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Guidelines on Standard Operating Procedures for Haematology

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Foreword

Standard Operating Procedures (SOPs) are an inseparable component of a quality assurance programme. They denote laboratory integrity, ensure consistency and reliability, minimize errors and are a reflection of the policy of a laboratory in regard to quality. SOPs are a pre-requisite for accreditation of a laboratory. However, they are not very widely used in the laboratories of our Region, the main reason being non-availability of guidelines for the formulation of SOPs adaptable to local conditions. The World Health Organization has attempted to remedy this deficiency and is providing guidance in the establishment of SOPs in various disciplines of laboratory services.

'Standard Operating Procedures for Haematology' is a step in this direction. It has been designed to be of use to haematology laboratories at different levels engaged in undertaking routinely used test methods. For SOPs to be useful, it is essential that the staff are well acquainted with them and adhere meticulously to the instructions given. New staff should be well trained in the use of SOPs and laboratory managements should periodically review and update these guidelines.

I am sure the publication will achieve its intended objectives.

Dr Uton Muchtar Rafei
Regional Director

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1. GENERAL PRINCIPLES

Introduction

Standard operating procedures (SOPs) are an essential part of good laboratory practice. Using SOPs is the best way to maintain the optimal quality of performance in the laboratory by providing a stable pattern of function for laboratory staff. By enabling everyone working in the laboratory to understand the various procedures, SOPs ensure consistent quality of work with appropriate quality assurance procedures 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 person in charge. Any subsequent change must be authenticated and authorized so that the precise procedure used on any day is always documented. SOPs should be prepared for every analytic test undertaken and for all significant activities relating to the practice of the laboratory. Thus, some are intended primarily for test procedures, whilst other documents should be prepared for specimen collection, specimen storage, laboratory safety, data processing, record storage, handling of urgent requests, and even for a telephone-answering policy.

SOPs should accurately reflect good laboratory practice and be sufficiently practical to be useable in a routine service laboratory. Some relate to general aspects of laboratory function (e.g. specimen reception, safety precautions, data storage); others must take account of (a) the tests which are undertaken, (b) specific instruments such as an automated blood-cell counter, and (c) any special local circumstances. The SOPs described in this publication should be regarded only as outlines, to be used as guidance to any laboratory in preparing its own SOPs. Where appropriate, the SOPs which relate to specimen collection and storage, safety in handling specimens, etc. should be cross-referenced or repeated in the SOPs for specific tests.

Every laboratory should have at least a small library of books and other publications relating to haematology. This should include technical documents which have been published by the WHO Regional Office and by WHO units concerned with laboratory practice (ee Section 23). The following are of particular interest in the context of standard procedures in haematology:

1. International Council for Standardization in Haematology: *Calibration and control of basic blood cell counters.* WHO/LAB/97.2

2. England JM, Lewis SM, Lloyd E & Rowan RM: *Calibration and maintenance of semi-automated haematology equipment* WHO/LBS/92.8
3. Lewis SM: *Quality assurance in haematology.* WHO/LAB/98.4
4. Poller L: *The prothrombin time.* WHO/LAB/98.3

Structure of an SOP

An SOP will usually include the following items:

Title page

- Document reference number and date of preparation
- Indication if original or revised version and date of revision
- Name of person responsible for writing the document
- Signature of person verifying the document
- Signature of person authorizing the use of the document
- Latest date for routine review of document

Scope

- Brief description of purpose of the SOP and indications for performing the procedure or test
- Training required and grades of staff permitted to perform the described task

Specimen requirements

- Type and amount of specimen; anticoagulant (if appropriate)
- Delivery arrangements
- Storage conditions and time/temperature restrictions

Specimen reception

- Registration and check of test request form
- Criteria for rejection of a specimen

Safety precautions

- Lists protective clothing, gloves, etc which are obligatory
- Prohibitions (e.g. smoking, food and drink in laboratory)
- Handling "high-risk" blood samples

Equipment and reagents

- Lists of all the equipment or apparatus, solutions, chemicals, controls, calibrators, disposable items, forms and other stationery

Procedure

- Describes the method for performing the test, calculation of results and internal quality control. These should be in sufficient detail, for the test to be undertaken without reference to a textbook. If there is a manufacturer's instruction manual, the relevant sections should be reproduced in sequential steps, in clearly understood terms to ensure no ambiguity. The level of training and/or qualifications required for undertaking the various steps must be indicated.

Quality control procedures

- Describes the procedures for internal quality control in accordance with the manufacturer's instructions and standard methods.
- When available, instructions for participating in an external quality assessment scheme.

Maintenance

- Lists the schedule for routine in-house maintenance (daily, weekly) and arrangements for maintenance servicing

Limitations

- Describes known sources of error (e.g. inherent counting error, effects of interfering substances), how to recognize them and what steps to take to avoid/correct them.

Reporting results

- Validation of test for releasing results
- Procedure for composing report.
- Procedure for rapid reporting of urgently required results
- Storage of results and other data documentation; retention of request forms

Specimen post-test

- Indicates period of retention and conditions for storage
- Instruction for disposal of diluted sub-samples and specimens

Clinical significance

- Reference range for healthy subjects
- Clinical significance of results

List of relevant literature

All SOPs should be reviewed once a year. If no changes have been made to the procedure, the document will be given a sequential *Version number* and dated. If a change has been made to a method or if equipment or apparatus has been superseded, the document should be amended at that time and the revision should be indicated in the heading of the document with the date.

2. SPECIMEN RECEPTION

Specimens are normally transported to the laboratory in a rack or in individual plastic bags accompanied by a request form. On arrival in the laboratory the following steps must be taken.

Specimen registration

This can be undertaken by a technician, laboratory assistant or clerk.

- ➢ Remove the sample from the rack or from the bag.
- ➢ Check that the tube is firmly stoppered and check for any leakage of the specimen. If there is, handle as described below
- ➢ Read the patient's full name from the request form and check that the sample is labelled with the same name. If the sample is unlabelled or has a different name to that on the request form, treat as a rejected specimen (see below)
- ➢ If the labelling is satisfactory, record the details in the day-register and assign a unique laboratory number; this number must be recorded in the register, on the specimen tube and on the request form.
- ➢ From the request form ascertain what tests are required. Record requests for any tests other than a blood count in a special register.
- ➢ Recheck that the sample is correctly labelled with the patient's name, that the laboratory number has been correctly assigned, and that the specimen tube and request form bear the same number. If possible this step should be carried out by a second person.
- ➢ For a blood count place the specimen in a dedicated rack. Requests for other tests should be noted and appropriate action taken (see *'Additional tests'* below).

On-call samples

These are samples received after the normal working day and during the week-end. The technician/technologist on duty must enter details in a special register and allocate a consecutive laboratory number from that register.

On the next working day a record of the on-call tests will be entered in the day-register, and renumbered as for routine specimens.

Additional tests

If the request is only for a special test (e.g. ESR, Sickle screen, Hb electrophoresis, IM screen, Coagulation screen, Direct antiglobulin test), check that the correct sample has been provided. (see below). Place the specimen in a dedicated rack and take with the request form to the appropriate section of the laboratory.

If the request combines additional test(s) with the blood count, make a copy of the request form and tick the original form to show that this has been done. Place the specimen in the blood-count rack with both request forms.

The technician or technologist will decide whether to separate part of the specimen for the additional test or to carry out the blood count, followed by the additional test on the same sample.

If the request is for malaria parasites, copy the request form, tick the original form to show that this has been done; take the specimen and copy of request form to the appropriate section of the laboratory and make sure that someone knows that it is there.

Urgent tests

These may be from an out-patient clinic, accident and emergency cases, obstetrics, surgery, or neonatal unit. The processing of urgent tests has priority over other samples. On arrival they must be immediately entered in the register. Special note must be made of the telephone number or other method of contact with the sender.

The specimen tube and request form must be clearly marked (e.g. a red label or red ink) and immediately taken to the blood-count bench, making sure that a technician or technologist knows that it is there.

The test must be carried out without delay. Normally it should be possible for provisional results (for some tests) to be telephoned to the clinic within 30 minutes of receiving the specimen (See # 4 *'Reporting urgent requests'*)

Rejection of specimens

There are several reasons why a specimen may be unacceptable. Only a senior technologist or medically qualified member of the laboratory staff may reject any specimen.

Unlabelled or incorrectly labelled samples

These must not be analysed and the doctor requesting the test must be informed without delay. If the doctor is confident that the sample can be correctly identified, it may be analysed after the tube has been correctly labelled by the doctor or his authorized representative, who must sign a form accepting responsibility for any error. If there is any doubt about the correct identity of the sample, it must be discarded.

Leaking samples

Where a small volume of blood has leaked from the specimen tube, the sample should be treated as potentially hazardous and gloves should be worn to handle the tube. If the request form is contaminated a new form must be completed and the original form discarded.

If a larger volume of blood has leaked from the tube, the sample should be discarded as the results may be misleading. The doctor requesting the test should be informed immediately and a fresh specimen requested.

Outdated samples

If the specimen is over-aged by the time it is received in the laboratory the doctor requesting the test(s) should be informed immediately by a technologist or medically qualified member of staff. If it is not possible to obtain a fresh specimen the laboratory staff member must discuss with the sender which tests, if any, might still be carried out on the old sample without compromising the reliability of the analysis.

Unsuitable samples

Some tests may be performed on either plain blood or anticoagulated blood, whereas only anticoagulated blood can be used for other tests (e.g. blood count, ESR); furthermore, when blood is anticoagulated, the reliability of analysis may be affected by the type and amount of anticoagulant and its concentration.

If there is an obviously wrong tube (clotted sample with a blood count request) the person in charge of specimen registration must immediately inform the doctor who requested the test(s).

If there is insufficient blood in the tube resulting in significantly excessive anticoagulant, a senior member of the laboratory staff must decide whether or not to carry out the requested test(s). He/she must immediately inform the doctor who requested the test(s) and discuss the potential analytic error. If the test(s) are carried out, the report must include a note of this limitation.

Specimen collection

As a rule, blood specimens will be collected by the clinical staff or phlebotomists under their direction. In some cases the collection of venous blood may be undertaken in the laboratory by or under the direction of a doctor. The laboratory director has the duty to ensure that all staff who collect specimens have been instructed on the correct procedure as follows:

1. Identify the patient
2. Fill out test request form(s) and ensure that the correct identification is marked on the specimen tube(s).
3. Prepare the patient and cleanse the venepuncture site with 70% alcohol or other antiseptic solution, followed by drying with a clean piece of cotton wool or gauze..
4. Avoid prolonged compression of the vein by tourniquet prior to collecting the blood; this should not be for more than one minute.
5. The correct anticoagulants for various tests.
6. Ensure adequate mixing of blood with the anticoagulant but avoid frothing by excessive shaking of the specimen.
7. Ensure that the tubes are securely stoppered and placed in plastic bags or a rack for delivery to the laboratory.
8. Ensure that adequate arrangements are made for rapid despatch of the specimens to the laboratory.

Capillary blood

Capillary blood may be collected by the laboratory staff either in the laboratory or in the wards. The same principles apply with regard to identifying the patient and filling out the request form. Performance of a skin puncture then requires the following procedures:

1. Select a suitable site for the puncture: blood samples may be taken from a capillary puncture of the heel [lateral or medial plantar surface] in young infants and a finger [palmar surface of the distal phalanx] in others.
2. Warm the site by gentle massage of the area or applying a warm wet cloth for a few minutes.
3. Clean the puncture site with 70% alcohol or other antiseptic solution and dry with a clean cotton-wool swab or piece of gauze.
4. Make a quick stab-puncture with a sterile lancet and remove the first drop of blood with a gauze pad.

5. Ensure free flow of blood; this will be facilitated by holding the puncture site downwards and applying *gentle* pressure to the surrounding tissue, but do not squeeze as this will cause contamination of the sample with tissue fluid.
6. Collect the blood into a capillary tube or pipette, etc. as required for the test.
7. After collecting the sample(s) apply gentle pressure with a gauze pad and, if necessary, cover the puncture site with an adhesive skin plaster.

3. LABORATORY SAFETY

Basic instructions

All samples received in the laboratory must be regarded as potentially hazardous. The following rules apply to all staff:

- Wear laboratory coat, fastened at the front, at all times
- Wear close-fitting disposable plastic gloves or thin rubber gloves when handling any specimens.
- Remove gloves when using the telephone or photocopier.
- Cuts or grazes, especially on the hands, must be covered with a waterproof dressing prior to starting work
- Never take personal items such as combs, cosmetics or handbags into the laboratory
- Never take food, drink or cigarettes into the laboratory
- Never perform any action which may bring your hands into contact with your face, eyes or mouth, such as eating, smoking or adjusting contact lenses.
- Keep workbenches clear of clutter
- Remove laboratory coat and gloves and wash hands before leaving the laboratory.
- Clerical/administrative staff must notify a member of the technical staff when a specimen container leaks or is broken.
- To avoid mechanical hazards do not wear pendant jewellery in the laboratory; ensure that long hair is contained and do not allow personal clothing to protrude beyond the sleeves of the laboratory coat.

Precautions during specimen handling

As stated above, all biological specimens must be regarded as potentially hazardous and the following precautions must be taken when handling them:

- Wear a plastic apron over the laboratory coat.

- ➢ Wear visor or glasses when there is a risk of splashing or aerosol spray, e.g. with centrifuges and cell washers.
- ➢ Mouth pipetting is forbidden at all times
- ➢ Clean up breakages or spillage immediately, using 10% hypochlorite
- ➢ If breakage occurs in a centrifuge, pick up all glass debris with forceps, put the buckets into a basin of 10% hypochlorite and leave overnight. Meanwhile, swab the centrifuge bowl with 10% hypochlorite, reswab it after 30 minutes, wash with water and dry.
- ➢ At the end of each day all surfaces must be swabbed down with 1% hypochlorite.

Waste disposal and cleaning of equipment

1. Place disposable glass and needles in a special bin for disposal without further handling
2. Put other discarded materials in robust plastic bags which should then be sealed and sent to a designated site (e.g. incinerator) for disposal.
3. Place re-usable glassware and plastic items in a bucket containing 2.5 % hypochlorite. Leave overnight before the items are washed and dried.
4. Soak pipettes in 2.5% hypochlorite for 30-60 minutes, wash in water and dry.

Safety officer

One senior member of the technical staff must be named as the safety officer who must inspect all parts of the laboratory at regular intervals to ensure that adequate safety precautions are maintained. The officer must maintain a record of all accidents and incidents affecting the health of laboratory staff and must report these to management. He or she must ensure that any resultant changes in laboratory procedure are implemented. Following an accident where a member of the staff is injured, the safety officer must ensure that the victim is seen promptly by a doctor.

4. REPORTING RESULTS

General

All reports must be validated by a qualified member of the laboratory staff

Where any of the tests on a specimen may result in a delay in completing the request, an interim report may be prepared of the results which have already been validated. This provisional report must be clearly marked as such, and must always be followed up with a final report.

At the end of the working day or, if appropriate, after the morning and afternoon shifts respectively, the clerk or assistant will sort out the batch of reports for delivery to different wards or clinics unless this procedure is undertaken by a central messenger service.

The head of the laboratory must establish liaison with the clinical administration to ensure that specimens are delivered promptly to the laboratory and that there is no delay between despatch of the reports from the laboratory until they reach their destination.

At regular intervals the head of the laboratory should meet with the clinical administration to check that the system is functioning well. Any delays that have occurred must be investigated and steps must be taken to avoid the problems in the future.

Reporting urgent requests

Urgent requests will normally be for haemoglobin, WBC, platelet count, malaria, coagulation screening and cross-matching.

Priority attention must be given to all requests from: accident and emergency units (A&E); intensive care unit (ICU); neonatal unit (NNU).

Urgent requests also include after-hours service and any other requests specifically telephoned to the laboratory prior to arrival of the specimen.

Urgent specimens must be marked with a conspicuous label as soon as they are received.

All urgent specimens must be recorded in the register and processed on receipt.

As soon as the technical validity of the results has been established by a senior technician/technologist, the requesting doctor must be contacted without delay. If the identity of the requesting doctor is not obvious from the request form, his or her identity must be ascertained from the ward. If this fails, urgent results can be phoned to the ward or clinic sister or the most senior nurse on duty.

With requests that are automatically classified as urgent (see above), abnormal results may be phoned directly to senior nursing staff in the specialist unit.

Blood group results must never be given by telephone.

When results are transmitted verbally in all cases, the request form must be signed to indicate when and to whom and by whom the results were communicated. This must always be followed by a written report.

5. DATA AND SPECIMEN RETENTION

Records

Test requests

Request forms are stored for three months

Reports of results

Copies of reports are stored for three months

Result work-books

Stored for at least five years.

If computerised, results are stored in the analyser database or on a PC for up to three months and then transferred to storage disks and stored for at least five years

Blood grouping and blood transfusion records

Stored for at least ten years

Specimens

EDTA blood samples

Stored for seven days at 4^0C – but suitable only for haemoglobin after 24 hours.

Citrated blood samples

Stored for three weeks at 4^0C

Blood films

Stored for five years

Plasma for coagulation tests

As coagulation tests must be carried out within a few hours, specimens are not stored at the end of the working day. If samples are required for specific purposes (e.g. repeating abnormal tests), the plasma can be kept for a few days frozen at –20°C; if a deep-freezer at -40°C is available, plasma can be stored for 2-3 months.

Serum or plasma for blood group serology

Kept for up to 1-2 weeks at 4°C until completion of antibody tests or transfusions. Samples may be stored frozen at –20°C for three months or longer if they are to be retained for subsequent cross-matching.

6. BLOOD COUNTS BY ELECTRONIC ANALYSERS

The commonly used counters are based on the aperture impedance principle in which blood cells which are non-conductors of electricity are diluted in a buffered electrolyte solution and allowed to pass through the orifice of an aperture tube between two electrodes. Interruption of the current by the non-conducting cells alters the electric charge and a pulse is produced. The amplitude of each pulse is proportional to the volume of the cell and the cell count is determined from the total number of pulses obtained from a measured volume of blood.

Many electronic counters are available of varying complexity. Earlier models required the users to calibrate the counters individually and to identify the appropriate settings for distinguishing red cells from leucocytes and platelets. Most modern analysers include measurement of haemoglobin, packed cell volume and absolute values (MCV, MCH and MCHC). These analysers are preset. It is, however, necessary to check their accuracy under certain circumstances and if necessary to adjust the settings by means of blood count calibrators.

Calibrators

These are usually produced by instrument manufacturers who provide stated values for the various blood count parameters. Suitable preparations of preserved blood can also be made by an individual laboratory as described in WHO document LAB/97.2 (See #1 Ref 1). Red cell count and leucocyte count must be established by careful measurement using calibrated pipettes to dilute the blood and haemocytometer chambers; a sufficient number of cells must be counted in the haemocytometer to reduce distribution error to 2%. This work is laborious and requires considerable technical skill; it should be undertaken by a senior technician. Haemoglobin concentration is obtained by the ICSH Reference method (See #7) and packed cell volume by a standardized method (See #8).

Calibration check is necessary when an analyser is first put into operation. It may also be required if the daily quality control tests show a drift. [*Sudden* loss of control indicates malfunction so that recalibration is inappropriate until the cause has been identified]. Calibration check is also necessary whenever any component is replaced and as a routine once or twice a year.

Controls

Stabilized blood preparations are available commercially or can be produced by an individual laboratory as described in the WHO documents LAB/97.2 and LAB /98.4 (See #1 Refs 1 and 3). They are easier to produce than calibrators as controls are intended only to check precision and reproduciblity of the analyser, so that the exact values are unimportant . Moreover, the stabilized preparations can be used for at least 2-3 months.

Procedures

The method for using any specific instrument will depend on the instructions provided by the manufacturer, and these should be incorporated in the laboratory SOP requirements. The following aspects must be included.

Start-up

- ➢ Check diluent/reagent levels and expiry dates
- ➢ Check that all plugs and cables are connected and that the electric current is stable
- ➢ Check that waste bottle is empty and that waste is draining adequately
- ➢ If results are automatically printed out, check that the printer has paper and ink supply.
- ➢ Turn on power supply in accordance with the instructions provided by the manufacturer
- ➢ Set date and test number
- ➢ Check instrument pressure gauges and vacuum

Run the counter with diluent alone to check background count (i.e. electronic noise and particulate material in diluent solution). This should be within acceptable limits specified by the manufacturer. Typical values are as follows:

RBC: $<0.03 \times 10^{12}/l$

WBC: $<0.04 \times 10^{9}/l$

Haemoglobin: <0.2g/dl

Platelets: $<5 \times 10^{9}/l$

- ➢ Use a fresh blood sample to prime the instrument in accordance with the instructions
- ➢ Carry out a count on the current control preparation(s). Check that results are within control limits (see # 20).
- ➢ The counter is then ready for handling the day's routine specimens.

Test procedure

All blood specimens must be mixed manually by inverting twenty times or left for 3-5 minutes on a mechanical mixer immediately before diluting. Count the diluted blood suspension immediately; if left to stand, make sure that the suspension is well mixed immediately before counting.

Specimens must not be left unstoppered.

Keep the aperture cell in a container filled with diluent when not being used.

Quality control

In addition to the control at the start of the day's work, include a control sample with the batch of tests once or twice during the day. Plot the results on a control chart. If there is evidence that the counter is out of control (see # 20), check possible sources of error as listed below and if necessary check the settings of the counter with calibrator.

Shut down

At the end of the day's work decontaminate the system by aspirating hypochlorite solution (25 ml diluted to 500 ml in distilled water) throughout the instrument *via* both sample and reagent lines. Then wash through with distilled water

Empty the waste bottle if this is a component of the system, or check that the connected drainage system is patent. Pour disinfectant into the waste trap to neutralise any bacterial growth in the effluent.

Turn off power supply in accordance with the instructions provided by the manufacturer.

Overnight keep the aperture in a container filled with distilled or deionized water. If the counter is not used every day, check regularly that the aperture has not been allowed to dry and make sure that no salt crust forms.

When not in use, keep the instrument covered to avoid dust contamination.

Maintenance schedule (weekly)

Check the quality control chart for evidence of drift (i.e. systematic errors) or sudden errors (i.e. random errors) - See # 20.

In a large hospital where at least 100 blood counts are performed each day check the daily averages of the absolute values (MCV, MCH and MCHC) for any drift or sudden change outside an established 2SD.

Clean the orifice and cell by gently agitating with a fine brush and flushing several times with diluent. If there is blockage of the orifice remove it from the instrument and backflush diluent under gentle pressure. **Never attempt to clear the orifice with a sharp device such as a needle or blade.**

For stubborn protein deposits soak the orifice and cell overnight in a detergent or other cleansing agent as recommended by the manufacturer; then wash in water before refitting onto the instrument.

- ➢ Check seals to determine the possibility of leakage and vacuum loss
- ➢ Check integrity of tubing
- ➢ Check stock of reagents, diluents, report forms and disposables

In a special logbook record the dates of all maintenance checks, replacements of components, down-time and identifiable causes of malfunction, servicing by manufacturer's agent, recalibrations, traceable batch numbers of calibrators and controls.

Sources of error

1. Inadequate mixing of specimens or diluted suspensions before counting
2. Pipetting fault giving inaccurate dilution
3. Inappropriate dilution, leading to excessive coincidence error
4. Delay in transferring suspension to a counting vial and performing the count
5. Electric interference or voltage fluctuation
6. External noise contamination e.g.,from other electrical equipment
7. Aperture blockage
8. Aspiration failure due to blockage, leaks or loss of vacuum
9. Contaminated diluent giving high background count
10. Air bubbles from vigorous shaking or dispensing
11. Incorrect threshold setting or faulty electric connection leading to deficient aperture current
12. Residual traces of lytic agent or detergent cleaner causing haemolysis or artefactual red-cell volume changes

Validation of Results

An initial check must be made that the report matches the request form.

A senior technologist or doctor must then ensure that:

- The results are technically valid and appropriate to the patients' clinical condition
- Blood film has been made if requested, or if indicated from the blood count and clinical information
- Other tests are being dealt with by the laboratory
- Quality control procedures have been carried out and are satisfactory

If previous blood counts have been carried out, the present results do not differ from the last count by more than the following amounts without clinical reason:

Haemoglobin – 2 g/dl
MCV – 6 fl
MCH – 5 pg
WBC – Normal to abnormal or Abnormal to very abnormal
Platelets – 50%

Transmission of the results can then be authorized as either a *final report* or a *provisional report* (if awaiting results of the blood film and /or other tests).

Blood Films

It is necessary to distinguish between a differential leucocyte count (DLC) and a blood film survey for which a general comment will be made on the cells. When a DLC has not been specifically requested, a film survey will be performed without a full DLC unless indicated by the blood count results and clinical circumstances. The decision to examine a blood film should be made by a suitably qualified and experienced technologist or doctor.

Blood films must always be examined in the following circumstances:

- First-time blood counts with any abnormal parameters
- Haematology out-patients at every visit
- Weekly on Oncology clinic patients
- Weekly on patients undergoing radiotherapy or cytotoxic drug treatment
- All neonatal and paediatric patients
- Patients with lymphadenopathy, hepatosplenomegaly, or with glandular fever or flu-like symptoms
- Patients with fever in or coming from malaria area (unless diagnosis of malaria has already been confirmed)
- When the blood count by an automated analyser has been flagged – e.g. because of microcytosis, agglutination, cell fragments, platelet clumps.

7. HAEMOGLOBINOMETRY

A reference preparation of haemoglobincyanide (HiCN) was developed by the International Council for Standardization in Haematology in 1965, and subsequently established by WHO as the internationalreference standard. This method is generally recommended for measuring haemoglobin in routine laboratories. Preparations equivalent to the international standard are available commercially.

Principle

Blood is diluted in the ICSH reagent based on Drabkin's reagent. This contains potassium ferricyanide, potassium cyanide and a non-ionic detergent. Red cells are lysed and the released haemoglobin is converted to cyanmethaemoglobin (HiCN). A fresh solution should be made every 2-3 weeks and stored in a dark bottle in a cupboard away from direct sunlight.

The absorbance of the solution is measured in a spectrometer/ spectrophotometer at a wavelength of 540nm; from the molecular weight and extinction coefficient this measurement can be converted to haemoglobin concentration. If a spectrometer is not available it can be measured in a simple colorimeter or photometer using an appropriate yellow-green filter. The concentration of haemoglobin in the test sample is then calculated by comparing the light absorbance with that of the reference standard (or a commercial equivalent). The advantages of this method over oxyhaemoglobin are that the reference preparation is stable and all forms of Hb are converted with the exception of sulphaemoglobin.

Use of spectrophotometer

Ensure that lenses and mirrors are free of dust and fingerprints. If necessary, clean with a tissue or soft cloth.

Connect to a stabilized power supply (batteries if stability of electric supply cannot be assured).

Avoid excessive exposure of the photocell to light. As a precaution, keep the light-path blocked by closing the shutter or inserting the cuvette cover in the holder in place of a cuvette when not actually performing a measurement.

Clean cuvettes by soaking for a few hours in a mild detergent. If necessary, clean the inside surfaces with a cotton wool swab. Rinse with distilled water, invert to dry and store cuvettes in a dust-free container. Discard cuvettes that are scratched or broken.

Operation procedures

Check dark current

Switch on and set the meter to read infinity on the absorbance scale with the light-path blocked. This setting should be checked each time it is switched on.

Check stability of readings

Fill a cuvette with distilled water, place in the cuvette holder, set the absorbance reading to a convenient part of the scale such as 0.100. Repeat this reading at 5-minute intervals until it is steady. In routine use never take measurements before this warm-up period is complete and readings are stable (usually 5-30 minutes).

Cuvettes

After filling a cuvette with solution, wipe the outside clean and dry and check that there are no bubbles or particles. Handle it by the top or sides and do not touch the optically transmitting surfaces. After inserting the cuvette into its holder check that minor movements do not affect readings.

If cuvettes are used in pairs (i.e. test solution and blank in two different cuvettes) they must first be matched. To do this, fill several cuvettes with HiCN solution. Set the first cuvette at a convenient reading point (e.g. 0.100). Read each cuvette in turn and select only those which differ by less than 0.005 units.

Solution volume

Check the volume of solution required by adding increasing volumes to a cuvette and noting the amount necessary to produce a constant reading. In routine practice it is advisable to use more than the minimum volume but it is not usually necessary to fill the cuvette to the top.

External illumination

With a filled cuvette in position, check that the reading is not affected by variations in external light from above the instrument. If this occurs the instrument should either be moved or the external light excluded by closing the lid or blocking off the top of the cuvette holder.

Photometers/colorimeters

These instruments are generally easier to use but the principle of operation is the same.

Test procedure

1. Add 20 µl of well mixed EDTA blood to 5 ml ICSH (Drabkin) reagent in a test-tube to give a 1-in-251 dilution. Stand for a minimum of five minutes to allow complete conversion to HiCN.
2. Pour into a cuvette which is paired with a second cuvette containing reagent alone.
3. Set the spectrometer to a wavelength of 540 nm or a photometer with a yellow-green filter, and measure the absorbance of the diluted test samples and reagent solution.
4. Calculate the haemoglobin concentration using the following formula:

   Hb (g/l) = (A540 test / A540 standard) x conc. of standard (mg/l)* x (251 / 100)

   *given on the label of the vial.

Reference standard and control preparation

A reference standard with a concentration of about 800 mg/l is available from WHO, and similar material is available commercially with stated haemoglobin concentration. The main purpose of the reference is as a check that the spectrophotometer is accurate

Control preparations of whole blood should be used alongside the batch of tests in order to check that the test is being performed reliably by means of a control chart (See #20).

Calibration curve

This is prepared in order to check linearity of response of the instrument.

Set up a series of five tubes. Into the tubes pipette sequentially 6, 4.5, 3.0, 1.5 and 0 ml of HiCN Reference standard. Make the volumes up to 6 ml in each tube by adding, in the sequence, 0, 1.5, 3.0, 4.5 and 6 ml of reagent, giving, respectively, 100%, 75%, 50% and 25% of the original concentration of the reference standard. Measure the absorbance at 540nm of each solution. Plot on arithmetic graph paper with Hb concentration on the x axis and absorbance on the y axis.

All points should fall on a line passing through zero. If there is irregularity in the graph, check the function of the spectrophotometer.

This check should be carried out whenever the spectrometer is serviced or repaired, and also routinely approximately every six months. .

Calibration Table

When many blood samples are tested each day it is convenient to read results from a calibration graph (or a table derived from it) that converts the readings of absorbance to haemoglobin concentration as described above. In practice, readings will be reliable only if they lie within the linear part of the curve. With most instruments the most accurate and precise readings are in the range 0.2-0.7 absorbance units. If a reading occurs outside this range the analysis should be repeated using a different, more appropriate, dilution of the sample. The calibration table is valid only for the instrument on which the values have been obtained. Each day the table should be rechecked by measuring a sample from a previous batch as well as a control preparation.

Sources of error

- Collection faults
- Inadequate mixing of specimen before sampling
- Pipetting or dilution errors
- Clots in specimen
- Reagent left on bench exposed to prolonged light or allowed to freeze
- Reference preparation out of date or deteriorating, especially if it has been left standing on the bench for some time after opening the vial.
- Improper instrument calibration
- Instrument fault
  - Insufficient warm-up time
  - Lamp failing or overheating – may require replacement
  - Failing photocell
  - Mains voltage variation
  - Non-linearity
  - Cuvettes incorrectly positioned
  - Cuvettes dirty or scratched

8. PACKED CELL VOLUME (MICROHAEMATOCRIT)

A capillary tube filled with blood and sealed at one end is centrifuged at high speed in a microhaematocrit centrifuge. The height of the resulting column of red cells is measured, and calculated as a fraction of the height of the entire column of blood.

There is a small but consistent error in all microhaematocrit determinations, about 1-3%, due to some plasma being trapped between red cells after the blood has been centrifuged. The International Council for Standardization in Haematology (ICSH) has recommended that in routine practice no correction should be applied

Method

Materials

Plain non-heparinized capillary tubes (75mm long with a bore of 1.155 ± 0.085mm) made from soda lime glass tubes are recommended. for blood in EDTA; heparinized capillary tubes should be used for blood collected directly from a skin puncture.

Sealing the base of the tube requires a clay material such as Cristaseal, Miniseal or Seal-Ease. These materials must be kept in a sealed container or plastic bag when not being used, as it should not be allowed to dry.

Procedure

1. When using EDTA anticoagulated blood, thoroughly mix the blood samples and fill capillary tubes to approximately three quarters of their length with the samples.
2. Wipe excess blood from the outside of each tube, then seal by pushing gently into the plastic material such as Cristaseal. Make sure that the top of the clay can be easily seen, and check that the seal has a flat surface. Discard any tube where the upper surface of the clay is noticeably uneven.

3. Place the tubes in the microhaematocrit centrifuge with the sealed end pointing outwards. Note the position and identity of each tube.
4. Firmly secure the inner lid of the centrifuge, then close the outer lid. Centrifuge for five minutes at 12000 *g* (but see below).
5. As soon as the centrifuge has stopped, remove the tubes and stand them upright until they are read. It is important that the cells are not allowed to settle and that reading takes place with the minimum of delay. If delay by more than a few minutes is unavoidable, seal the top of the tubes as well to avoid evaporation of the plasma.
6. Using arithmetic graph paper, measure the length of the red-cell column excluding the buffy coat and also the total length. Calculate the PCV as a fraction, i.e. ml/ml):

$$\frac{\text{Number of ruled lines in length of red-cell column}}{\text{Number of ruled lines of entire column}}$$

If the PCV is greater than 0.50, recentrifuge for a further five minutes to ensure minimal plasma trapping.

Maintenance

➢ Check the centrifuge for blood spillage at the end of the day. If there has been any breakage or leakage from the tubes during centrifuging, clean the instrument wearing gloves. Remove glass pieces with forceps and swab the rotor and other exposed parts with freshly prepared 10% solution of hypochlorite. Leave for 30 minutes and then swab with water and dry (see also #3 *'Precautions during specimen handling'*)

➢ Check the efficiency of centrifugation of a new instrument and thereafter at six-month intervals. For this, fill two capillary tubes with a normal blood sample and centrifuge for five minutes; remix the original blood sample, fill two more tubes and centrifuge for six minutes. Repeat the procedure consecutively for 7 and 8 minutes. When the centrifuge has an adequate force, all tubes will give the same reading ±2%. If this does not occur within five minutes, it will be necessary to centrifuge for a longer time to obtain the correct PCV. In this event the rotor should be checked by an expert.

Sources of error

➢ The EDTA (dipotassium salt for choice) should be in a concentration of 1.5 mg/ml. This concentration will be exceeded if a reduced amount of blood is added to a standard specimen container or if the blood is taken up in a capillary which contains anticoagulant. This will cause shrinkage of the red cells with falsely low PCV.

- Storing blood beyond 6-8 hours results in an artefactual increase in PCV, especially in hot climates
- Inadequate mixing of blood before sampling.
- A clot in the specimen.
- Inadequate packing in prescribed time (see '*Maintenance*' above)
- Including the buffy-coat layer in the reading of the red cell level.
- Continuous use of centrifuge for several hours, especially in hot climates, will result in its becoming over-heated, causing the samples to lyse.
- Evaporation of plasma during centrifugation, especially if the centrifuge over-heats, or if the spun sample is left for a time before being read.

9. MICROSCOPY

The modern compound microscope is an indispensable piece of apparatus in the laboratory and a theoretical knowledge of its working principles is essential. It is a precision instrument and its efficient use requires a degree of skill and some basic training. Time spent on the systematic setting-up of the microscope is vital, as the use of a poorly set-up microscope will result in fatigue, headaches and possible eye strain.

In essence, a microscope consists of an objective lens and eyepiece along with the mechanism necessary for focusing them, and an illumination system for passing a bright light through the object under examination and into the objective lens. The condenser (between the light source and specimen) collimates the light rays in the plane of the specimen; the objective produces a magnified image within the microscope; the ocular further enlarges this image and enables it to be seen by the eye.

Objectives

There are usually three objectives in a nosepiece. The commonly used objectives are x10, x40 and x100. As a rule, the x100 objective requires oil immersion.

Eyepieces

There may be one eyepiece (monocular) or two (binocular). Their magnifying power is marked on them; as a rule this is x6 and x10. Magnification of the object is obtained by multiplying the magnifying power of the objective by that of the eyepiece:

Objectives			
	x10	x40	x100
Eye-pieces			
x6	60	240	600
x10	100	400	1000

Illumination system

Preferably the light source should be a tungsten filament lamp. This may be reflected into the microscope by a mirror or provided directly by a low-voltage light bulb fitted into the base of the microscope. The microscope must not be

used in direct sunlight without a grey density filter to cut down the intensity of the light.

The condenser is a large convex lens which converges the light onto the object to be examined. It is placed between the light source and the stage; it can be raised for maximum illumination and lowered for minimum illumination.

The iris diaphragm, inside the condenser, is used to increase or reduce the amount of light that passes into the condenser.

Correct adjustment of the condenser lens is essential to produce a clear image. The condenser must be positioned in order to provide critical illumination of the field; light rays from the light source must be brought to focus in the plane of observation and the light transmitted must almost fill the objective lens.

Setting up a microscope for critical illumination

- Choose the x10 objective.
- Focus on a slide.
- Close the light source iris diaphragm down to its minimum.
- Close the iris diaphragm on the condenser down to its minimum.
- Centre the light source using the two centering screws on the condenser.
- Focus the condenser until a faint orange or red halo is seen around the central light source.
- Open the light source diaphragm until light fills the whole field of view.
- Remove the fixed eyepiece, look down the tube and open the iris diaphragm on the condenser so that light fills about 2/3 to 3/4 of the field of view.
- Adjust the light intensity to suit personal preference.

Focusing the object

Low-power objective (x10): Rack the condenser down. Lower the objective until it is just above the slide preparation. Raise the objective using the coarse adjustment screw until a clear image is seen in the eyepiece. If there is insufficient illumination, rack the condenser up slightly.

High-power objective (x40): Rack the condenser half way down. Lower the objective until it is just above the slide preparation. Using the coarse adjustment, raise the objective very slowly until a blurred image of the field appears. Bring into focus using the fine adjustment. Raise the condenser to obtain adequate illumination.

Oil-immersion objective (x100): Dry-stained preparations must be used. Place a drop of immersion oil on the part of the slide to be examined. Rack the condenser up. Lower the objective until it is in contact with the oil. Bring it as close as possible to the slide but avoid pressing on the preparation. Do not force the objective against the slide.

Look through the eyepiece and turn the fine adjustment very slowly until the image is in focus.

Precautions

- Cover the microscope when not in use.
- Remove all the immersion oil from the objective immediately after use.
- Before and after use clean optical parts with lens tissue, absorbent paper or soft cloth and cleaning fluid composed of equal volumes of ether and isopropyl alcohol
- Do not use xylol for cleaning the lenses, nor soak them in alcohol as this may dissolve the cement
- Do not rack the objective downwards to bring the object into rough focus whilst still looking down the microscope, as you may damage the lens if you crash through the slide.
- Do not attempt to dismantle the objectives.
- Do not lubricate the microscope with any old oils or sprays lying around - they will congeal and damage the mechanism.
- Turn the light power off when not using the microscope.
- In hot, humid climates, in order to prevent fungi growing on the lenses, place the microscope every evening in a warm cupboard heated by one or two 40-watt light bulbs which are left on continuously to achieve a constant dry, warm atmosphere.
- In hot, dry climates the main problem is dust. Apart from keeping the microscope covered when not in use, at the end of the day's work clean the microscope by blowing air on it with a rubber bulb; then finish cleaning the lenses with a lens brush or a fine paint brush. If dust particles remain on the surfaces of the objectives, remove with clean paper.

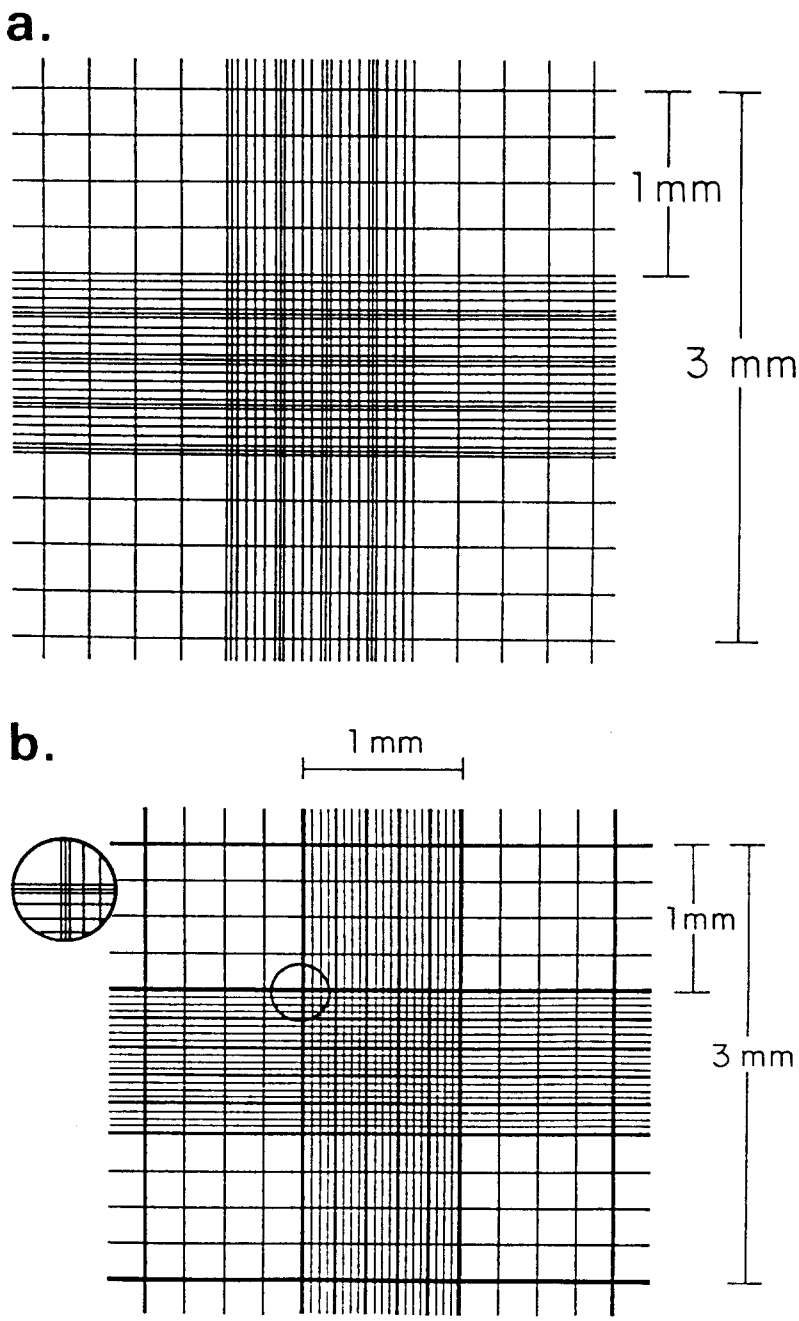
Guidelines on Standard Operating Procedures for Haematology

(SEA/HLM/320)

Corrigendum

In the document SEA/HLM/320, please *insert* the following figure after first para on page 35:

Figure 1: Hemocytometer counting chamber – (a) Neubauer and (b) Improved Neubauer



10. VISUAL HAEMOCYTOMETRY

Visual haemocytometry requires accurate dilution of a blood sample with precision pipettes, and a precision-made glass counting chamber consisting of a thick glass slide with a depressed central area which is converted into a volumetric chamber when overlaid by a cover glass. To facilitate counting, the chamber has a ruled grid. The type of chamber used in haematology for performing cell counts is usually the "improved Neubauer". This is illustrated in Figure 1. It has four corner squares and a central square, each 1x1 mm (=1 mm^2) and the total chamber area is 3x3 mm (= 9 mm^2). The corner squares are sub-divided into 16 smaller squares of 0.25x0.25 mm, while the central square is divided into 25 smaller squares, 0.2x0.2 mm, and each of these is further divided into 16 squares of 0.05x0.05mm, so that 80 of the smallest squares provide an area of 0.2 mm^2. The depth is 0.1 mm.

Care is needed to ensure the correct positioning of the special cover glass on top of the counting chamber. The cover glass itself must be optically flat and should provide a firm cover without bowing. When a cover glass is correctly applied to the surface of a counting chamber, Newton's rings first form and then disappear showing that the relative position of cover glass and chamber is sufficiently even to ensure a constant depth of chamber throughout its entire area.

Visual haemocytometry is an acceptable alternative to electronic counting for WBC and platelet counts. It is not recommended for routine RBC counts because the number of cells which can be counted within a reasonable time in the routine laboratory (e.g. about 400) will be too few to ensure a precise result (see below). However, it can be used as a reference method if a large number of cells is counted, thus reducing the inherent error to $<2\%$.

Method

1. Wash the chamber and the cover glass in running tap water immediately after use to remove all residual organic matter. Before use, wash in alcohol and dry.
2. Place the chamber on a flat surface. Press the cover glass down, checking that it is in the correct position by the presence of several Newton's rings that will appear on each side. If rings do not form, reclean the chamber and the cover glass. If rings still do not form, try again with another cover glass.

3. The blood, diluted in the appropriate fluid, is mixed on a mechanical mixer for 2 to 3 minutes, and taken up into a capillary tube.
4. Fill both sides of the chamber by allowing the diluted blood to flow under the upper edge of the cover glass in one smooth action . The chamber must be cleaned and the filling process repeated if any of the following defects occur:
   - The fluid overflows into the moat
   - The chamber area is not completely filled
   - Air bubbles occur anywhere in the chamber area
   - Any debris appears in the chamber area
5. Place the chamber into a petri dish which contains a small piece of damp blotting paper. Leave undisturbed in a horizontal position, not exposed to direct sun or other heat source and free from drafts and vibrations for a sufficient time for the cells to settle - red and white cells 2 to 3 min., platelets at least 20 min.
6. Scan the entire chamber area at low magnification to check that the cells are distributed evenly throughout the ruled grid.
7. Focus on the grid at a selected magnification (e.g. x400 for red cells and platelets; x250 for white cells) and count all the cells present in a sufficient number of squares to ensure an acceptable level of accuracy (see Fig. 2 & 3 below). Use a suitable convention to ensure that cells are not counted twice, e.g. count only cells that touch the top and right-hand margins of a square, omitting from the count of that square any cell that touches its bottom and left-hand margins. If during the counting period the chamber dries out, the preparation must be discarded. Evaporation can be delayed by setting up the microscope in a cool area away from direct sunshine. Avoid heating the chamber by exposing it to the microscope lamp light beam only during the counting procedure.

Count variance

In all particle counting procedures there is an inherent error due to the fact that the particles are distributed in a random way (Poisson distribution). This variance (σ) is calculated as ($\sqrt{n}$) ÷ n where n is the total number of particles counted and variance is expressed as a percentage. For example, a count of 100 will have a variance of 10%; there is a 95% chance that the count could be any number between mean $n+2\sigma$ and $n-2\sigma$ in the adjacent squares, i.e. between 80 and 120. By contrast, when 2500 particles are counted, the variance is reduced to 2%; clearly, this amount of labour for each blood count is impractical for a routine test, and it is necessary to define a count that is sufficiently accurate to be clinically valid.

Total Leucocyte (WBC) Count

Make a 1-in-20 dilution of blood by adding 0.02 ml of whole blood to 0.38 ml of diluting fluid (2% acetic acid lightly coloured with 1% crystal violet) and mix for 2-3 minutes.

The red cells are lysed but the leucocytes remain intact. Before filling the chamber, inspect the fluid to ensure it is clear, unless the white cell count is markedly raised, in which case the solution will appear turbid, and a greater dilution should be used. Fill the chamber and leave the cells to settle for 3 - 5 minutes.

Place the chamber on the microscope stage and, using the x25 objective, count the number of cells seen in the four large (1 mm^2) corner squares observing the criteria for inclusion and exclusion of cells touching the borders. The leucocytes will be seen more clearly by partially closing the condenser diaphragm.

Calculation

Cell count (/l): $= N \times (D/A) \times 10 \times 10^6$

where N = total number of cells counted, D = dilution of blood, A = total area counted (in mm^2), 10 = factor to convert area to volume (in μl), assuming a chamber of 0.1 mm depth, and 10^6 = factor to convert count per μl to count per litre.

Example

If 200 cells were seen in the four large corner squares (= 4 mm^2),

$$\text{Count} = 200 \times (20/4) \times 10 \times 10^6 = 1000 \times 10 \times 10^6 = 10.0 \times 10^9/l$$

Sources of error

- Insufficient anticoagulation with clot in specimen
- Inadequate mixing of specimen
- Incorrect dilution or faulty dilution, especially due to using chipped or dirty pipettes or failure to clean blood from outside of pipette
- Inadequate mixing of diluted sample before filling counting chamber
- Faulty chamber, especially due to using non-standard cover-glass
- Flooding of chamber with excess sample
- Air bubbles, fingerprints, oily surface or debris in chamber

- Failing to count all the cells in the squares or conversely including artefacts in the count
- Excessive distribution error due to too few cells counted
- Nucleated red cells included in count.

Platelet count

Platelet diluting fluid consists of 1% ammonium oxalate. This must be prepared using scrupulously clean glassware and glass distilled water. Not more than 500 ml should be prepared at a time. This solution must be filtered through a micropore filter and stored at 4^0C.

Make a 1:20 dilution of blood in the diluent. Mix on a mechanical mixer for about 10 minutes. Then fill the chamber as described for the WBC and leave the preparation for 20 minutes (but no longer than 30 minutes) for the platelets to settle, in a wet chamber (i.e. petri dish with a small piece of damp blotting paper).

Place the chamber on the microscope stage and, using the x40 objective, focus on the central square . The platelets appear as small (but not minute) refractile particles and this characteristic is greatly enhanced using phase-contrast microscopy

A count of about 200 will suffice, as this has a variance of 7%. When the count is normal this number of counts will be obtained in 80 of the smallest squares (=0.2 mm^2). If the platelet count is low it may be necessary to count the entire central area (=1mm^2), remembering to take this into account in the calculation.

Calculation

Platelet count (/l): = N x (D/A) x 10 x 10^6

where N = total number of platelets counted, D = dilution of blood, A = total area counted (in mm^2), 10 = factor to convert area to volume in (μl), assuming a chamber of 0.1 mm depth, and 10^6 = factor to convert count per μl to count per litre.

Example

If 200 cells were seen in 80 small squares (= 0.2 mm^2),

Count = 200 x (20/0.2) x 10 x 10^6
= 200 x 100 x10 x 10^6
= 200 x 10^9/l.

Sources of error

- Imperfect venepuncture with delay in adding blood to anticoagulant
- Inadequate amount of anticoagulant with clot formation
- Platelet clumps
- Diluent not particle-free
- Presence of red-cell fragments, stroma of haemolysed cells and other debris
- Other errors common to all cell counts: see Sources of error under "Total leucocyte (WBC) count".

Red Cell (RBC) Count

As referred to above, the labour in doing a visual RBC with a reasonable degree of precision is warranted only for establishing the count of a reference preparation and occasionally in special cases , e.g. when it is necessary to have a reliable measurement of MCV or MCH.

If the blood has been stored in a refrigerator, allow it to equilibrate at ambient temperature on the bench. Then mix well on a mechanical mixer for 3-5 minutes or by inverting the tube at least 20 times (Do not shake the tube as this will cause foaming, making accurate pipetting impossible).

Dilute the blood 1:200 in a solution of 10 ml 40% formalin in a litre of 32g/l trisodium citrate.

Fill the counting chamber in exactly the same way as for WBC, with the same precautions. Leave in a wet chamber (Petri dish with a small piece of damp blotting paper) for 2-3 minutes for the cells to settle. Then count the cells at a magnification of x40. A sufficient number of cells must be counted to minimize errors due to variable cell distribution. To obtain a variance of 2% it is necessary to count about 2500 cells. All the cells in the entire central square ($=1mm^2$) should be counted and, if necessary, the count should be continued on the other half of the chamber.

Calculation

Cell count (/l): $= N \times (D/A) \times 10 \times 10^6$

where N = total number of cells counted, D = dilution of blood, A = total area counted (in mm^2), 10 = factor to convert area to volume (in μl), assuming a chamber of 0.1 mm depth, and 10^6 = factor to convert count per μl to count per litre.

Example

If 2500 cells were seen in the central area (= 1 mm^2),

RBC = 2500 x (200/1) x 10 x 10^6 = 500 000 x 10 x 10^6 = 5.00 x 10^{12}/l.

The count variance is 2%. - i.e. 95% of counts will be between 4.8 and 5.2 x 10^{12}/l.

Red Cell Indices

These are Mean cell volume (MCV) in femtolitre (fl), Mean cell haemoglobin (MCH) in picogram (pg) and Mean cell haemoglobin concentration (MCHC) in g/dl or g/l (sometimes also expressed as percentage). They are calculated from Haemoglobin, RBC and Packed cell volume.

11. PREPARATION AND STAINING OF BLOOD FILMS

The film is made from a drop of blood spread evenly on a slide and stained. While blood film preparation is a simple and straightforward procedure, the quality of blood films in many laboratories is often poor. It is important to ensure that the film is well made, and stained with a Romanowsky stain, as the validity of the blood film evaluation, and the differential in particular, is dependent upon having a well-made and well-stained film.

Preliminary preparation

A. Spreaders

Select a glass microscope slide with at least one smooth end.

Using a glass cutter, break off one corner of this end, leaving width of c15 mm to serve as the spreader.

Each spreader can be used repeatedly provided that the spreading edge remains smooth. The edge must be wiped carefully and dried before and after each use, and the slide must be discarded if the spreading edge becomes chipped.

B. Clean slides

It is essential to use clean, dry, dust-free slides: remember that grease and residual detergent are equally liable to spoil a blood film.

New slides: Boxes of clean grease-free slides may be available. If not, proceed as follows. Leave overnight in a detergent solution. Then wash thoroughly in running tap-water, rinse in distilled water if available and wipe dry with a clean linen cloth. Before use, wipe the surface with methylated spirits (95% ethanol) or methanol and dry with a clean cloth; then keep covered to avoid having dust settle on the surface.

Used slides: Discard in detergent solution, heat to about 60°C for 20 minutes. Then wash in running tap-water, rinse in distilled water if available and treat as for new slides as described above.

Spreading the blood

Ideally, films should be prepared directly from a skin puncture or within one hour of blood collection into EDTA.

Adequate mixing of the specimen is necessary prior to film preparation. Using a wooden stick or glass capillary, place a small drop of well-mixed blood in the centre line of a slide about 1 cm from one end. Without delay, place a spreader in front of the drop at an angle of about 30° to the slide; move it backwards to make contact with the drop. The blood should run quickly along the contact line. With a steady movement of the hand, spread the drop of blood along the slide. The spreader must not be lifted off the slide until the last trace of blood has been spread out; with a correctly sized drop the film should be about 3 cm in length. It is important that the film of blood finishes at least 1 cm before the end of the slide. The thickness of the film can be regulated by varying the size of the drop of blood, by varying the pressure and speed of spreading, and by changing the angle at which the spreader is held: with anaemic blood the correct thickness is achieved by using a wider angle and, conversely with polycythaemic blood, the angle should be narrower.

After the film has been made, let it dry in the air. Write the patient's identification (and date if appropriate) in pencil on the edge of the film at its origin.

Fixing the films

To preserve the morphology of the cells, films must be fixed as soon as possible after they have dried. It is important to prevent contact with water before fixation is complete. Methyl alcohol (methanol) is the choice, although ethyl alcohol ("absolute alcohol") can be used. To prevent the alcohol from becoming contaminated by absorbed water, it must be stored in a bottle with a tightly fitting stopper and not left exposed to the atmosphere, especially in humid climates. Methylated spirit must not be used as it contains water.

To fix the films, place them in a covered staining jar or tray containing the alcohol for 2-3 minutes. In humid climates it might be necessary to replace the methanol 2-3 times per day; the old portions can be used for storing clean slides.

Romanowsky staining

Romanowsky stains are universally employed for staining blood films and are generally very satisfactory. The main components of a Romanowsky stain are:

1. A cationic or basic dye such as azure B, which binds to anionic sites and gives a blue-grey colour to nucleic acids (DNA or RNA), nucleoproteins, granules of basophils and weakly to granules of neutrophils
2. An anionic or acidic dye such as eosin Y, which binds to cationic sites on proteins and gives an orange-red colour to haemoglobin and eosinophil granules.

There are a number of different combinations of these dyes which vary in their staining characteristics. May-Grunwald-Giemsa is a good method for routine work. Wright's stain is a simpler method, whilst Leishman's is also a simple method which is especially suitable when a stained blood film is required urgently or the routine stain is not available (e.g. at night). Field's stain is a rapid stain used primarily on thin films for malarial parasites. Whichever method is used, it is important to select dyes that are not contaminated with other dyes or metallic salts.

Staining can be carried out in a jar or on a staining rack

May-Grunwald-Giemsa method

1. Air dry slides.
2. Fix in Methanol for 2-3 minutes at room temperature.
3. Stain for 15 mins in May-Grunwald stain freshly diluted with an equal volume of buffered distilled water, pH6.8
4. Stain for 10 minutes in Giemsa stain freshly diluted with buffered distilled water, pH 6.8 (1/9)
5. Wash in running tap-water and then leave for 3-4 minutes in buffered distilled water, pH 6.8
6. Allow to dry. Mount with a coverslip if required, using DPX neutral mounting medium.

Leishman's method

- ➢ Air dry slides
- ➢ Flood with neat Leishman's stain for three minutes; as the stain's formulation includes methanol, this will fix the cells.
- ➢ Dilute the stain on the slide with an equal amount of buffered water, pH 6.8, adding the water slowly with a plastic Pasteur pipette and mixing by sucking the stain up and down with the pipette.
- ➢ Leave the slide for approximately 12 minutes; the appearance of a polychromatic 'scum' on the surface of the slide is merely a result of oxidation of the dye components and can be ignored.

- ➢ Wash off excess stain with slowly-running tap water and flood slide for one minute with buffered water, pH 6.8.
- ➢ Dry the slide and mount with a coverslip if required, using DPX mounting medium.

Field's stain method

- ➢ Air dry the film and fix in methanol for 2-3 minutes.
- ➢ Dip the slide into Field's Stain Solution - B (red) and agitate gently for 10 seconds.
- ➢ Rinse slide with buffered distilled water, pH 6.8
- ➢ Drain excess water and dip slide into Field's Stain Solution - A (blue) and agitate gently for 15 seconds.
- ➢ Rinse slide with buffered water, pH 6.8, for 30 - 60 seconds.
- ➢ Dry the slide upright and mount with a coverslip if required, using DPX mounting medium

Microscopic Examination of Blood Films

Every film should first be inspected at low power (x10) before general examination is undertaken with the x 40 lens. The x100 oil-immersion lens should generally be reserved for examining unusual cells and for looking for fine details of cytoplasmic granules, punctate basophilia, etc. It is essential to cover the film with a coverglass as this permits the film to be examined with the x 10 and x 40 lenses Thus, when the film is completely dry cover it by a rectangular cover-glass either permanently with a neutral mountant (DPX) or with immersion-lens oil (e.g. oil of cedarwood) as a temporary mount so that the cover-glass can be removed for re-use with successive slides. The cover-glass must be wider than the film so that cells at the edges of the film can be properly examined.

Survey the film at x10 magnification to get a general impression of its quality. Then find an area where the red cells are evenly distributed, just touching but not overlapping, and study their morphology at x 40. At the same time, scan the film to get an impression whether the leucocytes are increased or decreased, identify any unusual or abnormal cells, estimate the relative proportion of platelets and note the presence of abnormally large platelets. Use the x 100 lens for studying the fine details of the cell morphology.

Staining characteristics of a correctly stained normal film

Nuclei	Purple
Cytoplasm	
Erythrocytes	Deep pink

Reticulocytes	Grey-blue
Neutrophils	Orange-pink
Lymphocytes	Blue; some small lymphocytes deep blue
Monocytes	Grey-blue
Basophils	Blue
Granules	
Neutrophils	Fine purple
Eosinophils	Red-orange
Basophils	Purple-black
Monocytes	Fine reddish (azurophil)
Platelets	Purple

Sources of error

Film preparation

- Irregular spread with ridges and long tail: Edge of spreader dirty or chipped; dusty slide
- Holes in film: Slide contaminated with fat or grease
- Irregular leucocyte and platelet distribution, especially in tail: poor film-making technique
- Film too short and too thick: spreader held at incorrect angle
- Film extending to end of slide: blood drop too large
- Short thin film: blood drop too small
- Film extends to edge of slide: spreader too wide or not positioned correctly
- Cellular degenerative changes: delay in fixing, inadequate fixing time or methanol contaminated with water

Staining faults

Too blue:	Smear too thick
	Inadequate time in buffer
	Buffer pH too high
	Staining time too long
	Diluted stain overused, requires replenishment
	Stock MGG solution incorrectly made or made from impure dye
	Stock solution left exposed to bright daylight

Too pink:	Excessive time in buffer
	Buffer pH too low
	Stock MGG solution incorrectly made or made from impure dye
	Coverslip mounted before film is completely dry
Too faint:	Staining time too short
	Excessive washing after staining
Stain deposit:	Stain solution left in uncovered jar or tray
	Stain solution not filtered
	Dirty slides
Blue background:	Inadequate fixation
	Prolonged storage before fixation
	Blood anticoagulated with heparin

Differential Leucocyte Count (DLC)

Check the film macroscopically to confirm its identity and assess the quality.

Using a low-power lens (x10 objective) on the microscope, check the approximate differential cell distribution and the presence of unusual cells or cells in bunches, suggestive of malignancy.

Using the x40 objective high-power lens, perform a 200 cell DLC. The x100 objective oil immersion lens should be reserved for examining fine intracellular details and when searching for parasites.

Move the slide along the stage of the microscope in a broad battlement track running transversely across the body of the film, avoiding the edges completely.

Record the numbers of each type of white cell. It helps to have a mechanical or electronic differential counter.

Calculate the percentage of each of the five basic leucocytes (Neutrophils, Eosinophils, Basophils, Lymphocytes, Monocytes). Report the DLC as percentage; if the total WBC is known, also report the DLC in absolute numbers x 10^6 or 10^9 / l. Note the presence of any immature cells, especially blast cells, and report these alongside the DLC. Note the presence of normoblasts – if there is a significant number, do not include in the DLC but record the number per 100 leucocytes.

12. MANUAL RETICULOCYTE COUNT

Reticulocytes are juvenile red cells. They contain remnants of ribosomes and ribonucleic acids which were present in larger amounts in their nucleated precursors. Because the number of reticulocytes in the peripheral blood is a fairly accurate reflection of erythropoietic activity, a reticulocyte count is one of the essential procedures in diagnostic haematology.

Ribosomes have the property of reacting with certain dyes (e.g. azure B or New methylene blue) to form a blue precipitate of granules or filaments. This reaction will only take place in supravitally-stained,unfixed preparations.

The most immature reticulocytes are those with the largest amount of precipitable ribosomal material, whilst in the least immature only a few dots or strands are seen.

Method for the Reticulocyte Count

Staining solution

1.0 gm of New methylene blue or Azure B is dissolved in 100 ml of citrate-saline solution (1 part 3% tri-sodium citrate to 4 parts 0.85% saline).

After dissolving the dye, the solution is filtered and is then ready for use. Store at 4°C.

Staining method

Deliver two or three drops of stain into a tube, and add approximately equal volume of patient's blood. If anaemic use a larger proportion of blood; use a smaller proportion of blood if polycythaemic.

Mix and leave in water batch or incubator at 37oC for 15-20 minutes.

At the end of this time, resuspend the red cells by gentle mixing and make a thin film in the usual way.

When dry, the films are examined without counter-staining. The reticular material should be stained deep blue and the non-reticulated cells shades of pale greenish-blue.

Counting

Choose an area of the field where the cells are undistorted and the staining is good.

Using the x100 oil-immersion lens, count the number of reticulocytes seen per 1000 red cells.

Counting the red cells can be helped by inserting into the eyepiece a paper or cardboard diaphragm in the centre of which has been cut a small square to reduce the optical field. An easier labour-saving method is to use a Miller ocular insert ; this consists of a large square inside which in one corner is a smaller square of one-ninth the area of the large square. Provided that the red cells are evenly distributed, the red cells need to be counted only in the small square as there will be approximately nine times that number in the complete large square.

Calculation

No. of reticulocytes in n fields = x

Average number of cells in each small square = y

Total number of cells in n fields = 9yn

Reticulocyte percentage = x / (9yn)

Absolute reticulocyte count = % Reticulocytes x RBC ($x10^{12}/l$)

Usually more convenient to report as $x10^9/l$.

As an example, if there were 18 reticulocytes per 1000 red cells, the percentage of reticulocytes is: 18/1000 x 100% = 1.8%

If the RBC = $4.5 \times 10^{12}/l$ Absolute reticulocyte count

$$= \frac{1.8 \times 4.5 \times 10^{12}}{100} = 81 \times 10^9/l$$

13. PLATELET ESTIMATE BY PROPORTIONAL COUNT

This is based on the number of platelets relative to the red cells in a stained blood film.

Using the same method as for reticulocytes (See # 12), count the number of platelets alongside 1000 red cells.

Calculation

No of platelets in n fields = x

Average number of red cells in each field = y

Total number of red cells in n fields = 9yn

Platelet proportion = x/9yn

Platelet count = Platelet proportion (as %) x RBC ($x10^9$/l)

If RBC is not available, an approximate estimate can be made from Haemoglobin or PCV:

PCV (l/l)	.45	.36	.30	.25	.15
Hb (g/l)	150	120	100	80	50
RBC Estimate ($x10^{12}$/l)	5.0	4.0	3.0	2.5	1.5

14. ERYTHROCYTE SEDIMENTATION RATE (ESR)

The ESR (erythrocyte sedimentation rate) measures the distance red blood cells will fall along the length of a vertical tube over a given time period. A raised ESR reflects an increased production of acute-phase proteins. Although it is a non-specific phenomenon, it is clinically useful in certain chronic disorders, e.g. rheumatoid arthritis or tuberculosis, as an index of progress of the disease.

A normal ESR does not exclude organic disease but, on the other hand, the vast majority of acute or chronic infections and most neoplastic and degenerative diseases are associated with changes in the plasma proteins which lead to an acceleration in sedimentation.

Principle

The recommended Westergren sedimentation tube is made from either glass or plastic, has a length of about 30 cm and a bore of 2.5 mm. Many of the tubes which are now available are intended to be disposed of after use. Glass tubes can be re-used provided that they are adequately cleaned by washing through with water, followed by alcohol, and then allowed to dry overnight.

Specially made racks with a scale graduated in mm from 0 to 140 are available and these have adjustable levelling screws for holding the tubes in an exactly vertical position.

It is conventional to set up sedimentation rates at room temperature (18 - 25°C).

Sedimentation is normally accelerated as the temperature rises and, if the test is to be carried out at a higher ambient temperature, a normal range should be established for that temperature. The test should be protected from direct sunlight and draughts, and it should never be set up near a radiator or on a bench where there is vibration, e.g. from a centrifuge.

The test is performed on venous blood diluted accurately with 31.3 g/l trisodium citrate in the proportion of one volume of citrate to four parts of blood. It should be carried out within two hours of collecting the blood, though a delay of up to six hours is permissible provided the blood is kept at 4°C.

Blood in EDTA can also be used for up to 24 hours after collection provided that it is kept at 4^0C and diluted with trisodium citrate immediately before setting up the test .

Method

- Mix the blood well and draw the sample into clean dry Westergren tube.
- Place the tube into the stand, taking care that the base is firmly positioned on the base pad to prevent leakage.
- Adjust the rack so that the tube rests in an exactly vertical position.
- Leave undisturbed for 60 min.
- At the end of the hour read the height of clear plasma above the upper margin of the column of sedimenting cells to the nearest millimetre.
- A poor delineation of the upper layer of red cells, so-called 'stratified' sedimentation, has been attributed to the presence of many reticulocytes.
- Report this measurement as the ESR (Westergren) in units of mm in 1 hour.

Sources of error

- Specimen older than specified time after collection and before testing (see above)
- Incorrect proportion of anticoagulant
- Incorrect type of anticoagulant
- Haemolysed sample
- Contaminated sedimentation tubes
- Tubes tilted during sedimentation
- Test set up near central heating or direct sunshine
- Test set up adjacent to centrifuge or other instrument causing vibration
- Failure to read at exactly one hour.

15. PROTHROMBIN TIME

This tests the extrinsic system in the coagulation pathway. It involves the addition of brain thromboplastin to plasma, with clot formation after the addition of calcium chloride.

Materials

Patient's plasma

Blood should be collected into 31.3 g/l trisodium citrate in a concentration of one volume citrate to 9 volumes blood. As soon as possible after collection, the specimen should be centrifuged at about 3000 rpm for ten minutes and the platelet-poor plasma separated into a plastic tube, using a plastic pasteur pipette. This sample must be analysed within four hours after collection, unless it is frozen and kept in the deep-freeze refrigerator until tested at a later date.

Normal control plasma

Freshly collected normal plasma obtained in the same way as that of the patient

Rabbit-brain thromboplastin, stored at 4°C

This reagent can be commercially obtained or home made

Calcium chloride solution ,0.025 mol/l (i.e. M/40); stored at 4°C

In some commercial reagents this has already been added to the thromboplastin

Method

1. Reconstitute a vial of thromboplastin in accordance with the manufacturer's instruction. Leave in water-bath at 37°C for 10 minutes.
2. Dispense 0.1 ml into a plastic tube and add 0.1 ml of pre-warmed calcium chloride (or dispense 0.2 ml if the thromboplastin-calcium is already combined)
3. Add 0.1 ml of pre-warmed plasma and start the stop-watch.

4. Tilt the tube gently every other second, keeping it as much as possible under water to maintain the temperature. Record the appearance of a fibrin clot as the end-point.
5. Perform the test on the patient's plasma in duplicate, and also in duplicate on the normal control plasma. Repeat the test if duplicate measurements differ by more than 5%.

The normal range should be in the region of about 13-17 seconds. However, this depends on the thromboplastin and should be established by testing a group of healthy subjects whenever a new reagent is introduced (See # 19).

16. ACTIVATED PARTIAL THROMBO-PLASTIN TIME (APTT)

This test measures the intrinsic system of the coagulation pathway. The essential reagent is a phospholipid substitute for platelet factor three, and contact activation is achieved by adding an activator such as kaolin (celite or ellagic acid can also be used).

Materials

Patient's plasma

As for the prothrombin time (# 15), blood should be collected into 31.3 g/l trisodium citrate in a concentration of one volume citrate to nine volumes blood. As soon as possible after collection the specimen should be centrifuged at about 3000 rpm for ten minutes and the platelet-poor plasma separated into a plastic tube, using a plastic Pasteur pipette. This sample must be analysed within four hours after collection, unless it is frozen and kept in the deep-freeze refrigerator until tested at a later date.

Normal control plasma

Freshly collected normal plasma obtained in the same way as that of the patient

Kaolin 5 g/l in barbitone buffered saline, pH 7.4

This is stable at room temperature but should be stored at 4°C.

Phospholipid

Available commercially. Stored at -20°C, it is stable for at least a year. It should be dispensed in small volumes and should not be refrozen after thawing.

Calcium chloride solution

0.025 mol/l (i.e. M/40); stored at 4°C.

Method

1. Mix equal volumes of the phospolipid reagent and kaolin suspension. Leave to warm up in a glass tube in the water-bath at 37°C.
2. Place 0.1 ml of plasma in another glass tube. Add 0.2 ml of the kaolin-phospholipid reagent and start the stop-watch. Leave at 37°C for 10 minutes with occasional shaking.
3. At exactly 10 minutes, add 0.1 ml of pre-warmed calcium chloride and start a second stop-watch.
4. Record the time taken for the mixture to clot.
5. Perform the test on the patient's plasma in duplicate and, also in duplicate, on the normal control plasma. Repeat the test if duplicate measurements differ by more than 5%.

The normal range should be in the region of about 30-40 seconds. But this depends on the reagents used and the preliminary incubation period. Each laboratory should establish its own normal range by testing a group of healthy subjects.

17. THROMBIN TIME (TT)

This measures the time taken for plasma to clot when thrombin is added. It is a function of the final phase of coagulation.

Materials

Patient's plasma

As for the prothrombin time (#15), blood should be collected into 31.3 g/l trisodium citrate in a concentration of one volume citrate to nine volumes blood. As soon as possible after collection the specimen should be centrifuged at about 3000 rpm for ten minutes and the platelet-poor plasma separated into a plastic tube, using a plastic pasteur pipette. This sample must be analysed within four hours after collection, unless it is frozen and kept in the deep-freeze refrigerator until tested at a later date.

Normal control plasma

Freshly collected normal plasma obtained in the same way as that of the patient

Barbitone buffered saline, pH 7.4

Thrombin

This is available commercially as a freeze-dried material. It must be reconstituted with saline to 100 NIH units/ml, dispensed in 1-ml aliquots and stored at - 20°C. For use a vial is thawed and diluted with barbitone-buffered saline, pH 7.4 to obtain a normal clotting time of about 17 seconds – usually about 7-8 units/ml.

Method

1. Add 0.1 ml buffered saline to 0.1 ml normal plasma and leave in the water-bath at 37°C for four minutes.
2. Add 0.1 ml thrombin, and mix by shaking and simultaneously start the stop-watch.
3. Measure the clotting time.

4. Repeat with the patient's plasma in duplicate followed by a second sample of the normal plasma.
5. Express results as the mean values for the patient and normal. Repeat the test if duplicate measurements differ by more than 5%.

A patient's Thrombin Time should be within two seconds of the control. Times of 20 seconds and over are definitely abnormal.

18. INTERPRETATION OF COAGULATION SCREENING TESTS

PT	APTT	TT	Possible conditions
N	N	N	Normal haemostasis Thrombocytopenia* Disorders of platelet function Vascular disorder Bleeding from severely damaged vessel Very mild Factor VIII deficiency
Long	N	N	Factor VII deficiency (rare) At start of oral anticoagulation therapy
N	L	N	Factors VIII, IX deficiencies Factors XI, XII deficiencies (rare) Von Willebrand disease Circulating anticoagulant Prekallikrein or kininogen deficiency (rare)
Long	Long	N	Vitamin K deficiency Oral anticoagulant drugs Factors II, V, X deficiency (rare) Liver disease* Massive blood transfusion*
Long	Long	Long	Heparin Fibrinogen deficiency Hyperfibrinolysis DIC * Acute liver disease*

N = Normal *Thrombocytopenia present
Factor II = prothrombin
Factor VIII deficiency = Haemophilia A
Factor IX deficiency = Haemophilia B

Sources of error in tests

- Error in blood collection: incorrect ratio of blood to citrate
- Wrong anticoagulant for blood collection
- Delay in separating plasma from cells
- Delay in performing tests
- Tests not performed with plasma and reagents kept at $37^{0}C$
- Reagents deteriorating: incorrectly stored or out of date
- Technique not constant – it is essential to use the same technique as incubation time and temperature, amount of contact activation, method of tilting the tubes, all greatly influence the results
- Inability to detect beginning of clot and/or slow response when using stop-watch

19. CONTROL OF ORAL ANTICOAGULANT THERAPY

The test for this is the prothrombin time, as described in #15. The only differences are in the use of a standardized thromboplastin, the selection of normal plasma for comparison, and how the results are expressed.

The usual method for expressing the degree of anticoagulation is as prothrombin ratio:

$$\frac{\text{Patient's prothrombin time}}{\text{Normal prothrombin time}}$$

To control long-term anticoagulant therapy reliably, it is necessary to compare results between laboratories and in the same laboratory using different batches of thromboplastin. To achieve this WHO has developed a procedure by which all thromboplastins are calibrated in comparison with a standard by means of an International Sensitivity Index (ISI); the original WHO primary standard was given an ISI of 1.0. Every batch of thromboplastin, both home made and commercial products, must be calibrated, and all manufactured thromboplastins should have their ISI clearly shown on the label. The method to calibrate a thromboplastin which can be undertaken in a laboratory is described in WHO document WHO/LAB/98.3 (#1 Ref 4).

Mean normal prothrombin time (MNPT)

The normal range for the PT test is affected by variation in the laboratory technique, the source of thromboplastin and its potency. The normal range must be established each time a brand of thromboplastin or a batch of the reagent is changed or the method is modified or when a coagulometer is replaced or adjusted. The normal range should be established on a mimimum of 20 healthy adults including both sexes over a wide age range of 20-80 years. The tests need not all be done on the same day. The mean normal prothrombin time is determined as the geometric mean PT of the 20 healthy subjects. This value is used for calculating the prothrombin ratio in subsequent tests.

Calculation of INR

This is the prothrombin ratio which would have been obtained if the WHO reference thromboplastin had been used to perform the test on the blood sample.

Calculation

$$INR = (\text{observed ratio})^{ISI \text{ of thromboplastin}}$$

An alternative way to calculate is to convert the PR to its logarithm, multiply by the ISI and then convert to the antilog – i.e.

$$INR = \text{antilog}[\log PR \times ISI].$$

The WHO document WHO/LAB/98.3 includes a nomogram by means of which the INR equivalent to the prothrombin ratio can be easily read on the scale for any thromboplastin with an ISI in the range 1.0-1.6.

In patients receiving oral anticoagulants the INR must be maintained within the clinically recommended therapeutic range, and the dose should be adjusted with regular checks on the prothrombin time, usually at 2-3 week intervals. There should be close collaboration between the clinician responsible for treating the patient and the laboratory.

20. BONE MARROW IRON

Principle

The presence of stainable iron in the bone marrow provides an indication of iron stores. Normally, it occurs as an accumulation of small granules of siderotic material (or haemosiderin) lying free and in phagocytes, and some of the normoblasts also contain 1-4 small iron granules ("sideroblasts"). The iron store will be reduced or absent in iron-deficiency anaemia, increased when there is iron overload, in infections, dyserythropoietic anaemias, sideroblastic anaemias and thalassaemia. In the last two conditions there is a significant increase in the number of sideroblasts; in sideroblastic anaemias the granules occur characteristically in a perinuclear ring whilst in thalassaemia there is an increased number of prominent large iron granules scattered throughout the cytoplasm.

The test is based on Perls' Prussian-blue reaction in which siderotic material reacts with potassium ferrocyanide to form the blue-coloured compound, ferriferrocyanide. The most important purpose for using this test on bone marrow is to distinguish between iron- deficiency anaemia and thalassaemia.

Method

Reagents

- O.2 mol/l (0.2N) hydrochloric acid in distilled or de-ionized water
- 20 g/l potassium ferrocyanide in distilled or de-ionized water
- 10 g/l aqueous neutral red or eosin in distilled or de-ionized water

Procedure

Mix equal volumes of the hydrochloric acid and potassium ferrocyanide solutions in a staining jar. Select a bone-marrow slide containing particles and place it in the mixture at room temperature for 10 minutes. Wash thoroughly in tap water for about 20 minutes and counterstain with the neutral red or eosin solution for 30 seconds. Rinse well in tap water and allow to dry in the air before examining the slide by microscopy.

It is important to avoid contaminating the slides and glassware with iron. Prepare the glassware by soaking in 2 mol/l HCl followed by washing in de-ionized water before use.

21. ESTIMATION OF FOETAL HAEMOGLOBIN

Principle

Foetal haemoglobin is more resistant to denaturation by strong alkali than are other haemoglobins. For the test alkali is added to a haemolysate containing a known amount of haemoglobin. After a specified time, the denaturation is stopped by adding saturated ammonium sulphate to lower the pH and precipitate the denatured haemoglobin. The amount of unaltered haemoglobin is measured and expressed as a proportion of alkali-resistant (i.e. foetal) haemoglobin. The recommended method is that of ICSH, based on Betke method.

Reagents and equipment

- ICSH (modified Drabkin) reagent (see Section 7). Stable for 2-3 weeks at room temperature if kept in a dark bottle, protected from light.
- Sodium hydroxide 1.2 mol/l. Stable for four weeks at room temperature.
- Saturated ammonium sulphate (NH4)SO4 500 g to one litre distilled or de-ionized water.
- Spectrophotometer with wavelength setting at 413 nm; can also be used at 540 nm.
- Stopwatch with a second hand.
- Whatman No. 42 filter paper and funnel.

Blood in any anticoagulant can be used; a normal and a raised HbF control should be included with each batch of samples. The raised control can be made by mixing one volume of cord blood with nine volumes of normal adult blood of the same ABO group to obtain a Hb F of 10%

Method

1. To prepare a lysate wash about 1-2 ml of the blood three times in isotonic saline. Add one volume of carbon tetrachloride and one volume of distilled or de-ionized water to two volumes of the washed cells.

Shake vigorously for 4-5 minutes in a stoppered centrifuge tube. Centrifuge at about 1200g (3000 rev/min) for 30 minutes. Pipette the clear supernatant into a test-tube and adjust its haemoglobin concentration to about 100 g/l with water.

2. Add 0.2 ml of the lysate to 4four ml reagent to make a haemiglobincyanide (HiCN) solution. Mix and stand for 10 minutes.
3. Pipette 2.8 ml of the HiCN solution into a 12x100-mm test tube, add 0.2 ml of sodium hydroxide, mix well and start a stop-watch.
4. At exactly two minutes add two ml of the saturated ammonium sulphate, mix well and allow to stand for five minutes to settle.
5. Filter the solution through Whatman No 42 filter paper and collect the filtrate. Filter again if the filtrate is not absolutely clear.
6. Add 3.5 ml distilled water to 0.1 ml of the HiCN solution as a standard.
7. Read the absorbance of the filtrate and the standard in a spectrophotometer at 413 nm (alternatively 540 nm).
8. Calculate % Hb F = $[A^{413}$ test x 100] ÷ $[A^{413}$ standard x 20.16].

Interpretation: Normal values for children at six months should be 2-3% or less, falling to less than 1% at one year; adults 0.2-1.0%.

Sources of errors

- Inaccurate pipetting
- Imprecise timing for denaturation
- Inadequate filtration, with turbidity in filtrate
- Reagent out of date or left exposed to light
- Quantification is only approximate if Hb F concentration is >20%

22. QUALITY ASSURANCE

An established programme of quality management is essential to ensure that the results of tests are reliable, reproducible and as accurate as possible, to achieve the necessary level of good practice and to be reassured that this standard is constantly maintained. Internal quality control (IQC) is central to this, and every member of the laboratory staff should be aware of the procedures in use in their laboratory.

There are several procedures which might be included in any laboratory. The choice depends on which tests are undertaken routinely, the number of specimens handled each day, the equipment (especially if this includes automated analysers), the size of the laboratory, and the level of training of its staff.

Control Chart

Any laboratory performing more than 20 blood counts per day must institute a quality control chart. Modern automated analysers have a programme which provides a print-out (also known as a Levey-Jennings chart) of the results on controls which may be included whenever a batch of tests is run. A similar chart can be prepared for semi-automated counters and for some tests which are performed manually.

Method

1. Initially, when a control preparation has been made and dispensed into a number of containers, it must be checked for homogeneity by measuring haemoglobin (or other appropriate component) in duplicate or triplicate on 4 or 5 samples from the batch.. The measurement must be performed with meticulous care by a technically skilled senior worker using calibrated pipettes for dilution. The results should vary by not more than 2%. The total ten (or more) results can then be used for establishing a control chart. Whenever a new batch of control material is made, the process must be repeated and a new chart established.

2. Calculate the mean and standard deviation (SD) of the control preparation by the following formulae:

Mean = $\Sigma x \div n$, where x = individual measurements; Σx = sum of measurements;

n = number of measurements

$SD = \sqrt{\Sigma (x - mean)^2 \div (n-1)}$

3. 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 haemoglobin. Draw two other lines above the mean to represent +2SD and +3SD, and two lines below the mean to represent –2SD and –3SD, respectively.

4. With each batch of routine tests include a sample of the control material and plot the results on the graph paper. This will give at least one value per working day. If the control is kept in the refrigerator, the sample to be used must be allowed to equilibrate to ambient temperature. and then mixed well by hand (at least 20 inversions) or on a roller mixer (at least 3-5 minutes). As the control is intended to simulate routine practice, it must be introduced into the batch of specimens and treated exactly like them.

5. Plot the results from the control on the chart and inspect the chart to assess whether the test is "in control" or "out of control". If the test is satisfactory, results will oscillate about the mean, and within the ± 2SD limits. A fault in technique, instrument, pipette or reagent will be suggested by one of the following:

   One result outside 2SD: Warning – Possibly early sign of SE or RE

   One result outside 3 SD: Reject – SE or RE

   Two consecutive results outside 2SD: Reject – SE

   Four consecutive results outside +1SD: Reject – SE

   Four consecutive results outside – 1SD: Reject – SE

   Six consecutive results on one side of mean: Warning – Possibly SE

   SE = Systematic error
   RE = Random error.

But first repeat the test on another sample of the control preparation and check that the material itself has not become infected or has begun to deteriorate.

Patients' data

In a large hospital where at least 100 blood counts are performed each day, there should be no significant variability in the means of red cell indices (MCV, MCH, MCHC) from day to day. Results are valid only if the population from whom the

specimens come does not vary significantly from day to day. A bias may occur if tests are carried out only on certain days on patients with, for example, iron deficiency or other conditions which affect the MCHC, MCH and/or MCV. To overcome this problem, results from these patients should be excluded or the median should be used instead of mean. It must be remembered that the component parameters of Hb, RBC and PCV are controlled only indirectly with this procedure.

Modern automated counters incorporate a computer programme which continually updates the calculation of the means so that a drift will soon be spotted, indicating the possibility of a change in instrument calibration or a fault in its function. With other counters results can be entered into a programmable calculator or personal computer and analysed at the end of each day.

To start the procedure it is first necessary to establish the mean and SD from the daily mean for each parameter over one to two weeks:

Mean of daily means = $\Sigma x \div n$, where x = daily means; Σx = sum of daily means;

n = number of days

$SD = \sqrt{\Sigma}$ (x- mean of daily means)$^2 \div (n-1)$

Thereafter the daily means for each parameter should fall within ±2SD. If a new counter is installed or repairs have been carried out a check must be made to see that the established results are unchanged; if they do change it is important to find out why and, if necessary, to recalibrate the counter.

Constancy of MCHC

A simple adaptation of the principle described above is useful for laboratories using manual methods and without computer facilities. It can be used for all the red-cell indices but it is especially adaptable for the MCHC.

The mean MCHC is calculated at the end of each day for 10 consecutive working days, and the daily mean and SD are calculated by the formula given above. Thereafter, the mean is calculated from all measurements obtained during the course of the day. If the test is being performed satisfactorily, this will not vary by more than 2SD on any day.

It is convenient and educational to plot the calculated result each day on a graph with MCHC on the vertical axis, days on the horizontal axis, and the +2SD and –2SD marked with horizontal lines.

Duplicate tests on patients' specimens

As far as possible, special tests should always be performed in duplicate. It is obviously impractical in a busy laboratory to do duplicate testing on all the routine blood counts; however, it is good practice to measure 2 or 3 specimens from each batch of specimens in duplicate.

The same procedure can be used to carry out repeat measurements on 2 or 3 specimens from the previous batch. This 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 themselves on storage. Thus, it 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 initial and repeat tests

It is first necessary to establish the standard deviation (SD) by measuring a set of 10 or more samples in duplicate. Calculate as follows:

$SD = \sqrt{\Sigma d^2 \div 2n}$, where d^2 = difference between duplicates squared;

Σ = summation;

n = number of specimens tested in duplicate.

None of the duplicate measurements should differ from each other by more than 2SD. This analysis detects random errors. If the test is always done badly the SD will be wide and the analysis will not be sensitive to individual minor faults.

Participation in External Quality Assessment

External quality assessment refers to a system in which laboratory results are scrutinized objectively by an outside agency in order to get a general impression of the standard of laboratory practice and to achieve inter-laboratory comparability. Each laboratory should strive to participate in any such scheme.

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The prothrombin time. WHO/LAB/98.3 (1998).

Requirements and guidance for external quality assurance programmes for health laboratories. WHO/LAB/ (1999)

Fundamental Diagnostic Hematology: Anemia (2nd edition). WHO/CDC 1992

Findamntal Diagnostic Hematology: The Bleeding and Clotting Disorders (2nd edition). WHO/CDC, 1992

Health Laboratory Services in support of primary health care in developing countries, 2nd edition. SEARO Regional Publication, 1999.

Bench-aid for diagnosis of Malaria. WHO Publication (1985).

Bench-aid for morphological diagnosis of anaemia. WHO Publication. In press (1999).

The following standard text book is available in low-price ELBS Edition in certain countries:

Dacie JV and Lewis SM. Practical Haematology, 8th edition. Churchill Livingstone, Edinburgh, 1996

Health Laboratory Services in support of primary health care in developing countries, SEARO Regional Publication No.24 (Revised edition).

Standards and Reference Materials

The following International standards are available from the WHO International Laboratories for Biological Standards:

National Institute for Biological Standards and Control
Blanche Lane, South Mimms, Potters Bar EN6 3QG UK

Ferritin, Human recombinant

Fibrinogen, Human

Folate, Whole blood

Haemoglobin (Haemiglobincyanide)

Haemoglobin A_2, lysate

Haemoglobin F, lysate

Vitamin B_{12}, Human serum

Central Laboratory of Netherlands Red Cross Blood Transfusion Service,
Plesmanlaan 125, Amsterdam, The Netherlands

Thromboplastin, Human recombinant

Thromboplastin, Rabbit