177
D	220	244 - 242 - 237 - 240 - 236 - 239 244 - 238 - 240 - 241 - 235 - 244
E	280	278 - 272 - 264 - 270 - 266 - 274 264 - 268 - 270 - 272 - 274 - 268

Exercise 9: Precision of pipetting

Fill one 20 μL pipette from the blood sample provided and dilute the measured volume in 4 mL of cyanide-ferricyanide reagent.

Repeat 10 times. Read the absorbance (A) of each in a spectrophotometer or photoelectric colorimeter at a wavelength of 540 nm or with an appropriate filter (e.g. Ilford 625). Convert results to Hb (g/L) by calculation from the formula:

$$\text{Hb (g/L)} = \frac{A \times m}{\epsilon \times d} \times 0.2$$

where A is Absorbance at 540 nm
m is molecular weight = 16000 g/mol*
ϵ is coefficient extinction = 11.0
0.2 is factor to correct for dilution (x 200)
and to convert mg/L into g/L (÷ 1000)
d = thickness of solution in light path of cuvette (usually 1.000 cm)

Test No.	Reading converted to (x) Hb g/L	$(x - \bar{x})$	$(x - \bar{x})^2$
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			

Calculate mean of Series (= $\bar{x}$)

Calculate standard deviation (SD) = $\sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}$

Calculate CV (%) = $\frac{100\ SD}{\bar{x}}$

*More exactly 16115 g/mol

Exercise 10: Assessment of linearity and reproducibility

Prepare the material for this exercise as follows:

(a) From one donor collect approximately 20 mL of venous blood in EDTA. Mix well and transfer approximately 8 mL, 8 mL and 4 mL into three centrifuge tubes, labelled 1, 2 and 3 respectively.
(b) Centrifuge tubes 1 and 2; take 2 mL of plasma from each and add to tube 3. Mix the contents of tube 3 and relabel as A.
(c) Mix the contents of tubes 1 and 2, pool together and relabel as E.
(d) Make 5 samples as follows:

Tube A - as above
Tube B - 2 mL of A + 1 mL of E
Tube C . 2 mL of A + 2 mL of E
Tube D - 1 mL of A + 2 mL of E
Tube E - as above

Carry out the exercise as follows:

(a) Using a 20 μL pipette make a 1:201 dilution (in duplicate) of each of the samples A - E in cyanide-ferricyanide reagent. Read the absorbance at 540 nm. By means of a haemoglobincyanide reference standard, convert the readings into Hb concentration (g/L).
(b) Measure microhaematocrit of each sample in duplicate.
(c) Record results on the chart.
(d) On arithmetic graph paper mark an arbitrary scale of 100-400 units on the horizontal axis. On the vertical axis mark one scale for A_{540} and another one for PCV.

Plot all the measurements obtained with samples A-E - plot A at 100, B at 200, C at 250, D at 300 and E at 400. Draw a line of best fit for the A_{540} measurements and another line for PCV measurements. From the results on the chart and the graph, note the following:

(1) Is any part of the line non-linear?
(2) Do any of the duplicate measurements not fall on or close to the line?

FRESH SAMPLES			
	A_{540}	Hb g/L	PCV
A 1 2			
B 1 2			
C 1 2			
D 1 2			
E 1 2			
HiCN std.			

(3) To what extent does the PCV parallel the Hb readings?

(4) From the measurements of Hb concentrations calculate the SD of the difference between duplicate measurements by the formula $SD = \sqrt{\frac{d^2}{2n}}$, where d = the differences between paired measurements, and n = number of paired readings. Have there been any significant errors in any of the duplicate measurements? This will be the case when the difference in the duplicate measurements is greater than the SD.

(5) Identify the reliable range of A_{540} in the colorimeter, and express this in terms of Hb concentration at the dilution used.

(6) Convert the readings of absorbance at each dilution into Hb in g/L by the formula on p.69. Are there any discrepancies from Hb obtained with the standard?

Exercise 11: Haemoglobinometry

Carry out haemoglobin estimation by haemiglobincyanide method on whole blood samples A and B; lysed samples C and D; and diluted sample E (as HiCN).

A standard will also be provided.

	HiCN A_{540}	Hb concentration (g/L)
HiCN standard		
Sample A		
Sample B		
Sample C		
Sample D		
Sample E		

Using all the class results, calculate s^2, SD and CV of Hb concentration for each specimen, and record below.

	s^2	SD	CV
Reference preparation			
Sample A			
B			
C			
D			
E			

Calculate F ratios of the following various sets. Compare the results and note whether there are significant differences in reliability of measurement of Hb:

	F-Ratio	Significance
1. High concentration (A) v Low concentration (B)		
2. High concentration (C) v Low concentration (D)		
3. Whole blood (A) v Lysate (C)		
4. Whole blood (B) v Lysate (D)		
5. Prediluted (E) v Self-diluted		

Exercise 12: Haemoglobinometry (cont.)

Repeat Exercise 11 on the same samples A, B, C and D using another method, e.g. oxyhaemoglobin. Using all class results, calculate s^2, SD and CV for each:

	s^2	SD	CV
Reference preparation			
Sample A			
B			
C			
D			

Calculate F ratios and determine whether there is a significant difference in precision in comparison with HiCN method:

	F-ratio	Significance
1. Sample A		
2. Sample B		
3. Sample C		
4. Sample D		

Exercise 13: Preparation of calibration graph/table for haemoglobinometry

A calibration graph should be prepared whenever a new photometer is put into use in the laboratory, and again every six months, by the method described on p. 57.

Check the validity of the graph for reading haemoglobin values. Select an appropriate dilution to be used when measuring haemoglobin on blood samples.

Dilute the samples of blood provided in cyanide-ferricyanide reagent and read the Hb concentration from the graph. Express the results in g/L.

	Absorbance/Transmittance	**Hb (g/L)**
Sample A		
Sample B		

Exercise 14: Red cell counts

Carry out a red cell count by electronic counter on the provided blood in EDTA (A) and preserved blood in ACD (B), using appropriate dilution.

Establish mean, SD, count variance (σ),and CV.

Keep results from this and subsequent exercises for use in Exercise 24.

Using an appropriate dilution, count the cells also in a counting chamber.

σ is the theoretical variation between consecutive counts. It conforms to a Poisson distribution and is given as $\sqrt{\lambda}$, where λ is the total number of cells counted in the defined volume. It must not be confused with the standard deviation (SD) which is a measure of the variation which actually occurs between results when the test is repeated consecutively.

Record the chamber counts and electronic counts on the appropriate forms below. For the chamber count the "class count" is the total number of cells counted by everybody as an accumulated entity.

Counting chamber

	Specimen	Dilution	No. of squares counted	No. of cells counted (λ)	Red-cell count (10^{12}/L)	σ of cells counted ($\sqrt{\lambda}$)	Range $\lambda \pm 2\sigma$	Count CV (%)
Self	A							
	B							
Total class	A							
	B							

Electronic counter

Specimen		A			B	
Sample	(1)	(2)	(3)	(1)	(2)	(3)
Dilution 1:						
Observed count (λ) (a) (b) (c) (d) (e)						
Individual Count precision Mean ($\bar{x}$) Variance (σ) = $\sqrt{\lambda}$ CV%						
Individual RBC (10^{12}/L)						
Mean of 3 samples						
Class results RBC (10^{12}/L) $\bar{x}$ SD CV%						
Class RBC SD CV%						

Exercise 15: Absolute values

Estimate MCV, MCH and MCHC on blood samples A and B.

Specimen		Hb g/L	RBC 10^{12}/L	PCV	MCV fL	MCH pg	MCHC g/L
A	Self result Class mean SD						
B	Self result Class mean SD						

The following results were obtained by reference methods:

Specimen	Hb g/L	RBC 10^{12}/L	PCV	MCV fL	MCH pg	MCHC g/L
A						
B						

Exercise 16: Total leukocyte counts

By counting chamber and electronic counter, estimate the total leukocyte count in blood samples A and B, making suitable dilutions of the blood. A is a fresh blood; B is a control material.

	Electronic counter	
Specimen	**A**	**B**
Sample	(1) (2)	(1) (2)
Dilution		
Observed		
Counts (λ)		
Count variance (σ)		
Count CV%		
WBC (10^9/L)		
Class results		
$\bar{x}$		
SD		
CV%		

	Counting chamber	
Specimen	**A**	**B**
Dilution		
Self count Chamber volume No. of cells counted (λ) Count variance (σ) WBC (10^9/L) Count CV%		
Total class count Chamber volume No. of cells counted (λ) Count variance (σ) WBC (10^9/L) Count CV%		
Class individual results $\bar{x}$ (10^9/L) SD CV%		

Exercise 17: Differential counts

Stain the film provided with a Romanowsky stain, and carry out a differential count. Total WBC (10^9/L) of the sample will be given.

	Neutrophils	**Eosinophils**	**Basophils**	**Lymphocytes**	**Monocytes**
As % of 100 cell count					
σ ($\sqrt{\lambda}$)					
As % of 200 cell count					
σ ($\sqrt{\lambda}$)					
As total count (s 10^9/L) 2 σ range					
Class results (Total count) $\bar{x}$ SD CV%					

Exercise 18: Effects of anticoagulant and storage on the blood

Three samples of the same blood are provided in different anticoagulants (two in each).

(a) EDTA 1.5 mg/mL
(b) EDTA 4 mg/mL
(c) Heparin

(A) Make blood films from each - fresh, and after leaving the blood at 4 °C and at room temperature for 24 hours. Stain by Romanowsky stain and compare the effects on blood cell morphology.

(B) At the same time measure the PCV of each sample. Note that the samples which have been kept at 4 °C must be well mixed and equilibrated to room temperature before being tested.

1. Comments on morphological changes at 24 hrs

EDTA 1.5 mg/mL
Red cells:
Leukocytes:
Platelets:

EDTA 4 mg/mL
Red cells:
Leukocytes:
Platelets:

Heparin
Red Cells:
Leukocytes:
Platelets:

2. Effects on PCV

Anticoagulant	**Fresh blood**	**Stored blood**	
		4 °C	**RT**
EDTA 1.5 mg/mL			
EDTA 4 mg/mL			
Heparin			

Exercise 19: Platelet count

(a) Haemocytometer method

Carry out platelet counts on fresh blood sample (A) and control preparation (B) by haemocytometer method, using ammonium oxalate diluent.

Also count by an electronic counter if available.

Specimen	No. of squares counted	No. of cells counted	σ ($\sqrt{\lambda}$)	Platelet count (10^9/L)	CV (%)
A					
B					

Platelet count (10^9/L)

		A	B
By accumulated class count	λ σ CV		
By class results	$\bar{x}$ SD CV		
By electronic counter method	$\bar{x}$ SD CV		

(b) Film method

Make and stain a blood film from the fresh blood sample provided.

Estimate the platelet: red cell ratio.

From the red cell count which is given, calculate the platelet count. Report your results.

No. of fields examined:	
Average number of red cells per field:	
Approximate total number of red cells surveyed:	
Total number of platelets counted:	
Proportion of platelets: red cells	
RBC (x 10^{12}/L)	
Platelet count (x 10^{9}/L)	

Exercise 20: Reticulocyte count

Make reticulocyte preparations from the blood provided and carry out a reticulocyte count. The total RBC will be given. Report your results as a percentage and as an absolute count.

Number of fields examined		
Average number of red cells per field (Determined from 10-15 fields)		
Approximate total number of red cells surveyed		
Total number of reticulocytes counted		
Reticulocyte percentage		
RBC (10^{12}/L)		
Absolute reticulocyte count (10^6/L)		

Calculate the following:

	Reticulocyte count	
	10^6/L	**%**
Individual participant Reticulocytes Range of 2 σ		
Accumulated class count Reticulocytes Range of 2 σ		
Class results Reticulocyte count SD Range of 2 SD CV %		

Exercise 21: Erythrocyte sedimentation rate

This test is discouraged in the clinic and health centre, but it may have a place in the hospital laboratory in the absence of an alternative method (e.g. C-reactive protein) for following the progress and response to treatment of a patient with a chronic disorder. For reliable results it must be performed by a standardized method and **great care must be taken to avoid biohazard of contamination by blood from the sample.** This exercise is intended to demonstrate the influence of various factors.

(a) Carry out the standard procedure using the Westergren method on the sample of blood which has been collected into trisodium citrate. Read at 10 min intervals for one hour. Plot the readings (mm) against time on arithmetic graph paper. Record the reading at one hour.

(b) A demonstration will be set up as follows:

Modification		Reading (mm/1h)
Citrated blood (1:5)	(1) Standard method at room temperature (2) Standard method at 37 °C (3) Standard method at 4 °C	
Normal blood in EDTA (room temperature)	(4) Blood diluted in citrate (1.5) (5) Blood diluted in plasma (PCV 0.33) (6) Blood diluted in fibrinogen (7) Blood diluted in albumin	

Reference method for quality control of ESR

With each batch of tests one quality control specimen should be included. For this collect a blood sample into two EDTA containers. Dilute one subsample (A) in trisodium citrate as for routine ESR. Measure the PCV of the other subsample (B) and then adjust its PCV to 0.33 adding an appropriate amount of autologous plasma as follows:

$$\text{Volume plasma to be added per 5 mL specimen} = \frac{5 \times \text{measured PCV}}{0.33} - 5$$

Perform the ESR on A by the routine method and on B by the reference Westergren method. Correct the measurement on B for lack of dilution:

Corrected ESR = (undiluted ESR x 0.86) - 12

If the routine method is performing satisfactorily the ESR by the routine method (A) should not differ from the corrected ESR (B) by more than:

12 mm/1 h when B is less than 60 mm
or 20 mm/1 h when B is higher than 60 mm.

This test can be performed with normal blood or with any patients' samples in which the PCV is in the range 0.30-0.36. In no case should the difference between the diluted (routine) test and the corrected undiluted (reference) test be more than the amount indicated above. A smaller difference would be expected for ESR values at the lower end of the range.

Exercises 22: Thromboplastin calibration graph for standardization of prothrombin time

The use of a standard and preparation of a linear calibration graph for haemoglobinometry were described in Exercises 10-13. The exercises which are described below are intended to illustrate the principles of standardization when the calibration graph is non-linear and logarithms of the measurements are used in the calculation. This is demonstrated by prothrombin times in which a local thromboplastin can be related to the WHO international standard by an appropriate procedure.

WHO has established the International Sensitivity Index (ISI) and the International Normalized Ratio (INR) so that when Prothrombin time is used for control of oral anticoagulants, the result obtained with any thromboplastin (commercial or home-made) can be adjusted so as to be equivalent to the primary international standard thrombosplastin. The ISI or a working preparation depends on the slope of the line of the calibration graph. This shape can be calculated accurately from the formula:

$$\text{Slope} = m + \sqrt{(m^2 + 1)}, \text{ where } m = \frac{\Sigma(Ly - \bar{Ly})^2 - \Sigma(Lx - \bar{Lx})^2}{2\Sigma(Ly - \bar{Ly})(Lx - \bar{Lx})}$$

where Ly = log PT for each plasma with the reference preparation (RP)[1]
Lx = log PT for each plasma with the working preparation (WP)
Ly = mean of log measurements of y
$L\bar{x}$ = mean of log measurements of x

When the slope has been determined, the ISI of the working preparation is then calculated as follows:

ISI of WP = ISI (RP) x b, where ISI(RP) = ISI reference preparation and b = slope.

This is a complex formula and calibration of a commercial thromboplastin requires prothrombin time to be measured in parallel with the new reagents and with a reference thromboplastin with an established ISI on a large number of plasmas from normal and from patients stabilized on oral anticoagulant treatment. To illustrate this the test results which are given below were obtained from six normal plasmas and 10 anticoagulated plasmas. The ISI of the reference preparation was 1.0.

[1]As a rule the horizontal (x) axis is used for reference results (see p. 38) but to conform with the WHO protocol for assigning ISI to a thromboplastin the reference preparation is plotted on the vertical (y) axis (WHO Technical Report Series No. 687, p.81-105, 1983).

Results of prothrombin tests (secs):

Test	Reference preparation (y)	Working preparation (x)
1	3	13
2	13	16
3	15	17
4	17	19
5	17	21
6	13	15
7	28	35
8	30	40
9	30	43
10	35	46
11	36	50
12	48	70
13	50	90
14	40	50
15	45	67
16	35	48

(1) Plot xy data on double-log graph paper, with reference preparation (y) on vertical axis and working preparation (x) on horizontal axis. Identify intercept of x on the y axis (= a). This may be positive or negative (see p. 41).

(2) Calculation of slope (b) from formula $y = a + bx$; $b = \frac{L\bar{y} \times La}{L\bar{x}}$

where $L\bar{y}$ = mean of logs of reference preparation
$L\bar{x}$ = mean of logs of working preparation
La = log intercept of x on y (by calculation or from graph)

(3) Calculation of slope from graph

Read off paired xy plots and intercept (a) on the graph.

$$\text{Calculate the slope} = \frac{\text{Log } y_2 - \text{Log } y_1}{\text{Log } x_2 - \text{Log } x_1}$$

Try this calculation at four different levels to check consistency.

	1	2	3	4
Log y				
Log x				
Slope				

(4) Calibration of an individual batch of thromboplastin

This can be carried out with three pooled plasmas (one normal pool and two pools with high INRs of 2-4).

You are provided with samples of these pools and with a working preparation (WP) of thromboplastin and a sample of thromboplastin standard or reference preparation (RP) with a stated International Sensitivity Index (ISI).

Carry out prothrombin time tests (PT) on the three plasmas (in duplicate) with both the thromboplastins,.

Record the mean times of each pair of tests. Plot these on double-log graph paper with the PT for RP on the vertical axis (y) and the PT for WP on the horizontal axis (x). Draw the best fit of a straight line; calculate the slope and the ISI of the working preparation (WP) as described above.

Exercise 23: Measurement of prothrombin time and international normalized ratio (INR)

Carry out prothrombin time tests (PT) on two plasmas A and B from patients receiving oral anticoagulants and on a pooled normal plasma provided, using a working preparation of thromboplastin whose ISI will be given. Record the means of duplicate tests. Convert the PT measurements to standardized PT (i.e. on the international scale) by calculation from the ISI. Record the INRs.

	Plasma A	Plasma B	Normal Pool
1st Measurement			
2nd Measurement			
Mean			
Prothrombin time ratio (PTR) $\frac{\text{PT Patient}}{\text{PT normal}}$			
Thromboplastin ISI			
INR (PTR^{ISI})*			

*Obtained with calculator which has an x^y function or calculate:

INR = Antilog of (log PT x ISI)

In practice mean normal prothrombin time (MNPT) should be derived locally from the prothrombin times of 20 healthy men and women. For the present exercise a normal plasma pool has been prepared from a smaller set of normal donors.

Exercise 24: External quality assessment

For this exercise, each student will be regarded as an independent laboratory. From the results obtained in Exercises 14-20, calculate the deviation index (DI):

$$DI = \frac{x - \bar{x}}{SD^1}, \text{ or } \frac{x - m}{SD^1}$$

where $\bar{x}$ is the the mean, m is the median and SD^1 is the adjusted SD (see p. 24).

Individual performance can be judged as follows:

DI	
<1.0	Satisfactory
1.0 - 2.0	Acceptable but borderline
2.0 - 3.0	Unsatisfactory; requires checking of technique and calibration
>3.0	Serious problem

Exercise 25: Control chart method for quality control

First, make a series of at least 15 measurements of the control material provided and calculate the mean and standard deviation (SD) of the results. Prepare a control chart, calibrating the vertical scale in appropriate units (e.g. Hb g/L), and indicate the levels of +2 and +3SD and -2 and -3SD (see p. 48).

For the exercise, measurements of the control material are provided. Ten replicate measurements of haemoglobin were obtained (Column A). Then haemoglobin was measured each day on one of the samples. Results over 20 days are shown in Columns B and C. All measurements are expressed in g/L. Plot these on the chart and comment.

A	Day	B	Day	C
142	1	142	11	145
141	2	144	12	148
146	3	143	13	148
144	4	143	14	149
143	5	141	15	151
145	6	143	16	151
140	7	145	17	152
143	8	143	18	154
142	9	144	19	154
144	10	142	20	154

Exercise 26: Statistical analysis of means of absolute values

This is the method of quality control based on the principle that the mean values of the blood count (and especially the absolute values) remain constant in a selected population (see p. 50). You are provided with the MCHC measurements obtained over several successive days from the routine blood counts.

Determine the mean and SD of the first day's data as a base line. Then determine the mean and SD on each subsequent day. Has there been a change in the mean (= systematic error) or an increase in the SD (= random error)?

Repeat the exercise using only data from the blood counts in which the Hb was more than 130 g/L.

DAY									
1		2		3		4		5	
28*	34	30*	34	33	32*	34	36	32	32
32	27*	29*	33	34	30*	34	33	31	31*
34	28*	29*	30*	33	32*	35	34	32	31
33	33*	33	26*	35	32	34	36*	30*	35*
27*	35*	34	30*	30*	36*	32*	35*	26*	30*
30*	34	32	23*	27*	36	34	32*	25*	39*
32	33	30*	30*	27*	38*	33*	33	28*	32
34*	33	32	32	25*	30*	32*	34	32	33
33	32	33	32	33	32	34	35*	33*	31
32	30*	34	32	32	34	35	34	31	31

*Hb <130 g/L

Exercise 27: Normal reference range

Results are given below for the haemoglobin measurements obtained from a group of apparently normal people in a population. Plot the data as a frequency histogram on arithmetical graph paper and by eye determine the best fit curve. Is the distribution of "normal" (i.e. Gaussian)?

Calculate mean ($\bar{x}$) and SD. Set a range of $\bar{x} \pm 2SD$.

Eliminate from the original data any values which are outside ±2SD.

Recalculate $\bar{x}$ and SD of the remaining values. Calculate the range which should include 95% or a normal population (i.e. 2SD). Does this differ from the original group?

Hb (g/L)	No.
70	2
80	0
90	3
100	4
110	4
120	7
130	9
140	13
150	8
160	5
170	3
180	0

Repeat this exercise with the following set of data:

Hb (g/L)	No.
60	2
70	4
80	5
90	3
100	7
110	10
120	13
130	11
140	7
150	4
160	1

Exercise 28: Normal reference values (cont.)

The data in the following Tables are the MCV values for a hospital population (men and women) with normal haemoglobin and no apparent haematological disorders.

Establish the reference intervals for this population and decide whether men and women can be combined to provide one reference interval.

	MALES MCV	FEMALES MCV		MALES MCV	FEMALES MCV
1	92.6	95.1	21	91.6	87.8
2	91.5	90.1	22	90.5	93.5
3	89.1	92.9	23	89.1	93.0
4	93.5	95.3	24	95.9	95.8
5	91.4	88.3	25	98.9	91.7
6	91.9	93.9	26	85.1	96.7
7	97.9	94.0	27	89.3	89.0
8	90.7	85.1	28	90.5	91.3
9	93.9	93.3	29	86.6	90.8
10	92.7	90.2	30	100.6	93.0
11	86.7	99.2	31	95.9	90.4
12	87.5	92.8	32	92.2	89.4
13	90.3	92.3	33	93.2	84.1
14	94.4	90.4	34	90.2	94.4
15	90.8	94.5	35	85.0	93.3
16	87.0	94.6	36	92.6	91.1
17	95.6	90.7	37	85.3	94.8
18	90.7	84.5	38	96.5	93.7
19	85.7	94.2	39	94.4	87.7
20	91.6	92.0	40	96.8	90.0

Exercise 29: Reference values

Serum bilirubin (total) was determined in a group of healthy young men. The results are recorded in mg/L.

Present the results as a histogram and calculate mean, median and standard deviation. Calculate the 95% reference range.

5.0	5.0	4.0	6.0
4.0	4.0	5.0	5.0
6.0	8.0	7.0	9.0
4.0	5.0	14	5.0
3.0	5.0	10	6.0
7.0	4.0	6.0	5.0
4.0	22	13	4.0
11	5.0	29	4.0
5.0	4.0	4.0	5.0
3.0	3.0	5.0	6.0
9.0	5.0	4.0	10
4.0	6.0	15	16

PART 3: STANDARDS OF LABORATORY PRACTICE

Quality extends beyond internal quality control, external quality assessment and the pre- and post-analytic phases all of which are concerned essentially with the delivery of reliable test results. In addition the laboratory requires good direction and good management. To this end every laboratory director and supervisor should encourage the use of standardized test procedures and should establish policies which will set good standards for the various functions involved in running the laboratory as described in Part 1 (p.16). Special attention must be paid to prompt action leading to quick solution of the problem when the results from an external quality assessment survey or internal quality control indicate unsatisfactory performance or when there are unexplained errors in the routine tests.

1. STANDARD OPERATING PROCEDURES

Standard operating procedures (SOPs) help to maintain quality of performance by providing a stable pattern of function and avoiding unauthorized individual variation. They ensure consistent quality of work, allowing procedures to be understood by everyone working in the laboratory providing a training basis for new staff, ensure that appropriate quality assurance procedures are in place and provide guidance for solving problems when results fail to meet the expected quality standards. By definition, an SOP is a written standard procedure that has been approved by the departmental head (and laboratory director). Any subsequent change must be authenticated so that the precise procedure used on any day is always documented. SOPs should be prepared for every analytic test undertaken by the laboratory. They are intended primarily for test procedures, but similar documents should be prepared for specimen collection, data processing, computer functions, handling of urgent requests, even for a telephone answering policy. An SOP will normally include the following items:

Title and document reference number
Contents
Introduction
Principle of test
Specimen requirements
Reagents
Description of equipment
Calibration
Test procedure/methodology
Training requirements
Calculations
Calibration
Reference interval
Clinical significance of results
Limitations of procedure
Result reporting procedure
Maintenance schedule
Trouble shooting
Quality control procedures
Safety requirements
References

Authorization signature and date
Amended procedure and date

2. PROBLEM SOLVING FOR UNSATISFACTORY PERFORMANCE

This may require recalibration, a check of the integrity of pipettes and autodiluters, trouble shooting with the manufacturer's instruction manual or expert help. As procedures for trouble shooting can vary considerably from instrument to instrument it may be necessary to rely on the technical service provided by the manufacturer of the specific instrument. Regular preventive maintenance of any instruments used in the laboratory will reduce the incidence of problems, minimizing the risk of breakdowns and ensuring satisfactory function.

It is not the intention in this document to provide instructions for carrying out maintenance procedures and undertaking repairs; calibration and maintenance of semi-automated haematology equipment are described in a WHO document WHO/LBS/92.8, and in some other publications listed in Appendix 1. The purpose of this Section is to illustrate initial steps which should be taken to distinguish between unsatisfactory control specimens, calibration error, poor technique and instrument failure. This programme may require cooperation from an External Quality Assessment Scheme (EQAS) and/or from a reference laboratory.

2.1 Haemoglobinometry

The first step is to check whether the error has occurred in the routine tests or in the control procedures, and to compare the Internal Quality Control (IQC) and EQAS results. If both the IQC and EQA results are correct the problem may be due to faulty specimen collection, errors in specimen identification or some other aspect of laboratory organization. If the IQC and/or EQA results are wrong, repeat the measurements on another sample of control preparation and on the EQA material if still available. If the same results still occur it becomes necessary to carry out measurements on the following set:

(a) Duplicate samples of a whole blood specimen with normal/high haemoglobin concentration
(b) Duplicate samples of a whole blood specimen with low haemoglobin concentration
(c) Two samples of lysate with normal/high haemoglobin concentration
(d) Two samples of lysate with low haemoglobin concentration
(e) Vial of haemiglobincyanide reference preparation
(f) Reagent blank
(g) Five or six repeat measurements on (a) and (b) hourly during one working day
(h) Repeat measurements on (a) and (b) after storage for 2-3 days.

Interpretation

If there's difference of more than 2% between the two samples of (a) or (b) this indicates imprecision: check pipettes and pipetting technique.

If results on (a) are wrong compared with (b): check the linearity and whether an appropriate dilution factor is used; also check pipettes.

If results on (a) and (b) are wrong compared with (c) and (d) this suggests inadequate mixing of whole blood specimens.

If (a) to (d) are all wrong compared with (e) this suggests that the reagent is faulty with inadequate conversion to HiCN and/or samples are not being lysed adequately. Possibility must be considered of faulty specimen collection resulting in partial clotting of specimens.

If (a) to (e) are all wrong there is likely to be a fault with the photometer, possibly incorrect, misaligned or dirty filter, incorrect wavelength setting; dirty cuvettes.

If blank (f) reads unusually high there may be turbidity in the solution or dirty or incorrectly positioned cuvettes.

If (g) shows different sequential results during the day this is likely to be due to instrument drift as a result of fluctuating current, failing lamp or failing photocell.

If (h) gives wrong results when compared with the original results the samples are deteriorating as a result of contaminated or infection; blood collection containers and specimen storage conditions should be checked, and a fresh batch of control material prepared.

2.2 Blood counting with electronic counter

Some of the procedures described above for haemoglobinometers can also be used for checking cell counters, but as a rule the latter will require more elaborate investigation. The following protocol is applicable to simple counters and also to more sophisticated automated systems.

1. Check condition of specimens as they are received in laboratory and the reliability of specimen identification.
2. Compare results of internal quality control and external quality assessment surveys.
3. If two or more counters are in use in the laboratory compare results by the different systems/methods (a) in EQA surveys, (b) with control preparations and (c) on fresh blood samples.
4. Check the history of the previous performance of the counter(s) for means of absolute values (p.50, Section 6.7.).
5. Check the condition of the EQAS and IQC specimens for clots, integrity of containers, leakage, adequacy of pre-analytic mixing.
6. Repeat measurements on the EQA preparation, a fresh EQA specimen if available, and a new sample of IQC material, from another batch if available.
7. Check instrument calibration (with an appropriate calibrant), specimen feed and the diluent; if necessary repeat the tests with another batch of diluent.
8. If still faulty, the instrument should be checked by the service engineer and recalibrated.
9. Repeat measurements with control preparation and in the next EQAS survey.

2.3 Packed cell volume by microhaematocrit centrifuge

The main cases of error are:

(a) Inadequate speed of centrifugation
(b) Fluctuating current resulting in variable centrifugal force
(c) Capillary tubes with variable bore
(d) Incorrect sealing of tubes
(e) Error in reading
(f) Overheating of blood during centrifugation

To check the various factors carry out the following exercise:

Collect five blood samples (normal and anaemic) in K_2EDTA in containers which are large enough so that after adding the blood there will be an equal space left to allow adequate oxygenation. Mix well by inverting at least 20 times and fill ten capillary tubes with blood from the samples in duplicate; seal the tubes and spin for 3, 5, 7, 8 and 10 minutes, reading the PCV after each time period (see below). There should be no differences between any of the PCV readings between 5 minutes and longer times.

Compare readings on paired samples as a check of variable bore and/or incorrect sealing of tubes.

Examine the sealed ends of the tubes to see if the blood lies horizontally or sloping.

Read the height of the packed cells as a proportion of the total column by placing the tubes against a sheet of arithmetic graph paper; compare the measurements with the usual method.

Read the total height of the sample in the capillary tube before and after centrifugation as a check whether any evaporation has occurred.

Check whether any haemolysis is visible in the supernatant plasma in any of the tubes. If lysis has occurred, to see if it is due to overheating of the centrifuge during operation, place it in a cold room, if available, and repeat the centrifugation on a fresh batch of 5 or more blood samples for 5 and 10 minutes.

3. TEST SELECTION

The purpose of laboratory tests is to produce features which provide either quantitative or qualitative information relevant to specific patients or to the general population. When selecting a new method (or analyzer) its performance should first be assessed for this function by two procedures: (1) Comparability by comparison with a reference method (so-called "gold standard"); and (2) Clinical utility evaluation with tests carried out by the new method in parallel with an established routine method with which the laboratory is familiar (or a reference method) on a batch or successive batches of specimens from a variety of patients as well as from normal subjects in order to answer the following questions:

(a) How well does the new method identify specimens with the abnormal feature - i.e. specificity?
(b) How well does it ensure a negative result when the abnormal feature is absent - i.e. specificity?
(c) What proportion of the tests gives the correct result (true positive and true negative) - ie. accuracy?

(d) What is the probability that a positive test occurs only when the disease is present - i.e. positive predictive value?
(e) What is the probability that a negative test occurs only when the disease is absent - i.e. negative predictive value?

3.1 Comparability with reference method

Test specimens are measured alternately or in batches by the new method (A) and the reference method (B). The differences between paired results [A-B] are plotted on the vertical axis of linear graph paper against the means of the pairs [(A+B) ÷ 2] on the horizontal axis. The extent of differences between the methods is readily apparent, and if any particular concentration of the analyte shows bias this is well demonstrated.

3.2 Clinical utility

Taking the results from the established or reference method as "truth", the results with the new method are recorded as True positive (TP); True negative (TN); False positive (FP); and False negative (FN). The following calculations are then carried out:

Diagnostic sensitivity: $TP \div [TP + FN]$

Diagnostic specificity: $TN \div [TN + FP]$

Positive predictive value: $TP \div [TP + FP]$

Negative predictive value: $TN \div [TN + FN]$

The diagnostic sensitivity and specificity should be near 100% if the test is unique for a particular diagnosis. A lower level of diagnostic sensitivity or specificity may still be satisfactory, if the result can be interpreted in conjunction with other tests as part of an over-all pattern of results.

PART 4: PREPARATION OF CONTROL MATERIALS

1. INTRODUCTION

In this part methods are given for preparation of control materials. All laboratories should be able to make a lysate as well as preserve and stabilize blood specimens for internal quality control of their blood counts. The staff of a central laboratory should be able to make all the preparations for a national external quality assessment scheme. In some situations larger district general hopsitals may be expected to provide EQA and IQC material for smaller units, and also be responsible for control of haemoglobinometry in the health clinics of their area.

Accordingly, at least some laboratory workers will be expected to accept this responsibility and must be familiar with the preparation of quality control materials.

Some of these materials are required for the exercises described in Part 2. Organizers of training courses must be able to prepare small batches of the various materials for use by the course participants.

2. GENERAL NOTES

1. Human blood for use as calibration and control material for blood counts should be HBsAg and HIV and hepatitis C virus (HCV) antibody negative. Anticoagulated blood is usually available from Blood Transfusion Services; the anticoagulant is either citrate-phosphate-dextrose (CPD) or acid citrate dextrose (ACD-NIH A)[2]. For lysates blood in other anticoagulants (e.g. EDTA or heparin) can also be used.
2. In the following procedures care should be taken at all stages to avoid contamination. Where possible sterile glassware and reagents should be used and aseptic handling procedures observed.
3. Broad spectrum antibiotics should be added to aid sterility, e.g. 25-50 mg of penicillin and 25-50 mg of gentamycin per 500 mL material has been found to be satisfactory.

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[2]CPD: Trisodium citrate, dihydrate 26.3 g; citric acid, monohydrate 3.27 g; sodium dihydrogen phosphate, monohydrate 2.22 g; dextrose 25.5 g; water to 1 litre. The solution is sterilized by autoclaving at 126 °C for 30 minutes. Its pH is 5.6-5.8. For use,7 volumes of blood are added to 1 volume of solution.

ACD (NIH-A): The following formula was recommended by the United States National Institute of Health; hence referred to as "NIH-A" solution. Trisodium citrate, dihydrate 22 g; citric acid, monohydrate 8 g; dextrose 25 g; water to 1 litre. The solution is sterilized by autoclaving at 126 °C for 30 minutes. Its pH is 5.4. For use, 10 volumes of blood are added to 1.5 volumes of solution.

3. PREPARATION OF HAEMOLYSATE

1. Centrifuge anticoagulated blood in bottles of appropriate size (e.g. 30 mL screw-cap "universal" containers). Remove the plasma and buffy coat aseptically.

2. Add to each red cell deposit an excess of physiological saline (9 g/L NaCl), mix well, and re-centrifuge. Discard the supernatant and any remaining "buffy coat".

3. Repeat saline wash two times to ensure complete removal of plasma, white cells and platelets, each time removing the top layer of packed red cells.

4. Add to the washed cells an equal volume of water and 0.5 volumes of carbon tetrachloride or chloroform[3], cap the containers and then shake vigorously on a mechanical shaker or vibrator for one hour. Refrigerate overnight to allow the lipid/cell debris to form a semi-solid interface between carbon tetrachloride and lysate.

5. On the following day centrifuge at about 2500 g for 20 minutes. Transfer the upper lysate layers into universal containers and centrifuge at about 3000 g for an hour. Collect the upper 95% and pool into a clean bottle.

6. To each 70 mL of lysate add 30 mL of glycerol. After the addition of antibiotics (see paragraph 2.3), this stock material may be stored at 4 °C for short periods or frozen at -20 °C for a longer period until required for dispensing.

7. If a lower concentration is required add an appropriate volume of 30% (v/v) glycerol in 9 g/L NaCl to the stock. Mix well.

8. With continuous mixing, dispense aseptically into sterile containers. Cap and seal.

9. **Method for assigning value of Hb concentration:** see Section 7.1 The CV should be less than 2%. Stored frozen the product should maintain its assigned value for several years.

4. PREPARATION OF PRESERVED BLOOD

Human blood is collected in blood collection bags containing CPD or ACD from donors of the same ABO blood group. Equine blood may be collected in bags of up to 2 litre capacity. If only packed red cells are available resuspend in an equal volume of compatible (fresh frozen) plasma.

1. Run the blood through blood administration sets directly into a round-bottom flask, mixing at the same time, and continue to mix for at least 20 minutes after the addition of the last unit of blood or other material.

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[3]May not be available in the laboratory; an alternative is 0.4 volumes of toluene.

If the preparation is to be used also as a leukocyte and/or platelet control first dispense the blood into a set of bottles (see Section 3.1); centrifuge at about 1500 *g* for 20 minutes, discard the buffy layer and then remix the red cells and supernatant to reconstitute the blood. To ensure total removal of the leukocytes it may also be necessary to pass the blood through a leukocyte filter.

2. Cell levels may be adjusted, as follows:

   a) To increase red cell count - sediment cells over exit vents of bag and run into the flask with minimum of plasma.
   b) To lower red cell count - add fresh frozen plasma or a solution of K_2EDTA anticoagulant in 9 g/L NaCl; the anticoagulant:saline ratio must be the same as the usual anticoagulant:blood ratio.
   c) For white cell count - add fixed cells (see Section6.2). These can be human or selected avian blood.
   d) For platelet count - add fixed human platelets (see Section 6.3).

3. Add antibiotics (see Section 2.3).

4. With continuous mixing dispense in sterile containers; cap and seal. Refrigerate at 4 °C until needed.

5. For analysis, the sample should be gently mixed on a roller mixer or by hand before opening. Unopened vials of human blood keep in good condition for about three weeks at 4 °C, and those of equine blood for up to two months.

6. **Methods for assigning values**

   a) RBC: see Section 7.3. The CV should be less than 2%.
   b) PCV: see Section 7.2. The CV should be less than 2%.

5. PREPARATION OF STABILIZED WHOLE BLOOD CONTROL[4]

5.1 Reagent

Formaldehyde 37-40%	6.75 mL
Glutaraldehyde 50%	0.75 mL
Trisodium citrate	26 g
Distilled water to	100 mL

[4]Reardon, D.M., Mack, D., Warner, B and Hutchinson, D. Med. Lab Sciences, 1991; 48 : 19-26.

5.2 Method

1. Obtain preserved whole blood in CPD or ACD. This should be as fresh as possible and not more than 48 hours old. Filter through a 40 μm blood filter and measure the volume.

2. Add 1 volume reagent to 50 volumes of the blood.

3. Add antibiotics (see Section 2.3).

4. Mix continuously with a magnetic stirrer for one hour at room temperature. Then leave to stand overnight at 4 °C.

5. To obtain a different value remove 50 mL of supernatant plasma, and keep in reserve.

6. Remix the remainder and then with continuous mixing dispense part of the stock in 2 mL volumes into sterile containers.

7. Return the plasma (see 5) to the remaining stock, remix and dispense the rest of the stock in the same manner in 2 mL volumes into sterile containers.

8. Cap and seal; refrigerate at 4 °C until needed.

9. For analysis the sample should be gently mixed on a roller mixer or by hand before opening. Unopened vials keep in good condition for all blood count parameters for 2 - 3 years.

10. **Methods for assigning values (see Section 7)**

PCV obtained by centrifugation is likely to differ from measurement of the same sample by a blood cell counter.

6. PREPARATION OF FIXED BLOOD CELLS

Chicken and turkey red blood cells are nucleated and, when fixed, their size, as recognized by some electronic cell counters, is within the leukocyte size range on these counters. They are suitable for use as a leukocyte control in preserved whole blood. They may, however, give inconsistent results with some counting systems, especially the more sophisticated automated systems which do not identify them as leukocytes. Human red cells may also be used. A preparation of fixed human platelets can be used as a platelet control when added to preserved blood as described in Section 4.2.

6.1 Reagent

0.15M iso-osmotic phosphate buffer (pH 7.4)

(A) - 23.4 g/L sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)
(B) - 21.3 g/L anhydrous disodium hydrogen phosphate (Na_2HPO_4) or
53.7 g/L $Na_2HPO_4.12H_2O$.

Both stock solutions keep well when refrigerated.

For use, mix in proportion 18 mL A + 82 mL B. Check that the pH is 7.4.

0.25% glutaraldehyde fixative

To 1 litre of phosphate buffer, add 5 mL 50% glutaraldehyde solution (commercially available), mix and use at once.

6.2 Method for preparation of surrogate leukocytes

1. Collect about 25 mL of blood into any anticoagulant.

2. Centrifuge the blood. Remove the plasma and buffy coat.

3. Add an excess of phosphate buffer to the red cells, mix and transfer to centrifuge bottle; re-centrifuge and discard supernatant and buffy coat.

4. Repeat wash and centrifugation twice.

5. Add to the washed cells 10 times their volume of glutaraldehyde fixative, mix by vigorous shaking to ensure complete resuspension and rotate slowly on a mechanical mixer for one hour. To test for complete fixation, centrifuge 2-3 mL of the suspension, discard supernatant, add 2-3 mL water to the deposit, mix and centrifuge; if haemolysis occurs, fixation is incomplete. Either more time is needed or the stock glutaraldehyde requires replacement.

6. When fixation is complete, centrifuge the suspension and discard supernatant.

7. Add an excess of distilled water to the fixed cell deposit, resuspend and mix by stirring and shaking: again centrifuge and discard supernatant. Repeat twice.

8. Resuspend the washed fixed cells to approximately 30% concentration in 9 g/L NaCl. Mix well by vigorous shaking.

9. Carry out a rough count by routine method to determine the approximate concentration.

10. If it is intended to keep this preparation as a stock, autoclave at 121 °C for 15 minutes and then store at 4 °C. If aliquots are to be removed subsequently by opening the capped container it is advisable to add antibiotic (see Section 2.3) and then store at 4 °C.

11. Before use re-suspend by vigorous hand shaking (or by a vortex mixer) until no clumps remain at the base of the container, then roller mix for at least 20 minutes. If available, sonication for 1-2 minutes should also be used.

12. For use as a WBC surrogate mix the stock as described above and then transfer an appropriate amount to a volume of preserved blood (see Section 4.2) from which the leukocytes have been removed.

13. Mix well for 20 minutes and with continuous mixing dispense into sterile containers. Cap and seal.

14. For analysis resuspend by vigorous hand shaking followed by leaving on a mechanical mixer for at least 15 minutes before opening the tube.

15. **Method for assigning WBC value:** see Section 7.4. The CV should be less than 5%.

At 4 °C the shelf-life will be several months.

6.3 Preparation of fixed platelets

1. Obtain 50 mL of fresh platelet concentrate. Centrifuge at 1000 g for 10 minutes and remove the supernatant.

2. Wash the residual deposit in phosphate buffer and resuspend in 2 mL buffer.

3. Add 10 mL of glutaraldehyde fixative, mix by vigorous shaking to ensure complete resuspension and then rotate on a mechanical mixer for one hour.

4. Centrifuge, discard the supernatant, wash the deposit twice in phosphate buffer and resuspend in about 10 mL of CPD (see Section 2.1) or glycerol saline (3 mL glycerol with 7 mL of 9 g/L NaCl).

5. Resuspend by vigorous shaking.

6. Carry out a rough platelet count by routine method to determine the approximate concentration.

7. Add the appropriate amount of the suspension to the preserved blood as described in Section 4.2.

8. Mix well for 20 minutes and with continuous mixing dispense into sterile containers. Cap and seal.

9. For analysis resuspend by vigorous hand shaking followed by leaving on a mechanical mixer for at least 15 minutes before opening the tube.

10. Method for assigning platelet count value: see Section 7.5.

The CV should be less than 10%.

At 4 °C the shelf life will be several months.

7. METHODS FOR ASSIGNING VALUES

7.1 Haemoglobin (Hb)

Haemiglobincyanide (HiCN) method with five replicate measurements from each of two vials in accordance with ICSH recommendations for reference method for haemoglobinometry in human blood (ICSH Standard 1995):

Journal of Clinical Pathology 1996; **49**:271-274 or *Recommended method for the determination of the haemoglobin concentration of blood.* Geneva, World Health Organization, 1991 (unpublished document LAB/84.10 Rev. 1).

7.2 PCV

Microhaematocrit method on five replicate samples from each of two vials from the batch in accordance with ICSH recommended method for the determination of packed cell volume:

In Fundamental Diagnostic Haematology: Anemia, 2nd ed., p.67-70. For details see Appendix 1: WHO documents and publications, Reference 6.

7.3 Red cell count (RBC)

Electronic counting with 10 replicate counts on each of two vials from the batch in accordance with ICSH reference method for the enumeration of erythrocytes and leukcocytes:

Clinical and Laboratory Haematology 1994; **16**:131-138.

7.4 Leukocyte count (WBC)

Electronic counting with 10 replicate counts on each of two vials from the batch in accordance with ICSH reference method for the enumeration of erythrocytes and leukocytes.

Clinical and laboratory haematology 1994, 16:131-138

7.5 Platelet count

Haemocytometry (with phase contrast microscopy if available) - 5 replicate counts on two vials from the batch; dilution by precalibrated pipettes.

APPENDIX 1

REFERENCES

GENERAL

1. Dacie JV, Lewis, SM. *Practical Haematology*, 8th ed. Edinburgh, Churchill Livingstone, 1994. [Low-price ELBS Edition also available]

2. Lewis SM, Verwilghen RL. *Quality assurance in haematology*. London, Ballière Tindale (W.B. Saunders), 1988

3. Lewis SM, Koepke JA. *Hematology Laboratory Management and Practice*. Oxford, Butterworth Heinemann, 1995

4. Cavill I. (Ed). Quality Control. *Methods in Hematology*, 1990, 22:1-217

5. Westgard JO. *Quality control topics are a regular feature in a website on the internet*: http//www.Westgard.com

WHO DOCUMENTS AND PUBLICATIONS[5]

1. *WHO Expert Committee on Biological Standardization., Thirty-third Report. Annex 3, Requirements for thromboplastin and plasma used to control oral anticoagulant therapy.* Geneva, world Health Organization, 1983 (WHO Technical Report Series, No. 687).

2. *Recommended method for the determination of the haemoglobin concentration of blood.* Geneva, World Health Organization, 1984 (unpublished document LAB/84.10 Rev.1).

3. *Standardized Romanowsky staining of blood and bone marrow films.* Geneva, World Health Organization, 1986 (unpublished document LAB/86.1).

4. *Recommended methods for the visual determination of white cell and platelet counts.* Geneva, World Health Organization, 1988 (unpublished document WHO/LAB/88.3).

5. *Biological substances: International standards and reference reagents.* Geneva, World Health Organization, 1991.

APPENDIX 1

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[5]Available from the Unit of Health Laboratory Technology (LAB), WHO, 1211 Geneva 27; Fax: 41 22 791 4836, except References 1 and 5 which are available from the Unit of Biologicals (BLG), WHO, 1211 Geneva 27; Fax: 41 22 791 0746.

APPENDIX 1

6. Evatt BL, Gibbs WN, Lewis SM, McArthur JR. *Fundamental diagnostic hematology: Anemia*, 2nd ed. US Department of Health and Human Services, Centers for Disease Control, Atlanta, USA and the World Health Organization, Geneva, Switzerland, 1992.

7. Evatt BL, Gibbs WN, Lewis SM, McArthur JR. *Fundamental diagnostic hematology; Bleeding and clotting disorders*, 2nd ed. US Department of Health and Human Services, Centers for Disease Control, Atlanta, USA and World Health Organization, Geneva, Switzerland, 1992.

8. *Guidelines for reticulocyte counting by microscopy on supravitally stained preparations*. Geneva, World Health Organization, 1992 (unpublished document WHO/LBS/92.3).

9. *Calibration and maintenance of semi-automated haematology equipment*. Geneva, World Health Organization, 1992 (unpublished document WHO/LBS/92.8).

10. *Recommendations for standardization, safety and quality control of erythrocyte sedimentation rate*. Geneva, World Health Organization, 1993 (unpublished document WHO/LBS/93.1).

11. *Safety in health-care laboratories*. Geneva, World Health Organization, 1997 (unpublished document WHO/LAB/97.1).

12. *Calibration and control of basic blood cell counters*. Geneva, World Health Organization, 1997 (unpublished document WHO/LAB/97.2).

ICSH PUBLICATIONS

1. Recommendations for reference method for haemoglobinometry in human blood [(ICSH) Standard] and specifications for international haemiglobincyanide reference preparation. *Clinical and Laboratory Haematology*, 1987, 57:73-79.

2. The assignment of values to fresh blood used for calibrating automated blood cell counters. *Clinical and Laboratory Haematology,* 1988, 10:203-212.

3. ICSH reference method for staining of blood and bone marrow films by azure B and eosin Y (Romanoswky stain). *British Journal of Haematology,* 1984, 57:707-710.

4. Recommendation for reference method for determination by centrifugation of packed cell volume of blood. *Journal of Clinical Pathology,* 1980, 33:1-2.

5. Recommendation for reporting prothombin time in oral anticoagulant control. *Thrombosis and Haemostasis*, 1985, 53:155-156.

6. Reference method for the enumeration of erythrocytes and leukocytes. *Clinical and Laboratory Haematology*, 1994, 16:131-138.

APPENDIX 1

7. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobincyanide standard. *Journal of Clinical Pathology*, 1996, 49:271-274.

8. ICSH Recommendations for measurement of erythrocyte sedimentation rate. *Journal of Clinical Pathology*, 1993, 46:198-203.

APPENDIX 2

SYMBOLS AND ABBREVIATIONS USED IN THIS MANUAL

ICSH	International Council for Standardization in Haematology
IQC	Internal quality control
EQA	External quality assessment
NEQAS	National external quality assessment scheme
$\sqrt{}$	Square root
$\sum$	Sum
SD	Standard deviation
σ	Count variance
λ	number of cells counted
$\bar{x}$	Mean (of set of results x_1 x_2 x_3 .....x_n)
m	Median
CV	Coefficient of variation
d	Difference of measurement from mean. It is calculated from $x - \bar{x}$
delta (δ)	Difference between two sequential measurements