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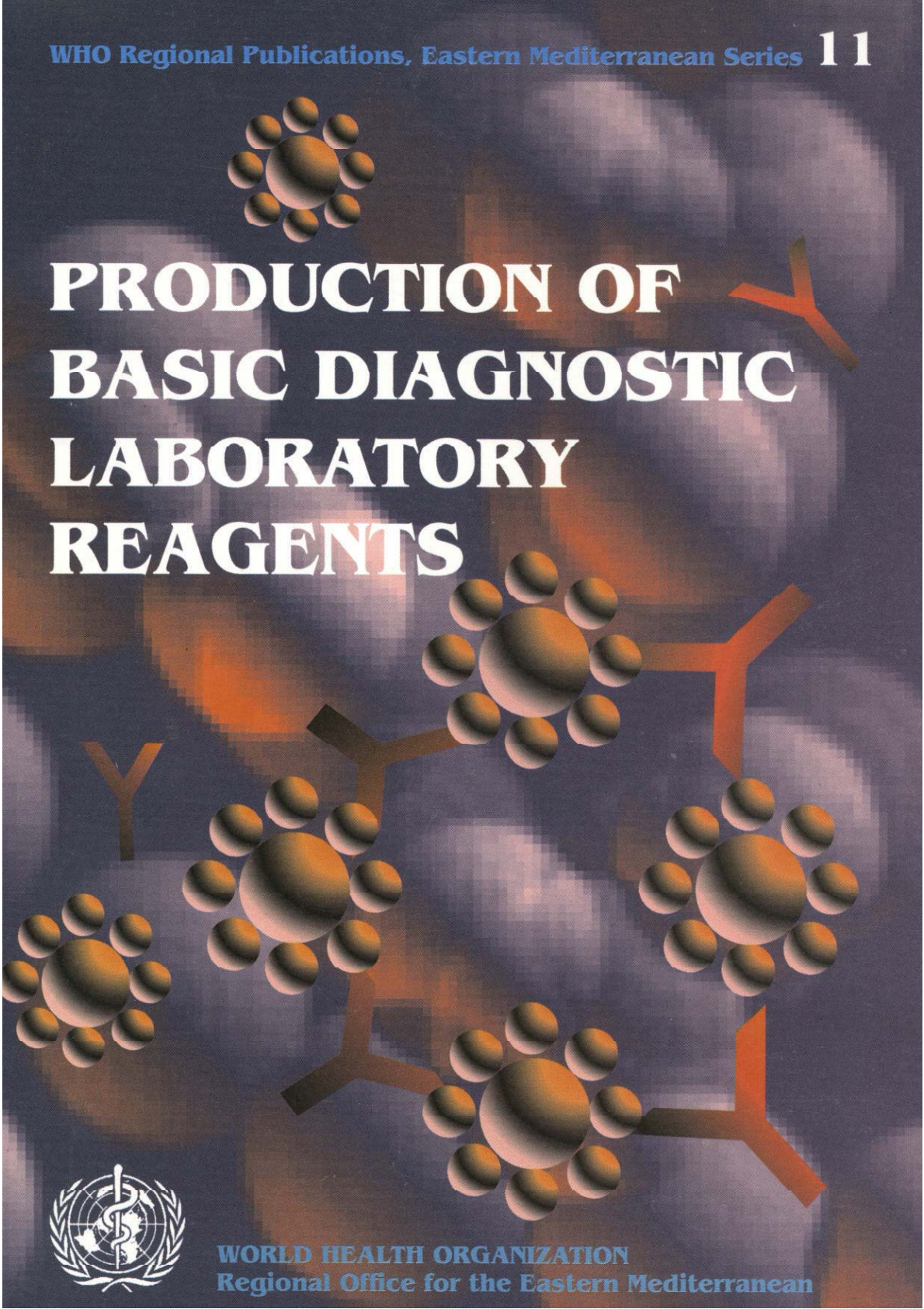

PRODUCTION OF BASIC DIAGNOSTIC LABORATORY REAGENTS

WORLD HEALTH ORGANIZATION
Regional Office for the Eastern Mediterranean

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Production of Basic Diagnostic Laboratory Reagents

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Foreword

In many countries, a major problem for those responsible for providing good laboratory services is the quality and constancy of supply of laboratory reagents. This publication addresses that problem and at the same time provides invaluable information on many other facets of good laboratory practice, thus helping to achieve the goal of the World Health Organization, Health for All by the Year 2000.

The contribution that laboratory medicine makes to the health programmes of a country is dependent upon the clinical usefulness of the service provided to the health-care structure, whether it is at the intermediate or peripheral level. Good laboratory practice in all disciplines of laboratory medicine is the cornerstone of such service but good laboratory practice is impossible without a constant supply of quality reagents. This document, describing the organization necessary for the production of laboratory reagents, follows the publication by the Regional Office for the Eastern Mediterranean, of another excellent publication: *Basics of Quality Assurance for Intermediate and Peripheral Laboratories*. In both cases, the discussion addresses laboratory medicine in general rather than a specific discipline. The techniques of good laboratory practice are applicable to all disciplines and these two documents will assist in breaking down the professional barriers within laboratory medicine. For their efforts to be more effective there is a need for greater cooperation between the professional bodies representing the various disciplines and the discussions should concentrate on laboratory practice in laboratory medicine and not be restricted to individual disciplines.

The range of reagent systems included in this publication is not exhaustive but the general proforma, used to describe each, can be applied by national and regional groups wishing to extend the range to meet their needs. The first two chapters provide excellent guidelines for individual countries in organizing their own reagent production. The list of equipment included provides an invaluable checklist for those who may be considering the practicality of undertaking a similar project. I am not aware of another document as complete as this one.

This publication is intended to encourage, nationally or within a region, the production of laboratory reagents, in those situations where the quality of the laboratory service is adversely affected by the lack of constant supply of good commercially prepared products. It is also possible to establish a situation in which the production of reagents and the provision of other aspects of good laboratory practice could be corporatized and contracted to a commercial organization.

For a number of years I have been fortunate to have worked with colleagues and for organizations representing countries from various regions of the world. A number of these countries do have problems with laboratory practice and this publication will assist with their efforts. Changes in laboratory practice will provide an improvement in the clinical usefulness of the service provided. Considerable emphasis has been placed upon the implementation of internal and external quality control programmes and their role as a means of monitoring the competency of laboratories. However, when the laboratory practice is such that the results of such programmes can be reliably predicted, then it may be more effective for programmes to address matters such as instrument maintenance, quality of water, temperature control and reagent quality. Greater emphasis should be placed upon improving the system which produces the analytical results than upon monitoring its performance. This publication provides a means of improving one of these aspects, reagent production.

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Preface

In agreement with the targets of the World Health Organization's Global Medium Term Programme, the Eastern Mediterranean Regional Office (EMRO) is encouraging countries of the Region to start their own local production of simple basic reagents for health laboratory services. Laboratory reagents production has already started in some EMR Member States, by initiatives from either governmental or private companies. This activity is either entirely independent or a satellite activity of pharmaceutical companies. Some companies have started by buying bulk reagents with local repackaging, others have established complete local production of reagents from imported raw materials.

The preparation of this manual on the production of reagents used in most common analyses was identified as a priority in the WHO/EMRO Regional Meeting of Directors of Health Laboratory Services in November 1988 in Rabat, Morocco. We do not claim that this manual is complete nor that it will solve all the problems which may be encountered in local production of reagents. However, it represents, in our opinion, an important step to help some countries, not only in the Eastern Mediterranean Region but also in other Regions, to start their own local production of some essential diagnostic reagents.

The authors are grateful to Dr Hussein A. Gezairy, Regional Director of the Eastern Mediterranean, and Dr M.H. Khayat, Director, Programme Management, for their valuable support and encouragement. Our thanks are due to T.D. Geary, Oakbank Consultants, Oakbank, South Australia, Australia, Dr S. Sufi, WHO Collaborating Centre for Research and Reference Services in Immunoassays of Hormones in Human Reproduction, Hammersmith Hospital, London, United Kingdom, Dr H. Kuffer, Central Institute of Vally Hospitals, Sion, Switzerland, Mr Geoff Cowburn, Microbiology Department, Derbyshire Royal Infirmary, UK, Mr P. Mugg, Medvet Science PTY Ltd., Adelaide, Australia and Dr V. Thamdrup-Rosdahl, Statens Seruminstitut, Artillerivej, Copenhagen, Denmark, for their valuable comments and constructive suggestions.

We would also like to extend our thanks to Dr Kruse, Bonn, Germany, for providing the procedure for the preparation of blood cells for quality control, and Dr Sievers, Helsinki, Finland, for providing the procedure for the preparation of human quality control serum. Dr T. Yixun, Shanghai, People's Republic of China for providing information on production of antistreptolysin O reagents, and Dr D. Kutter, Luxembourg for review and providing the manufacturing recipe of albumin reagent (Annex 1).

Introduction

Self-reliance in health, at the individual, community and national levels, was strongly endorsed in the Alma-Ata Declaration of 1978. in order to achieve the global goal of Health for All by the Year 2000. However, there are many instances where Member States are not self-reliant in some facets of health development; they depend on external assistance almost on a permanent basis. The provision of laboratory reagents is one of the important factors in laboratory services where most developing countries are dependent on external supply. Self-reliance through local production of reagents for health laboratories is one of the targets of the World Health Organization's Global Medium Term Programme.

Self-reliance may be either at a national level or at the level of a group of countries having a common interest, which may be more realistic and cost-effective. Some countries are very cautious about this approach, having seen the results of the fragmentation of certain larger federations into smaller countries, some of which lost access to industries which had originally been built and developed for the common interest of a group of countries.

Some believe that, if financial resources are available, it may be more cost-effective to import reagents rather than to produce them locally. Whether a country should start local production or not depends on local resources, facilities and the availability of the necessary infrastructure and required skills. These factors also determine the range of reagents to be produced, their quantities and their level of sophistication.

Faced with limited resources, there is no alternative for many countries but to establish reagent production which will be most appropriate for their needs, starting with relatively simple reagents. To minimize the difficulties that might be encountered, local production of basic reagents should be introduced in phases, focusing on reagents for tests in most common use and those considered essential. Establishing centralized production units should be encouraged in countries where the required infrastructure exists. Two important factors must be considered: cost-effectiveness and quality.

Local production of basic reagents and diagnostics will help to reduce dependence on foreign sources. The establishment of national centres for the production of laboratory diagnostic reagents will lead to the development of health laboratory services and ensure their sustainability. Although sometimes it appears to be cheaper to buy than to produce in the short term, this will not be true in the long run with local production. Money will remain within the country and savings in hard currency may be used for other purposes.

1

ORGANIZATION OF A PRODUCTION UNIT FOR LABORATORY REAGENTS

1. ORGANIZATION OF A PRODUCTION UNIT FOR LABORATORY REAGENTS

A well organized unit for the preparation of reagents is a prerequisite for establishing good manufacturing practice. A production unit will have different sections that are responsible for administrative, preparative, analytical and technical work . The size of and the number of personnel working in each section will vary, but their work should be coordinated and managed so that the production will be cost-effective. The production unit may include the following sections:

- administration
- section for the preparation of distilled water
- section for cleaning glassware and plastics used as containers for reagents
- section for quality control of raw materials
- section for preparation of reagents and reagent solutions
- section for dispensing reagent solutions into containers
- section for quality control of prepared reagents
- section for reagent kit assembly and packaging
- storeroom for reagents, including a refrigerated room (2-4°C)
- section for waste disposal

Administration includes coordination of the work in the different sections involved in reagent production, as well as ordering materials, registration of incoming and outgoing products, and filing of technical reports on the production process of each batch of reagent produced. This work should be simplified by using standard forms for orders, registration, and documentation, so that all information can be easily read and understood (see also Section 2.3.4).

Problems may occasionally arise when chemicals of insufficient quality are obtained or when the production of a reagent batch has failed for technical reasons. This may require the disposal of chemicals that cannot be re-purified or otherwise used. However, safe waste disposal of chemicals is not always easy and must be done cautiously. The disposal of chemicals must take into account their possible reaction during this process; for example, the explosion of chemicals, the chemical properties of the final products and their polluting effect on the environment. Procedures of safe disposal must be carefully selected. They may include burning of certain, but not all, organic chemicals as well as potassium cyanide under controlled conditions. Inorganic chemicals may be

neutralized with acids or alkalis to form an insoluble salt that can be buried in a safe location. For example, H_2SO_4 can be neutralized with $CaCO_3$ or $Ca(OH)_2$ to form $CaSO_4$, gypsum, which can be disposed of without polluting the environment.

The production of a laboratory reagent may include the preparation of various reagent components and also several intermediate steps. The sequence of these steps must be adhered to during the production in order to avoid failures, and monitored with each step so that possible errors can be traced to their origin. The continuous monitoring of a production process is an effective mechanism to ensure a good quality product. Therefore, the importance of monitoring a production process cannot be overestimated.

The production process must be standardized and the description of this process should be available as a written document. Instruction documents, meticulously describing each step in the production line, are most helpful. The written instructions can be either contained in a single manual or made up of several documents, depending on the organization and the local arrangement of the production unit. Such documents must be thoroughly studied by the supervising managers and by the operators working at the bench. They should contain the following information:

1. **Detailed description of the basic chemicals.** Basic chemicals can be prepared using different procedures and by various manufacturers. The different ways of preparation imply that the quality of a chemical obtained from different manufacturers will be different. This is why the consumer should insist that the supplier of chemicals also provides in its catalogue all relevant information, including the chemical name, the chemical formula, the content of impurities and the number.

   All this information should be included in the instruction document, so that a correct order can be given for further procurement. Also, the name of the supplier and/or the manufacturer must be given. Finally, the document should provide information on the hazards of each chemical (usually provided in the manufacturer's catalogue, or as a data sheet obtainable from the manufacturer/supplier).

2. **List of equipment for production.** The equipment will be selected according to the quantities of reagent to be prepared and according to the production process. Smaller quantities are usually produced in a discontinuous process. For these, convenient laboratory equipment (such as 5 L flasks or 10 L beakers) may be used. The preparation of larger volumes of a reagent may sometimes be easier in a continuous process, for which more sophisticated equipment (such as a stirring machine for larger volumes, or a temperature-controlled mixing device) will be required. These items should be clearly described in the list of equipment.

3. **Principle of the reaction for which the reagent under production will be used.** This applies to those reagents that are used for quantitative measurement in clinical chemistry.

4. **Detailed manufacturing recipe.** This section must state the exact amount of each basic chemical (in grams/kilograms or millilitres/litres) that is required for the production of a given quantity of a reagent. The description must outline the exact working conditions and sequence of steps of production. It must also include information about possible dangers that may occur during the production process.

It is important to remember that scaling up a production process from small volumes to larger volumes may require an entire revision of the method of preparation. For example, when mixing chemicals that react in an exothermic reaction (e.g. concentrated sulfuric acid and water), heat will be set free to a degree that may cause sudden evaporation or even boiling of the reaction mixture. This can be avoided by replacing water with ice, prepared with distilled water, when larger volumes must be prepared. If pure ice cannot be made available, the reaction mixture must be cooled from outside, while adding the chemicals at a slower rate.

Efficient mixing of the solution is important. It is also important to remember that some chemicals develop fumes which may be hazardous when large quantities are used. For example, sodium azide, NaN_3 is hazardous when used in large quantities. An alternative, less toxic preservative should be used for the preservation of large volumes of a reagent solution.

The instructions should also include information on the filling of the reagent or reagent component into the final container for distribution.

Annex 1 gives an example of a manufacturing recipe.

5. **Specifications of the final reagent.** The characteristics of the final reagent or reagent component may include the following:

   - appearance (e.g. clear, colourless solution)
   - viscosity
   - surface tension (dyn/m)
   - pH (if buffer or buffered reagent)
   - UV-visible light spectrum

6. **Description of control methods for each component of the reagent.** The analytical methods used for controlling the reagent preparation are usually chemical test procedures, such as colorimetric, titration, potentiometry, chromatography, etc.

7. **Measurement procedure for which the reagent has been prepared.** The measurement procedure must be the same as that for which the reagent has been produced. It must also be identical with the method on the insert for the reagent kit that is provided as a package to the consumer. The description should mention the analytical instrument for which the reagent can be used for measurement.

8. **Reference materials.** These materials are used for the calibration of the test system for which the reagent has been prepared. They can be obtained from international organizations (e.g. WHO, M & T) or national institutions (e.g. NBS, NIBSC, ATCC).

The filling of reagents into containers for distribution should be done within four hours of preparation to avoid inhomogeneity of solutions. This is particularly important when solutions containing polymeric compounds (including biologicals) must be bottled.

An important part of the production line is packaging. It may be worth mentioning that in commercial production, packaging may account for up to 20% of the total expenditure. It is obvious that cleanliness and suitability of containers as well as their correct labelling must be controlled and assured.

2

GENERAL CONSIDERATIONS AND REQUIREMENTS FOR REAGENT PRODUCTION

2. General considerations and requirements for reagent production

2.1 Equipment for reagent production

The establishment of a production unit for laboratory reagents should be based on the results of an assessment providing sound information of local needs. This information should include the kind of laboratory tests which are being used locally, the amount of annual consumption of reagents and the quantities that should be made available within a certain period of time. Such data will influence the decision about the scale of production, the purchase of equipment for production and the organization of the production unit. The kind and size of equipment that will be used for large-scale production will differ from equipment used for production at a laboratory level. Also, problems of technical and environmental safety will differ from one situation to another. There is, however, some basic equipment that should be available in any production unit. This includes:

- equipment for the purification of water
- refrigerators and freezers
- washing machine for cleaning glassware and plasticware
- balances
- dispenser systems for reagent solutions
- dispenser systems for reagent powders
- mixers and magnetic stirrers
- heaters
- refrigerated centrifuges for centrifugation of large volumes
- stills for liquid chemicals
- equipment for sterile filtration
- laminar flow cabinet for sterile work
- volumetric pipettes, micropipettes, burettes and flasks (calibrated and certified by the manufacturer).

The size and the number of each item and the degree of automation will depend on the volume of production.

Refrigerators and freezers are used for the storage of certain chemicals and biochemicals. These storage facilities should be equipped with an alarm system indicating a breakdown of the cooling system to avoid the danger of fire or explosion of

volatile and flammable chemicals. A variety of semi-automated and automated washing machines are commercially available. Some systems use vapour sterilization for glassware and plastic containers. Centrifuges are required for the preparation of quality control material from blood or serum. Some procedures work at low temperature for which a refrigerated centrifuge must be used.

Stills for distillation of chemicals should be made of glass. They are used for the purification of liquid chemicals which sometimes cannot be purchased at analytical grade. The still should enable distillation at low pressure, either at water vapour pressure or at oil vapour pressure under manometric control; in the latter case the still should be supplemented with an oil pump.

Crystalline chemicals can often be purified by recrystallisation using organic or inorganic solvents. This process requires simple laboratory devices such as beakers or flasks. Desiccators are useful to remove the solvents under low pressure.

Equipment for sterile filtration is needed for the preparation of sterile water, buffer solutions and liquid control materials of biological origin. A simple filtration device comprises a suction apparatus and a filter system. The pore size of the filters which may be of glass or filter membranes, should not exceed 0,2 µm, otherwise bacteria will not be retained.

To avoid microbial contamination during the working process it is recommended to install and use a laminar flow cabinet. This is particularly important in the production of culture media and quality control materials of biological origin.

Control and maintenance of equipment for production and analytical investigation is a part of the production process which cannot be overestimated. Continuity of good manufacturing practice will be best accomplished with well-functioning equipment. This is why the function of each instrument must be controlled prior to, and during the course of the production process, and immediate steps should be taken in case of failure. This is of particular importance when handling chemicals, otherwise a situation may occur which may not only result in products of low quality, but may also be hazardous to the laboratory worker during the working process.

It is not the purpose of this document to provide a detailed description of control and maintenance of each item of equipment which may be used. Some information is provided in the WHO publication *Maintenance and Repair of Laboratory, Diagnostic Imaging, and Hospital Equipment.*[1] However, it is recognized that the more sophisticated

[1] *Maintenance and Repair of Laboratory, Diagnostic Imaging, and Hospital Equipment,* WHO, Geneva, 1994.

equipment that may be used for reagent production, such as large washing machines or filling devices, will require special training on maintenance.

An important component in the production of good quality reagents is the provision of purified water. The quantity of purified water should not be underestimated, since it must be used for cleaning of materials and containers as well as for the preparation of solutions. Therefore, considerable quantities of distilled water may be needed. However, one should also be aware that tap water may be good enough for certain steps of the production line. The preparation of large quantities of sterile and deionized water is more economical using an ion exchange system rather than a water still. On the other hand, the costs of the preparation of deionized water increase with the degree of purification, and high investment is needed for the preparation of water with low conductivity (<10 μS). It is particularly important to eliminate heavy metal ions, which catalyze a number of reactions that may cause deterioration of reagent solutions (see also Section 2.5) and may inhibit enzyme reactions.

2.2 Cleaning of glassware

Most analyses can be influenced by impurities, especially enzyme tests (determination of enzyme activities or when enzymes are used as reagents) or immunological reactions. Thus it is essential to use very clean glassware: note that new glassware is never clean.

The cleaning of newly purchased glassware should include the following steps:

- Dipping in or filling with detergent solution.
- Rinsing with tap water; then with deionized or distilled water.
- Rinsing with NaOH (1-2 mmol/L).
- Rinsing with tap water; then with deionized or distilled water.
- Rinsing with HNO_3 (1/3 concentrated)[1]. **Caution: highly corrosive.**
- Rinsing thoroughly with deionized or distilled water[2].
- Drying either in an oven or with acetone (redistilled or analytical grade).
  Note: pipettes and volumetric flasks should never be heated over 50°C: optical glass cuvettes must only be dried with pure acetone.
- All glassware should be protected from dust; open containers, such as beakers, Erlenmeyer flasks, cylinders, etc., must be covered with aluminium, parafilm or supple plastic foils.

[1] Potassium dichromate should be avoided as it is difficult to eliminate completely.

[2] In the case of enzyme tests, in addition rinse with water that has been deionized and then distilled.

Cleaning of glassware after use will depend on the reagents for which it was used; some of the above-mentioned steps may be omitted. Used pipettes must be soaked in detergent solution for several hours and then treated according to the steps described above using a water pump. In some cases organic solvents must be used: analytical grade or redistilled benzene, ethyl ether (after the use of water insoluble solvent the glassware must be rinsed with a water soluble solvent, e.g. alcohol or acetone).

2.3 Quality control

2.3.1 Analytical equipment for quality control

The quality of reagents will be as good as, and cannot be better than, the quality of each individual component. The quality of the chemicals to be used for the preparation of a diagnostic reagent should therefore be optimal. Only in a few countries is good manufacturing practice of diagnostic reagents kept under regulatory control. However, some countries are taking steps to establish mechanisms for their quality assurance and protection of laboratory workers and the environment, similar to those for the production of pharmaceuticals.

A production unit for diagnostic reagents should have a laboratory for quality control. The laboratory should be separate from the production areas and each incoming batch of a chemical should be routinely investigated with validated test methods. since it will be more economical to identify the quality of each isolated component prior to the preparation of a reagent than the causes of the resulting suboptimal quality. The results of these investigations must be properly documented. The records should be made at the time of each action.

There exists a great variety of instruments and techniques for quality control of chemicals and biochemicals. Some of the instruments can only be used with highly purified solvents or reagents which may be very expensive. Sometimes the application of a technique and maintenance of an instrument require special skills. For example, the purchase of atomic absorption spectrometry or high pressure liquid chromatography will be a waste of resources unless laboratory personnel have been specially and extensively trained in the use of these techniques.

The selection of one or another commercially available analytical system will depend on the workload and financial implications of its use. It may be worth remembering that the calculation of the expenses for an instrument should include the cost of purchase, the running costs and cost of periodic maintenance. The basic equipment of a laboratory for quality control of chemicals and biochemicals is shown in Table 2.3.1.

TABLE 2.3.1 Equipment for quality control of chemicals and biochemicals

General laboratory equipment

- analytical balance
- centrifuge
- drying oven
- heating plates with magnetic stirrers
- analytical balance
- microscope
- refrigerator with freezer compartment or cold room with freezer compartment
- shaker
- thin layer chromatography equipment including an ultraviolet (UV) lamp
- titrimeter
- vortex mixers
- vacuum pump
- vacuum rotary evaporator
- water-bath
- water purification system

Analytical instruments

- pH meter
- infrared spectrometer with recorder
- UV/visible spectrometer with recorder
- melting point apparatus
- refractometer
- flame photometer

Optional analytical instruments

- gas chromatograph
- fluorometer
- viscosimeter
- densitometer
- high pressure liquid chromatograph
  atomic absorption spectrometer
- osmometer

Equipment for microbiology

- autoclave
- incubator
- centrifuge with refrigeration
- sterilizer

2.3.2 Quality control of chemicals and biochemicals

The purchase of starting materials is an important operation and should be done with great care and thorough knowledge of the products. Starting materials should be purchased directly from the producer, whenever possible, and with the relevant specifications. All incoming materials should be checked to ensure that the consignment corresponds to the order. Chemical substances are available in many different qualities. Chemicals suitable for the production of diagnostic reagents must be of high quality. Many manufacturers supply a grade according to the specifications of the American Chemical Society or to those described in Analytical Standards for Laboratory Chemicals (Analar Standards Ltd). Manufacturers use a variety of names for this 'analytical grade' material. In addition, they may also supply the same chemicals but at a lower grade or a lower price, which may still be pure enough for an intended purpose. These materials are often called 'general purpose' or 'laboratory grade' chemicals. Many biochemical substances are supplied without detailed specifications.

Some of the chemicals (e.g. picric acid and 2,4-dinitrophenylhydrazine) are supplied moistened with water. Such chemicals must not be dried because of the danger of explosion, and the water content mentioned by the manufacturer must be considered when calculating the amount of chemical to be weighed out.

The choice of chemicals with different specifications or of different origin for the preparation of a diagnostic reagent should be based both on economical criteria and on purity. Although the latter information may be provided in detail by the supplier, it may not always be correct, the reason being suboptimal production or deterioration of the chemical during transportation and storage. This is particularly true for biochemicals. In fact, the quality of chemicals and biochemicals that are obtained from the same producer may vary from batch to batch.

Raw chemicals must be investigated by visual inspection and by a variety of physical and chemical procedures that allow confirmation of their correct chemical composition (e.g. infrared and ultraviolet) and purity (e.g. TLC and HPLC). Raw biochemicals should be investigated for the following:

- physical appearance
- test for major activities (e.g. enzyme activities)
- test for contaminants or contaminating activities
- protein content
- stability of the major product

If a chemical has been found to be of insufficient quality, it is necessary to decide how to process the material. Sometimes it may be faster and more economical to purify

the raw chemical in the laboratory rather than to reject the batch and wait for a new order. Methods for purification of chemicals include recrystallization, distillation or chromatographic techniques. Purification of biochemicals is more difficult than purification of raw chemicals and may require special chromatographical and immunological techniques. Highly purified biochemicals are usually expensive and may not always be required for the preparation of a reagent. However, they must be tested for the absence of interfering substances or impurities which may cause an unwanted reaction. For example, certain enzyme preparations may contain other enzymes as impurities interfering with the intended principle of investigation for which the reagent will be prepared. Also, antisera may not be monospecific enough for the detection of an antigen. Only substances that have been released by the quality control laboratory and that are within their shelf-life should be processed for the production of reagents.

2.3.3 Quality control of diagnostic reagents

The final step in the preparation of diagnostic reagents is to ensure that they can be used to correctly measure the test substance in an appropriate quality control sample. The procedures used for quality control are those which are used in a clinical laboratory, and for which the reagents were prepared. The concentration of the analyte (or catalytic concentration of an enzyme) to be determined must be within acceptable limits. In addition, the analyst must ensure that the reagents are working correctly in other ways as well; for example, the absorbance change for the calibrator should also be within a predetermined range. It is, of course, essential that all methods used for quality control of reagents be calibrated with the appropriate reference materials. Sufficient amounts of each reagent batch are randomly selected and stored under recommended conditions. These samples are tested for performance at intervals during the period of indicated stability.

WHO documents give detailed instructions for the preparation of control materials: WHO/LAB/86.5 *Quality Assurance in Haematology* describes the preparation of a haemolysate for calibration and control of haemoglobin measurements; WHO/LAB/86.4 *Preparation of Stabilised Liquid Quality Control Serum to be used in Clinical Chemistry* describes the preparation of bovine serum, stabilized with ethanediol for the control of albumin, alkaline phosphatase, amylase, aspartate aminotransferase, bilirubin, calcium, creatinine, glucose, potassium, sodium, total protein and urea measurements. Further information is given in Section 3.17, Section 5.3 and Section 8.10.

Lyophilized animal serum with values assigned by manual methods has also been produced by WHO and is available from WHO Collaborating Centre for Research and Reference Services in Clinical Chemistry, Wolfson Research Laboratories, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom.

When purchasing commercial quality control sera, it is important to ensure that the values for particular constituents have been assigned by methods comparable to those described in this manual. For enzymes, both the assay method and the assay temperature should be identical to your procedure.

To be confident of the quality of the solutions ('kits') that have been prepared, both accuracy and precision must be tested. Accuracy is best assessed by confirming that the correct result can be obtained when a commercial assayed serum (with an assigned value by an appropriate method) is used as a sample. Precision should be assessed by confirming that the coefficient of variation under optimal and routine conditions (within and between batches) are acceptable using the prepared reagents and an internal quality control sample [1,2].

2.3.4 Documentation

Good documentation is an essential part of quality assurance. Its purpose is to ensure the continuity of good manufacturing practice, to prevent errors during the production process, to provide an audit trail for identifying possible failures and to allow for advanced planning. Documents should have an unambiguous content and be laid out in an orderly fashion so that each batch of starting material can be traced to the final product, and that the observations of each quality control check can be easily understood. Documentation should be made at all steps of the production line, including the receipt of materials, intermediate steps in the production, labelling, packaging, storing and mailing of the final reagents.

The labelling of each batch of reagent on the container or kit must be identical to the label stated on the register form, so as to allow for a comparison and control of the production process at any time, and also to reply correctly to incoming complaints from a consumer. It should mention:

- the trivial name of the product
- the major reagent components
- the batch number
- the date of production and/or the expiry date of the reagent.

2.3.5 References

[1] Whitehead TP. *Principles of Quality Control.* WHO, LAB/76.1, Geneva, World Health Organization, 1976.

[2] Stamm D. *Guidelines for a basic programme for internal quality control of quantitative analyses in clinical chemistry.* WHO, LAB/81.3, Geneva, World Health Organization, 1981.

2.4 Calibration of volumetric pipettes and flasks (gravimetric procedure)

For the preparation of standard solutions or of solutions needed for the calibration of methods or instruments, accurate calibrated pipettes and volumetric flasks are essential; such pipettes and flasks are commercially available (a guarantee must be requested). The following procedures describe how pipettes and volumetric flasks can be calibrated to ensure that their precise volumes are known.

Pipettes can be calibrated by weighing water (to the nearest 0,1-0,5% of the weight corresponding to the delivered volume) at a known room temperature and barometric pressure. Micropipettes of the constriction type (and others) are weighed filled with distilled water and empty (or drained) but wet. The difference in weights corresponds to the content (by weight) of the pipettes. This procedure is used for the calibration of volumetric pipettes. (NB. graduated pipettes should not be used for the preparation of standard solutions). For micropipettes (Sanz/Beckman type, self-made plastic type), pneumatic or positive displacement pipettes, or automatic dispensers or diluters (Hamilton or similar) the delivered water is weighed in a weighing bottle which is stoppered tightly after the water has been delivered. As a rule the weighing is repeated 8 - 10 times; the means of the weights or of the weighing differences are used for the calculation of the actual volumes of the water, taking into account the temperature of the water and the barometric pressure according to Table 2.4.1 [1]; the individual weights are used for the assessment of precision (standard deviation and coefficient of variation).

For the calibration of volumetric flasks, these are weighed when filled with water to the line and when empty (dry) to the nearest 0,1%; the weighing is repeated 8 - 10 times. The calculation of the actual volume is performed as for the pipettes, using Table 2.4.1.

Recalibration of pipettes and volumetric flasks will be necessary if they are dried in an oven at >50°C; to avoid the problem of recalibration, a set of calibrated pipettes and volumetric flasks for the preparation of standards should be kept separately from other pipettes and flasks.

Table 2.4.1 Calculation of the volume of a measured weight of water at various temperatures and atmospheric pressures [1]

Temp. °C	Barometric pressure					
	600 800 80.0	640 853 85.3	680 907 90.7	720 960 96.0	760 1013 101.3	800 mm Hg 1067 mbar 106.7 kPa
15	1.0018	1.0018	1.0019	1.0019	1.0020	1.0020
15.5	18	19	19	20	20	21
16	19	20	20	21	21	22
16.5	20	20	21	22	22	23
17	21	21	22	22	23	23
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	22	23	24	24	25	25
18.5	23	24	25	25	26	26
19	24	25	25	26	27	27
19.5	25	26	26	27	28	28
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	27	28	28	29	30	30
21	28	29	30	30	31	31
21.5	30	30	31	31	32	32
22	31	31	32	32	33	33
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	33	33	34	35	35	36
23.5	34	35	35	36	36	37
24	35	36	36	37	38	38
24.5	37	37	38	38	39	39
25	1.0038	1.0038	1.0039	1.0039	1.0040	1.0041
25.5	39	40	40	41	41	42
26	40	41	42	42	43	43
26.5	42	42	43	43	44	45
27	43	44	44	45	45	46
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	46	46	47	48	48	49
28.5	47	48	48	49	50	50
29	49	49	50	50	51	52
29.5	50	51	52	52	52	52
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

The figures given are in mL/g (or μL/mg), i.e. reciprocal of density.

Example using Table 2.4.1

Mean measured weight of water	4,978 g
Room (and water) temperature	19,5°C
Barometric pressure:	680 mm Hg
Correction factor from Table 2.4.1:	1,0026 mL/g

The actual volume (V) = 4.978 g x 1.0026 mL/g = 4,991 mL

The tolerances permitted in this test method for the parameters involved in calculating V are as follows:

Parameter	Maximum deviation
Water temperature	±0,25°C
Air pressure	±20 mm Hg

2.4.1 References

[1] NCCLS. Determining performance of volumetric equipment (proposed guideline), Villanova, PA, 1984.

2.5 Preparation of common reagent solutions

Caution: The concentrated solutions of acetic acid, hydrochloric acid, perchloric acid, sodium hydroxide and sulfuric acid mentioned here are corrosive. Eyes must be protected with safety glasses when using these solutions. If skin contamination occurs, wash with large volumes of water.

This section describes the preparation of standardized solutions of hydrochloric acid and sodium hydroxide. However, if standardized solutions are required infrequently, then it may be more convenient to purchase commercial volumetric solutions.

1. Acetic acid: glacial, specific gravity 1.048-1.051. CH_3COOH. Mr 60.05

   To prepare acetic acid, 1 mol/L, dilute 58 mL to 1 litre with water.

2. Hydrochloric acid; concentrated, specific gravity 1,18; HCl, Mr 36,45

Table 2.5.1 shows the volumes of concentrated hydrochloric acid which must be diluted to 1 litre with distilled water to give solutions with the approximate molarities indicated.

More dilute solutions, such as 0,05 mol/L are best prepared by appropriate dilution of 0,5 or 0,1 mol/L solutions.

Table 2.5.1 Preparation of solutions with defined HCl concentration

Volume of concentrated HCl	Dilute with distilled water to:	Approximate molarity
9 mL	1 litre	0.1 mol/L
45 mL	1 litre	0.5 mol/L
90 mL	1 litre	1 mol/L
450 mL	1 litre	5 mol/L
540 mL	1 litre	6 mol/L

For adjusting the pH of buffers, solutions of approximate molarity are adequate, but the exact molarity can be determined (when required) by standardizing with sodium carbonate solution (0,1 mol/L) as follows:

Sodium carbonate solution, 0,1 mol/L. Accurately weigh out 1,06 g of dry sodium carbonate, transfer to a 100 mL volumetric flask, dissolve and dilute to 100 mL with distilled water. (Sodium carbonate must be dried by heating at 275°C for 2 hours and then cooled to room temperature in a desiccator before weighing).

The hydrochloric acid solution must be standardized with the sodium carbonate solution as follows: transfer 10,0 mL of sodium carbonate solution (0,1 mol/L) into a 25 mL Erlenmeyer flask. Add 2 drops of methyl orange indicator solution (0,15 g methyl orange dissolved in 100 mL water). Titrate with hydrochloric acid solution (0,1 mol/L) from a 10 mL burette until the colour changes from yellow to orange. The molarity of the hydrochloric acid solution is calculated as follows:

molarity of HCl (mol/L) = (100 x 10)/ volume of HCl required

Adjust the molarity of the hydrochloric acid to exactly 100 mmol/L, if necessary as follows:

Example: If the molarity of the hydrochloric acid is found to be 103 mmol/L, then add 15 mL of distilled water to 500 mL of hydrochloric acid to adjust the molarity to exactly 100 mmol/L, as shown in the following formula:

$$\frac{\text{(volume of HCl x measured molarity)}}{\text{exact molarity required}} = \text{volume of HCl after dilution with water}$$

for the example above: $\frac{500 \times 103}{100} = 515$

3. Perchloric acid: available as 70% (specific gravity 1,67) or 60% (specific gravity 1,53), $HClO_4$, Mr 100,46

To prepare perchloric acid, 1 mol/L, dilute 80 mL of 70% perchloric acid or 109 mL 60% perchloric acid to 1 litre with water. To prepare perchloric acid, 0,33 mol/L, dilute 250 mL perchloric acid (1 mol/L) with 500 mL water.

4. Sodium chloride solution ('normal' or 'physiological saline' 0,154 mol/L); NaCl, Mr 58,44

Weigh 9,00 g NaCl and make up to 1 litre with water.

5. Sodium hydroxide; NaOH, Mr 40,0

For most purposes, such as adjusting the pH of buffers, sodium hydroxide solution can be prepared by weighing the required amount of sodium hydroxide pellets and dissolving in water. When preparing sodium hydroxide solutions, dissolve small amounts of pellets at a time in water to avoid the production of excessive heat. Table 2.5.2 shows the amounts of NaOH required for 1 litre of solution with the approximate molarities indicated.

NOTE: The volumes must be adjusted to 1 litre after the solutions have cooled to room temperature.

Table 2.5.2 Preparation of sodium hydroxide solutions

NaOH (g)	Volume of final solution	Approximate molarity (mol/L)
4	1 litre	0.1
8	1 litre	0.2
10	1 litre	0.25
16	1 litre	0.4
20	1 litre	0.5
40	1 litre	1
200	1 litre	5

More dilute solutions, such as 10 mmol/L, are best made by an appropriate dilution of one of the above solutions.

When accurately standardized solutions of sodium hydroxide are required, precautions must be taken to ensure that carbonates are absent. Carbonate may be present on the pellets, particularly if the bottle of pellets is old or carbon dioxide may be absorbed from the atmosphere into solutions of sodium hydroxide.

Carbonate-free sodium hydroxide solutions can be prepared from a saturated solution of sodium hydroxide by diluting, in carbon dioxide-free water, as follows:

Carbon dioxide-free water. Water free from carbon dioxide can be prepared by boiling distilled water for 30 minutes to expel the carbon dioxide. The boiled water may then be bottled while still warm and stored in a tightly stoppered bottle (preferably screw-cap).

Saturated sodium hydroxide solution. Carefully prepare about 100 mL of a saturated solution of sodium hydroxide in distilled water; about 50 g will be required. Allow the carbonates to settle out, then use the supernatant for preparing the stock solution, 0,1 mol/L. The saturated solution should be stored in a plastic bottle with a tightly-fitting top. It is stable indefinitely at 20-25°C.

Sodium hydroxide 0,1 mol/L. Using a rubber bulb, carefully pipette 2,7 mL of the saturated sodium hydroxide solution and dilute to 500 mL using carbon dioxide-free water. Standardize by titration against 4 mL of the 0.1 mol/L hydrochloric acid using 0,1 mL of phenol red indicator. Adjust to exactly 0,1 mol/L. Store this solution in a tightly-stoppered plastic bottle and renew weekly.

6. Sulfuric acid; concentrated, specific gravity 1,84, H_2SO_4, Mr 98,08

To prepare sulfuric acid, (1 mol/L) dilute 54,5 mL to 1 litre with water. Always prepare the dilution by slowly adding the sulfuric acid, while mixing, to the water.

Never add water to concentrated sulfuric acid (see also Section 1 of Chapter 1).

Allow the solution to cool to room temperature before adjusting the volume to 1 litre.

7. Water, pure, specific gravity 1,00, H_2O, Mr 18,02

Water is the most widely used chemical in the laboratory and it is essential to use water of adequate quality if errors are to be avoided. Water may contain significant amounts of calcium, sodium and potassium salts which may interfere with the analysis of these cations in blood. In addition there may be other substances that will inhibit enzymes (e.g. from moulds and bacteria or heavy metals and detergents); such inhibition may affect enzymes being measured in blood or may inhibit enzymes used in reagents. Several methods are available for purifying water (see Section 2.1).

Specifications for the quality of water required in clinical laboratories have been given by NCCLS [1] as shown in Table 2.5.3:

Table 2.5.3 Specifications for water quality

Property	Type I	Type II	Type III
Bacterial content: Colony forming units/mL(maximum)	10	1000	not applicable
pH	not specified	not specified	5,0-8,0
Silicates mg/L	0,05	0,1	1,0
Resistivity-minimum (MOhm/cm at 25°C)	10	1,0	0,1
Conductance-maximum (µS/cm at 25°C)	0,1	1,0	10

Type I water must be free of particulate matter >0,6 µm; this can be achieved by passing the water through a membrane filter with a mean pore size no greater than 0.6 µm. The specification for Type I water also requires treatment with activated charcoal to remove organic material.

In laboratories with limited resources, it will be difficult to achieve the specifications for water of Type I or Type II. For many purposes water with a conductivity of less than 20 µS will be adequate. To achieve greater reagent stability, the prepared reagent should be filtered to remove bacteria and other organisms using, for example, a filter with a pore size of 0,22 µm (see also Section 3.1.2.2).

2.5.1 References

[1] NCCLS. Preparation and testing of reagent water in the clinical laboratory (tentative guidelines), 2nd edition, Villanova, PA, 1988.

2.6 Reference buffers

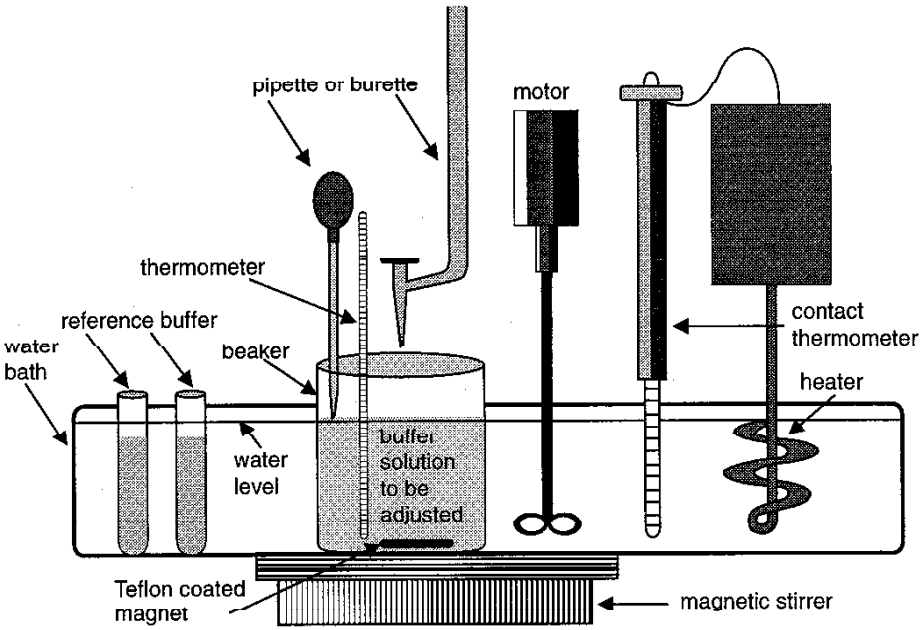
2.6.1 The use of reference buffers and their purpose

The purpose of reference buffers is to calibrate pH meters used to adjust the pH of buffers, various solutions and other media used in the clinical laboratory.

The reliability required for the pH adjustment of solutions depends on the precision and accuracy needed. As the pH of buffers is highly temperature-dependent this parameter must be considered in most instances, especially for the determination of the

catalytic concentration of enzymes, where usually an accuracy of ±0,05 (sometimes ±0,01) pH units is necessary. The use of a thermostabilized water-bath (+/- 0,05°C) is essential. The temperature of the reference buffer must be the same as that of the media to be adjusted: the procedure is illustrated in Figure 2.1.

Figure 2.1 Assembly of equipment for the preparation of standard buffers



* Use pipette or burette

Sometimes, if the pH to be adjusted is too distant from the pH of the reference buffer (especially in the alkaline region), two reference buffers must be used for the calibration of the pH meter, one reference buffer with a lower, the other with a higher pH than the pH to be adjusted.

2.6.2 Preparation of reference buffers

Chemicals should always be of analytical grade. Water should be of high purity (Type I, filtered through 0,22 µm filter), CO_2-free (especially for buffers with pH over 7,0). The water is gently boiled in an open Erlenmeyer (conical) flask for 30 minutes; then cooled to room temperature, the flask tightly closed with a clean rubber stopper, or preferably with a screw cap.

The reference buffers are prepared according to the procedures described by the National Bureau of Standards [1].

1. Potassium hydrogen phthalate buffer (0,05 mol/L)

Chemicals

Potassium hydrogen phthalate: analytical grade, $KHC_8H_4O_4$, Mr 204,22
The salt should be dried for 2 hours at 110°C and cooled to room temperature before use.

Potassium hydrogen phthalate, dried	10,211 g
Distilled H_2O	200 mL

- Dissolve

Distilled H_2O to	1000,0 mL

- Mix well by shaking.
  Store in a tightly stoppered polyethylene bottle in order to protect from evaporation and contamination by moulds.

It should be replaced every 2-3 months or when mould is apparent. The buffer needs no protection from atmospheric carbon dioxide.

pH at various temperatures

Temperature (°C)	pH
15	3,996
20	3,999
25	4,004
30	4,011
37	4,024
40	4,030

2. Potassium-sodium phosphate buffer (39,125 mmol/L)

KH_2PO_4: 8,695 mmol/L
Na_2HPO_4: 30,43 mmol/L

Chemicals

Potassium dihydrogen phosphate, anhydrous, analytical grade , KH_2PO_4, Mr 136,13

Disodium hydrogen phosphate, anhydrous, analytical grade, Na_2HPO_4, Mr 141,982

The salts should be dried for 2 hours at 110°C and cooled to room temperature in a desiccator before use.

Potassium dihydrogen phosphate, dried	1,184 g
Disodium hydrogen phosphate, dried	4,320 g

- Dissolve;
  Distilled H_2O to 1000,0 mL
- Mix well by shaking.
- Store the solution in a tightly stoppered polyethylene bottle and replace after four weeks or sooner if moulds or sediment appear or if it has been exposed repeatedly to air.

This buffer is sensitive to contamination with atmospheric carbon dioxide; if the solution is to maintain the assigned pH for a few weeks, exclusion of carbon dioxide is essential.

pH at various temperatures

Temperature (°C)	pH
15	7,449
20	7,430
25	7,415
30	7,403
37	7,392
40	7,388

3. Sodium bicarbonate/carbonate buffer 0,05 mol/L (0,025 mol/L for both $NaHCO_3$ and Na_2CO_3)

Chemicals

Sodium hydrogen carbonate (bicarbonate), anhydrous, analytical grade, $NaHCO_3$, Mr 84.02. should not be dried by heating.
Sodium carbonate, anhydrous, analytical grade, Na_2CO_3, Mr 106,00, should be dried for 2 hours at 275°C and cooled in a desiccator before use.

Sodium hydrogen carbonate (bicarbonate)	2,101 g
Sodium carbonate, dried	2,65 g
Distilled H_2O	200 mL

- Dissolve
  Distilled H_2O to 1000,0 mL
- Mix well by shaking.

Although elaborate precautions to prevent contamination of the buffer with atmospheric carbon dioxide are usually unnecessary, the container (polyethylene bottle)

should be kept tightly stoppered. Replace the stopper rapidly after use. The buffer should be renewed after 3-6 months or sooner if moulds appear.

pH at various temperatures

Temperature (°C)	pH
15	10,120
20	10,064
25	10,014
30	9,968
35	9,928
37	9,906
40	9,891

4. Sodium tetraborate buffer 10 mmol/L

Chemicals

Sodium tetraborate, decahydrate (borax), analytical grade,
$NaB_4O_7 \cdot 10\ H_2O$, Mr 381,44; the salt should not be dried in an oven before use.

Borax (crush large lumps)	3,814 g
Distilled H_2O	300 mL

- Dissolve;

Distilled H_2O to	1000,0 mL

- Mix well by shaking.
- Store in a tightly stoppered polyethylene bottle to prevent contamination with atmospheric carbon dioxide.
- Replace the stopper rapidly after use.
  The buffer should be replaced after 3-6 months or sooner if moulds appear.

pH at various temperatures

Temperature (°C)	pH
15	9,276
20	9,227
25	9,183
30	9,143
37	9,093
40	9,074

5. Tris(hydroxymethyl) aminomethane/HCl buffer (TRIS)

Reference electrodes containing linen fibre or dialysis membrane junctions should not be used with this buffer. For this reason it is not included as a reference buffer.

2.7 REFERENCES

[1] Durst R. *Standard Reference Materials: Standardization of pH Measurement,* National Bureau of Standards, U.S. Department of Commerce, December 1975.

3

REAGENTS AND METHODS FOR CLINICAL CHEMISTRY

3. Reagents and methods for clinical chemistry

The choice of the analytes included in this chapter has been dictated by the needs of the countries for which the manual is intended. Similar considerations have prevailed in the choice of the methodology. Simple tests, mainly those described in the WHO document "Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories" LAB/86.3, as well as more sophisticated methods, often close, or even identical, to existing reference methods have been included. Though this manual is devoted to the preparation of reagents and solutions, short descriptions of the methods of analysis are included, as the original documents are not always easily accessible. Reference methods have not been considered, except for glucose, as they are often too complicated for routine use. In the case of the alanine/aspartate aminotransferases [ALAT (GPT), ASAT (GOT)] the chosen Société française de Biologie clinique (SFBC) methods are close to the International Federation of Clinical Chemistry (IFCC) methods but easier to perform. The reference and/or the usual ranges (previously called the normal ranges) are not mentioned as these may vary from region to region (or may be subject to regional variations).

The volumes mentioned in the short procedures correspond to those indicated in the original publications; these volumes can be changed (reduced or increased) as long as the proportions of solutions and samples are identical and the reliability of the methods is not altered.

In the preparation of reagents, a zero after the comma indicates the need for analytical precision in the measurement of volumes or weighing (e.g. 1000,0 mL, or 13,0 g); if no zero is shown after the comma, this precision is not essential.

In the case of enzymes, in order to simplify the presentation, the catalytic activity is expressed in U (international unit) corresponding to µmol/minutes and sometimes for practical reasons as KU corresponding to 10^3 µmol/minutes. The catalytic concentrations are expressed in units per litre as follows:

U/L = (µmol)/L

The derived SI unit katal (mol/s, kat) or its subunits (µkat, nkat), only adopted in a few countries, has not been considered. Nevertheless, the conversion of international units (U) into SI units can be performed, if wished, as follows:

1 µkat = 1U/60 = 0,016 67 U
1 nkat = 10^{-3} U/60 = 0,000 016 67 U

The same would apply for the catalytic concentrations.

The specific activity (SpA, U/mg of enzyme protein) per milligram of lyophilisate of an enzyme preparation may vary from manufacturer to manufacturer or from batch to batch. Therefore the user must always take into account the information given on the label or in the catalogue to calculate the catalytic concentration of the enzyme for the preparation of the reagent.

3.1 Alanine aminotransferase (ALAT, ALT)

L-alanine: 2-oxoglutarate aminotransferase, (EC 2.6.1.2)

Previously: glutamate-pyruvate transaminase (GPT)

3.1.1 Methods

An IFCC reference method is available [1], but not described here.

The method according to the recommendation of the SFBC [2] is described here. It is a spectrometric, continuous monitoring method, derived from the IFCC method.

In the IFCC and SFBC methods the temperature of 30°C for the measurement of the catalytic concentration of ALAT is included in the physical and chemical measurement conditions. However, for practical reasons, essential in hot countries, the temperature of 37°C is more suitable; also in many modern analysers 37°C is the only one which can be used. Fortunately, the abovementioned methods work equally well at 37°C [3]. For this reason the pH of the buffer is mentioned at 30 and 37°C but catalytic activities of the reagent enzymes are expressed at 30°C (in excess).

Also described here is the colorimetric method of Reitman and Fränkel [4].

3.1.2 The SFBC method for the measurement of ALAT

3.1.2.1 REAGENTS

1. Tris (hydroxymethyl) amino methane (TRIS), $C_4H_{11}NO_3$, Mr 121,14
2. L-Alanine, $C_3H_7NO_2$, Mr 89,1
3. 2-Oxoglutaric acid (α-ketoglutaric acid), $C_5H_6O_5$, Mr 146,10
4. ß-Nicotinamide-adenine dinucleotide, reduced, disodium salt, (NADH) $C_{21}H_{27}N_7O_{14}P_2Na_2$, Mr709,4

5. L-Lactate:NAD oxidoreductase, (EC 1.1.1.27, lactate dehydrogenase, LDH) from pig skeletal muscle; specific activity higher than 540 U/mg, (30°C), in glycerol (reagent 8)
6. Pyridoxal-5-phosphoric acid monohydrate, (Pyridoxal phosphate, P5P) $C_8H_{10}O_6NP \cdot H_2O$, Mr 265,2
7. Hydrochloric acid, (1 mol/L)
8. Glycerol, 50% (v/v) in aqueous solution, $C_3H_8O_3$, Mr 92,10
9. Sodium chloride (0,154 mol/L)
10. Sodium hydroxide (1 mol/L)
11. Ammonium acetate, $C_2H_3O_2(NH_4)$, Mr 77,08
12. Albumin, bovine serum
13. Sodium pyruvate, $C_3H_3O_3Na$, Mr 110,0

3.1.2.2 SOLUTIONS

To prevent the growth of microorganisms in the solutions, sterilize the containers (about 1 hour at 150°C) in a dry oven. Prepare all solutions in volumetric flasks and with volumetric pipettes with fresh distilled or deionized water [1]. The pH of solutions must be adjusted at 30°C or 37°C ±0.1°C (according to the measurement temperature) with a pH meter calibrated at one of these temperatures with reference buffers (Section 2.6).

I. TRIS (109 mmol/L), L-Alanine (624 mmol/L) buffered, pH 7,5 (30°C) 7,35 (37°C)

TRIS	1,32 g
L-Alanine	5,56 g
Distilled H_2O about	80 mL
pH adjusted with HCl (1 mol/L) to 7,5 (30°C), or 7,35 (37°C)	
Distilled H_2O to	100,0 mL

Stable for at least six months at 0-4°C; bacterial contamination can be prevented by addition of sodium azide 8 mmol/L (46,5 mg in the 100 mL of solution I).

II. TRIS (109 mmol/L)/HCl (approx. 80 mmol/L), buffer pH 7,5 (30°C), or 7,35 (37°C)

TRIS	2,64 g
Distilled H_2O about	160 mL
pH adjusted with HCl (1 mol/L) to pH 7,5 (30°C), 7,35 (37°C)	
Distilled H_2O to	200,0 mL

The solution has the same stability as Solution I.

III. Pyridoxal phosphate (6.24 mmol/L)

Pyridoxal phosphate	0,0165 g
TRIS/HCl buffer	10,0 mL

Stable for two weeks at 0-4°C in a dark bottle.

IV. NADH (11,2 mmol/L)

NADH	0,0159 g
TRIS/HCl buffer	2,0 mL

Stable for 2 weeks at 0-4°C in a dark bottle.

V. LDH 151,2 KU/L at 30°C and 211,7 KU/L at 37°C

Mix the enzyme solution in glycerol (reagent 8, volume fraction 0,5) according to its original catalytic concentration and adjust with glycerol (volume fraction 0,5) to give the above-mentioned concentration (see Section 3.1.2.1 and Section 3.1.2.3).

Stable for at least 6 months at 0-4°C if no bacterial contamination.

VI. Working solution

TRIS/L-Alanine buffer (Solution I)	100,0 mL
Pyridoxal phosphate (Solution III)	2,0 mL
NADH (Solution IV)	2,0 mL
LDH (Solution V)	1,0 mL

Stable for one day at 0-4°C in a dark bottle.

VII. TRIS (109 mmol/L); 2-oxoglutarate (180 mmol/L)

pH 7,5 (30°C), 7,35 (37°C)	
TRIS	1,32 g
2-Oxoglutaric acid	2,63 g
Distilled H_2O about	80 mL
pH adjusted with NaOH (1 mol/L) to 7,5 (30°C), or 7,35 (37°C)	
Distilled H_2O to	100,0 mL

Stable for two weeks at 0-4°C if no bacterial contamination.

3.1.2.3 MEASUREMENT OF THE CATALYTIC CONCENTRATION OF THE REAGENT ENZYME

The measurement conditions (temperature, pH, ionic strength, type of buffer, concentration of substrates and cofactors) as well as the equipment must be the same as those used for the assay of overall ALAT determination and at the chosen temperature (30°C or 37°C).

LDH measurement

Prepare two samples from reagent V with catalytic concentrations of LDH of approximately 300 and 600 U/L, (30°C), by diluting them with sodium chloride (0,154 mol/L) containing albumin (10 g/L).

The solutions for the LDH measurement are prepared as for ALAT measurement except that solution V (LDH) is excluded from the working solution (solution VI); 2-oxoglutarate (solution VII) is replaced by sodium pyruvate (36 mmol/L) and used as starting reagent (40 mg of Na pyruvate dissolved in 10 mL of H_2O).

The catalytic concentration of LDH is determined in the two samples of diluted LDH reagent (reagent V); for reagent blank NaCl (0,154 mol/L) is used.

In the measurements proceed as follows:

Temperature 30°C or 37°C (± 0,1°C);

- Add in an optical glass cuvette of 10 mm lightpath:
  Working solution (solution VI) (without LDH (solution V) 2,00 mL
  Sample (diluted LDH solutions, or NaCl,
  0,154 mol/L for sample blank) 0,20 mL
- Mix and ensure temperature equilibrium at 30°C or 37°C, then add:
  Na pyruvate 0,20 mL
- Record the change of absorbance at 340 nm (or 334 nm, or 365 nm) immediately after adding pyruvate.
- Use the change of absorbance ($\Delta A/\Delta t$) in the time period of 20-60 seconds, corrected for the reagent blank when calculating the catalytic concentrations b of LDH in the sample.

b in μmol/(min.L), Δt in minutes

340 nm : $b = 1{,}905 \times 10^3 \times (\Delta A/\Delta t)$ U/L

334 nm : $b = 1{,}942 \times 10^3 \times (\Delta A/\Delta t)$ U/L

365 nm : $b = 3{,}529 \times 10^3 \times (\Delta A/\Delta t)$ U/L

3.1.2.4 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS [1]

1. Measurement of contaminant enzymes in the reagent LDH

There are two possibilities of contamination by interfering enzymes in the LDH preparations: ALAT and glutamate dehydrogenase (GlDH, EC 2.6.1.2).

ALAT

The contamination by ALAT corresponds to the conversion rate of the reagent blank of the ALAT measurement (in the absence of sample solution). The relative contamination by ALAT in the LDH preparation should not exceed 5.10^{-5} (relative activities).

GLUTAMATE DEHYDROGENASE

93 mg ammonium acetate is dissolved in 10 mL of working solution (solution VI) (121 mmol/L); final concentration in the test: 0,1 mol/L.

The same equipment is used as for the ALAT measurement.

Temperature 30°C or 37°C (±0,1°C);

- Add in an optical glass cuvette with a light path of 10 mm:

Working solution (solution VI) with ammonium acetate	2,0 mL
(or for blank reaction solution VI, without ammonium acetate)	2,0 mL

- Mix and ensure temperature equilibrium, then add:

2-Oxoglutarate (solution VII)	0,20 mL

A lag phase of 120 seconds may occur.

- Record the change of absorbance at 340 nm (or 334, or 365 nm).

Calculate the catalytic concentration, using the formula as for LDH (Section 3.1.2.3).

The relative contamination by GlDH in the LDH preparation should not exceed $3x10^{-5}$ (relative activities).

2. 2-Oxoglutarate

Must be free of pyruvate and lactate

3. Pyridoxal phosphate (P5P)

The purity of pyridoxal phosphate is estimated from its absorbance at 388 nm and at 25°C in a solution of P5P in NaOH (0.1 mol/L). The purity must be higher than 0.995 based on the molar absorbance of fully ionized P5P of 650 m^2/mol.

4. NADH

The value for the absorbance of NADH under the conditions of this method is 630 m^2/mol at 340 nm and 30°C (see also reference 5).

3.1.2.5 ALAT MEASUREMENT BY SFBC METHOD

Principle

$$\text{2- Oxoglutarate + L-Alanine} \xrightarrow[\text{P5P}]{\text{ALAT}} \text{L- Glutamate + L-Lactate}$$

$$\text{L-Lactate + NADH+ H}^{+} \xrightarrow{\text{LDH}} \text{Pyruvate + NAD}^{+}$$

during preincubation: exhaustion of interfering endogenous lactate.

The decrease of absorbance is measured at 340, 334 or 365 nm.

Measurement conditions

Temperature: 30°C or 37°C.

pH: 7,35 ±0,05 at 37°C; 7,5 ±0,05 at 30°C.

Wavelength: 339 nm (340); alternately with Hg line photometer only at 334 or 365 nm.

Lightpath in square thermostabilized cuvettes: 10 mm.

Sample: serum or heparin plasma.

- Read absorbance against water or air.

	Blank	Samples
Working solution (solution VI) (mL)	1.0	1.0
NaCl, 154 mmol/L (mL)	0,1	-
Sample (mL)	-	0,1

- Mix thoroughly, preincubate for 10 minutes at 30°C or 37°C.

TRIS/oxoglutarate (solution VII) (mL)	0,1	0,1

- Mix thoroughly, let equilibrate for 120 seconds, monitor change of absorbance during 200 seconds.

Use: Δ absorbance (ΔA)/min for calculation.

Calculation of catalytic concentration b:

Molar lineic absorbance of NADH at pH 7,5:

339 nm (340) :	630 m²/mol
334 nm:	618 m²/mol
365 nm:	340 m²/mol

$$b_{339\,(340)} = (\Delta A_{Sample}/\text{min} - \Delta A_{Blank}/\text{min}) \times 1905\ \text{U/L}$$

b_{334} = (ΔA_{Sample}/min - ΔA_{Blank}/min) x 1942 U/L

b_{365} = (ΔA_{Sample}/min - ΔA_{Blank}/min) x 3529 U/L

Limit of detection: 2,5 U/L

Linearity of measurement: 288 U/L at 30°C and 350 U/L at 37°C. Above this value the sample must be diluted with NaCl (0,154 mol/L), the measurement repeated and the value multiplied by the dilution factor.

3.1.3 ALAT measurement by Reitman and Fränkel method

3.1.3.1 REAGENTS

1. Orthophosphoric acid, disodium salt, anhydrous, Na_2HPO_4, Mr 141,98
2. Orthophosphoric acid, monopotassium salt, anhydrous, KH_2PO_4, Mr 136,13
3. 2-Oxoglutaric acid (α-ketoglutaric acid), $C_5H_6O_5$, Mr 146,1
4. D,L-Alanine, $C_3H_7NO_2$, Mr 89,1
5. Sodium hydroxide (1 mol/L and 0,4 mol/L)
6. 2,4-Dinitrophenylhydrazine (with water), $C_6H_6N_4O_4$, Mr 198,14 (dry substance)
   Caution: may explode violently when dry. Do not dry the wet substance.
7. Hydrochloric acid (1 mol/L)
8. Sodium pyruvate, $C_3H_3O_3Na$, Mr 110,0
9. Triethanolamine, $C_6H_{14}NO_3$, Mr 149,23
10. Ethylenediamine tetra-acetic acid, disodium salt, $C_{10}H_{14}N_2O_8Na_2.2H_2O$, Mr 372,24

3.1.3.2 SOLUTIONS

The same precautions should be followed as those described in Section 3.1.2.2.

I. Phosphate buffer (0,1 mol/L).pH 7,4

Na_2HPO_4 (anhydrous)	11,9 g
KH_2PO_4 (anhydrous)	2,2 g
Distilled H_2O	800 mL
pH adjusted with the appropriate phosphate to 7,4	
Distilled H_2O to	1000,0 mL

Stable for two months at 2-8°C.

II. Buffered substrate: 2-oxoglutarate (2 mmol/L), D,L-Alanine (0,2 mol/L)

2-Oxoglutarate	0,029 2 g
D,L-Alanine	1,78 g
NaOH (1 mol/L) until the chemicals are dissolved, pH adjusted with NaOH (1 mol /L) to 7.4	
Phosphate buffer (Solution I) to	100,0 mL

III. Colour reagent: 2,4-dinitrophenylhydrazine (1 mmol/L)

Note that the weight of 2,4-dinitrophenylhydrazine must be adjusted to take into account the water content (indicated by the manufacturer).

2,4-Dinitrophenylhydrazine, if dry it would be	0,019 8 g
HCl (1 mol/L) to	100,0 mL

Stable for 2 months at 2-8°C.

IV. Triethanolamine (51 mmol/L), ethylene-diamine tetra-acetate (EDTA, 5,373 mmol/L) buffer

Triethanolamine	3,8 g
EDTA	0,5 g
Distilled H_2O about	100 mL
HCl (1 mol/L)	15 mL
- *Check that pH is 7,5-7,6*	
Distilled H_2O to	500,0 mL

Stable indefinitely at 2-8°C.

V. Pyruvate standard (4 mmol/L)

Na pyruvate	0,044 g
Triethanolamine buffer (Solution IV)	100,0 mL

- Mix well and divide into small portions of about 1 mL and store in a freezer. Stable for 6 months when frozen and for 1 week at 2-8°C.

3.1.3.3 ALAT MEASUREMENT BY REITMAN AND FRÄNKEL METHOD

- Label sufficient tubes for reagent blank (B), standard (S), controls (C1, C2) and patient's samples (1, 2, 3, etc.), control blanks (C1B, C2B) and patient's sample blanks (1B, 2B, 3B, etc.).
- Remove one portion of pyruvate standard from the freezer, thaw and mix well.
- Pipette into the tubes as follows:

	B	S	C1,C2 1,2,3, etc.	C1B,C2B 1B,2B,3B
Buffered substrate (solution II)(mL)	0,5	0,5	0,5	0,5
- Put buffered substrate (solution II) back in refrigerator.				
- Warm all tubes at 37°C for 5 minutes, then add:				
Distilled H_2O (mL)	0,1	-	-	-
Pyruvate standard (mL)	-	0,1	-	-
Control's or patient's sample (mL)	-	-	0,1	-
- Mix and keep all tubes at 37°C for 30 minutes, then add:				
Colour reagent (mL)	0,5	0,5	0,5	0,5
Control's or patient's sample (mL)	-	-	-	0,1
- Mix: after 20 minutes at room temperature add:				
Sodium hydroxide, 0,4 mol/L (mL)	5,0	5,0	5,0	5,0

- Mix thoroughly; wait for 5 minutes, then measure the absorbances.
  Colorimeter: green filter, Ilford number 604 (520 nm).
  Spectrometer: 505 nm.

  Set the instrument to zero with tube B.
- Calculate the amount of pyruvate formed and use Table 3.1.1 to convert to ALAT (U/L).
- Check the control results.

Table 3.1.1 Conversion of pyruvate formed (µmol/L) in 30 min into ALAT activity (U/L) at 37°C

PYRUVATE FORMED (µmol/L)/30 min	ALAT ACTIVITY U/L at 37°C	PYRUVATE FORMED (µmol/L)/30 min	ALAT U/L at 37°C
60	2	1560	40
120	3	1620	42
180	4	1680	44
240	5	1740	46
300	7	1800	47
360	8	1860	49

(Continued.)

PYRUVATE FORMED (μmol/L)/30 min	ALAT ACTIVITY U/L at 37°C	PYRUVATE FORMED (μmol/L)/30 min	ALAT U/L at 37°C
420	9	1920	53
480	11	1980	55
540	13	2040	56
600	14	2100	60
660	15	2160	62
720	16	2220	64
780	17	2280	66
840	18	2340	67
900	20	2400	69
960	22	2460	71
1020	24	2520	73
1080	25	2580	76
1140	27	2640	80
1200	29	2700	84
1260	31	2760	87
1320	33	2820	91
1380	35	2880	95
1440	36	2940	98
1500	38	3000	102
		3060	109

3.1.4 Certified reference materials

In preparation by Measurement and Testing Programme (M & T) of the European Community.

3.1.5 References

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[2] Mathieu M, Guidolet J, Junien C, Lalégerie P et al. (Société Française de Biologie Clinique, Commission Enzymologie). Recommendations for determining the catalytic concentration of alanine aminotransferase in

human serum at +30°C. *Ann. Biol. Clin,* 1982, 40:154-157. Mathieu M, Aubry A, Bailly M, Braun J-P et al. Additional information regarding the recommended method for measuring the catalytic concentration of alanine aminotransferase in human serum at +3°C. *Ann. Biol. Clin.,* 1982, 40:158-159.

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[4] Reitman S and Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.* 1957, 28:56-63.

[5] Gerhardt W, Kofoed B, Westlund L and Pavlu B. Quality Control of NAD. Evaluation of methods for detection of inhibitors and specifications for NAHD quality. *Scand. J. Clin. Lab. Invest, Supplement.* 1974, 139: 1-51.

3.2 Albumin

3.2.1 Introduction

There are no internationally agreed methods for albumin measurement. If a specific method is essential, then immunochemical methods are required. For routine use dye-binding methods are appropriate.

Albumin has the ability to bind certain dyes. When bromocresol green binds to albumin there is a shift in the dye's peak absorption wavelength. Serum is diluted with buffered bromocresol green (BCG) at pH 4,2. The measurement of absorbance at 632 nm (filter No. 607) within 30 seconds of mixing the serum and BCG largely avoids the problem of the non-specific reaction of BCG with globulins.

3.2.2 Bromocresol green method [1,2]

3.2.2.1 REAGENTS

1. Bromcresol green (BCG, 3,3',5,5'-tetra-bromo-m-cresol sulfonephthalein, sodium salt) $C_{21}H_{13}Br_4O_5SNa$, Mr 720,02

2. Sodium azide, NaN_3, Mr 65,02
   Caution: handle with care; poison, danger of explosion especially by conversion to heavy metal azides (e.g. lead azide in lead pipes).
3. Sodium chloride (0,154 mol/L)
4. Succinic acid, $C_4H_6O_4$, Mr 118
5. Sodium hydroxide, NaOH, Mr 40,01, and sodium hydroxide (0,250 mol/L)
6. Brij-35 (polyoxyethylene lauryl ether)
7. Bovine serum albumin or other calibrators, such as a reference or control serum with assigned values for albumin or human serum albumin (Cohn fraction V)

3.2.2.2 SOLUTIONS

I. Succinic acid (424 mmol/L)

Succinic acid	1,0 g
Distilled H_2O to	20,0 mL

- Prepare as required; discard after use.

II. Brij-35 (250 g/L)

Brij-35, solid	25,0 g
Distilled H_2O about	25,0 mL

- Warm to dissolve at 60°C; let cool and add:

Distilled H_2O to	100,0 mL

III. Working dye solution: succinic acid (47,5 mmol/L); BCG (80,6 µmol/L); NaN_3 (1,54 mmol/L); buffered at pH 4,2

Succinic acid	5,6 g
BCG, sodium salt	0,058 g
NaOH	1,0 g
Brij 35 (solution II)	2,5 mL
Distilled H_2O about	800 mL

- Dissolve:

NaN_3	0,1 g
Distilled H_2O about	100 mL

- Dissolve and mix both solutions.
- Adjust pH with NaOH (0,25 mol/L) or succinic acid (solution I) to pH 4,20.
- Transfer slowly (avoid frothing) to a 1 litre volumetric flask.

Distilled H_2O to	1000,0 mL

- Filter through 0,6 µm glass filter and fill into appropriate polyethylene containers. The solution is stable for several months at 2-8°C.
  This solution requires careful preparation. Several commercial BCG solutions are available; ensure you select a BCG solution in succinate buffer at pH 4,2. For the control of BCG solutions see Section 3.2.2.3.

IV. Succinate (47,5 mmol/L), pH 4,2

Prepare the succinate solution by the same procedure as for the working dye solution (solution III) but without BCG.

V. Sodium chloride (0,154 mol/L); Sodium azide (15,4 mmol/L)

NaN_3	1,0 g
NaCl (0,154 mol/L) to	1000,0 mL

Stable indefinitely at 20-25°C.

VIa. Albumin standard solution (100 g/L)

- Dry about 5,3 g of bovine serum albumin (BSA) powder overnight at about 60°C.
- After drying, cool in a desiccator and weigh out in a small beaker exactly: BSA 5,0 g
- Float the powder on the surface of NaCl solution (solution V), about 30 mL
- After the albumin has dissolved, transfer quantitatively and slowly into a 50 mL flask (down the side of the flask to avoid frothing); then add:
  NaCl solution (solution V) to 50,0 mL

The solution VIa is the same as the standard used for total protein (100 g/L) and is diluted to serve as standard for the determination of albumin (solution VIb):

VIb. Diluted albumin solution (50 g/L)

Albumin standard solution (solution VIa)	5,0 mL
$NaCl/NaN_3$ solution (solution V)	5,0 mL

Stable for six months at 2-8°C.

NOTE. Reference or control serum or commercial calibrators with assigned values for albumin may also be used; such material should not be turbid.

3.2.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

BCG working solution

In a 10 mm lightpath cuvette this solution should have the following absorbance readings (zero the instrument on distilled water):

Spectrometer		Colorimeter	
(nm)	absorbance	Ilford filter	absorbance
430	about 1,4	601	about 1,1
615	about 0,25	607	about 0,2

The pH should be 4,15 to 4,25.

3.2.2.4 MEASUREMENT OF SERUM/PLASMA ALBUMIN: BROMOCRESOL GREEN

Absorbance readings for this method are taken immediately for each tube after mixing the standard, control or patient's sample with the working dye solution.

Colorimeter: orange filter, Ilford 607 (600 nm)
Spectrometer: 632 nm

- Set the instrument to zero absorbance with the working dye solution.
- Label sufficient tubes for the batch including standard (S), control (C1,C2) and patients' samples (1, 2, 3, etc.).
- Pipette into the tubes as follows:

	S	C1, C2	1,2,3, etc.
Working dye solution (mL)	4,0	4,0	4,0
Standard, 50 g/L (mL)	0,02	-	-
Controls (mL)	-	0,02	-
Patient's sample (mL)	-	-	0,02

- Mix tube 'S' well immediately after adding the 0,02 mL volume and read the absorbance.
- Proceed to add 0,02 mL of control to tube 'C1'
- Mix well and read the absorbance. Proceed in this way adding the 0,020 mL volume to each tube and reading the absorbance before going on to the next sample.
- Calculate the results in g/L and check the control results.

NOTE: A serum/plasma blank is required if the patients' sample is very turbid (use 4,0 mL solution IV and add 0,02 mL sample).

3.2.3 Certified reference material

Available as bovine serum albumin from the National Bureau of Standards (NBS); SRM 926 and 927.

3.2.4 References

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[2] Spencer K and Price CP. Influence of reagent quality and reaction conditions on the determination of serum albumin by the bromocresol green dye-binding method. *Ann.Clin.Biochem.*, 1977, 14:105-115.

3.3 Alkaline phosphatase (AP)

Orthophosphoric-monoester phosphohydrolase, alkaline optimum (EC 3.1.3.1)

3.3.1 Methods

A provisional reference method has been proposed by IFCC [1]. As an alternative, the recommendation of the SFBC [2], a widely accepted method, is presented here. This is a continuous, monitoring, spectrometric method. In addition, a two-point method suitable for routine use is described [3].

In the IFCC and SFBC methods the temperature of 30°C for the measurement of the catalytic concentration of AP is included in the physical and chemical measurement conditions. However, for practical reasons, e.g. in warm climates, the temperature of 37°C is more suitable. Furthermore, in many modern analysers this temperature is the only one which can be used. Fortunately, the methods work equally well at 37°C [4]. However, the pH of the buffer must be adjusted at 30°C as there is currently no specification for the pH of the SFBC buffer at 37°C.

3.3.2 SFBC method for the measurement of alkaline phosphatase

3.3.2.1 REAGENTS

1. 2-Amino-2-methyl-1-propanol (AMP), $C_4H_{11}NO$, Mr 89,14
2. 4-Nitrophenylphosphate, disodium salt, hexahydrate (4NPP), $C_6H_4NO_6PNa_2 \cdot 6\ H_2O$, Mr 371,2
3. Magnesium sulfate, heptahydrate, $MgSO_4 \cdot 7\ H_2O$, Mr 246,49
4. Hydrochloric acid (1 mol/L)
5. 4-Nitrophenol (4NP), high purity, $C_6H_5NO_3$, Mr 139,1
6. Sodium hydroxide, (10 mmol/L)

3.3.2.2 SOLUTIONS

To prevent the growth of microorganisms in the solutions, sterilize the containers (for about 1 hour at 15°C in a dry oven). Prepare all solutions in volumetric flasks (or with volumetric pipettes) with fresh distilled or deionized water [1]. The pH of solutions must be adjusted at 30 or 37°C ±0,1°C (according to the measurement temperature, but note that solution I must be adjusted at 30°C) with a pH meter calibrated at one of these temperatures with reference buffers (Section 2.6).

I. AMP (1,93 mol/L), pH 10,5 (30°C)

Warm the AMP to approximately 37°C until it is completely liquified.

AMP	86 g
Distilled H_2O	200 mL
pH adjusted with HCl (1 mol/L) to 10,5 (30°C)	
Distilled H_2O to	500 mL

Stable for at least one year at 4°C in a polyethylene bottle.

II. Magnesium sulfate (2,15 mmol/L)

$MgSO_4 \cdot 7\ H_2O$	0,265 g
Distilled H_2O to	500,0 mL

Stable for at least one year at 4°C in a polyethylene bottle.

III. Magnesium/AMP buffer

AMP (solution I)	1 volume
$MgSO_4$ (solution II)	1 volume

Stable for at least 3 months [1].

IV. 4NPP (0,48 mol/L)

4NPP	17,8 g
Distilled H_2O to	100,0 mL

The pH of the solution should be 8-9.

This solution is stable for at least one year if stored in small portions at -20°C. The Expert Panel on Enzymes of the IFCC has recommended to prepare smaller volumes freshly each day; stable 8 hrs at 20 -26°C. The pH must be kept between 8 and 9.

V. 4NP (1 mmol/L), stock standard (1)

- Place approximately 400 mg of high purity 4NP into a 10 mL beaker and put into an oven at 50°C for 24 hours;
- Allow to cool in a desiccator for 2 hours.

4NP (weigh quickly to prevent H_2O absorption)	0,139 g
Distilled H_2O to	1000,0 mL

Stable for at least 3 months if stored at 2-6°C and protected from light and evaporation.

VI. 4NP (0,04 mmol/L) in magnesium buffer (solution III) [1]

4NP (solution V), exactly	10,0 mL
Magnesium buffer (solution III) to	250,0 mL

- Measure the absorbance of this solution in a 10 mm cuvette at 405 nm at 30 or 37°C against a buffer blank (prepared by diluting 10,0mL of twice-distilled or deionized and distilled water to 250,0 mL with magnesium buffer [solution III]), using the same instrument as for the measurement of the catalytic concentration of AP.

The absorbance of 4NP is temperature-dependent [5], thus the absorbance measurement must be made at 30 or 37°C according to the temperature used for AP measurement. This net absorbance reading is used to confirm the molar absorbance for the calculation of the catalytic activity of AP. If the 4NP is of analytical purity it can also be used for controlling the optical performance of the photometer (see Section 3.3.2.3).

The 4NP solutions (solutions V and VI) should be prepared at least twice with absorbance readings performed in triplicate. The mean reading must agree within 0,99 - 1,01 of the mean molar absorbance of 4NP under the described condition which is within the range 1827 - 1860 m^2/mol at 30°C. The molar absorbance at 37°C is not defined; therefore if the value is required at 37°C, it must be measured, having first confirmed that the value at 30°C is within the above range.

3.3.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS [1]

AMP buffer (1,2,6)

The AMP (reagent 1), contains variable amounts of contaminants (diamines, 5-amino-3-aza-2,2,5-trimethylhexanol) inhibiting the catalytic activity of the AP. A functional test for the indirect determination of the presence of inhibitors is recommended.

The same reagents are used as for the determination of the overall AP reaction; the final conditions are identical. The presence of inhibitors can be detected by preincubating the reagent solution for 10 minutes prior to the addition of 4 NPP. The catalytic concentration of AP will be lower than in a reagent solution which has not been exposed to preincubation. Values of catalytic concentrations should be >98% after 15 minutes of preincubation compared to those from measurements without preincubation. AMP can be purified according to reference 6.

4NPP

Some commercial 4NPP preparations may contain an unacceptable excess of 4NP, resulting in a too high blank absorbance or a reduced AP catalytic activity. The preparations must meet the following criteria:

- the enzymatic conversion of 4NPP to 4NP should be >98%.
- the molar absorbance of 4NPP at 311 nm in NaOH (10 mmol/L), at 25°C 986,7 ±7,6 m^2/mol.
- the mol fraction of 4NP to 4NPP must be less than $0,3 \cdot 10^{-3}$*.
- the mol fraction of inorganic phosphate to 4NPP must be less than 0,01*.

4NP

The crystalline 4NP preparations must meet the following criteria:

- colourless to slightly yellow
- melting point 113-114°C*
- mass fraction of H_2O in 4NP < 0,001*
- molar absorbance at 401 nm in NaOH (10 mmol/L) at 24°C 1838 ±9 m^2/mol

4NP can be purified by recrystallization or sublimation. Many commercial preparations meet these criteria. 4NP is also available as clinical standard (SRM 938) from the NBS.

*Should be mentioned on the label of the purchased reagent.

3.3.2.4 AP MEASUREMENT BY THE SFBC METHOD

Principle

$$\text{4-Nitrophenylphosphate} + H_2O \xrightarrow{AP} \text{4-Nitrophenolate} + \text{phosphate}$$

The increase of absorbance is measured at 405 nm

Measurement conditions

Temperature 30 or 37°C
pH 10,3 ± 0,05 (37°C); 10,5 ± 0,05 (30°C)
Wavelength: 405 nm
Lightpath in square thermostabilized cuvettes: 10 mm.

Samples: serum or heparinized plasma	Blank	Sample
Magnesium/AMP buffer (solution III)	2,8	2,8
NaCl 0,154 mol/L (mL)	0,10	-
Samples (mL)	-	0,1
Mix thoroughly, ensure that correct temperature is reached.		
4 NPP (solution IV) (mL)	0,10	0,10

- Mix thoroughly, after 30-60 seconds monitor change of absorbance A during 180 seconds;
- Use ΔA/min for calculation.

Calculation of the catalytic concentration b:

Lineic molar absorbance of 4-nitro phenolate at 405 nm, pH 10,5 (30°C): 1860 m²/mol [1].

$b = (\Delta A_{Sample}/min - \Delta A_{Blank}/min) \times 1\,626$ U/L

Limit of detection: 2.2 U/L

Limit of linearity: 390 U/L (above this value dilute the sample with NaCl 0,154 mol/L).

3.3.3 Two-point method

3.3.3.1 REAGENTS

1. 2-Amino-2-methyl-1-propanol (AMP), $C_4H_{11}NO$, Mr 89,14

2. Magnesium sulfate, heptahydrate, analytical grade, $MgSO_4 . 7 H_2O$, Mr 246.49

NOTE: Magnesium sulfate is recommended instead of magnesium chloride since the latter compound is hygroscopic.

3. 4-Nitrophenyl phosphate, disodium salt, hexahydrate (4NPP), $C_6H_4NO_6PNa_2 . 6 H_2O$, Mr 371,2
4. 4-Nitrophenol (4NP), $C_6H_5NO_3$, Mr 139,1
5. Hydrochloric acid (1 mol/L)
6. Sodium hydroxide, (0,25 mol/L)

3.3.3.2 SOLUTIONS

See Section 3.3.2.2 for general comments.

I. AMP (0,88 mol/L) buffer, pH 10,3

- Warm the AMP to approximately 37°C until it is completely liquified.

AMP	78,5 g
Distilled H_2O about	700 mL
pH adjusted with HCl* (1 mol/L) to pH 10,3	
Distilled H_2O to	1000,0 mL

Stable for one month at 20-25°C if stored in tightly stoppered polyethylene bottle.

II. $MgSO_4$ (1,5 mmol/L)

$MgSO_4 . 7 H_2O$	0,370 g
Distilled H_2O to	1000,0 mL

Stable indefinitely at 20-25°C.

III. 4NPP (225 mmol/L) in solution II

$4NPPNa_2 . 6 H_2O$	83,5 mg
Magnesium solution (solution II)	1,0 mL

Stable for one working day.

IV. 4NP stock standard solution (10,8 mmol/L)

4NP	0,150 g
Distilled H_2O to	100,0 mL

*In the original WHO method concentrated HCl 37 % (v/w) is used to adjust the pH.

Stable for about six months at 4°C in the dark.

V. 4NP working standard solution (54 µmol/L)

Stock 4NP solution (solution IV)	0,5 mL
NaOH (0,25 mol/L) to	100,0 mL

To be prepared freshly before use.

3.3.3.3 MEASUREMENT OF AP WITH TWO-POINT METHOD

- Label sufficient test-tubes for reagent blank (B), control (C1,C2) and patients' samples (1, 2, 3, etc.).
- Pipette into the tubes as follows:

	B	C1,C2	1,2,3, etc.
AMP buffer (mL)	1,4	1,4	1,4
- Warm all tubes at 37°C for 5 minutes			
Distilled H_2O (mL)	0,05	-	-
Control or patient's serum/plasma (mL)	-	0,05	0,05
- Mix all tubes, leave at 37°C, add at timed intervals:			
substrate solution (mL)	0,10	0,10	0,10
- Incubate at 37°C for 15 minutes; add at timed intervals:			
NaOH (mL)	4,0	4,0	4,0

- Mix well, cool to room temperature and measure the absorbance.
  Colorimeter: violet filter, Ilford number 600 (410 nm).
  Spectrometer: 410 nm.
- Set the instrument to zero absorbance with the reagent blank.
- Calculate the results in U/L from the calibration graph.
- Check the control results.

3.3.3.4 PREPARATION OF THE CALIBRATION GRAPH

Tube No.	1	2	3	4	5	6
4-Nitrophenol, 54 µmol/L(mL)	1	2	4	6	8	10
Sodium hydroxide, 0,25 mol/L(mL)	9	8	6	4	2	0
Activity (U/L)	40	80	160	240	320	400

- Mix well and measure the absorbance of each tube at 410 nm (violet filter, Ilford 600) setting the spectrometer to zero with sodium hydroxide solution (0,250 mol/L). Plot the absorbance of each tube against the activity.

3.3.3.5 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Taking into account the concentrations, the purity and presence of possible inhibitors of the AP catalytic activity in the reagents and solutions (AMP, 4NPP, 4NP) can be controlled with the techniques described in Section 3.3.2.3.

3.3.4 Certified reference material

Reference material for alkaline phosphatase is available from the M & T.

3.3.5 References

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3.4 α-AMYLASE

1,4-α D glucan glucano hydrolase (EC 3.2.1.1)

A reference method is not yet available. Several routine methods with auxiliary enzymes and synthetic substrates are available. Most of these methods are protected by patents. At the moment it is difficult on scientific grounds to select the best of these methods. For these reasons only the reagents of the amyloclastic starch iodine method proposed by WHO [1] will be described here.

3.4.1 Amyloclastic starch-iodine method

3.4.1.1 REAGENTS

1. Soluble starch, pharmaceutical grade, $(C_6H_{10}O_5)_n$
2. Potassium iodide, analytical grade, KI, Mr 166,02
3. Potassium iodate, analytical grade, KIO_3, Mr 214,02
4. Orthophosphoric acid, disodium salt, anhydrous, Na_2HPO_4, Mr 142,03
5. Sodium chloride, NaCl, Mr 58,45
6. Benzoic acid, $C_7H_6O_2$, Mr 122,12
7. Hydrochloric acid, concentrated (37% w/v) analytical grade, HCl, Mr 36,47
   Caution: highly corrosive.

3.4.1.2 SOLUTIONS

I. Starch paste (40 g/L)

- Prepare in a 50 mL beaker

Soluble starch	0,4 g
Distilled H_2O, cold	10,0 mL

Mix to form a paste.

II. Buffered starch substrate : Na_2HPO_4 (187 mmol/L) ; NaCl, (29,9 mmol/L); benzoic acid (70,4 mmol/L); starch (400 mg/L);

pH 6,9 - 7,1	
Orthophosphoric acid, disodium salt, anhydrous	26,6 g
Sodium chloride	1,75 g
Benzoic acid	8,6 g
Distilled H_2O, about	500 mL

- Dissolve and heat to boiling.
- Add all of starch paste (solution I) and rinse the beaker with distilled water.

Continue to boil for 1 minute, cool to room temperature.

Distilled H_2O to	1000,0 mL

Stable for at least for one year at 20-25°C.

III. Stock iodine solution: KIO_3 (16,7 mmol/L); KI (278 mmol/L)

Potassium iodate	3,57 g
Potassium iodide	45,0 g
Distilled H_2O, about	800 mL
HCl concentrated	9,0 mL
Distilled H_2O to	1000,0 mL

Stable for one year at 20-25°C if stored in a dark bottle.

IV. Working iodine solution

In a graduated 100 mL cylinder:	
Stock iodine solution (solution III)	10 mL
Distilled H_2O	90 mL

Stable for two months at 2-8°C if stored in a dark bottle.

3.4.1.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Buffered starch substrate solution: The pH should remain at 6,9 - 7,1.

The stability of the solution is monitored by noting the absorbance at 660 nm (or red filter Ilford Number 608) of the reagent blank with each set of test samples.

3.4.1.4 METHOD FOR MEASUREMENT OF α-AMYLASE IN SERUM/ PLASMA

- Label sufficient test tubes for reagent blank (B), control (C1,C2) and patients' samples (1, 2, 3, etc.).
- Pipette into the tubes as follows:

	B	C1,C2	1,2,3, etc.
Buffered starch substrate (mL)	1,0	1,0	1,0

- Warm all tubes at 37°C for 5 minutes

Control or patient's sample (mL)		0,02	0,02

- Mix and incubate at 37°C for exactly 7 minutes and 30 seconds

Working iodine solution (mL)	1,0	1,0	1,0

- Mix well and add 8 mL of distilled water to each tube.
- Mix well and read the absorbance without delay.
  Colorimeter: red filter Ilford number 608 (680 nm) Spectrometer: 660 nm
- Set the instrument to zero absorbance with distilled water.
- Calculate the results in U/L.
- Check the absorbance of the tube B to monitor the stability of the substrate solution.
- Check the control results.

Calculation:

$$\text{Amylase activity (U/L)} = \frac{(B - T)}{B} \times 1470$$

B = absorbance of reagent blank
T = absorbance of test
1470 = factor to express values in U/L

If the result is greater than 735 U/L (i.e. there is little or no blue colour in tube T), then the sample must be diluted with saline (0,02 mL serum + 0,1 mL saline) and the analysis repeated using 0,02 mL of the diluted sample. The measured value must be multiplied by 6 to calculate the amylase activity of the sample to take into account the dilution factor.

3.4.2 Certified reference materials

None available.

3.4.3 References

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3.5 Aspartate aminotransferase (ASAT, AST)

L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1)
Previously: glutamate-oxaloacetate transaminase (GOT)

3.5.1 Methods

A reference method for aspartate aminotransferase has been recommended by the IFCC [1]. The method described here follows the recommendation of the SFBC [2]. It is a spectrometric, continuous monitoring method derived from the IFCC method.

In the IFCC and SFBC methods the temperature of 30°C for the measurement of the catalytic concentration of ASAT is included in the physical and chemical measurement conditions. However, for practical reasons, essential in hot countries, the temperature of 37°C is more suitable; also in many modern 37°C analysers this temperature is the only one which can be used. Fortunately, the above-mentioned methods work equally well at 37°C [3]. For this reason the pH of the buffer is mentioned at 30°C and 37°C, but the catalytic activities of the reagent enzymes are expressed at 30°C. Also described here is a modified colorimetric method of Reitman and Fränkel [4].

3.5.2 The SFBC method

3.5.2.1 REAGENTS

1. Tris (hydroxymethyl) aminomethane, TRIS, $C_4H_{11}NO_3$, Mr 121,14
2. L-Aspartic acid, $C_4H_7NO_4$, Mr 133,1
3. 2-Oxoglutaric acid (α-ketoglutaric acid), $C_5H_6O_5$, Mr 146,10
4. ß-Nicotine adenine dinucleotide, reduced; disodium salt (NADH) $C_{21}H_{27}N_7O_{14}P_2Na_2$, Mr 709,4
5. L-Malate: NAD oxidoreductase (EC1.1.1.37), malate dehydrogenase (MDH) from pig heart, specific catalytic activity higher than 1020 U/mg at 30°C, in glycerol (reagent 9).

6. L-Lactate: NAD oxidoreductase (EC.1.1.1.27, lactate dehydrogenase, LDH) from pig skeletal muscle; specific catalytic activity higher than 480 U/mg at 30°C, in glycerol (reagent 9).
7. Pyridoxal-5-phosphoric acid monohydrate (pyridoxal phosphate) $C_8H_{10}O_6NP \cdot H_2O$, Mr 265.2
8. Hydrochloric acid, 5 mol/L
9. Glycerol volume fraction 0,5 in aqueous solution (50% v/v), $C_3H_8O_3$, Mr 92,10
10. Sodium hydroxide; 5 mol/L and 0,5 mol/L
11. Sodium chloride; 154 mmol/L
12. Oxalacetic acid, $C_4H_4O_5$, Mr 132,1
13. Sodium pyruvate, $C_3H_3O_3Na$, Mr 110,0
14. Sodium azide, NaN_3, Mr 65,0
15. Ammonium acetate, $C_2H_3O_2(NH_4)$, Mr 77,08
16. Albumin, bovine serum

3.5.2.2 SOLUTIONS

To prevent the growth of microorganisms in the solutions, sterilize the containers (for about 1 hour at 150°C, in a dry oven). Prepare all solutions in volumetric flasks (or with volumetric pipettes, class A), with fresh distilled or deionized H_2O. The pH of solutions must be adjusted at 30°C or 37°C ±0,1°C (according to the measurement temperature) with a pH meter calibrated at 30°C or 37°C with reference buffers (Section 2.6) [1].

I. TRIS (96 mmol/L), L-aspartate (0,25 mol/L), pH 7,8 (30°C), or 7.65 (37°C)

TRIS	1,16 g
L-Aspartic acid	3,32 g
Distilled H_2O	80,0 mL
pH adjusted with NaOH 5 mol/L to 7,8 (30°C) or 7,65 (37°C)	
Distilled H_2O	100,0 mL

- Filter through a 0,6 µm glass-filter, fill into polyethylene bottles and label.

Stable for at least six months at 0-4°C; bacterial contamination can be prevented by addition of sodium azide 8 mmol/L (46,8 mg in the 100 mL of solution I) [1].

II. TRIS (96 mmol/L)/HCl buffer, pH 7,8 (30°C) or 7,65 (37°C)

TRIS	1,16 g
Distilled H_2O	80,0 mL
pH adjusted with HCl (5 mol/L) to pH 7,8 (30°C) or 7,65 (37°C)	
Distilled H_2O to	100,0 mL

- Filter through a 0,6 µm glass-filter, fill into polyethylene bottles and label.

Stability as for TRIS/ASP (solution I).

III. Pyridoxal phosphate (6,24 mmol/L), TRIS (96 mmol/L)

Pyridoxal phosphate	0,0165 g
TRIS/HCl (solution II)	10,0 mL

Stable for one week if stored in a refrigerator between 0-4°C in a dark container [1].

IV. NADH (11,3 mmol/L); TRIS (96 mmol/L)

NADH	0,016 g
TRIS/HCl buffer (solution II)	2,0 mL

Stable for one week if stored in a refrigerator between 0-4°C in a dark container [1].

V. Working solution

TRIS/ASP (solution I)	100,0 mL
Pyridoxal phosphate (solution III)	2,0 mL
NADH (solution IV)	2,0 mL
MDH*	720 U/L
LDH*	1080 U/L

Stable for one day at 0-4°C in a dark container [1].

VI. 2-Oxoglutarate (0,144 mol/L)

2-Oxoglutaric acid	0,210 g
Distilled H_2O	10,0 mL

- Dissolve and adjust pH with NaOH (0,5 mol/L) to pH 7,8 (30°C) or, 65 (37°C).
- Filter through a paper filter.

Stable for 1 week at 0-4°C.

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* Amount added according to the specific catalytic activities in the enzyme reagents 5 and 6. Predilution in glycerol (reagent 9) can be performed if necessary, but the change in the final volume of solution V must be less than 0,1%.

3.5.2.3 MEASUREMENT OF THE CATALYTIC CONCENTRATION OF THE REAGENT ENZYMES

The measurement conditions (temperature, pH, ionic strength, type of buffer, concentration of substrate and cofactors) as well as the equipment must be the same as those used for the assay of overall ASAT determination, and at the chosen temperature (30°C or 37°C).

For MDH and LDH measurement prepare a stock enzyme solution:

Dilute the enzyme reagents 5 and 6 together in glycerol (reagent 9) according to their original catalytic concentrations to give the following concentrations at 30°C: MDH 52,8 KU/L and LDH 75,6 KU/L; or at 37°C: MDH 75,6 KU/L and LDH 113 KU/L*.

MDH MEASUREMENT

Prepare two samples from the above enzyme stock solution (MDH/LDH) with catalytic concentrations of MDH of about 300 and 600 U/L at 30°C, respectively about 430 and 860 U/L at 37°C** by diluting with NaCl (154 mmol/L) also containing albumin (10 g/L).

The solution for the MDH measurement is prepared as for ASAT except that the enzymes are excluded from the working solution (solution V). 2-Oxoglutarate (solution VI) is replaced by oxalacetic acid, (4,8 mmol/L). 6,3 mg oxalacetic acid (reagent 12) are dissolved in 10 mL of HCl (10 mmol/L) at 4°C. Stored in ice this solution is stable for one hour. This solution is used as starting solution. The catalytic concentration of MDH is determined in the two samples of the diluted stock solution; for the reagent blank, NaCl (0,154 mol/L) is used.

For the measurements at temperature 30°C or 37°C (±0,1°C). proceed as follows:

- Add in an optical glass cuvette of 10 mm lightpath:
  Working solution (solution V) (without enzymes) 2,0 mL
  Sample (diluted MDH solutions, or NaCl,
  0,154 mol/L for reagent blank) 0,2 mL
- Mix and ensure temperature equilibrium at 30°C or 37°C, then add:
  Oxalacetic acid solution 0,2 mL

---

* Amount added according to the specific catalytic activities in the enzyme reagents 5 and 6. Predilution in glycerol (reagent 9) can be performed if necessary, but the change in the final volume of solution V must be less than 0,1%.

** Calculations from values as in references 1 and 3.

- Record the change in absorbance at 340 nm (or 334 nm, or 365 nm) immediately after adding oxalacetic acid. Use the change in absorbance ($\Delta A/\Delta t$) in the time period of 20-60 seconds corrected for the reagent blank rate when calculating the catalytic concentration b of MDH in the sample:

  340 nm: $b = 1{,}905 \times 10^3 (\Delta A/t)$
  334 nm: $b = 1{,}942 \times 10^3 (\Delta A/\Delta t)$
  365 nm: $b = 3{,}529 \times 10^3 (\Delta A/\Delta t)$
  b in U/L, t in min.

LDH MEASUREMENT

Prepare two samples from the above enzyme stock solution (MDH/LDH) with catalytic concentrations of LDH of about 300 and 600 U/L (30°C), respectively about 450 and 900 U/L at 37°C*, by diluting with NaCl (0,154 mol/L), also containing albumin (10 g/L).

The solution for the LDH measurement is prepared as for ASAT except that the enzymes are excluded from the working solution (solution V). 2-Oxoglutarate (solution VI) is replaced by sodium pyruvate (36 mmol/L). 40 mg of Na pyruvate are dissolved in 10 mL of distilled H_2O. This solution is used as starting reagent.

The catalytic concentration of LDH is determined in the two samples of the diluted stock solution; for the reagent blank, NaCl (154 mmol/L) is used.

For the measurements at temperature 30°C or 37°C (± 0,1°C) proceed as follows:

- Add in an optical glass cuvette of 10 mm lightpath:
  Working solution (solution V) (without enzymes) 2,0 mL
  Sample (diluted LDH solutions, or NaCl,
  0,154 mol/L for reagent blank)
- Mix and ensure temperature equilibrium at 30°C or 37°C; then add:
  Na pyruvate 0,2 mL
- Record the change in absorbance at 340 nm (or 334 nm or 365 nm) immediately after adding pyruvate.
- Use the change of absorbance ($\Delta A / \Delta t$) in the time period of 20-60 seconds corrected for the reagent blank rate when calculating the catalytic concentration b of LDH in the sample:

  339 nm: $b = 1{,}905 \times 10^3 (\Delta A/\Delta t)$
  334 nm: $b = 1{,}942 \times 10^3 (\Delta A/\Delta t)$

* Calculations from values as in references 1 and 3.

365 nm: $b = 3{,}529 \times 10^3 (\Delta A/\Delta t)$
b in U/L, t in minutes.

3.5.2.4 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS [1]

1. Measurement of contaminant enzymes in the reagent enzymes (MDH,LDH)

Glutamate dehydrogenase (GlDH, EC 1.4.1.3) is a possible contaminating enzyme. Any contamination of the MDH/LDH in solution V with ASAT is corrected by measurement of blank activity. If the value of $\Delta A/\Delta t$ exceeds 0.0012/min different preparations of the reagent enzymes should be used.

Measurement of possible contamination with GlDH

The same equipment must be used as for the ASAT measurement.

- Dissolve 93 mg ammonium acetate in 10 mL of solution V (0,121mol/L). Final concentration in the test: 0,10 mol/L.

For the measurement at temperature 30°C or 37°C (±0,1°C) proceed as follows:

- Add in an optical glass cuvette with a light path of 10 mm.:
  Working solution (solution V) (with ammonium acetate) 2,0 mL
  (or for blank reaction solution V, without ammonium acetate)
  NaCl (0,154 mol/L)) 0,2 mL
- Mix and ensure temperature equilibrium at 30°C or 37°C, then add:
  2-Oxoglutarate (solution VI) 0,2 mL

  A lag phase of 120 seconds may occur. Then record the change of absorbance at 340 nm (or 334 nm or 365 nm) $\Delta A/\Delta t$ for 60-120 seconds.
- Calculate the catalytic concentration of GlDH as described for MDH and LDH (Section 3.5.2.3). The ratio between the catalytic concentration of GlDH, and the concentration of either reagent enzyme shall not exceed 3.10^{-5} (relative activities).

2. 2-Oxoglutarate: Must be free of pyruvate and malate.
3. Pyridoxal phosphate: See Section 3.1.2.4 (ALAT).
4. NADH: See Section 3.1.2.4 (ALAT).

* Calculations from values as in references 1 and 3.

3.5.2.5 ASAT MEASUREMENT BY THE SFBC METHOD

Principle

$$\text{2-Oxoglutarate} + \text{L-Aspartate} \xrightarrow[\text{P5P}]{\text{ASAT}} \text{L Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+$$

during preincubation: exhaustion of interfering pyruvate and other endogenous compounds.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

The decrease of absorbance is measured at 339 (340), 334 or 365nm, if a spectrometer is used.

Measurement condition

pH: 7,65 ±0,05 at 37°C; 7,8 ±0,05 at 30°C.
lightpath in square thermostabilized cuvette: 10 mm
Sample: serum or heparinized plasma

	Blank	Samples
Working solution (solution V) (mL)	1,0	1,0
NaCl, 154 mmol/L (mL)	0,10	-
Sample (mL)	-	0,10

- Mix thoroughly, preincubate for 10 minutes at 30 or 37°C.

Oxoglutarate (solution VI) (mL)	0,10	0,10

- Mix thoroughly, let equilibrate for 120 seconds, monitor for change of absorbance during 200 seconds.

Use: absorbance (ΔA)/min for calculation.

Calculation of catalytic concentration b:

Molar lineic absorbance of NADH at pH 7,5, 30°C.

339 (340) nm: 630 m²/mol
334 nm: 618 m²/mol
365 nm: 340 m²/mol

$b_{339(340)\ nm} = (\Delta A_{Sample} - \Delta A_{Blank}) \times 1905\ U/L$
$b_{334\ nm} = (\Delta A_{Sample} - \Delta A_{Blank}) \times 1942\ U/L$
$b_{365\ nm} = (\Delta A_{Sample} - \Delta A_{Blank}) \times 3529\ U/L$

Limit of detection: 2.5 U/L

Limit of linearity: 400 U/L. Above this value dilute the sample with NaCl, 0,154 mol/L, and multiply the value by the dilution factor.

3.5.3 Reitman and Fränkel method [4]

3.5.3.1 REAGENTS

1. Triethanolamine, $C_6H_{15}O_3N$, Mr 149,19
2. Ethylenediamine tetra-acetic acid, disodium salt, dihydrate
   (Ethylenedinitrilotetracetic acid disodium salt, dihydrate, EDTA)
   $C_{10}H_{14}N_2O_8Na_2 \cdot 2\ H_2O$, Mr 372.24
3. Orthophosphoric acid, disodium salt, anhydrous, Na_2HPO_4, Mr141,98
4. Orthophosphoric acid, monopotassium salt, anhydrous, KH_2PO_4, Mr 136,13
5. 2-Oxoglutaric acid (α-ketoglutaric acid), $C_5H_6O_5$, Mr 146,10
6. D,L-Aspartic acid, $C_4H_7NO_4$, Mr 133,11
7. Sodium hydroxide (1 mol/L and 0,4 mol/L)
8. 2,4-Dinitrophenylhydrazine (with water), $C_6H_6N_4O_4$, Mr 198,14 (dry substance)
   Caution: may explode violently when dry. Do not dry the wet substance.
9. Hydrochloric acid (1 mol/L)
10. Chloroform, $CHCl_3$, Mr 119,38
11. Sodium pyruvate , $C_3H_3O_3Na$, Mr 110,0

3.5.3.2 SOLUTIONS

I. Phosphate buffer, pH 7,4 (100 mmol/L)

Na_2HPO_4 (anhydrous)	11,9 g
KH_2PO_4 (anhydrous)	2,2 g
Distilled H_2O about	800 mL
pH adjusted with appropriate phosphate to 7,4	
Distilled H_2O to	1000,0 mL

Stable for two months at 2-8°C.

II. Buffered substrate reagent, 2-Oxoglutarate (2 mmol/L), D,L-Aspartate (200 mmol/L)

2-Oxoglutaric acid	0,0292 g
D,L-Aspartic acid	2,66 g
NaOH (1 mol/L)	20 mL

pH adjusted with NaOH (1 mol/L) to 7,4
Phosphate buffer (I) to 100,0 mL

- Add 1 mL of chloroform as preservative and store at 4-8°C.

Stable for two weeks at 2 8°C, but must be discarded sooner if it becomes turbid.

III. Colour reagent, 2,4-Dinitrophenylhydrazine (1 mmol/L)

NOTE: that the weight of 2,4-dinitrophenylhydrazine must be adjusted to take into account the water content, indicated by the manufacturer.

2,4-Dinitrophenylhydrazine, if dry it would be	0,019 8 g
HCl (1 mol/L)	100 mL

Stable for two months at 2-8°C.

IV. Triethanolamine (0,051 mol/L), ethylene-diamine tetra-acetate (EDTA,5,373 mmol/L) buffer

Triethanolamine	3,8 g
EDTA	1,0 g
Distilled H_2O about	200 mL
HCl (1 mol/L)	15 mL

- Check that pH is 7,5-7,6

Distilled H_2O to 500,0 mL
Stable indefinitely at 2-8°C.

V. Pyruvate standard (4 mmol/L)

Na pyruvate	0,044 g
Triethanolamine/EDTA buffer (solution IV)	100,0 mL

Mix well, divide into small portions of about 1 mL and store in a freezer. Stable for six months when frozen, or for one week at 2-8°C.

3.5.3.3 QUALITY CONTROL

Control serum with assigned values

3.5.3.4 ASAT MEASUREMENT BY REITMAN AND FRÄNKEL METHOD

- Label sufficient tubes for reagent blank (B), standard (S), control (C1, C2) and patients' samples (1, 2, 3, etc.), control blanks (C1B, C2B) and patients' sample blanks (1B, 2B, 3B, etc.).

- Remove one portion of pyruvate standard from the freezer, thaw and mix well.
- Pipette into the tubes as follows:

	B	S	C1,C2 1,2,3, etc.	C1B,C2B 1B,2B,3B1
Buffered substrate reagent (mL)	0,5	0,5	0,5	0,5
- Put buffered substrate reagent back in refrigerator				
- Warm all tubes at 37°C for 5 minutes, add:				
Distilled H_2O (mL)	0,1	-	-	-
Pyruvate standard (mL)	-	0,1	-	-
Controls or patients' sample (mL)	-	-	0,1	-
- Mix and keep all tubes at 37°C for 60 minutes, then add:				
Colour reagent (mL)	0,5	0,5	0,5	0,5
Controls or patients' sample (mL)	-	-	-	0,1
- Mix; after 20 minutes at room temperature add:				
Sodium hydroxide (0,4 mol/L)(mL)	5,0	5,0	5,0	5,0

- Mix thoroughly, wait for 5 minutes, then measure the absorbances.
  Colorimeter: green filter, Ilford number 604 (520 nm)
  Spectrometer: 505 nm
- Set the instrument to zero with tube B.
- Calculate the amount of pyruvate formed and use Table 3.5.1 to convert to ASAT(U/L).

Table 3.5.1 Conversion of pyruvate formed [(μmol/L)/ 60 min] into ASAT activity (U/L) at 37 °C

Pyruvate formed [(μmol/L)/60 minutes]	ASAT activity (U/L at 37°C)	Pyruvate formed [(μmol/L)/60 minutes]	ASAT activity (U/L at 37°C)
120	4	1680	52
240	6	1800	56
360	10	1920	60
480	12	2040	64
600	15	2160	69
720	19	2280	73
840	23	2400	77

(Continued)

Pyruvate formed [(μmol/L)/60 minutes]	ASAT activity (U/L at 37°C)	Pyruvate formed [(μmol/L)/60 minutes]	ASAT activity (U/L at 37°C)
960	27	2520	81
1080	31	2640	85
1200	35	2760	92
1320	40	2880	98
1380	42	3000	106
1440	44	3120	114
1560	48	3240	125

3.5.4 Certified reference material

Certified reference material is not yet available, but SRM 909 (human serum) in which the catalytic concentration of ASAT has been established can be obtained from NBS.

3.5.5 References

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3.6 Bilirubin

Bilirubin-IXa; total and conjugated (glucuronidated, direct reading) bilirubin.

3.6.1 Methods

No internationally agreed reference method is available, although a candidate reference method has been proposed [1]. The reagents for the caffeine activator method of Jendrassik and Grof [2] are described here.

3.6.2 Jendrassik and Grof method

3.6.2.1 REAGENTS

1. Bilirubin, non-glucuronidated, powder. $C_{33}H_{36}N_4O_6$, Mr 584, or commercial bilirubin standards.
2. Caffeine (1,3,7-trimethylxanthine), $C_8H_{10}N_4O_2$, Mr 194,19
3. Sodium benzoate, $C_7H_5O_2Na$, Mr 144,11
4. Sodium acetate, trihydrate, $C_2H_3O_2Na \cdot 3\ H_2O$, Mr 136,09
5. Sulfanilic acid (aniline-4-sulfonic acid), $C_6H_7NO_3S$, Mr 173,19
6. Hydrochloric acid (concentrated, 37% w/v and 0,05 mol/L)
7. Sodium nitrite, $NaNO_2$, Mr 69,01
8. Sodium hydroxide (0,1 mol/L)
9. Potassium sodium tartrate, tetrahydrate, $C_4H_4O_6KNa \cdot 4\ H_2O$, Mr 282,23
10. Ethylenediamine tetra-acetic acid, disodium salt, dihydrate (ethylene dinitrilo tetra-acetic acid, disodium salt, dihydrate, EDTA) $C_{10}H_{14}N_2O_8Na_2 \cdot 2\ H_2O$, Mr 372,24
11. Ascorbic acid, $C_6H_8O_6$, Mr 176,13
12. Sodium carbonate, anhydrous, Na_2CO_3, Mr 106,00

3.6.2.2 SOLUTIONS

I. Caffeine (196 mmol/L); Na benzoate (389 mmol/L); Na acetate (683 mmol/L); EDTA (2,69 mmol/L)

Na acetate . $3H_2O$	93 g
Na benzoate	56 g
EDTA	1 g
Distilled H_2O about	500 mL

- Dissolve, add:

Caffeine	38 g
Distilled H_2O to	1000,0 mL

- Mix well and filter.

Stable for 6 months at 20-25°C.

II. Sulfanilic acid (26,2 mmol/L) in HCl

Sulfanilic acid	2,5 g
Distilled H_2O about	200 mL

- Pipette with a rubber bulb pipette

HCl concentrated	7,5 mL

- Dissolve, add

Distilled H_2O to	500,0 mL

Stable for 6 months at 20-25°C.

III. Sodium nitrite (72,5 mmol/L)

Na nitrite	0,5 g
Distilled H_2O to	100.0 mL

Stable for one month if stored at 2-8°C.

IV. Diazo reagent

Sulfanilic acid (solution II)	4,0 mL
Sodium nitrite (solution III)	0,1 mL

Leave for 2 minutes, then use within 5 hours. Store at 2-8°C.

V. Alkaline tartrate (1,24 mol/L); NaOH (1,88 mol/L)

NaOH	75 g
KNa tartrate	350 g
Distilled H_2O about	800 mL

- Dissolve, let cool

Distilled H_2O to	1000,0 mL

Stable for 6 months at 20-25°C.

VI. Ascorbic acid (227 mmol/L)

Ascorbic acid	0,2 g
Distilled H_2O to	5,0 mL

Must be prepared daily. Necessary only for conjugated bilirubin.

VII. Bilirubin-free serum

Leave pooled serum in the sunlight for one day.
Do not use pooled serum to which Na azide has been added.

VIII. Na carbonate (100 mmol/L)

Na_2CO_3, anhydrous	1,06 g
Distilled H_2O about	50 mL

- Dissolve, add:

Distilled H_2O to	100,0 mL

Keep tightly stoppered in a plastic bottle.

IX. Bilirubin standards (342 µmol/L, 0,2 g/L, 20 mg/dL)

This solution must be prepared in dark ambient light, since bilirubin is destroyed by sunlight.

Bilirubin	0,020 g
Na carbonate (solution VIII)	2,0 mL
NaOH (0,1 mol/L)	1,5 mL
Bilirubin-free serum (solution VII) to	100,0 mL

- Divide into small volumes and store at -20°C.

This solution must be protected from light, e.g. with aluminium foil or black carbon paper.

NOTE: Commercial bilirubin standards can also be used and may be more reliable than the home-made solution.

3.6.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Molar absorbance of bilirubin in various solvents:

Solvents	wavelength nm	m^2/mol	ref
Chloroform	453	6070 ±80	3,4
(range of acceptance)		(5910-6230)	
Chloroform	453	>5900	5
Dimethyl sulfoxide	456	6290	6

Stability of bilirubin solutions

Bilirubin is always unstable in solutions. This applies to all standard solutions (e.g. in chloroform) or in biological material: serum, plasma, urine. Bilirubin is extremely sensitive to light (daylight, UV light, artificial light); the degradation of the compound is retarded in albumin solutions.

3.6.2.4 METHOD FOR MEASUREMENT OF TOTAL AND CONJUGATED (DIRECT) BILIRUBIN

Total bilirubin

- Label sufficient tubes for the batch, including standard (S), control (C1, C2), and patients' samples (1, 2, 3, etc.). These are for the 'tests'.
- Label 'blank' tubes corresponding to each "test" tube, i.e. SB, C1B, C2B, 1B, 2B, 3B, etc.
- Pipette as follows:

	S	SB	C1,C2	C1B,C2B	1,2,3 etc.	1B,2B etc.
Caffeine (solution I)	1,0	1,0	1,0	1,0	1,0	1,0
Bilirubin Standard (solution IX)(mL)	0,1	0,1	-	-	-	-
Controls (mL)	-	-	0,1	0,1	-	-
Patient sample (mL)	-	-	-	-	0,1	0,1
Diazo reagent (solution IV) (mL)	0.5	-	0.5	-	0,5	-
Sulfanilic acid (solution II) (mL)	-	0,5	-	0,5	-	0,5

- Mix well, leave at room temperature for 10 minutes, then add:

Alkaline tartrate (solution V) (mL)	1,0	1,0	1,0	1,0	1,0	1,0

- Mix well, and read absorbance of SB, C1B, C2B, 1B, 2B, 3B, etc.
- Read absorbances of S, C1, C2, 1, 2, 3, etc.

Conjugated bilirubin

The procedure is the same as described above for total bilirubin except that:

- for each patient's sample add 1,0 mL hydrochloric acid (50 mmol/L) to 'test' and 'blank' tubes instead of caffeine benzoate reagent;
- 5 minutes after addition of diazo reagent (to 'test') and sulfanilic acid (to 'blank'), add 50 µL of ascorbic acid solution to each tube; then add 1,0 mL alkaline tartrate to each tube; mix and read the absorbance immediately.

NOTE: results must be calculated using the 'total' bilirubin procedure for the standard.

Colorimeter: Orange filter, Ilford 607 (600 nm)
Spectrometer: 600 nm

- Set the instrument to zero absorbance with distilled water.
- Calculate the results in µmol/L.
- Check the control results.

3.6.3 Certified reference material

Bilirubin reference material is available from the National Bureau of Standards (NBS), Ordering Number SRM 916.

3.6.4 References

[1] Doumas BT, Kwok-Cheung PP, Perry BW et al. Candidate reference method for determination of total bilirubin in serum; development and validation. *Clin. Chem.* 1985, 31:1779-89.

[2] de Cediel N, Déom A, Hill PG, Sarkar AK et al. 4.8 Measurement of serum/plasma total bilirubin. Method: Jendrassik-Grof. *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories.* LAB/86.3, Geneva, World Health Organization, 1986, p. 83-87.

[3] American Association of Clinical Chemists and College of American Pathologists. Joint Commission Report. *Clin. Chem,* 1962, 8:4405-407 and *Pediatrics*, 1963, 31:878-880.

[4] Billing B, Haslam R and Wald N. Bilirubin standards and the determination of bilirubin by manual and Technicon Auto Analyzer methods. *Ann. Clin. biochem.*, 1971, 8:21-30.

[5] Dybkaer R and Hertz H. A reference for determination of bilirubin concentration in serum using bilirubin in cyanide-formamide for enrichment of serum. *Scand. J. Clin. Lab. Invest.*,1970, 25:151-160.

[6] Heime W and Tittelbach-Helmrich W. Zur Darstellung stabiler Bilirubin-Standard Lösungen mit Dimethylsulfoxyd. *Z. Med. Labortechnik*,1973, 14:332-337.

3.7 Calcium

3.7.1 Methods

The reference method uses atomic absorption. However, this method is not normally used for routine investigations. The photometric method described here, uses o-cresolphthalein complexone; it is a widely used method and recommended by WHO [1].

3.7.2 o-Cresolphthalein complexone method

3.7.2.1 REAGENTS

1. Hydrochloric acid (concentrated, 37% w/v and 0,5 mol/L)
2. o-Cresolphthalein complexone (CPC, o-Cresolphthalexon, Metallphthalein) $C_{32}H_{32}N_2O_{12}$, Mr 636,6
3. Ethanediol (ethylene glycol), $C_2H_6O_2$, Mr 62,07, d = 1,11 kg/L
4. 2-Amino-2-methyl-1-propanol (AMP); purity: at least 95%, $C_4H_{11}NO$, Mr 89,14, d = 0,93 kg/L
5. 8-Hydroxyquinoline, C_9H_7NO, Mr 145,16
6. Calcium carbonate, analytical grade, $CaCO_3$, Mr 100,09

3.7.2.2 SOLUTIONS

All glassware must be thoroughly cleaned, soaked overnight in HCl (0,5 mol/L) to remove traces of calcium, thoroughly rinsed with distilled or deionized H_2O, and finally dried before use.

I. Stock CPC solution (47,1 µmol/L); ethanediol (1,36 mol/L); AMP (271 mmol/L)

Ethanediol	42,18 g	(38 mL)
AMP	12,09 g	(13 mL)
Distilled H_2O about	400 mL	
CPC	0,015 g	

Dissolve, add:

Distilled H_2O to	500,0 mL

Stable for 3 weeks at 4-6°C.

II. Working CPC solution (47,1 µmol/L); ethanediol (1,36 mol/L); AMP (0,27 mol/L), 8-Hydroxyquinoline (6,89 mmol/L)

8-Hydroxyquinoline	0,1 g
Stock CPC solution (solution I)	100,0 mL

The 8-hydroxyquinoline dissolves slowly.
Stable for one week at 4-6°C.

III. Calcium stock standard (0,025 mol/L)

- Dry about 300 mg of calcium carbonate in a dry container in an oven for 4 hours at 80 -100°C. After heating, remove it from the oven and immediately close with a lid, let cool in a desiccator; when cool weigh rapidly.

$CaCO_3$	0,25 g
HCl concentrated about	0,5 mL
Distilled H_2O to	100,0 mL

IV. Calcium working standard (2,5 mmol/L)

Calcium stock standard (solution III)	10,0 mL
Distilled H_2O to	100,0 mL

Calcium working standards for calibration graph.

Ca++ mmol/L	Calcium stock standard mL	distilled H_2O mL
0,50	0,20	9,80
1,75	0,70	9,30
2,50	1,00	9,00
3,00	1,20	8,80
3,75	1,50	8,50

3.7.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Working CPC solution

The solution should have an absorbance at 575 nm of about 0,2 when measured with a spectrometer set to zero with distilled H_2O. An absorbance higher than 0,2 indicates that either the reagent has deteriorated or that it is contaminated with calcium.

3.7.2.4 METHOD FOR MEASUREMENT OF SERUM/PLASMA TOTAL CALCIUM

- Check the absorbance of the working CPC reagent first.
  Colorimeter: Yellow filter, Ilford 606 (580 nm)
  Spectrometer: 575 nm

The instrument is set to zero with distilled water, and the absorbance of the working CPC reagent is measured. If it is more than 0,2 then a fresh working CPC reagent must be prepared.

- Label sufficient tubes for reagent blank (B), standard (S), control (C1, C2) and patients' samples (1, 2, 3, etc.). All three samples should be done in duplicate.
- Pipette into the tubes as follows:

	B	S	C1,C2	1,2,3, etc.
Working CPC reagent (mL)	5,0	5,0	5,0	5,0
Distilled water (mL)	0,05	-	-	-
Calcium standard, 2,5 mmol/L(mL)	-	0,05	-	-
Control or patient's sample (mL)	-	-	0,05	0,05

- Mix well and measure the absorbance.
  Colorimeter: Yellow filter, Ilford 606 (580 nm)
  Spectrometer: 575 nm
- Set the instrument to zero with distilled water.
- Calculate the results in mmol/L; report the average of the duplicates.
- Check the control results.

3.7.3 Certified reference material

Reference materials with certified values (low and normal) for calcium in human serum are available from: M & T: (Ordering Number CRM 303 and CRM 304), or as dry calcium carbonate from the NBS (Ordering Number SRM 915).

3.7.4 References

[1] de Cediel N, Déom A, Hill PG, Sarkar AK et al. Calcium method: o-cresolphthalein complexone *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories..* LAB/86.3, Geneva, World Health Organization, 1986.

3.8 Cholesterol (total)

Δ5-cholestene-3 ß-ol

3.8.1 Methods

Center for Disease Control (Atlanta) is evaluating a candidate reference method derived from the Abell et al. procedure [1].

The reagents for an end-point enzymatic procedure, (CHOD-PAP) are described here [2]. Methods based on the Liebermann-Burchard reaction will not be described. The specific method of Abell et al., with extraction, is too complicated for routine work; the direct method, without extraction, is not specific enough.

3.8.2 Enzymatic (CHOD-PAP) method

3.8.2.1 REAGENTS

1. Tris (hydroxymethyl) aminomethane, (TRIS), $C_4H_{11}NO_3$, Mr 121,14
2. Phenol, analytical grade, C_6H_6O, Mr 94,11
   Caution: highly corrosive.
3. 4-Aminoantipyrine (4-aminophenazone), $C_{11}H_{13}N_3O$, Mr 203,25
4. 3,4-Dichlorophenol, $C_6H_4Cl_2O$, Mr 163,01
5. Sodium cholate (cholic acid, sodium salt), $C_{24}H_{39}O_5Na$, Mr 430,57
6. Fatty alcohol polyglycol ether (e.g. Thesit, n-dodecanol polyglycol ether from Desitinwerke, Hamburg, Germany)
7. Magnesium sulfate, heptahydrate, $MgSO_4 \cdot 7\ H_2O$, Mr 246,49
8. Cholesterol esterase (EC 3.1.1.13) from *Pseudomonas fluorescens* in ammonium sulfate (3,2 mol/L), pH about 6 or in NaCl (3,0 mol/L), pH about 6. Specific activity: about 55 U/mg (37°C)
9. Cholesterol oxidase (EC 1.1.3.6) from *Nocardia erythropolis* in ammonium sulfate (1 mol/L), pH about 6 or in NaCl (3,0 mol/L), pH about 6. Specific activity: about 45 U/mg (37°C)
10. Peroxidase (EC 1.11.1.7) from horseradish ; specific activity: >250 U/mg (25°C)
11. Hydrochloric acid, 0,1 mol/L

12. Sodium hydroxide, 0,1 mol/L
13. Cholesterol, $C_{27}H_{46}O$, Mr 386,64

3.8.2.2 SOLUTIONS

I. TRIS (100 mmol/L); $MgSO_4$ (50 mmol/L); phenol (6 mmol/L); 4-aminoantipyrine (1 mmol/L); 3,4-dichlorophenol (4 mmol/L); sodium cholate (10 mmol/L); fatty alcohol polyglycol ether (3g/L); cholesterol esterase (800 U/L); cholesterol oxidase (500 U/L); peroxidase (400 U/L); pH 7,7

TRIS	1,21 g
Phenol	0,0565 g
4-Aminoantipyrine	0,0203 g
3,4-Dichlorophenol	0,063 g
Sodium cholate	0,430 g
Fatty alcohol polyglycol ether	0,300 g
pH with HCl (100 mmol/L) to 7,7	(about 80 mL)
$MgSO_4 . 7 H_2O$	1,23 g
Cholesterol esterase	80 U
Cholesterol oxidase	50 U
Peroxidase	40 U

- Readjust pH, if necessary either with HCl (0,1 mol/L) or NaOH (0,1 mol/L)

Distilled H_2O to 100,0 mL

Stable for four weeks at 2-8°C or one week at 15-25°C.

II. Cholesterol standard (5,17 mmol/L, 2,0 g/L, 200 mg/dl) in fatty alcohol polyglycol ether (100 g/L)

- Dissolve by gentle heating at about 40°C :

Cholesterol	0,1 g
fatty alcohol polyglycol ether	5,0 g

- Mix at temperature 40-60°C with

Distilled H_2O 30 mL

- Cool to 20°C, add:

Distilled H_2O to 50,0 mL

Stable for about one month at 20-25°C.

3.8.2.3 QUALITY CONTROL

Control serum with assigned values

3.8.2.4 METHOD FOR THE ENZYMATIC MEASUREMENT OF CHOLESTEROL

Principle:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{fatty acid} + \text{cholesterol}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{4-Cholestenone} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{4-Benzoquinone-monoamino-4-phenazone} + 4\,H_2O$$

The chromogen shows a broad absorbance maximum at about 500 nm; the increase of absorbance measured between 470-550 nm (546 nm with a mercury lamp) is proportional to the quantity of total cholesterol.

MEASUREMENT CONDITIONS

Temperature: see below.
Read absorbance at 470-550 nm, (at 546 nm with a mercury lamp).
Samples: Serum or plasma

	Standard	Samples
Standard solution (solution II) (mL)	0,02	-
Samples (mL)	-	0,02
Enzyme solution (solution I) (mL)	2,0	2,0

- Mix thoroughly, incubate for 10 minutes at 20-25°C or 5 minutes at 37°C.
- Within a further 60 minutes at 20-25°C read absorbance A against solution I in reference cuvette.

Calculation

$$\text{molar concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 5{,}17 \text{ mmol/L};$$

$$\text{mass concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 2 \text{ g/L}$$

3.8.3 Certified reference material

Cholesterol reference material is available from NBS, Ordering Number: SRM 911a.

3.8.4 References

[1] Abell LL, Levy BB, Birdie B and Kendall FE. A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.*, 1952, 195:357-366.

[2] Siedel J, Rollinger N, Roschau P and Ziegenhorn J. Cholesterol. *Methods of Enzymatic Analysis,* 3rd edition, Volume III, Metabolic 3. Bergmeyer HV ed. Weinheim, Verlag Chemie, 1985.

3.9 Cholinesterase

Acylcholine acylhydrolase, E.C. 3.1.1.8 (ChE, pseudo-cholinesterase, plasma or serum cholinesterase, non specific cholinesterase, butyryl cholinesterase or benzoylcholinesterase)

3.9.1 Methods

No reference method for cholinesterase is available.

Two widely used methods are described here. The difference lies essentially in the substrates used which are butyrylthiocholine iodide [1] and benzoylcholine [2]. The reagents for phenotyping are also included (2).

3.9.2 Procedure with butyrylthiocholine iodide as substrate

3.9.2.1 REAGENTS

1. Orthophosphoric acid, disodium salt, dihydrate, $Na_2HPO_4 \cdot 2H_2O$, Mr 178,05
2. Orthophosphoric acid, monopotassium salt, KH_2PO_4, Mr 136,13
3. 5,5'-Dithio-bis-(2-nitro benzoic acid)(DTNB), $C_{14}H_8N_2O_8S_2$, Mr 396,21
4. Butyrylthiocholine iodide (BTI), $C_9H_{20}NOSI$, Mr 317,23.
   Solid BTI should be stored in the dark in a desiccator.

3.9.2.2 SOLUTIONS

All solutions should be stored in plastic bottles. Water must be twice distilled or deionized and distilled.

I. Phosphate buffer (67 mmol/L); pH 7,4 at 37°C

$Na_2HPO_4 . 2 H_2O$	9,595 g
KH_2PO_4	1,74 g
Distilled H_2O to	1000,0 mL

- Check that pH is 7,4 at 37°C, adjust if necessary with one of the above salts (67 mmol/L).

Stable at 0-4°C, if it is stored tightly stoppered.

II. DTNB (0,27 mmol/L)

DTNB	0,011 g
Phosphate buffer (solution I)	100,0 mL

Store in a dark bottle at least overnight at 4°C before use.

Stable for four weeks if stored in the dark at 0-4°C.

III. Butyrylthiocholine iodide (90 mmol/L)

Butyrylthiocholine iodide	0,143 g
Distilled H_2O	5,0 mL

Stable for four weeks if stored at 0-4°C in a dark bottle.

3.9.2.3 QUALITY CONTROL

Control serum with assigned values

3.9.2.4 MEASUREMENT OF CHE USING BUTYRYLTHIOCHOLINE IODIDE

Principle

$$\text{Butyrylthiocholine iodide} + H_2O \xrightarrow{\text{ChE}} \text{Butyrate} + \text{Thiocholine}$$

$$\text{Thiocholine} + \text{5,5'-Dithio-bis-(2-nitrobenzoic acid)} \longrightarrow \text{5-Thio-2-nitrobenzoate} + \text{2-Nitrobenzoate-5-mercapto-thiocholine}$$

The rate of production of the yellow anion, 5-thio-2-nitrobenzoate, read at 410 nm, corresponds to the catalytic concentration of ChE.

Measurement conditions

Temperature: 37°C

Lightpath in a square thermostabilized cuvette: 10 mm

Wavelength: 410 nm
Molar lineic absorbance of 5-thio-2-nitrobenzoate: 1 360 m^2/mol
Sample: serum or plasma (heparin)

DTNB (solution II)	2,9 mL
Butyrylthiocholine (solution III)	0,1 mL
Sample	0,02 mL

- Mix by inversion.
- Read absorbance and start stopwatch.
- Use ΔA/min for the calculation of the catalytic concentration b of CHE:
  $b = 11,1 \times 10^3 \times \Delta A/min$ (U/L)

3.9.3 Procedure with benzoylcholine chloride as substrate and dibucaine inhibition (dibucaine number) and fluoride inhibition (fluoride number)

3.9.3.1 REAGENTS

1. Orthophosphoric acid, dihydrogen salt, dihydrate, $Na_2HPO_4 . 2H_2O$, Mr 178,05
2. Orthophosphoric acid, monopotassium salt, KH_2PO_4, Mr 136,13
3. Benzoylcholine chloride, $C_{12}H_{18}ClNO_2$, Mr 243,74
4. Dibucaine hydrochloride (Nupercaine CIBA, Cinchocaine), $C_{20}H_{29}N_3O_2 . HCl$, Mr 379,73
5. Sodium fluoride, NaF, Mr 42,00

3.9.3.2 SOLUTIONS

I. Phosphate buffer (67 mmol/L); pH 7,4 at 25°C

$Na_2HPO_4 . 2 H_2O$	47,55 g
KH_2PO_4	9,09 g
Distilled H_2O to	5000,0 mL

- Check that pH is 7,4 at 25°C; adjust if necessary with one of the above salts (67 mmol/L).

Stable at 0-4°C, if it is tightly stoppered.

II. Benzoylcholine chloride (0,2 mmol/L)

Benzoylcholine chloride	0,025 g
Phosphate buffer (solution I)	500,0 mL

Should be prepared daily.

III. Dibucaine hydrochloride (0,04 mmol/L)

Dibucaine hydrochloride	0,015 g
Phosphate buffer (solution I)	1000,0 mL

Stable for one week at 4°C.

IV. Sodium fluoride (0,2 mmol/L)

NaF	0,042 g
Phosphate buffer (solution I)	100,0 mL

- Dilute 5 mL of this solution to 250 mL with phosphate buffer (solution I)

V. Benzoylcholine (0,1 mmol/L) (for the assay of cholinesterase)

- Mix equal volumes of phosphate buffer (solution I) and benzoyl choline chloride solution (solution II).

VI. Benzoylcholine (0,1 mmol/L); dibucaine (0,02 mmol/L) - for dibucaine inhibition

- Mix equal volumes of benzoyl choline chloride solution (solution II) and dibucaine hydrochloride (solution III) .

VII. Benzoylcholine (0,1 mmol/L); sodium fluoride (0,1 mmol/L) - for fluoride inhibition

- Mix equal volumes of benzoyl choline chloride (solution II) and sodium fluoride (solution IV).

3.9.3.3 QUALITY CONTROL

Control serum with assigned values

3.9.3.4 MEASUREMENT OF CHE AND PHENOTYPING USING BENZOYLCHOLINE CHLORIDE AND DIBUCAINE AND FLUORIDE INHIBITION

Principle

$$\text{Benzoylcholine} + H_2O \xrightarrow{\text{ChE}} \text{benzoic acid} + \text{choline}$$

The maximum difference in the absorption spectra of the substrate and the reaction product occurs at 235 nm. Measurements are made at 240 nm because of the absorbance of diluted plasma; the change of absorbance at 240 nm is proportional to the catalytic concentration of ChE.

Some patients are not able to metabolize muscle-relaxant drugs of the succinylcholine type used in anaesthesia; dramatic incidents may occur. In these cases some

cholinesterase isoenzymes are decreased. The addition of dibucaine or fluoride as inhibitors to the assay system enables the identification of inborn error in metabolism (dibucaine number or fluoride number).

Measurement conditions

Temperature: 25 ±0,05°C, if the test has to be performed at 37°C the results are divided by 1,83 in order to express them at 25°C.

Lightpath in square thermostabilized cuvettes: 10 mm
Wavelength: 240 nm
Molar lineic absorbance of benzoylcholine at 25°C: 660 m^2/mol
Samples: serum or plasma (heparin) diluted 1+99 with phosphate buffer (solution I).

Blood cells should be separated as soon as possible after blood collection. Haemolysis must be avoided. ChE is extremely stable in undiluted plasma, even at room temperature or at temperatures experienced during mailing.

Benzoyl choline (solution V)	2,0 mL
Sample, diluted	2,0 mL

- Mix thoroughly.
- Read absorbance at 30 second intervals during at least 4 minutes, or until linearity is reached.

Calculation

Plot the absorbance values as a function of time and draw the best straight line through the points. Calculate the change of absorbance per minute $(\Delta A/min)_S$. The catalytic concentration b of ChE is obtained as follows:

$$b = 30{,}3 \times 10^3 \times (\Delta A/min)_S \text{ U/L}$$

Dibucaine and fluoride numbers

If the dibucaine number or the fluoride number is required, repeat the procedure with solution VI or VII instead of solution V.

The rates $(\Delta A/min)_D$, in presence of dibucaine, and $(\Delta A/min)_F$ in presence of fluoride are calculated as for $(\Delta A/min)_S$. Then numbers are calculated as follows:

$$\text{dibucaine number} = 100 \times \frac{(\Delta A/min)_S - (\Delta A/min)_D}{(\Delta A/min)_S}$$

$$\text{fluoride number} = 100 \times \frac{(\Delta A/min)_S - (\Delta A/min)_F}{(\Delta A/min)_S}$$

3.9.4 Certified reference material

Not yet available.

3.9.5 References

[1] Whittaker M. Cholinesterase. *Methods of Enzymatic Analysis*, 3rd ed. Bergmeyer HV ed. volume IV, Enzymes 2. Weinheim, Verlag Chemie, 1984, p. 63-67.

[2] Whittaker M. Cholinesterase *Methods of Enzymatic Analysis,* 3rd ed. Bergmeyer HV ed. Volume IV, Enzymes 2.. Weinheim, Verlag Chemie, 1984, p. 68-74.

3.10 Creatinine

(2-Amino-1,5-dihydro-1-methyl-4-H-imidazole-4-one)

3.10.1 Methods

At present, there is no international agreement on a reference method. Many variants of the alkaline picrate (Jaffé) method have been published. The reagents for an end point method using the alkaline picrate reaction [1] are described here. Several enzymatic methods have also been proposed but they will not be discussed here.

3.10.2 End-point Jaffé method

3.10.2.1 REAGENTS

1. Sodium dodecyl sulfate (sodium lauryl sulfate), $C_{12}H_{25}NaO_4S$, Mr 288,38
2. Orthophosphoric acid, trisodium salt, dodecahydrate $Na_3PO_4 \cdot 12\ H_2O$, Mr 380
3. Di-sodium tetraborate (BORAX), $Na_2B_4O_7 \cdot 10\ H_2O$, Mr 381,44
4. Sodium hydroxide (1 mol/L)
5. Picric acid, $C_6H_3N_3O_7$, Mr 229,11

NOTE: For safety reasons, during transport and storage, water is added to the product.

Caution: do not dry the reagent. Danger of explosion.

6. Acetic acid, glacial, $C_2H_4O_2$, Mr 60,05

   Caution: highly corrosive.

7. Creatinine, anhydrous, pure, $C_4H_7N_3O$, Mr 113.12
   or creatinine zinc chloride, $C_8H_{14}N_6O_2ZnCl_2$, Mr 362,52

8. Hydrochloric acid (100 mmol/L)
9. Benzoic acid, $C_7H_6O_2$, Mr 122,12

3.10.2.2 SOLUTIONS

I. Dodecyl sulfate (139 mmol/L)

Dodecyl sulfate, sodium salt	20,0 g
Distilled H_2O to	500,0 mL

Stable indefinitely at room temperature. If the reagent becomes turbid, it must be discarded.

II. Picric acid solution, saturated (about 56,75 mmol/L, 13 g/L)

As picric acid is supplied as a moist chemical, ensure that the picric acid and the water are correctly mixed. The proportion of water should be mentioned by the manufacturer.

Picric acid (as supplied), equivalent of dry picric acid	7 g
Distilled H_2O to	500 mL

- Stir for several hours to obtain a saturated solution. Stable indefinitely at room temperature if stored in a dark bottle.

III. Na_3PO_4 (0,05 mol/L); borax (0,05 mol/L), pH 12,8

Na_3PO_4 . 12 H_2O	9,5 g
Borax	9,5 g
pH adjusted with NaOH (1 mol/L) to 12,8	
Distilled H_2O to	500.0 mL

Stable indefinitely at room temperature if stored in a tightly stoppered polyethylene bottle.

IV. Diluted picric acid solution (about 19,9 mmol/L)

Picric acid (solution II)	175,0 mL
Distilled H_2O to	500,0 mL

V. Working reagent: dodecyl sulfate (46,3 mmol/L); phosphate (16,7 mmol/L); borax (16,7 mmol/L); picric acid (6,63 mmol/L)

- Mix equal volumes of dodecyl sulfate (solution I), borax (solution III) and diluted picric acid (solution IV) . To be prepared just before use.

VI. Acetic acid (60%, v/v)

Acetic acid, glacial	60,0 mL

Distilled H_2O to	100,0 mL

Caution: corrosive.

Stable indefinitely at 20-25°C.

VII. Stock creatinine standard (10 mmol/L)

Creatinine, anhydrous	0,113 g
(or creatinine zinc chloride	0,181 g)
HCl (0,1 mol/L) to	100,0 mL

Stable for 2 months at 2-8°C.

VIII. Benzoic acid (8,2 mmol/L)

Benzoic acid	1,0 g
Distilled H_2O to	1000,0 mL

Stable indefinitely at 20-25°C.

IX. Working creatinine standard (500 µmol/L, 56,5 mg/L)

Stock creatinine standard (solution VII)	5,0 mL
Benzoic acid (solution VIII) to	100,0 mL

For a calibration curve (graph):

Stock creatinine standard (solution VII)	Creatinine concentrations	
mL	mmol/L	mg/L
0	0	0
1,0	0,1	11,3
2,0	0,2	22,6
3,0	0,3	33,9
4,0	0,4	45,2
5,0	0,5	56,5
8,0	0,8	90,4
10.0	1.0	113.0

Benzoic acid (solution VIII) to	100 mL

Stable for 2 months at 2-8°C.

3.10.2.3 MEASUREMENT OF SERUM/PLASMA CREATININE METHOD: END-POINT JAFFÉ

- Label sufficient tubes for the batch, including reagent blank (B), standard (S), control (C1, C2) and patients' samples (1,2,3, etc.).
- Prepare sufficient working creatinine reagent.

- Pipette into the tubes as follows:

	B	S	C1,C2	1,2,3, etc.
Distilled water (mL)	0,2	-	-	-
Standard, 500 µmol/L (mL)	-	0,2	-	-
Control (mL)	-	-	0,2	-
Patient's sample (mL)	-	-	-	0,2
Working creatinine Reagent (mL)	2,0	2,0	2,0	2,0

- Mix well and leave at room temperature for 90 minutes.
  Colorimeter: blue-green filter, Ilford number 603 (490 nm)
  Spectrometer: 490 nm
- Set the instrument to zero with the reagent blank, then transfer the reagent blank solution back again into tube B.
- Measure the absorbance of each tube (reading 1); remember to transfer each solution back into the tube after measuring the absorbance.
- Add 0,05 mL of acetic acid, 60%, to each tube. Mix well, leave for 6 minutes at room temperature and then read the absorbance of each tube again (reading 2); set the instrument to zero with tube B.
- Calculate the corrected absorbance for each tube (reading 1 - reading 2).
- Calculate the results in µmol/L.
- Check the control results.

NOTE: prepare duplicate tubes if you use an instrument with a flow-through cell.

3.10.3 Certified reference material

Creatinine as reference material is available from the NBS, Ordering Number SRM 914.

3.10.4 References

[1] de Cediel, N. Déom A, Hill PG, Sarkar AK et al. Measurement of serum/plasma creatinine, Method: Jaffé reaction. *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories.* LAB/86.3, Geneva, World Health Organization, 1986, p. 93-97.

3.11 D-Glucose (D-glucopyranose)

3.11.1 Methods

The hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) method is internationally considered to be the reference method [1]. In addition, two other enzymatic methods will be described here, one using glucose dehydrogenase [2] and another with glucose oxidase and peroxidase [3]. These three enzymatic methods are widely used as routine methods. The non-enzymatic o-toluidine method [4] is also described.

3.11.2 Glucose standard solutions

For all four methods a common standard can be used. Therefore, the preparation of the glucose standard is described first.

Reagents

1. Glucose, anhydrous, $C_6H_{12}O_6$, Mr 180,16
2. Benzoic acid, $C_7H_6O_2$, Mr 122,12

Solutions

I. Benzoic acid solution (20 mmol/L)

Benzoic acid	2,5 g
Distilled H_2O to	1000 mL

II. Stock glucose (100 mmol/L), benzoic acid (20 mmol/L) solution

Glucose	18,0 g
Benzoic acid (solution I)	1000,0 mL

- Divide into 100 mL portions and store at -20°C in tightly capped plastic bottles. The solution is stable under these conditions for more than 1 year.
- Thaw completely and mix well before preparing working standards. Before weighing, the glucose should be dried at 60-80°C for 4 hours. Allow to cool in a closed container before weighing.

III. Working glucose standard solutions

Allow the stock glucose solution (solution II) to attain room temperature. Then prepare the working standards as follows:

Glucose concentrations (mmol/L)	(g/L)	Stock glucose (solution II) (mL)	Benzoic acid (solution I) to mL
2,5	0,45	2,5	100,0
5,0	0,90	5,0	100,0
10,0	1,80	10,0	100,0
20,0	3,60	20,0	100,0
25,0	4,50	25,0	100,0

The working glucose standards are stable for at least 1 month at 2-8°C when stored in amber glass bottles under sterile conditions.

3.11.3 Hexokinase/G6PDH method

3.11.3.1 REAGENTS

1. Orthophosphoric acid, monosodium salt, monohydrate, $NaH_2PO_4 . H_2O$, Mr 138,05
2. Magnesium sulfate, heptahydrate, $MgSO_4 . 7 H_2O$, Mr 246,49
3. Perchloric acid (0,33 mol/L)
4. Adenosine-5'-triphosphate, disodium salt, trihydrate (ATP)
   $C_{10}H_{14}N_5O_{13}P_3Na_2 . 3 H_2O$, Mr 605,2
5. ß-Nicotine adenine dinucleotide phosphate, disodium salt (NADP)
   $C_{21}H_{26}N_7O_{17}P_3Na_2$, Mr 787,4
6. Hexokinase (HK, EC 2.7.1.1) from yeast, lyophilized, specific activity > 40 U/mg lyophilisate (25°C)
7. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) from yeast or *Leuconostoc mesenteroides*, lyophilized, specific activity >15 U/mg lyophilisate (25°C)
8. Glycerol; volume fraction 0,5 in aqueous solution (50% v/v), $C_3H_8O_3$, Mr 92,09
9. Sodium hydroxide (1 mol/L)

3.11.3.2 SOLUTIONS

I. Phosphate (70 mmol/L), $MgSO_4$ (4 mmol/L), ATP (1,6 mmol/L), NADP (1,6 mmol/L)

$NaH_2PO_4 . H_2O$	0,483 g
$MgSO_4 . 7 H_2O$	0,049 g

ATP	0,048 g
NADP	0,063 g
Distilled H_2O about	30 mL
pH adjusted with NaOH (1 mol/L) to 7,7	
Distilled H_2O to	50,0 mL

Stable 14 days at 4°C if growth of microorganisms is avoided.

II. HK (>100 KU/L), G6PDH (>180 KU/L), $MgSO_4$ (0,1 mol/L)

HK*	0,025 g
G6PDH*	0,095 g
$MgSO_4 . 7 H_2O$	0,148 g
Glycerol 50% (v/v)	6,0 mL

Stable for one month if stored at 4°C.

3.11.3.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

1. The enzymes HK and G6PDH should not contain more than the following amounts of other contaminant enzymes (relative to the HK and G6PDH catalytic activities):

Contaminants in %	HK	G6PDH
Glucose-6-phosphate isomerase, (EC 5.3.1.9)	0,002	0,02
6-Phosphogluconate dehydrogenase (EC 1.1.1.44)	0,01	0,01
Glutathione reductase (EC 1.6.4.2)	0,01	0,05
Glutamate dehydrogenase (EC 1.4.1.3)	0,05	-
Phosphoglucomutase (EC 5.4.2.2)		0,01

At present most of the commercial enzyme preparations meet these requirements.

2. Glycerol must be free from glucose

3.11.3.4 MEASUREMENT OF GLUCOSE WITH HK AND G6PDH

Principle

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{D-Glucose-6-phosphate (G6P)} + \text{ADP}$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{Glucose-6-phosphate-dehydrogenase}} \text{6-Phosphate-D-gluconate} + \text{NADPH} + \text{H}^+$$

*The specific activity of the enzyme preparations may vary from batch to batch, which must be taken into account for calculation.

The production of NADPH, measured by the increase of absorbance at 339 nm (340), or at 334 or 365 nm if a spectrometer is used, is proportional to the amount of glucose present.

Preparation of samples and standard

Whole blood (finger tip or ear lobe) (with heparin, EDTA, citrate, oxalate or fluoride as anticoagulants)

Blood	0,1 mL
Perchloric acid (0,33 mol/L) (ice cold)	1,0 mL

- Mix thoroughly with a glass rod, centrifuge 10 minutes at 3000 rpm.
  Use the supernatant for the assay.
  Serum or plasma must immediately be separated from blood cells.
  Deproteinize as for whole blood.

Glucose standard	
Working glucose standard (5,0 mmol/L)	0,1 mL
Perchloric acid (0,33 mol/L)	1,0 mL

Measurement conditions

	Standard	Samples
Standard (diluted with perchloric acid [mL])	0,1	-
Supernatant of deproteinized samples (mL)	-	0,1
Phosphate buffer (solution I) (mL)	2,0	2,0
- Mix thoroughly and read absorbance A_1 at 339 nm (340), 334 or 365 nm with a spectrometer at room temperature.		
HK/G6PDH solution (solution II) (mL)	0,02	0,02

- Mix thoroughly and after 5-30 minutes read absorbance A_2;
- use $A_2-A_1 = \Delta A$ for calculation.

Calculation

$$\text{molar concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 5{,}0 \text{ mmol/L};$$

$$\text{mass concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 0{,}9 \text{ g/L}$$

3.11.4 Glucose dehydrogenase method

3.11.4.1 REAGENTS

1. Orthophosphoric acid, disodium salt, dihydrate, $Na_2HPO_4 . 2 H_2O$, Mr 178,05
2. Orthophosphoric acid, monosodium salt, monohydrate, $NaH_2PO_4 . H_2O$, Mr138,05
3. Sodium chloride, NaCl, Mr 58,45
4. Ethylenediamine tetra-acetic acid, disodium salt, dihydrate (Ethylene dinitrilo-tetra-acetic acid, disodium salt, dihydrate, EDTA) $C_{10}H_{14}N_2O_8Na_2 . 2 H_2O$, Mr 372,24
5. Oxamic acid, $C_2H_3NO_3$, Mr 89,05
6. Sodium azide, NaN_3, Mr 65,02
7. ß-Nicotine adenine dinucleotide, free acid (NAD), $C_{21}H_{27}N_7O_{14}P_2$, Mr 663,4
8. Mutarotase (MR, EC 5.1.3.3) for analytical purpose from hog kidney suspension in ammonium sulfate (3,2 mol/L); specific activity about 5 KU/mg, or from *Acinetobacter calcoaceticus* lyophilized; specific activity about 1 KU/mg (25°C)
9. Glucose dehydrogenase (GDH, EC 1.1.1.47) for biochemical use from *Bacillus megaterium*, lyophilized; specific activity about 220 U/mg (25°C)
10. Glucose, $C_6H_{12}O_6$, Mr 180,16
11. Perchloric acid (1 mol/L)
12. Sodium $NaClO_4 . H_2O$, Mr 122,45
13. Sodium hydroxide (1 mol/L)
14. Hydrochloric acid (1 mol/L)

3.11.4.2 SOLUTIONS

I. Phosphate (0,12 mol/L), NaCl (0,15 mol/L), EDTA (3,0 mmol/L), Oxamic acid (25 mmol/L), NaN_3(15,4 mmol/L)

$Na_2HPO_4 . 2 H_2O$	1,0 g
$NaH_2PO_4 . H_2O$	0,055 g
NaCl	0,444 g
EDTA	0,056 g
Oxamic acid	0,11 g
NaN_3	0,050 g
Distilled H_2O about	40 mL

pH adjusted with NaOH or HCl (1 mol/L) to 7,6	
Distilled H_2O to	50,0 mL

Stable at room temperature for 4 weeks.

II. NAD (2,2 mmol/L), mutarotase (45 KU/L)

NAD	0,058 g
Mutarotase	1800 U
Buffer solution (solution I)	40,0 mL

Stable at 2-8°C for 4 weeks.

III. Glucose dehydrogenase (500 KU/L)

GDH	250 U
Buffer solution (solution I)	0,5 mL

Stable at 2-8°C for 4 weeks.

IV. $HClO_4/NaClO_4$ (0,33 mol/L together)

$NaClO_4$	0,52 g
$HClO_4$ (1 mol/L)	4,0 mL
Distilled H_2O to	25,0 mL

Stable indefinitely.

3.11.4.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Glucose dehydrogenase must contain less than 0,005% NADH oxidase.

3.11.4.4 MEASUREMENT OF GLUCOSE WITH GLUCOSE DEHYDROGENASE

Principle

$$\alpha\text{-D-glucopyranose} \xrightarrow{\text{mutarotase}} \text{ß-D-glucopyranose}$$

$$\text{ß-D-glucopyranose} + NAD^+ \xrightarrow[+\ NADH + H^+]{\text{glucose dehydrogenase}} \text{D-gluconolactone}$$

$$\text{D-gluconolactone} + H_2O \longrightarrow \text{D-gluconate} + H^+$$

The production of NADH, measured by the increase of absorbance at 339 nm (340), or at 334 or 365 nm if a spectrometer is used, is proportional to the amount of glucose present.

Preparation of samples and standard

Whole blood is taken from finger tip, heel or ear lobe, with heparin as anticoagulant. Immediately after blood collection proceed with deproteinization as described in Section 3.11.3.4.

Serum or plasma

Store in refrigerator for not longer than 30 minutes at 2-8°C; if an immediate preparation of serum or plasma is not possible, add 2 mg sodium azide and 1 mg EDTA per mL blood.

Standard for whole blood assay

Working glucose standard (5,0 mmol/L)	0,1mL
Perchloric acid (0,33 mol/L) (ice cold)	1,0mL

In serum or plasma assay no dilution of the standard (5,0 mmol/L) is required.

a) Whole blood assay

	Standard	Samples
NAD/mutarotase (solution II) (mL)	2,0	2,0
Standard, treated as samples (mL)	0,2	-
Samples deproteinized (mL)	-	0,2

- Mix thoroughly, read absorbance A_1 against water

Glucose dehydrogenase (solution III) (mL)	0,02	0,02

- Mix thoroughly, read absorbance A_2 after 15 minutes against distilled water,
- use $A_2 - A_1 = \Delta A$ for calculation.

Calculation

$$\text{molar concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 5{,}0 \text{ mmol/L};$$

$$\text{mass concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 0{,}9 \text{ g/L}$$

b) Serum or plasma assay

Read absorbance at 339 nm (340), 334 or 365 nm against water in a reference cuvette at room temperature.

	Standard	Samples
NAD/ mutarotase (solution II) (mL)	2,0	2,0
Standard, 5,0 mmol/L (mL)	0,02	-
Samples (mL)		0,02

- Mix thoroughly, read absorbance A_1

Glucose dehydrogenase (solution III) (mL)	0,02	0,02

- Mix thoroughly, read absorbance A_2 after 15 minutes
- Use $A_2 - A_1 = \Delta A$ for calculation.

Calculation

$$\text{molar concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 5{,}0 \text{ mmol/L};$$

$$\text{mass concentration} = \frac{\Delta A_{Sample}}{\Delta A_{Std}} \times 0{,}9 \text{ g/L}$$

3.11.5 Glucose oxidase method without protein precipitation [3]

3.11.5.1 REAGENTS

1. Orthophosphoric acid, di-sodium salt, anhydrous, Na_2HPO_4, Mr 141,96
2. Orthophosphoric acid, monopotassium salt, anhydrous, KH_2PO_4, Mr 136,09
3. Sodium chloride. NaCl, Mr 58,45
4. Phenol. C_6H_6O. Mr 94.11
   Caution: highly corrosive.
5. 4-Aminophenazone (4-amino antipyrine), $C_{11}H_{13}N_3O$, Mr 203,25
6. Sodium azide, NaN_3, Mr 65,02
7. Glucose, anhydrous, $C_6H_{12}O_6$, Mr 180,16
8. Glucose oxidase (GOD, EC 1.1.3.4)

Various units of activity are used by manufacturers to define units of activity of GOD. The following products have been found to be satisfactory:

(i) Fermcozyme[1] 653 AM

(ii) Fermcozyme[1] 952 DM; this product contains both glucose oxidase and peroxidase.

(iii) BDH[2] glucose oxidase (750 Scott units/mL)

[1] Fermcozyme 653 AM and 952 AM are available from Hughes and Hughes Limited.
[2] BDH Diagnostics.

(iv) Sigma[3] glucose oxidase, type X, lyophilized, specific activity 100 - 150 U/mg) (35°C).

9. Peroxidase (POD, EC 1.11.1.7) from horseradish, specific activity: not less than 500 U/mg, (25°C).

3.11.5.2 SOLUTIONS

I. Phosphate (67 mmol/L); NaN_3 (15,4 mmol/L); 4-Aminophenazone (1,67 mmol/L), pH 7,0

KH_2PO_4	3,54 g
Na_2HPO_4	5,78 g
4-Aminophenazone	0,34 g
NaN_3	1,0 g
Distilled H_2O to	1000 mL

Stable for at least 4 weeks at 20-25°C.

II. Buffered enzyme solution:

The different glucose oxidase reagents have been found to be satisfactory when diluted with solution I as follows:

(1) Using Fermcozyme 653 AM:

Phosphate (63 mmol/L); NaN_3 (14,4 mmol/L); 4 Aminophenazone (1,56 mmol/L); GOD (about 30 KU/L); POD (at least 30 KU/L), pH 7,0

Fermcozyme 653 AM	6,4 mL
Peroxidase	0,006 g
Aminophenazone (solution I) to	100,0 mL

(2) Using Fermcozyme 952 DM:

Phosphate (64 mmol/L); NaN_3 (14,8 mmol/L); 4-Aminophenazone (1,6 mmol/L); GOD (about 20 KU/L)]; POD (not specified), pH 7,0

Fermcozyme 952 DM	4,00 mL

(3) Using BDH glucose oxidase (750 Scott units/mL):

Phosphate (66 mmol/L); NaN_3 (15,2 mmol/L);

4-aminophenazone (1,65 mmol/L); GOD (about 6 KU/L); POD (at least 30 KU/L); pH 7,0

Glucose oxidase (750 Scott units/mL)	1,2 mL

[3] Sigma Chemical Company.

Aminophenazone (solution I) to	100,0 mL
Peroxidase	0,006 g

(4) Using Sigma glucose oxidase, type X.
Phosphate (67 mmol/L); NaN_3 (15,4 mmol/L);
4-aminophenazone (1,67 mmol/L); GOD (at least 15 KU/L); POD (at least 30 KU/L); pH 7,0.

Glucose oxidase (type X)	0,015 g
Aminophenazone (solution I) to	100,0 mL
Peroxidase	0,006 g

The buffered enzyme solutions are stable for 4 weeks at 2-8 °C.

III. Phenol solution (3,61 mmol/L)

Phenol	0,34 g
Distilled H_2O to	1000,0 mL

This solution is stable indefinitely at 20-25 °C.

IV. Working enzyme solution

Phosphate (about 33 mmol/L); NaN_3 (about 7,6 mmol/L);
4-aminophenazone (about 0,83 mmol/L); GOD (at least 3 KU/L); POD (at least 15 KU/L); phenol (1,8 mmol/L).

- Mix equal volumes of phenol solution (solution III) and buffered enzyme solution (solution II).
This solution is stable for about 1 hour at 20-25°C.

3.11.5.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

Glucose oxidase and peroxidase: the enzyme preparation should not contain more than 200 U/mg of catalase and less than 0,1% amylase, saccharase, ß-galactosidase or ß-glucosidase (relative to specific activity of glucose oxidase and peroxidase).

3.11.5.4 MEASUREMENT OF GLUCOSE WITH GLUCOSE OXIDASE

a. Pipette 0,1 mL of sample or working glucose standard (10 mmol/L) into centrifuge tubes containing 1,0 mL of distilled water. Mix well.
b. Pipette 0,2 mL of the diluted sample or standard into a test tube for the colour reaction.
c. For blank, pipette 0,2 mL of distilled water in another test tube.
d. To each tube, add 2,0 mL of working enzyme solution. Mix and keep at 37°C for 15 minutes. Shake the tubes briefly two or three times during the period to ensure adequate aeration.

e. Remove the tubes from the water-bath and allow them to cool to room temperature.
f. Transfer to cuvettes and read the absorbance without delay at 515 nm (Ilford filter 604), setting the spectrometer to zero with the reagent blank.
g. Calculate the glucose concentration in the patients' specimens from the absorbance of the 10 mmol/L standard as follows:
Concentration of glucose in mmol/L = (T x C)/S
where T = absorbance reading of patient's test
S = absorbance reading of working standard
C = concentration of working standard

NOTE 1: For high glucose samples (more than 25 mmol/L), repeat steps (b) to (f) above, but use only 0,1 mL of the diluted sample. Remember to multiply the result by 2 to obtain the glucose concentration of the sample.

NOTE 2: Laboratories able to accurately dispense 0,02 mL volumes of samples and standards may omit steps (a) to (c) above and add 0,02 mL of sample or standard to 2,0 mL of working enzyme solution. Use 2,0 mL working enzyme solution as blank. If the glucose concentration is more than 25 mmol/L, dilute the sample with water (0,1 mL + 0,1 mL) and use 0,02 mL. Remember to multiply the result by 2.

3.11.6 o-Toluidine method

The o-toluidine solution is unpleasant to use because of the high concentration of acetic acid. However, the method has proved useful and cheap and is therefore retained in the manual, although enzymatic methods for glucose are preferable.

3.11.6.1 REAGENTS

1. Glucose, anhydrous, analytical grade, $C_6H_{12}O_6$, Mr 180,16
2. Acetic acid, glacial, analytical grade, $C_2H_3O_2$, Mr 60,05
Caution: highly corrosive.
3. 2-Toluidine (o-toluidine), density 0,998, C_7H_9N, Mr 107,15
Caution: highly corrosive, do not pipette by mouth, use dispenser.
4. Thiourea, CH_4N_2S, Mr 76,12

3.11.6.2 SOLUTIONS

o-Toluidine (0,559 mol/L); thiourea (19,7 mmol/L)

Thiourea	1,5 g
Glacial acetic acid (glass cylinder)	940,0 mL
o-Toluidine (with dispenser or burette)	60,0 mL

- Mix well; store in amber bottle.

Stable for up to 3 months at 20-25°C.

NOTE: The solutions should not be used immediately after preparation; allow to stand for 24 hours before using.

3.11.6.3 MEASUREMENT OF GLUCOSE WITH o-TOLUIDINE

Label sufficient test tubes for the batch, including reagent blank (B), standard (S), control (C1, C2) and patients' samples (1,2,3, etc.).

Pipette into the tubes as follows:

	B	S	C1, C2	1,2,3, etc.
Distilled water (mL)	0,05	-	-	-
Standard, 10 mmol/L (mL)	-	0,05	-	-
Control (mL)	-	-	0,05	-
Patient's plasma (mL)	-	-	-	0,05
o-Toluidine reagent (mL)	3,0	3,0	3,0	3,0

- Mix well, cover and incubate all tubes at 100°C for 12 minutes.
- Cool rapidly to room temperature and measure absorbance.
  Colorimeter: orange filter, Ilford number 607 (600 nm)

Spectrometer: 630 nm

- Use a dry cuvette or rinse with glacial acetic acid and drain.
- Set the instrument to zero with the reagent blank (B).
- Calculate the results in mmol/L.
- Check the control results.

NOTE: Prepare a protein-free filtrate if the sample is grossly haemolysed or icteric; see full method for details.

3.11.7 Certified reference material

Glucose as reference material can be obtained from NBS, Ordering number SRM 917.

3.11.8 References

[1] Kunst A, Draeger B and Ziegenhorn J. D-glucose: UV-method with hexokinase and glucose-6-phosphate dehydrogenase. *Methods of Enzymatic Analysis,* 3rd ed. Bergmeyer HV ed. Volume VI, Metabolites 1: Carbohydrates. Weinheim, Verlag Chemie, 1984, p. 163-172.

[2] Vormbrock R. D-Glucose:UV-method with hexokinase and glucose-6-phosphate dehydrogenase in *Methods of Enzymatic Analysis,* 3rd ed. Bergmeyer HV ed. Volume VI, Metabolites 1: Carbohydrates. Weinheim, Verlag Chemie, 1984, p. 172-178.

[3] Hill PG and Swaminathan S. A simple manual method for plasma glucose without protein precipitation. Report to WHO, April 1989, ref. H5/181/58.

[4] de Cediel N, Déom A, Hill PG, Sarkar AK et al. Measurement of plasma glucose. Method: o-toluidine *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories.* LAB/86.3, Geneva, World Health Organization, 1986, p. 98-102.

3.12 Iron (Fe)

3.12.1 Methods

The ICSH has proposed recommendations [1] for the measurement of iron in serum but no reference method is available. Methods using bathophenanthroline disulfonate [2] and FerroZine [3] will be described here.

Caution: For all procedures, all glassware must be entirely cleaned of iron, by immersion overnight in iron-free HCl (6 mol/L), and then rinsed with water, free of measurable iron; the reagents must be selected in the catalogue for iron analysis.

3.12.2 Bathophenanthroline procedure without deproteinization

3.12.2.1 REAGENTS

1. Sodium dithionite, (hyposulfite), $Na_2S_2O_4$, Mr 174,11
2. Magnesium sulfate, heptahydrate, $MgSO_4 \cdot 7\ H_2O$, Mr 246,49

3. Acetic acid, glacial, $C_2H_3O_2$, Mr 60,05 (density : 1,049 g/L)

   Caution: highly corrosive.

4. Teepol-610, (Shell, Fluka)
5. Sodium hydroxide, (1 mol/L)
6. Bathophenanthroline disulfonic acid, disodium salt $C_{24}H_{14}N_2O_6S_2 \cdot 3H_2O$, Mr 590,55
7. Iron, pure, wire, Fe, Mr 55,85
8. Hydrochloric acid (6 mol/L)

3.12.2.2 SOLUTIONS

I. Teepol reagent, iron free

Dithionite	50 g
Magnesium sulfate	10 g
Distilled H_2O	925 mL

- Add and mix immediately

Teepol-610	1200 mL
NaOH (1 mol/L)	375 mL

- After 10-15 minutes centrifuge the turbid solution;

All the contaminant iron is converted to $Fe(OH)_2$, which is carried down with the $Mg(OH)_2$ precipitate.

- Separate the supernatant. Adjust pH with glacial acetic acid to 5,4 - 6,2.
- Divide in portions for daily use in completely filled, tightly stoppered bottles.

Stable for at least 2 months if stored at 4°C. The solution loses its reducing power when exposed to air.

II. Bathophenanthroline (28,8 mmol/L)

Bathophenanthroline disulfonate	1,7 g
Distilled H_2O to	100,0 mL

Stable indefinitely.

III. Iron stock standard (17,91 mmol/L)

In an Erlenmeyer flask:	
Iron wire	1,0 g
HCl (6 mol/L) about	25 mL

- Heat with gentle mixing on a hot plate under a hood.
- After complete dissolution, transfer quantitatively to a 1 litre volumetric flask.
  Distilled H_2O to 1000,0 mL

Stable indefinitely if well stoppered.

IV. Iron working standards (17,9 and 35,8 µmol/L; 1,0 and 2,0 mg/L)

Iron stock standard (solution III) (mL) + water to 1000,0 mL

mL	µmol/L	mg/L
1,0	17,9	1,0
2,0	35,8	2,0

To be prepared fresh each day.

3.12.2.3 MEASUREMENT OF IRON USING BATHOPHENANTHROLINE PROCEDURE

Principle

$$\text{Transferrin } (Fe^{3+})_2 \xrightarrow{\text{Teepol}} \text{Transferrin} + 2\,Fe^{3+}$$

$$Fe^{3+} + \text{dithionite} \longrightarrow Fe^{2+} + \text{sulfite}$$

$$Fe^{2+} + 3\,\text{Bathophenanthroline sulfonate} \longrightarrow Fe^{2+}(\text{bathophenanthroline})_3$$

Teepol is added to split the transferrin $(Fe^{3+})_2$; Fe^{2+} reacts with the bathophenanthroline to form an iron complex; the absorbance is proportional to the iron concentration and is read between 500 - 550 nm (546 nm if a spectrometer is used).

Condition of measurement

Samples: serum or plasma

Haemoglobin below 5,0 g/L does not interfere.

- Pipette directly into the cuvettes (lightpath: 10 mm).

	Blank(B)	Standards(St)	Samples(S)
Water (mL)	0,2	-	-
Teepol reagent (solution I)(mL)	0,5	-	-

- Mix thoroughly and set absorbance at 0,
  add Bathophenantroline (solution II) (mL) 0,01 - -
- Mix thoroughly and read off absorbance A_B
  Iron working standards (mL) - 0,2 -

Teepol reagent (solution I) (mL)	-	0,5	-
- Mix thoroughly and set absorbance at 0			
Bathophenantroline (solution II) (mL)	-	0,01	-
- Mix thoroughly and read off absorbance A_{St1}			
and A_{St2} Samples (mL)	-	-	0,2
Teepol (solution I)(mL)	-	-	0,5
- Mix thoroughly and set absorbance at 0, add:			
Bathophenantroline (solution II) (mL)	-	-	0,01

- Mix thoroughly and read off absorbance A_S

Note: Follow the whole procedure in the sequence mentioned: blank, standard 1, standard 2, sample 1, sample 2, sample 3, etc. (complete one after the other).

Calculation of the iron concentration C

A. in molar concentration

$$f_1 = \frac{17,9}{A_{St1} - A_B} \quad ; \quad f_2 = \frac{35,8}{A_{St2} - A_B}$$

f_1 and f_2 are the factors calculated from the measurements with standards with 17,9 and 35,8 µmol/L iron concentrations,

$$fm = \frac{f_1 + f_2}{2}$$

where fm is the mean of factors f_1 and f_2

$C = (A_s - A_B) \times fm$ (µmol/L)

Note that f_1 and f_2 should not differ from fm by more than ±5%.

C = sample iron concentration (µmol/L) in sample

B. in mass concentration C

$$f_1 = \frac{1}{A_{St1} - A_B} \quad ; \quad f_2 = \frac{2}{A_{St2} - A_B}$$

$$fm = \frac{f_1 + f_2}{2}$$

$C = (A_s - A_B) \times fm$ (mg/L)

f_1 and f_2 are the factors calculated from the measurements with standards 1 mg/L and 2 mg/L iron concentration.

Note that f_1 and f_2 should not differ from f_m by more than ±5%.

3.12.3 FerroZine method

3.12.3.1 REAGENTS

1. FerroZine(R) [3-(2-pyridyl)-5,6-bis(phenyl sulfonic acid)1,2,4 triazine] disodium salt $C_{20}H_{12}N_4O_6S_2Na_2$, Mr 514,44
2. Thiourea (thiocarbamide), CH_4N_2S, Mr 76,12
3. Sodium hydroxide (12,5 mol/L);
   Caution: highly corrosive.
4. Acetic acid, glacial, $C_2H_4O_2$, Mr 60,05
   Caution: highly corrosive.
5. Sodium dodecyl sulfate (sodium lauryl sulfate, SDS), $C_{12}H_{25}O_4S$ Na, Mr 288,38
6. L-ascorbic acid, $C_6H_8O_6$, Mr 176.12
7. Sodium metabisulfite (pyrosulfite), sodium dithionite, $Na_2S_2O_5$, Mr 190,11
8. Iron, pure, wire, Fe, Mr 55,85
9. Hydrochloric acid, (6 mol/L)

3.12.3.2 SOLUTIONS

I. FerroZine (7,78 mmol/L); Thiourea (328 mmol/L)

FerroZine	0,4 g
Thiourea	2,5 g
Distilled H_2O to	100,0 mL

Filter insoluble particles if necessary.

Stable for at least 2 months if stored in a dark brown bottle.

II. Stock acetate buffer (1 mol/L)

Acetic acid, glacial	60 mL
Distilled H_2O about	500 mL
pH adjusted with NaOH (12,5 mol/L) to 4,5	
Distilled H_2O to	1000,0 mL

Stable indefinitely if protected from bacterial growth.

III. Solution A: acetate (100 mmol/L); SDS (20,8 mmol/L)

Stock acetate buffer (solution II)	50,0 mL
Distilled H_2O about	300 mL
Lauryl sulfate	3,0 g

- Mix until the powder is completely dissolved

Distilled H_2O to	500,0 mL

IV. Solution B: acetate (100 mmol/L); ascorbic acid (341 mmol/L); sodium metabisulfite (10.5 mmol/L)

Stock acetate buffer (solution II)	50,0 mL
Ascorbic acid	30 g
Na metabisulfite	1 g
Distilled H_2O to	500,0 mL

Stable for 2 weeks if stored in a dark brown bottle at 4°C.

V. Iron buffer solution

Acetate /SDS (solution III)	1 volume
Acetate/ascorbic acid/sodium metabisulfite (solution IV)	1 volume

Must be prepared daily.

VI. Iron stock standard (17,91 mmol/L)

See Section 3.12.2.2, solution III.

VII. Iron working standards (17,9 and 35,8 µmol/L, 1,0 and 2,0 mg/L).

See Section 3.12.2.2, solution IV.

3.12.3.3 MEASUREMENT OF IRON WITH THE FERROZINE METHOD

Principle

The serum (plasma) sample is treated with buffer reagent which prevents precipitation of proteins, provides an acid medium for the dissociation of the Fe^{3+} transferrin complex, and reduces $Fe^{3+} \longrightarrow Fe^{2+}$. Addition of FerroZine reagent results in formation of a deeply coloured FerroZine-Fe^{2+} complex with an absorbance maximum at 562 nm proportional to the iron concentration. The presence of thiourea in the colour reagent prevents formation of FerroZine-Cu^{+} complex.

Measurement

Temperature: see below.
Wavelength: 562 nm (546 or 578 with a mercury lamp).
Samples: serum or plasma.

Specimens showing definite haemolysis are unsuitable, whereas those with barely visible haemolysis are suitable since iron bound to haemoglobin is not detected by the procedure.

Bilirubin and turbidity do not interfere.

	Blank(B)	Standards(St)	Sample(S)
Distilled H_2O (mL)	0,5	-	-
Working standards (mL)	-	0,5	-
Samples (mL)	-	-	0,5
Iron buffer (solution V)(mL)	4,5	4,5	4,5

- Mix thoroughly on a vortex mixer.
- Incubate for 15 minutes at 37°C in a water-bath.
- Read the absorbance A_1, against the blank at 562 nm (A 546 or 578 nm with a mercury lamp).

Ferrozine (solution I) (mL)	0,10	0,10	0,10

- Mix thoroughly on a vortex mixer.
- Incubate 15 minutes at 37°C in a water-bath.
- Read absorbance A_2 against blank at 562 nm (A546 or 578 nm with a mercury lamp), use
  $A_2 - A_1 = \Delta A_S$ and ΔA_{St} for calculation.

Calculation

As described in Section 3.12.2.3

3.12.4 Certified reference material

Available from NBS as Iron metal; Ordering number SRM 937.

3.12.5 References

[1] Lewis SM. International Committee for Standardization in Haematology. Proposed recommendations for measurement of serum iron in human blood. *Am. J. Clin. Pathol.*, 1971, 56:543-545.

[2] Lauber, K. Iron. *Clinical Chemistry, Theory and Practice*, 3rd ed. Richterich R and Colombo J-P ed., Chichester, John Wiley and Sons, 1981, p. 335-339.

[3] Tietz NW. Serum iron and iron binding capacity. *Fundamentals of Clinical Chemistry,* Tietz NW ed, Philadelphia, WB Saunders Company, 1976, p. 921-928.

3.13 Potassium and sodium

3.13.1 Methods

There are no agreed reference methods for potassium and sodium, but flame emission photometry with internal standardization is widely used. As an example, the reagents for the IL 343 and IL 543* flame photometer will be described here [1], even though the reagents are largely instrument specific. For other instruments, the manufacturers' instructions should be followed. Ion selective electrodes are available for potassium and sodium measurement, but as the reagents are entirely instrument dependent, they will not be described here. Colorimetric and enzymatic methods have recently been described, but have been insufficiently evaluated in routine use to be described here.

3.13.2 Flame emission spectrometry

3.13.2.1 REAGENTS

1. Lithium nitrate, analytical grade, $LiNO_3$, Mr 68,94
2. Potassium chloride, KCl, Mr 74,55
3. Sodium chloride, NaCl, Mr 58,44
4. Brij 35, [polyoxyethylene(23) lauryl ether], $C_{58}H_{118}O_{24}$, Mr 1199,57
5. Triton X-100; density 1,06 - 1,08

3.13.2.2 SOLUTIONS

A. Method without automatic diluter

I. Brij 35 (20% v/v)

Brij	20,0 mL
Distilled H_2O to	100,0 mL

- Heat and stir with a glass rod until homogeneity is achieved.
- Cool to room temperature.

II. Internal lithium standard: lithium nitrate (15 mmol/L)

For zero setting of the instrument, and for dilution of stock standard and sample.

Li nitrate	2,069 g
Distilled H_2O , about	1900,0 mL
Brij 35 (solution I)	2,0 mL

*Instrumentation Laboratory.

- Mix without foaming; add
  Distilled H_2O to 2000,0 mL
- Filter, if necessary.

Standards for serum, plasma, CSF, sweat, dialysate

III. Stock standard: KCl (5,0 mmol/L); NaCl (140 mmol/L)

To dry, heat an excess of the necessary amounts of KCl and of NaCl overnight at about 100°C in a dry oven, let cool to room temperature in a desiccator, then weigh rapidly:

KCl	0,373 g
NaCl	8,182 g
Distilled H_2O to	1000,0 mL

IV. Working standard: KCl (0,0249 mmol/L); NaCl (0,696 mmol/L)

Use a dry 1 litre volumetric flask or rinse it three times with lithium nitrate (solution II).

Lithium nitrate (solution II) to the mark	1000,0 mL
Stock standard (solution III)	5,0 mL

Standards for urine

V. Stock standard: KCl (0,1 mol/L); NaCl (0,1 mol/L)

Dry KCl and NaCl as mentioned for reagent III, then weigh rapidly.

KCl	7,455 g
NaCl	5,844 g
Distilled H_2O to	1000,0 mL

VI. Working standard; KCl (0,497 5 mmol/L); NaCl (0,497 5 mmol/L)

Use a dry 1 litre volumetric flask or rinse it three times with lithium nitrate (solution II).

Lithium nitrate (solution II)	1000,0 mL
Stock standard (solution V)	5,0 mL

B. Method with automatic diluter

VII. Brij 35 (30%, v/v)

Brij 35	30,0 mL
Distilled H_2O to	100,0 mL

Heat and stir with a glass rod until homogeneity is reached, cool to room temperature.

VIII. Lithium nitrate (3,0 mol/L)

Lithium nitrate	413,6 g
Distilled H_2O about	1800,0 mL
Brij 35 (solution VII)	2,0 mL

- Mix without foaming
  Distilled H_2O to 2000,0 mL
- Filter if necessary.

IX. Rinsing solution

Triton X-100	20,0 mL
Distilled H_2O to	2000,0 mL

Standards for serum, plasma, CSF, dialysate

X. Stock standard; KCl (38,4 mmol/L); NaCl (1,435 mol/L)

KCl	2,863 g
NaCl	83,861 g
Distilled H_2O to	1000,0 mL

XI. Working standard; KCl (3,84 mmol/L); NaCl (143,5 mmol/L)

Stock standard (solution X)	50,0 mL
Distilled H_2O to	500,0 mL

Standards for urine

XII. Stock standard; KCl (2,0 mol/L); NaCl (2,0 mol/L)

KCl	149,1 g
NaCl	116,9 g
Distilled H_2O to	1000,0 mL

XIII. Working standard: KCl (100 mmol/L); NaCl (100 mmol/L)

Stock standard (solution XII)	50,0 mL
Distilled H_2O to	1000,0 mL

3.13.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

The purity of lithium, potassium and sodium salts can be investigated by flame spectroscopy, but in principle the information on the labels for the chemicals should be reliable.

3.13.3 Certified reference material

Available from NBS; Ordering numbers: KCl: SRM 918; NaCl: SRM 919

3.13.4 References

[1] Frei J, Michod J and Weissbrodt A-L. Department of Clinical Chemistry, University Hospital (Laboratoire Central de Chimie Clinique), Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland (unpublished document).

3.14 Proteins (total)

3.14.1 Methods

The Kjeldahl method for protein nitrogen used to be the classical procedure for protein measurement. It is time-consuming and rarely necessary now that certified reference materials and commercial calibrators are available. Many methods have been described using the biuret reaction. The method described here has previously been recommended by WHO [1].

3.14.2 Biuret method

3.14.2.1 REAGENTS

1. Sodium chloride (154 mmol/L)
2. Sodium azide, NaN_3, Mr 65,02
   Caution: handle with care, poison, danger of explosion.
3. Sodium hydroxide, NaOH, Mr 40,01 and 5 mol/L
4. Copper[II] sulfate, pentahydrate, $CuSO_4 \cdot 5H_2O$, Mr 249,68
5. Potassium sodium tartrate, tetrahydrate, $KNaC_4H_4O_6 \cdot 4\ H_2O$, Mr 282,23
6. Potassium iodide, KI, Mr 166,02
7. Bovine serum albumin (BSA) or other calibrator, such as reference or control serum with assigned values for total proteins or human serum albumin (HSA, Cohn factor V)

3.14.2.2 SOLUTIONS

I. Biuret reagent: copper sulfate (12 mmol/L); KNa tartrate, (31,9 mmol/L); KI (30 mmol/L); NaOH (0,6 mol/L)

Copper sulfate	3,0 g
KNa tartrate	9,0 g
KI	5,0 g
Distilled H_2O about	500 mL

- Dissolve completely; then add:

NaOH (5 mol/L)	120 mL
Distilled H_2O to	1000,0 mL

Stable indefinitely at 20-25°C if stored tightly stoppered in a polyethylene bottle.

II. Blank biuret reagent: KNa tartrate (31,9 mmol/L); KI (30 mmol/L); NaOH (0,6 mol/L)

KNa tartrate	9,0 g
KI	5,0 g
Distilled H_2O about	500 mL

- Dissolve completely

NaOH (5 mol/L)	120 mL
Distilled H_2O to	1000,0 mL

Stable indefinitely at 20-25°C, if stored tightly stoppered in a polyethylene bottle.

III. NaCl (0,154 mol/L); NaN_3 (15,4 mmol/L)

NaN_3	1,0 g
NaCl (0,154 mol/L)	1000,0 mL

Stable indefinitely at 20 - 25 °C.

IV. Protein stock standard (100 g/L)

Weigh out about 5,3 g of BSA powder and dry it overnight at about 60°C. After drying and cooling in a desiccator weigh out exactly:

BSA	5,0 g
NaCl (solution III) about	30 mL

- Float the powder in a small beaker on the surface of the NaCl solution. After the BSA has dissolved, transfer quantitatively and slowly into a 50 mL volumetric flask (down the side of the flask to avoid frothing); add:

NaCl (solution III) to	50,0 mL

Stable for 6 months if stored at 2-8°C.

Commercial total protein standards, or control material with assigned values for total proteins are available and may be more convenient for laboratories performing few analyses.

V. Working standards

Proteins (g/L)	Protein stock (solution IV)(mL)	NaCl (solution III) (mL)
20,0	0,2	0,8
40,0	0,4	0,6
60,0	0,6	0,4
80,0	0,8	0,2
100,0	1,0	0

Working standards can be made similarly from commercial total protein standards (or from control sera).

3.14.2.3 CONTROL OF REAGENTS AND SOLUTIONS

The purity of each salt is controlled by flame spectroscopy.

Turbid solutions must be discarded.

3.14.2.4 MEASUREMENT OF SERUM/PLASMA TOTAL PROTEINS

- Label sufficient tubes for the batch including reagent blank (RB), standard (S), controls (C1, C2) and patient's samples (1,2,3,etc.). These are for the reagent 'blank' and 'tests'.
- Label a blank tube corresponding to each 'test' tube, i.e. SB, C1B, C2B, 1B, 2B, 3B, etc; these are the 'blanks'.

Pipette into the tubes as follows:

	RB	S	C1,1,2, etc.	SB	C1B,1B,2B, etc.
Biuret reagent (mL)	2,5	2,5	2,5	-	-
Blank biuret reagent (mL)	-	-	-	2,5	2,5
Distilled water (mL)	0,05	-	-	-	-
Protein standard,100 g/l (mL)	-	0,05	-	0,05	-
Controls' or patient's samples (mL)	-	-	0,05	-	0,05

- Mix well, leave at 37°C for 10 minutes or at room temperature for 30 minutes.
  Colorimeter: yellow-green filter, Ilford number 605 (550 nm)
  Spectrometer: 540 nm
- Set the instrument to zero with blank biuret reagent.
- Measure absorbance first of SB and C1B, 1B, 2B, etc., then of RB, then measure absorbance of S, C1, 1, 2, etc.
- Calculate the results in g/L.
- Check the control results.

3.14.3 Certified reference material

From NBS: No. SRM 926 (BSA lyophilized powder extremely hygroscopic) or easier to use: No. SRM 927 (70 g/L (w/v) BSA solution)

From NCCLS: No. ASC-1 (BSA solution)

From IFCC: HSA, international liquid standard

3.14.4 References

[1] de Cediel N, Déom A, Hill PG, Sarkar AK et al. Measurement of serum/plasma total protein; Method: Biuret. *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories.* LAB/86.3, Geneva, World Health Organization, 1986, p. 108-112.

3.15 Triglycerides (TG)

3.15.1 Methods

There is no reference method for triglyceride measurement available. The reagents for two end-point procedures will be described here, an enzymatic UV method (with lipase, glycerokinase, pyruvate kinase, and lactate dehydrogenase) [1] and an enzymatic colorimetric method (with lipase, glycerokinase, glycerol phosphate oxidase, peroxidase, and Trinder reaction) [1, p 12-18].

3.15.2 Enzymatic UV method

3.15.2.1 REAGENTS

1. Tris (hydroxymethyl) aminomethane (TRIS), $C_4H_{11}NO_3$, Mr 121,14
2. Magnesium sulfate, heptahydrate, $MgSO_4 \cdot 7\ H_2O$, Mr 246,49

3. Isotridecanol polyglycol ether (Genapol X-080, Hoechst AG)
4. 3,4-Dichlorophenol, $C_6H_4OCl_2$, Mr 163,01
5. Sodium cholate (cholic acid, sodium salt), $C_{24}H_{39}O_5Na$, Mr 430,57
6. ß-Nicotine-adenine dinucleotide, reduced form, disodium salt (NADH) $C_{21}H_{27}N_7O_{14}P_2Na_2$, Mr 709,2
7. Adenosine triphosphate, disodium salt, trihydrate (ATP) $C_{10}H_{14}N_5O_{13}P_3Na_2 \cdot 3\ H_2O$, Mr 605,2
8. Phosphoenolpyruvate, tricyclohexylammonium salt (PEP), $C_3H_2O_8P(C_6H_{14}N)_3$, Mr 465,3
9. Lipase (EC 3.1.1.3) from *Pseudomonas* species, lyophilized; specific activity per mg lyophilisate: > 40 U/mg (37°C)
10. Glycerokinase (EC 2.7.1.30, GK) from *Bacillus stearothermophilus*: in Tris buffer pH 7,3, stabilized; specific activity: about 85 U/mg (25°C)
11. Pyruvate kinase (EC 2.7.1.40, PK) from rabbit muscle, suspended in ammonium sulfate solution (3,2 mol/L); specific activity: >200 U/mg (25°C), about 500 U/mg (37°C)
12. Lactate dehydrogenase (EC 1.1.1.27, LDH) from pig heart, suspended in ammonium sulfate solution, (3,2 mol/L); specific activity: >300 U/mg (25°C), 750 U/mg (37°C).
13. Sodium bicarbonate (sodium hydrogen carbonate), $NaHCO_3$, Mr 84,01
14. Triglyceride standard; Precilip control from Boehringer, Mannheim, or other calibrators are suitable.
15. Hydrochloric acid (5 mol/L)

3.15.2.2 SOLUTIONS

I. TRIS (100 mmol/L); $MgSO_4$ (0,030 mol/L); Sodium cholate (8 mmol/L); Isotridecanol polyglycol ether (2 g/L); 3,4-Dichlorophenol (3mmol/L); pH 8,2.

TRIS	1,21 g
$MgSO_4 \cdot 7\ H_2O$	0,739 g
Sodium cholate	0,345 g
Isotridecanol polyglycol ether	0,200 g
3,4-Dichlorophenol	0,049 g
Distilled H_2O about	80 mL
pH adjusted with HCl (5 mol/L) to 8.2	
Distilled H_2O to	100,0 mL

Stable for one year at 4°C if growth of microorganisms is avoided.

II. Sodium bicarbonate (0,595 mol/L)

$NaHCO_3$	5 g
Distilled H_2O to	100,0 mL

III. NADH (6 mmol/L); ATP (2,5 mmol/L); PEP (7,5 mmol/L)

NADH	0,017 g
ATP	0,006 g
PEP	0,014 g
Sodium bicarbonate (solution II)	4,0 mL

To be prepared fresh every week; store at 4°C.

IV. Lipase (1000 KU/L); PK (>150 KU/L); LDH (>150 KU/L)

Dilute the stock suspensions of PK and LDH to the required activity concentration with water. Add the appropriate amount of lipase and mix well. To be prepared fresh every week; store at 4°C.

V. TRIS (2 mol/L); HCl buffer, pH 7,3

TRIS	24,23 g
Distilled H_2O about	60 mL
pH adjusted with HCl (5 mol/L) to 7,3	
Distilled H_2O to	100,0 mL

VI. Glycerokinase (GK) (>350 KU/L)

Dilute stock GK solution to the required activity with TRIS/HCl buffer (solution V).

3.15.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

The enzymes must be reasonably free of hexokinase (<0,01% relative to enzyme activity) as well as from other interfering kinases and phosphatases. ATP and PEP should be reasonably free from ADP and pyruvate.

3.15.2.4 MEASUREMENT OF TG WITH THE ENZYMATIC UV METHOD

Principle

$$\text{Triglyceride} + H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + 3\ \text{R-COOH (fatty acids)}$$

$$\text{Glycerol} + ATP \xrightarrow{GK} \text{Glycerol-3-phosphate} + ADP$$

$$ADP + PEP \xrightarrow{PK} ATP + Pyruvate$$

$$Pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-}Lactate + NAD^+$$

The disappearance of NADH, measured by the decrease of absorbance at 339 nm (340), or at 334 or 365 nm if a spectrometer is used, is proportional to the amount of triglyceride present.

Measurement conditions

Room temperature
Lightpath in square cuvettes: 10 mm

- Read absorbance at 339 nm (340), or at 334 or 365 with a spectrometer.

Sample: serum or plasma (heparin, fluoride, EDTA) should be separated from blood cells within 1 hour of blood collection: dilute lipaemic samples with NaCl (154 mmol/L), if they contain more than 7,9 mmol/L of TG.

Serum or plasma can be frozen until ready for analysis, but can also be kept for 2 days at 4°C.

	Blank	Samples
TRIS (solution I) (mL)	2,5	2,5
NADH (solution III) (mL)	0,1	0,1
Lipase (solution IV)(mL)	0,05	0,05
NaCl, 0,154 mol/L (mL)	0,05	-
Samples	-	0,05

- Mix thoroughly,
- After 10 minutes read absorbance A_1 against water or air

Glycerokinase (solution VI) (mL)	0,01	0,01

- Mix thoroughly.
  After 10 minutes read absorbance A_2; use A_1-A_2 = ΔA and subtract A_{Blank} to give $A_{corrected}$

Calculation

Molar concentration:	339 (340) nm :	$A_{corrected}$ x 8,60 mmol/L
	334 nm :	$A_{corrected}$ x 8,77 mmol/L
	365 nm :	$A_{corrected}$ x 15,94 mmol/L

Mass concentration:	339 (340) nm .	$A_{corrected}$ x 7,614 g/L
	334 nm :	$A_{corrected}$ x 7,762 g/L
	365 nm :	$A_{corrected}$ x 14,108 g/L

3.15.3 Enzymatic colorimetric method

3.15.3.1 REAGENTS

1. TRIS (hydroxymethyl) aminomethane (TRIS), $C_4H_{11}NO_3$, Mr 121,14
2. Ethylenediamino tetra acetic acid, disodium salt, dihydrate (ethylene dinitrilo tetra-acetic acid, disodium salt, dihydrate, EDTA), $C_{10}H_{14}N_2O_8Na_2 . 2 H_2O$, Mr 372,24
3. Sodium cholate (cholic acid, sodium salt), $C_{24}H_{39}O_5Na$, Mr 430,57
4. Magnesium sulfate, heptahydrate, $MgSO_4 . 7 H_2O$, Mr 246,49
5. Isotridecanol polyglycol ether (Genapol X-080, Hoechst AG)
6. 4-Chlorophenol, C_6H_5OCl, Mr 128,56
7. Sodium azide, NaN_3, Mr 65,02
8. Adenosine triphosphate, disodium salt, trihydrate (ATP), $C_{10}H_{14}N_5O_{13}P_3Na_2 . 3 H_2O$, Mr 605,2
9. Lipase (EC 3.1.1.3) from *Pseudomonas* species, lyophilized; specific activity about 1200 U/mg (37°C) (40 U/mg lyophilisate)
10. Glycerokinase (EC 2.7.1.30, GK) from *Bacillus stearothermophilus* in TRIS buffer pH 7,3; specific activity: about 85 U/mg (25°C)
11. L-Glycerol-3-phosphate oxidase (EC 1.1.3.21, GPO) from microorganisms, lyophilized; specific activity about 50 U/mg (25°C) (about 40 U/mg lyophilisate)
12. Peroxidase (EC 1.11.1.7, POD) from horseradish, lyophilized, activity 100 U/mg lyophilisate (25°C)
13. 4-aminophenazone (4-aminoantipyrine), $C_{11}H_{13}N_3O$, Mr 203,25
14. Potassium hexacyanoferrate (II) trihydrate (potassium ferrocyanide trihydrate) $K_4[Fe(CN)_6] . 3 H_2O$, Mr 422,38
15. Glycerol, $C_3H_8O_3$, Mr 92,09
16. Hydrochloric acid, 5 mol/L

3.15.3.2 SOLUTIONS

I. TRIS (0,1 mol/L); $MgSO_4$ (17,5 mmol/L); EDTA (10 mmol/L); Na Cholate (3,5 mmol/L);Isotridecanol polyglycol ether (1,2 g/L); 4-Chlorophenol (3,5 mmol/L), NaN_3 (15,4 mmol/L); ATP (1,0 mmol/L); Lipase (>20 KU/L); GK (>1 KU/L); GPO (>8 KU/L); POD (>2 KU/L) pH 7,6

TRIS	1,21 g
$MgSO_4 . 7 H_2O$	0,431 g
EDTA	0,372 g
Isotridecanol polyglycol ether	0,120 g
4-Chlorophenol	0,045 g
NaN_3	0,100 g
ATP	0,061 g
Distilled H_2O, about	80 mL
pH adjusted with HCl (5 mol/L) to 7,6	
Lipase	0,050 g
GK	0,2 mL
GPO	0,020 g
POD	0,002 g
Distilled H_2O to	100,0 mL

Stable for 2 days at 20-25°C, two weeks at 4°C.

II. TRIS buffer (100 mmol/L); pH 7,6

TRIS	1,21 g
Distilled H_2O, about	80 mL
pH adjusted with HCl (5 mol/L) to 7,6	
Distilled H_2O to	100,0 mL

Stable for 6 months at 4°C.

III. 4-Aminophenazone (14 mmol/L); Potassium hexocyanoferrate (II) (0,24 mmol/L); TRIS (100 mmol/L); pH 7,6

4-Aminophenazone	0,029 g
K-hexocyanoferrate (II) . $3 H_2O$	0,001 g
TRIS (solution II)	10,0 mL

Stable for 4 weeks at 4°C.

IV. Glycerol standard (2,26 mmol/L, 208 mg/L)

Glycerol	0,208 g
Distilled H_2O to	1000,0 mL

Stable for several weeks, in well-stoppered bottle at 4°C.

3.15.3.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

The enzymes, especially GPO, must be free from hydrogen peroxide-producing enzymes such as uricase, glucose oxidase and cholesterol oxidase. The lactate oxidase content of GPO should not exceed 0,005% relative to GPO activity. No further information is available.

3.15.3.4 MEASUREMENT OF TRIGLYCERIDES WITH THE ENZYMATIC/COLORIMETRIC METHOD

Principle

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + 3\text{R-COOH (3 fatty acids)}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$\text{4-chlorophenol} + \text{4 aminophenazone} + H_2O_2 \xrightarrow{\text{POD}} \text{4-(4-benzoquinone monoimine)-phenazone} + HCl + 2H_2O$$

In the last reaction, the Trinder reaction, a red quinonemonoimine dye is produced which has a maximum absorbance at 505 nm. The increase of absorbance measured at 500 nm, or 546 nm if a mercury lamp is used, is proportional to the amount of triglyceride.

Measurement conditions

Room temperature

Wavelength: 500 nm (546 with a mercury lamp), absorbance read against air or water.

Samples: serum or plasma (as described in section 3.15.2.4)

	Blank(B)	Standard(St)	Samples(S)
Solution I (mL)	2,0	2,0	2,0
Solution III (mL)	0,05	0,05	0,05
NaCl, 0,154 mol/L (mL)	0,02	-	-
Standard, solution IV (mL)	-	0,02	-
Samples	-	-	0,02

- Mix thoroughly, allow to stand for 10-70 minutes, read absorbance; use for calculation:

  $A_S - A_B = \Delta A_S$ and $A_{St} - A_B = \Delta A_{St}$

Calculation (as tripalmitin)

molar concentration: $\frac{\Delta A_S}{\Delta A_{St}}$ x 2,26 mmol/L;

mass concentration: $\frac{\Delta A_S}{\Delta A_{St}}$ x 2 g/L

3.15.4 Certified reference material

Tripalmitin reference material is available from NBS.

3.15.5 References

[1] Nägele U, Wahlefeld AW and Ziegenhorn J. Triglycerides. *Methods of Enzymatic Analysis*, 3rd ed. Bergmeyer HV ed., Volume VIII. Metabolites III, Weinheim, Verlag Chemie, 1985, p. 2-12 & 12-18.

3.16 Urea (Carbamide, carbonic acid diamide)

3.16.1 Methods

No international agreement exists as yet, but the end-point version of the UV urease/ glutamate dehydrogenase method is widely regarded as a reference method [1]; the reagents are not described here. A colorimetric urease method combined with the Berthelot reaction [2] and a diacetyl monoxime method [3] are described here.

3.16.2 Urease Berthelot method

3.16.2.1 REAGENTS

1. Urease (EC 3.5.1.5) from jack bean (*Canavalia ensiformis*) specific activity: ca 250 U/mg enzyme protein, >4 U/mg (25°C)
2. Orthophosphoric acid, monosodium salt, monohydrate, $NaH_2PO_4 \cdot H_2O$, Mr 138,05
3. Urea, crystalline, CH_4N_2O, Mr 60,06
4. Phenol, C_6H_6O, Mr 94,11
5. Sodium nitroprusside, dihydrate, $Na_2(NO)Fe(CN)_5 \cdot 2\ H_2O$, Mr 297,96

6. Hypochlorite, sodium salt with 10-14% (w/v) available chlorine, NaClO, Mr 74,45
   Caution: highly corrosive.
7. Sodium hydroxide, NaOH, Mr 40,0 and 0,2 mol/L
8. Glycerol, $C_3H_8O_3$, Mr 92,09

3.16.2.2 SOLUTIONS

I. Phosphate buffer (20 mmol/L), pH 7,0

$NaH_2PO_4 . H_2O$	0,276 g
Distilled H_2O about	70 mL
pH adjusted with NaOH (0,2 mol/L) to 7,0	
Distilled H_2O to	100,0 mL

II. Urease (10 KU/L)

Urease (100 U)	0,025 g
Phosphate buffer (solution I)	5,0 mL
Glycerol	5,0 mL

Stable one year if stored at 4°C.

III. Urea standard solution (10,0 mmol/L, 600 mg/L, 60 mg/dL)

Urea	0,600 g
NaN_3	0,100 g
Distilled H_2O to	1000,0 mL

Stable for one year at 4°C.

IV. Phenol (0,106 mol/L); nitroprusside (0,17 mmol/L)

Nitroprusside	0,025 g
Phenol	5,0 g
Distilled H_2O to	500,0 mL

Stable for 6 months at 4°C.

V. Hypochlorite (at least 0,05% w/v available chlorine); NaOH (0,125 mol/L)

NaOH	2,5 g
Distilled H_2O about	100,0 mL
Na hypochlorite	2,5 mL
Distilled H_2O to	500,0 mL

Stable for 6 months at 4°C.

3.16.2.3 CONTROL OF PURITY OF REAGENTS AND SOLUTIONS

All reagents should be of analytical grade and free of ammonium ions. In humid climates, some batches of analytical grade urea have been found to contain free ammonia. If such urea is used as a standard for methods employing urease, then calculated values for samples will be low because of the additional colour in the standard due to the ammonia. Ammonia may be removed by drying the urea at 37-40°C overnight.

Sodium hypochlorite (NaOCl)

The concentration is expressed in terms of the available chlorine content, i.e. a solution containing 10% (w/v) available chlorine contains 10 g available chlorine in 100 mL, which is equivalent to 2,8 mol/L (Mr for chlorine: 35,45). On storage, the available chlorine will decrease due to decomposition of the sodium hypochlorite. The available chlorine content may be measured volumetrically as follows:

Solutions

1. Acetic acid (approximately 5 mol/L)

   - Add 30 mL glacial acetic acid to 70 mL of distilled water

2. Sodium thiosulfate (0,1 mol/L), $Na_2S_2O_3 . 5 H_2O$, Mr 248,17

$Na_2S_2O_3 . 5 H_2O$	12,41 g
Distilled H_2O to	500 mL

Sufficient accuracy is obtained by preparing a fresh solution before use from analytical grade sodium thiosulfate.

3. Potassium iodide (1,20 mol/L), KI, Mr 166,0

KI	20 g
Distilled H_2O to	100 mL

4. Starch indicator

   - Add 0,2 g soluble starch to 5 mL distilled water to form a paste.
   - Heat about 95 mL distilled water to boiling and add the starch paste.
   - Continue boiling for about 1 minute.
   - Cool to room temperature.

   The solution is stable only for a few days at room temperature.

Measurement procedure

- Quantitatively dilute the sodium hypochlorite solution to approximately 1% (w/v) available chlorine. For example, dilute 10,0 mL of a 10-14% solution to 100 mL with distilled water.
- Pipette 5,0 mL of the approximately 1% available chlorine solution into a volumetric flask and add about 20 mL distilled water.
- Add 4 mL acetic acid (approximately 5 mol/L) and 5 mL potassium iodide solution (1,2 mol/L).
- Titrate with sodium thiosulfate solution (0,1 mol/L) until the solution has a pale yellow colour.
- Add about 2 mL starch indicator and continue to titrate with sodium thiosulfate until the solution turns from blue to colourless. Note the volume of thiosulfate solution used.

Calculation

$$\% \text{ available chlorine} = \frac{V \times 0{,}003545 \times 100}{5};$$

where V is volume of thiosulfate solution used.

Note that 1,0 mL sodium thiosulfate (1,0 mol/L) is equivalent to 0,003545 g available chlorine.

Approximately 14 mL of sodium thiosulfate (0,1 mol/L) will be required to titrate 5 mL of hypochlorite solution containing approximately 1% (w/v) available chlorine. Multiply by the initial dilution factor to obtain the percentage available chlorine concentration in the original sodium hypochlorite solution.

3.16.2.4 MEASUREMENT OF UREA WITH THE UREASE BERTHELOT METHOD

Principle

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2\,NH_3 + CO_2$$

$$2\,NH_3 + 2\,NaClO \longrightarrow 2\,NH_2Cl + 2\,NaOH$$

$$2NH_2Cl + 2\,\text{phenol} + 2OH^- \longrightarrow 2,4\text{-aminophenol} + 2\,Cl^- + 2\,H_2O$$

$$2,4\text{-aminophenol} + 2\,\text{phenol} + O_2 \longrightarrow 2\text{-indophenol} + 2\,H_2O$$

The blue dye indophenol produced in the Berthelot reaction absorbs light between 530 nm and 650 nm proportional to the initial urea concentration.

Measurement conditions

Temperature: 37°C.

Wavelength: 530-580 nm, (546 or 578 nm with a mercury lamp).

Samples: serum or plasma (all anticoagulants, except ammonium heparinate and fluoride can be used): urine (fresh) has to be diluted 100-fold with NaCl (0,154 mol/L).

	Blank(B)	Standard(St)	Samples(S)
Urease (solution II) (mL)	0,1	0,1	0,1
Distilled H_2O (mL)	0,02	-	-
Standard (solution III) (mL)	-	0,02	-
Sample (mL)	-	-	0,02

- Mix thoroughly, stopper tubes with clean stoppers or Parafilm.
- Incubate for 15 minutes at 37°C.

Phenol (solution IV) (mL)	5,0	5,0	5,0
Hypochlorite (solution V) (mL)	5,0	5,0	5,0

- Mix thoroughly and immediately, incubate for 30 minutes at 37°C.
- Read absorbance A_{St} and A_S against the blank.

Calculation

Serum or plasma

$$\text{molar concentration:} \frac{\Delta A_S}{\Delta A_{St}} \times 5 \text{ mmol/L}$$

$$\text{mass concentration:} \frac{\Delta A_S}{\Delta A_{St}} \times 300 \text{ mg/L}$$

Urine

$$\text{molar concentration:} \frac{\Delta A_S}{\Delta A_{St}} \times 500 \text{ mmol/L}$$

$$\text{mass concentration:} \frac{\Delta A_S}{\Delta A_{St}} \times 30 \text{ g/L}$$

3.16.3 Diacetyl monoxime method

3.16.3.1 REAGENTS

1. Benzoic acid, $C_7H_6O_2$, Mr 122,12
2. Orthophosphoric acid (85% w/v), H_3PO_4, Mr 98,04
   Caution: highly corrosive.
3. Sulfuric acid, concentrated (95-97% w/v), H_2SO_{42}, Mr 98,08
   Caution: highly corrosive.
4. Diacetyl monoxime, $C_4H_7NO_2$, Mr 101,11
5. Thiosemicarbazide, CH_5N_3S, Mr 91,14
6. Trichloracetic acid, $C_2HO_2Cl_3$, Mr 163,4
   Caution: highly corrosive.
7. Urea, crystalline, analytical grade, CH_4N_2O, Mr 60,06

3.16.3.2 SOLUTIONS

I. Acid solution: thiosemicarbazide (1,1 mmol/L), urea (7,5 µmol/L)

Distilled H_2O about	200 mL
- Add slowly H_2SO_4 (concentrated)	44 mL
- Mix and add slowly H_3PO_4 (concentrated)	66 mL
- Mix; let the solution cool to room temperature but do not use ice water for cooling; add: Thiosemicarbazide	0,05 g
- Dissolve and add: Working standard urea solution (2,5 mmol/L)	1,5 mL
Distilled H_2O to	500,0 mL

Stable for at least six months if stored at 2-8°C in an amber bottle. The presence of a small amount of urea in the reagent improves the linearity of the calibration curve.

II. Diacetyl monoxime (39,6 mmol/L)

Diacetyl monoxime	2,0 g
Distilled H_2O to	500,0 mL

Stable for at least six months at 2-8°C.

III. Working solution

Use a graduated cylinder

Acid solution (solution I)	50 mL
Diacetyl monoxime (solution II)	50 mL

Must be prepared daily.

IV. Benzoic acid (8,2 mmol/L)

Benzoic acid	0,5 g
Distilled H_2O to	500,0 mL

Stable for several months at 20-25°C.

V. Stock urea solution (125 mmol/L)

Urea	0,75 g
Benzoic acid (solution IV) to	100,0 mL

Stable for several months at 2-8°C.

VI. Working urea standard solutions

- Prepare working standards in a 50 ml volumetric flask:

Urea		Stock urea solution
mmol/L	mg/L	mL
2,5	150	1,0
5,0	300	2,0
7,5	450	3,0
10.0	600	4.0
15,0	900	6,0
20,0	1200	8,0

- Add benzoic acid (solution IV) up to 50 mL for each.

Stable for several months at 2-8°C.

VII. Trichloroacetic acid (0,306 mol/L)

Trichloroacetic acid	25 g
H_2O to	500,0 mL

Stable for several months at 20-25°C

3.16.3.3 MEASUREMENT OF UREA BY DIACETYL MONOXIME METHOD

- Label sufficient centrifuge tubes for the batch including standard (S), control (C1, C2) and patients' samples (1, 2, 3, etc.).
- Prepare sufficient working solution (mix equal volumes of acid reagent and diacetyl monoxime reagent) for the number of samples expected during the day.

- Pipette into the tubes as follows:

	S	C1, C2	1,2,3, etc.
Standard, 10 mmol/L (mL)	0,05	-	-
Control (mL)	-	0,05	-
Patients' samples (mL)	-	-	0,05
Trichloroacetic acid (solution VII) (mL)	0,5	0,5	0,5

- Mix well and centrifuge.
- Label a second set of tubes, including reagent blank (Bl), standard (St), control (C1, C2) and patients' samples (1, 2, 3, etc.).
- Pipette into the tubes as follows:

	Bl	St	C1,C2	1, 2, 3, etc.
Trichloroacetic acid (solution VII) (mL)	0,1	-	-	-
Supernatant from tubes above (mL)	-	0,1	0,1	0,1
Working solution (solution III) (mL)	3,0	3,0	3,0	3,0

- Mix well: place all tubes in a boiling water-bath or 100°C heating block for 15 minutes.
- Cool tubes to room temperature, mix well, read the absorbance without delay.
  Colorimeter: green filter, Ilford number 604 (520 nm)
  Spectrometer: 530 nm
- Set the instrument to zero with tube Bl.
- Calculate the results in mmol/L.
- Check the control results.

3.16.4 Certified reference material

Urea as reference material can be obtained from the NBS; Ordering number SRM 912a.

3.16.5 References

[1] Kerscher L and Ziegenhorn J. Urea: UV-method. *Methods of Enzymatic Analysis,* 3rd ed. Bergmeyer HV ed. Volume III. Metabolites 3, Weinheim, Verlag Chemie, 1985, p. 444-449.

[2] Kerscher L and Ziegenhorn J. Urea: Colorimetric methods. *Methods of Enzymatic Analysis,* 3rd ed. Bergmeyer HV ed. Volume VIII. Metabolites 3, Weinheim, Verlag Chemie, 1985, p. 449-453.

[3] de Cediel N, Déom A, Hill PG, Sarkar AK et al. Measurement of serum/plasma urea. Method: diacetyl monoxime *Methods recommended for essential clinical chemical and haematological tests for intermediate hospitals laboratories.* LAB/86.3, Geneva, World Health Organization, 1986, p. 113-117.

3.17 Liquid serum calibrator and control materials

Introduction

The lack of appropriate materials for calibration and control of routine investigations poses a major problem in many laboratories. For some analytes aqueous calibrators can be used, as described in this manual, but in other cases it is essential to use serum-based calibrators. Most commercial quality control sera are expensive and may not always be available in all countries. Therefore, WHO has published the preparation of bovine serum quality control material to overcome difficulties that seriously impede the control of laboratory performance [1]. Although in this document the use of animal sera is recommended rather than using human sera, for some analytes, particularly those involving the application of immunoassays, the use of human-serum based materials cannot be avoided. The following section provides a summary of the method for preparing bovine serum stabilized with ethanediol and the procedure for the preparation of liquid human serum control and calibration material.

3.17.1 Summary of method for the preparation of stabilized bovine serum using ethanediol (ethylene glycol)

The WHO document LAB/81.4 [2] describes the collection of animal serum and full details for the preparation of low, medium and high concentration serum for the analytes listed in Table 3.17.1.

3.17.1.1 PREPARATION OF MEDIUM CONCENTRATION QUALITY CONTROL SERUM

Start with 1 litre of fresh bovine serum.

- Carefully mix to ensure homogeneity and freeze at -20°C. It is important to avoid possible deterioration of the serum by allowing it to remain at ambient temperature too long. The remainder of the process must be completed within one working day.
- Allow to thaw at room temperature. Do not disturb or mix.

- When completely thawed remove 150 mL of the top layer of fluid. This is 15% of the total volume and consists mainly of water or very dilute serum.
- Replace this volume of fluid by adding 150 mL of ethanediol.
- Mix very carefully and filter through non-absorbent cotton wool to remove any large aggregates.
- Measure the concentration of the analytes in which you are interested and construct a table, filling in your own results and the desired concentrations.
  It is anticipated that adjustments of the bovine serum material will be required for certain analytes, such as alkaline phosphatase, amylase, bilirubin, glucose and urea:
- Alkaline phosphatase is commercially available as powder. It can also be prepared from human placenta [2].
- Human saliva, which can be mixed in appropriate volumes with the bovine QC material, contains amylase about 1000 U/mL [1].
- Bilirubin is commercially available as powder and can be mixed with the QC material.
- Glucose is commercially available and can be mixed with the QC material.
- Urea is commercially available and can be mixed with the QC material.
- Mix the final product thoroughly and dispense into suitable, clean, dry, amber-coloured bottles, capped firmly and store at 4°C or at -20°C.

Table 3.17.1 Concentration of analytes in stabilized bovine quality control serum (1)

Analyte	Unit	Approximate concentration		
		low	medium	high
Albumin	g/L	30	38	45
Alkaline phosphatase	U/L	40	150	250
α-Amylase	U/L	-	300	700
ASAT	U/L	60	80	95
Bilirubin	µmol/L	-	35	150
Calcium	mmol/L	2,0	2,5	3,0
Creatinine	µmol/L	70	90	450
Glucose	mmol/L	3,0	5,6	20
Potassium	mmol/L	3,2	4,1	6,0
Sodium	mmol/L	125	135	159
Total protein	g/L	51	65	76
Urea	mmol/L	3,2	7,0	15

The stability of these materials has been demonstrated over 12 months.

3.17.2 Liquid human serum calibrator and control materials

Today, many laboratories prefer to use calibrators and control materials of human origin. A major drawback of these materials is their potential hazard to the user, because they may contain infectious agents. This danger can be reduced by careful selection of the material to be prepared. The procedure described in this section has been used by the Finnish Red Cross Blood Transfusion Service for more than 10 years. The material has also been distributed in the Finnish External Quality Assessment Scheme. The following analytes have been investigated in the scheme:

Albumin
Serum α-amylase
Thyroid stimulating hormone
Total and free thyroxine
HDL cholesterol
Transferrin
Immunoglobulins A,E,G and M
Haptoglobin
Cortisol

Materials

Blood bags for serum preparation, 300 mL
Plastic bottles, sterile polypropylene 1500 mL
Stainless steel or glass vessels, 10-15 L
Pressure vessels, 10-15 L
Prefilters, 0,2 µm (CWSS 004T4, Millipore Corp, U.S.A).
Sterile filters (NRP 7002, 0,22 µm, Pall Ultrafine Filtration Co., U.S.A.)
Injection vials, 2 ml (without silicone treatment)
Rubber stoppers for injection vials
Flip-off caps for injection vials

Equipment

Refrigerated centrifuge
Equipment for sterile filtration, preferably under pressure (small batches can be manually filtered)
Equipment for aseptically dispensing serum into vials (small batches can be dispensed manually)
Laminar air flow cabinet

Reagents

Sodium azide (analytical grade), NaN_3, Mr 65,01

Donors

Healthy volunteers with normolipaemia

Preparation of serum

- Collect blood from at least 10 healthy donors into bags that do not contain any anticoagulant.
- Keep the blood units at 20-23°C during the day of collection and transfer the bags to 2-6°C overnight.
- Test each unit for HIV, HBV,and HCV. Only negative units must be used for further processing.
- Centrifuge the bags the following morning at +5°C for 7 minutes at 6500 *g* (alternatively for 5 minute at 5000 *g* or for 20 minutes at 4200 *g*).
- Transfer the serum to a 300 mL separation bag in a laminar flow cabin and squeeze most of the air out of the bag.
- Centrifuge the bag for 5 minutes at 5000 *g*.
- Transfer the serum to a sterile plastic bottle and freeze it at -20°C until further use.

Preparation of control material

- Thaw the serum-containing bottles at room temperature and pool the serum in a sterile weighed vessel of glass or stainless steel.
- Test for HIV, HBV and HCV negativity.
- Mix the pooled serum for 15 minutes and weigh again to calculate the total amount (W) of available material. (Alternatively, measure the volume (V) of the pooled serum and calculate the weight using the formula:
  W(g) = V(mL) x 1,025 (g/mL).
- Calculate the amount (A) of sodium azide to obtain a 0,02% solution in serum using the formula:
  A(g) = 0,02 x W/100.
- Add the calculated amount of sodium azide to the serum and mix for 15 minutes.
- Transfer the serum to a pressure vessel and prefilter into another pressure vessel or prefilter manually under aseptic conditions in a laminar flow cabinet.
- Filter the serum through a 0,22 µm filter to obtain sterile material.
- Dispense the liquid control material under sterile conditions in 1 mL portions into sterile injection vials, stopper the vials and fix the stoppers with flip-off caps.
- Label the vials.

Storage

The vials can be stored at -70°C for 10 years, at -20°C for one year and at +4°C for one month.

3.17.3 References

[1] Browning DM, Hill PG and Olazabal DAVR. *Preparation of stabilized liquid quality control serum to be used in clinical chemistry*, LAB/86.3, Geneva, World Health Organization, 1986.

[2] Kenny AP and Eaton RH. *Practical guidelines for the preparation of quality control sera for use in clinical chemistry.* LAB/81.4, Geneva, World Health Organization, 1981.

4

REAGENTS FOR DRY CHEMISTRY TESTS FOR URINE AND STOOL ANALYSIS

4. Reagents for dry chemistry tests for urine and stool analysis

4.1 Introduction

Reagents for so-called 'dry chemistry' tests offer several advantages to reagents in solutions:

- reagents retain their stability better in powder form than in solution;
- the reagents can be used for the preparation of tablets or test strips which are a simple means of semiquantitative analysis.

The reagents are particularly useful for urine analysis. The production of dry chemistry reagents for urine analysis is described here, while reagents for serum and blood analysis are beyond the scope of this manual.

Simple reagent powders can be prepared even in small quantities with little equipment, i.e. mortar and pestle. Reagent powders can be used on glass plates placed upon a sheet of white paper, white porcelain, or glass tiles, the latter permitting the observation of both colour reactions and precipitations. Test papers are easily prepared by impregnation of larger sheets of absorbent material such as filter paper or chromatographic paper.

4.2 Test for glucose

4.2.1 Dry variation of the Benedict reaction

The Benedict reaction is based on the reduction of bivalent blue copper-citrate ions to monovalent red cuprous oxide by glucose in boiling water.

Copper sulfate and citric acid are provided as stable reagent powder.

Reagents

1. $CuSO_4 . 5 H_2O$	20 g
2. Citric acid	300 g

- Grind the chemicals together in a mortar.
- Store the powder in a screw-capped container or in snap-cap vials. The powder is not hygroscopic.

Method for testing

- Place a pea-sized quantity of powder in a test-tube and moisten with 3 drops of urine.
- Add one pellet of dry sodium or potassium hydroxide and mix. Continue shaking throughout the spontaneous boiling of the mixture, which is caused by the strong exothermic reaction.

Olive-brown precipitates turning rapidly dark brown:	20 g/L of glucose or more
Orange precipitate turning brown after starting:	10 g/L of glucose
Persistent orange precipitate:	5 g/L of glucose
Slight orange precipitate with blue supernatant:	about 2 g/L of glucose
Unchanged blue solution:	< 2 g/L of glucose

False positive reactions may be caused by other reducing substances, such as vitamin C.

4.3 Test for ketone bodies

A test powder based upon Legal's classic reaction with sodium nitroprusside is very widely used. Nitroprusside yields a violet coloured complex with enolizable keto-groups such as acetoacetic acid and acetone which are the ketone bodies excreted during metabolic acidosis.

Reagents

Sodium nitroprusside	1 g
Na_2CO_3 (anhydrous)	200 g
$(NH_4)_2SO_4$	200 g

Preparation and testing

- Grind the chemicals thoroughly in a mortar.
  The powder is only very mildly hygroscopic and is stored in screw-cap or snap-cap vials.
- Moisten a pea-sized heap of powder with one drop of fresh urine.

  Violet colour within one minute: 50 mg/L acetoacetic acid or 500 g/L acetone or more orange colour: phenylpyruvic acid (excreted in phenylketonuria)

4.4 Combined test for urinary protein and urobilinogen

A piece of filter paper impregnated with sulfosalicylic acid and 4-dimethylamino-benzaldehyde (4-DMAB = Ehrlich reagent) is placed in the urine to be tested. Sulfosalicylic acid denatures the proteins, leading to turbidity or some precipitation, while 4-DMAB yields a cherry-red colour with urobilinogen.

Reagents

Sulfosalicylic acid	200 g
4-DMAB	20 g

- Dissolve the chemicals in distilled water and make up to 1 litre.
- Dip sheets of filter paper into this solution and dry in air.
  Cut the dry sheets into 1 x 2 cm pieces and store in screw-cap or snap-cap vials.

Testing

- One piece of each reagent paper is placed into about 1 mL of urine. The reaction may be performed in a micro test-tube or on a glass tile.
- Place the tube or tile first on a white paper to observe the colour reaction, and afterwards on a black paper to observe turbidity.

4.5 Test for bilirubin

Detection of urinary bilirubin is important, as bilirubinuria generally precedes clinical jaundice. Bilirubin is concentrated by adsorption of urine in a tablet of plaster. Bilirubin remains on the surface of the tablet, where it is detected by oxidation to blue-green biliverdin by acid ferric chloride.

Preparation

Ordinary white plaster ($CaSO_4 . 2 H_2O$) is mixed with sufficient water to yield a thick paste which is cast into tablets by pouring it into appropriate moulds (e.g. a plastic cover of a strip of tablets can be used). Once hardened the tablets may be stored without any special precautions.

Reagents (Fouchet reagent)

Trichloracetic acid	20 g
Ferric chloride	5 g
Distilled H_2O to	1000 mL

- The solution is stable for many months when properly stored in a stoppered flask or bottle.

Method for testing

- Place 10 drops of well mixed urine one by one upon the tablet. Take care to place every drop only after the preceding drop has been absorbed.

Bilirubin concentrates at the surface of the tablet leaving a yellow spot which turns to bluish green after reaction with one drop of Fouchet reagent. This reaction is highly sensitive (detection limit approximately 2 mg/dL bilirubin) and specific. Normal urine does not react.

4.6 Test for nitrite detection

Detection of urinary nitrite indicates urinary tract infections caused by nitrate-reducing bacteria provided adequate precautions are taken, e.g. allowing sufficient reaction time and rapid analysis after collection of urine. The reaction powder suggested by Jaeggy and Lanz is based upon the classical Griess reaction.

Reagents

α-Naphthylamine	6,20 g
Sulfanilic acid	1,0 g
Citric acid	25,0 g

- Grind the chemicals thoroughly in a mortar.

The powder is barely hygroscopic and may be stored in screw-cap or snap-cap vials.

Method for testing

- Moisten a pea-sized heap of powder with one drop of fresh urine.

Nitrite in concentrations as low as 1 mg/L yields a carmine red colour highly specific for the presence of nitrate-reducing bacteria. The test is positive in over 80% of urinary tract infections, although a negative test does not rule out bacteriuria.

4.7 Test for haemoglobin and blood in urine and faeces

There exist several old formulations for test powders to detect haemoglobin and blood in urine and faeces. They all use benzidine or o-toluidine, both known to be carcinogenic. Therefore old test-tube methods (the guaiac test), also based on the pseudo-peroxidase property of haemoglobin, will be described [1,2,3,4].

Reagents

Gum guaiac
Ethanol (96%)
Hydrogen peroxide (30%)
Diethyl ether
Caution: highly flammable.
Glacial acetic acid
Caution: corrosive.

Solutions

I. Gum guaiac (16,7 g/L)

Gum guaiac	0,1 g
Ethanol (95%)	6,0 mL

- Dissolve. To be prepared freshly before use.

II. Hydrogen peroxide (3%)

H_2O_2 (30%)	1 mL
Distilled H_2O to	10 mL

To be prepared freshly before use.

III. Acetic acid (30%, v/v)

Glacial acetic acid	30 mL
Distilled H_2O to	100 mL

IV. Gum guaiac/hydrogen peroxide solution

Place in a test-tube:

Hydrogen peroxide (solution II)	5 mL
Gum guaiac (solution I) a few drops (about 0.3 mL)	

The solution should be prepared freshly before use and will be slightly opalescent; it should not turn blue.

Method for testing

a) In urine

- Place 5 mL of urine in a test-tube.
- Pipette the gum guaiac (solution I) into the fluid until a turbidity results.
- Add up to 10 drops of hydrogen peroxide (solution II).

The test is positive if a blue colour is obtained both before and after boiling the urine for 15-20 seconds; pus does not respond after boiling (destruction of the true leukocyte peroxidases).

b) In faeces

- Place a portion of faeces, about the size of a small walnut, in a Pyrex test-tube.
- Add about 3 mL of acetic acid (solution III).
- Emulsify, using a glass rod and then boil over a low flame for 2 minutes or heat in a boiling water-bath for 10 minutes (this destroys the true peroxidases).
- After cooling at room temperature add about 3 mL of diethyl ether and extract the haeme compounds (soluble in acid ethyl ether) by inverting the tube gently several times. The separation of the ether layer from the solid compounds can be accelerated by the addition of a few drops of ethanol.
- Layer a few mL of ether extract of faeces onto about 5 mL gum guaiac/hydrogen peroxide (solution IV).
  A positive reaction is shown by the appearance of a deep blue ring at the interface of the two fluids.

4.8 References

[1] Hawk PB, Osler BL and Summerson WH. *Practical Physiological Chemistry,* 12th ed., Philadelphia, Toronto, The Blakiston Company, 1947, p. 774.

[2] Hawk PB, Oser BL and Summerson WH. *Practical Physiological Chemistry,* 12th ed., Philadelphia, Toronto, The Blakiston Company, 1947, p. 767-768 and 1227.

[3] Lynch, MJ, Raphael SS, Mellor LD, Spare PD et al. *Medical Laboratory Technology,* Philadelphia and London, WB Saunders Company, 1963, p. 89.

[4] Hallman L. *Klinische Chemie und Mikroskopie,* 11th ed. Stuttgart, New York, George Thieme Verlag, 1980, p. 113.

5

REAGENTS AND METHODS FOR HAEMATOLOGY AND COAGULATION

5. Reagents and methods for haematology and coagulation

5.1 Glucose-6-phosphate dehydrogenase

D-Glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49 (G6PDH)

5.1.1 Methods

A reference method as such does not exist; however, a quantitative method recommended by the ICSH[1] could be considered, at least provisionally as a candidate reference method for the determination of G6PDH in whole blood, but will not be described here.

The WHO has recommended [2] two screening tests; the brilliant cresyl blue (BCB) test in tubes and the 3-(4,5-dimethylthiazolyl-1,2)-2,3-diphenyl tetrazolium bromide (MTT) spot test. The reagents for both of these tests will be described here.

5.1.2 Brilliant cresyl blue (BCB) test

5.1.2.1 REAGENTS

Glucose-6-phosphate, monosodium salt (G6P), $C_6H_{12}O_9PNa$, Mr 282,2
ß-nicotine-adenine dinucleotide phosphate, disodium salt (NADP) $C_{21}H_{26}N_7O_{17}P_3Na_2$, Mr 787,4
Brilliant cresyl blue (BCB)

NOTE: some commercial BCB dyes do not decolorize. Therefore the dye should be tested for its suitability.

Tris (hydroxymethyl) amino methane (TRIS), $C_4H_{11}NO_3$, Mr 121,14
Hydrochloric acid (5 mol/L)
Oil (kerosene)

5.1.2.2 SOLUTIONS

I. G6P (0,292 mol/L)

G6P, monosodium salt	0,82 g
Distilled H_2O to	100,0 mL

II. NADP (0,635 mmol/L)

NADP	0,05 g
Distilled H_2O to	100,0 mL

III. BCB

BCB	0,032 g
Distilled H_2O to	100,0 mL

IV. TRIS buffer (0,740 mol/L); pH 8,5

TRIS	8,96 g
Distilled H_2O about	50 mL
pH adjusted with HCl (5 mol/L) to 8,5	
Distilled H_2O to	100.0 mL

5.1.2.3 G6PDH TESTING WITH BCB

Principle

$$G6P + NADP^+ \xrightarrow{G6PDH} 6\text{-phosphogluconate} + NADPH + H^+$$

$$NADPH + H^+ + BCB \longrightarrow \text{colourless dye}$$

Testing conditions

Sample: whole blood (capillary blood can be used).

Refrigerated ACD blood will keep for many weeks; if the blood is haemolysed immediately after finger puncture, the haemolysate may be kept at 4-6°C for up to 7-8 hours before the test is performed. At tropical room temperatures, tests on haemolysed specimens should not be delayed for more than four hours.

Preparation of haemolysate

Whole blood	0,02 mL
Distilled H_2O	1,0 mL
- Premix:	
G6P (solution I)	0,1 mL
NADP (solution II)	0,1 mL
BCB (solution III)	0,25 mL
TRIS (solution IV)	0,2 mL

- Add the haemolysate (1,02 mL), mix by rolling the tube between the palms.
- Top the mixture with oil to prevent contact of the test solution with air.
- Incubate at 37°C in a water-bath.
- Observe the decolorization time by noting when most of the blue colour of the solution has disappeared and the red colour of haemoglobin can be seen.
  It is most convenient to examine the tubes every 5-10 minutes, starting at 40 minutes of incubation. Normal specimens will usually be decolorized in 65 minutes.

5.1.3 MTT-linked spot test

5.1.3.1 Reagents

1. 3-(4,5-Dimethylthiazolyl-1.2)-2,3-diphenyl tetrazolium bromide, (thiazolyl blue, MTT), $C_{18}H_{16}N_5SBr$, Mr 414,33
2. Phenazine methosulfate; (N-methylphenazonium methosulfate) $C_{14}H_{14}N_2O_4S$, Mr 306,34
3. ß-nicotine-adenine dinucleotide phosphate, disodium salt (NADP) $C_{21}H_{26}N_7O_{17}P_3Na_2$, Mr 787,4
4. Glucose-6-phosphate, monosodium salt (G6P), $C_6H_{12}O_9PNa$, Mr 282,2
5. Hydrochloric acid (0,1 mol/L)
6. Orthophosphoric acid monopotassium salt, anhydrous, KH_2PO_4, Mr 136,13
7. Sodium hydroxide (1 mol/L)
8. DEAE paper

5.1.3.2 SOLUTIONS

I. MTT

MTT	0,01 g
Distilled H_2O	1,0 mL

II. Phenazine methosulfate (0,32 mmol/L), pH approximately 3,8

Phenazine methosulfate	0,002 g
Distilled H_2O	20,0 mL
HCl (0,1 mol/L)	0,2 mL

III. Phenazine methosulfate (0,32 mmol/L); NADP (6,35 mmol/L)

NADP	0,005 g
Phenazine methosulfate (solution II)	1,0 mL

IV. Phosphate buffer (0,2 mol/L); pH 7,0

KH_2PO_4	2,723 g
Distilled H_2O about	50 mL
pH adjusted with NaOH (1 mol/L) to 7,0	
Distilled H_2O to	100,0 mL

V. G6P (approximately 0,425 mol/L)

G6P monosodium salt	0,12 g
Phosphate buffer (solution IV)	1,0 mL

VI. Phosphate buffer (0,260 mol/L); pH 5

KH_2PO_4	3,54 g
Distilled H_2O about	80 mL
pH adjusted with NaOH (1 mol/L) to 5,0	
Distilled H_2O to	100,0 mL

VII. Glass tube preparations

- Apply 20 µL of MTT solution (solution I) to the inner surface of a small vial and dry at 90°C for approximately 90 minutes.
- Apply 20 µL of the phenazine methosulfate-NADP solution (solution III) to the bottom of the tube and dry for approximately 90 minutes.
- Apply 10 µL of the G6P solution (solution V) to the side of the tube and dry overnight at 40°C.

The tubes are stable for at least one year at room temperature if protected from dust and moisture.

VIII. DEAE paper impregnated with phosphate buffer (0,26 mol/L, solution VI)

- Pipette 20 µL of solution VI per cm^2 of DEAE paper.
- Let dry at 90°C.

The impregnated paper is indefinitely stable under dry storage conditions.

5.1.3.3 METHOD FOR THE MTT SPOT TEST

Principle

$$G6P + NADP^+ \xrightarrow{G6PDH} 6\text{-phosphogluconate} + NADPH + H^+$$

$$NADPH + H^+ + MTT \longrightarrow \text{purple insoluble formazan derivative}$$

Since the dye reacts non specifically with haemoglobin, this pigment is separated from G6PDH by adsorption of G6PDH on to DEAE paper.

Testing conditions

Samples

Blood samples collected into heparin, balanced oxalate, EDTA or ACD may be used. Heparinized capillary tubes are very satisfactory. ACD or heparinized blood may be used after several weeks' storage. The blood sample is allowed to sediment or is centrifuged.

Test

- Pipette a small circle of red cells (approximately 5 mm) on to both sides of the paper and allow to dry.
- Place the paper in distilled water to elute the haemoglobin from the paper. This may be expedited by blotting DEAE paper several times with filter paper until the spots are a pale red colour.
- Dissolve the contents of the prepared glass tubes in two drops of distilled water.
- Pipette the solution on to the spots.
- Read the test after two minutes.
  Normal samples give a deep purple colour.
  Faint colour is observed with G6PDH deficient blood.

No colour is observed with glucose dehydrogenase negative samples. Heterozygotes may often be detected because intermediate degrees of colour are observed. Anaemia has no effect on the results of the test because packed cells are used.

5.1.4 Certified reference material

Not available.

5.1.5 References

[1] Beutler E, Blume KG, Kaplan JC, Lohr GW et al (International Committee for Standardization in Haematology). Recommended methods for red-cell enzyme analysis. *British J. Haematol.* 1977, 35:331-340.

[2] Betke K, Brewer GY, Kirkman HN, Luzzato L et al. *Standardization of procedures for the study of glucose-6-phosphate dehydrogenase,* WHO Technical Report Series No. 366, Geneva, World Health Organization, 1967, p. 35-36 and 38-39.

5.2 Haemoglobin (in whole blood)

The internationally recommended method for haemoglobin measurement is the cyanhaemoglobin method. Only the reagents for this method will be described here [1,2].

5.2.1 Reagents

1. Potassium ferricyanide (potassium hexacyanoferrate III), $K_3Fe(CN)_6$, Mr 329,24
2. Potassium cyanide, KCN, Mr 65,11
   Caution: highly poisonous. Great care must be taken when handling this reagent and to ensure safe storage.
3. Orthophosphoric acid, monopotassium salt, anhydrous, KH_2PO_4, Mr 136,13
4. A suitable detergent, e.g. Sterox SE, Nonidet P40
5. Cyanhaemoglobin (cyanmethaemoglobin) standard, HiCN
   Photometer calibrator
6. Haemoglobin
   Method calibrator and standard; prepared only by the laboratories preparing the reagents or by reference centres
7. Citric acid, trisodium salt, $C_6H_5O_7Na_3 \cdot 2H_2O$, Mr 298,09
8. Toluene, C_7H_8, Mr 92,14

5.2.2 Solutions

I. Reagent solution

$K_3Fe(CN)_6$	0,2 g
KCN	0,05 g
(Handle with care, highly poisonous)	
KH_2PO_4	0,14 g
Sterox SE	0,5 mL
(or Nonidet P40	1,0 mL)
Distilled H_2O to	1000,0 mL

Stable for several months if stored between 4 and 25°C in a brown borosilicate bottle. Although containing cyanide, the prepared reagent is comparatively harmless. About 4 litres would have to be taken to have a lethal effect.

The reagent should be clear and pale yellow in colour. When measured against water as blank in a photometer (or spectrometer) at a wavelength of 540 nm, the absorbance must read zero. The pH of the solution must lie between 7,0-7,4 and must be checked regularly, at least once a month, using a pH meter.

The solution should be discarded if:

- the pH is outside the range 7,0-7,4.
- the absorbance at 540 nm against water as blank is other than zero:
- it becomes cloudy or discoloured;
- it should accidently freeze.

NOTE: Drabkin's solution or similar products, some of which are available in powder or tablet form, are also used instead of the solution described above. It should, however, be borne in mind that complete conversion of haemoglobin derivatives to HiCN takes some 20 minutes with these reagents and that the resulting solution is often slightly turbid, giving results that are too high.

II. Cyanhaemoglobin solution

Cyanhaemoglobin is recommended for use as a standard in haemoglobin measurement. It is available as WHO international reference material for the calibration of a locally prepared standard.

Solutions

a) Citrate solution (3,2% w/v)

Citric acid, trisodium salt	32,0 g
Distilled H_2O to	1000,0 mL

b) NaCl (0,154 mol/L)

c) NaCl(0,2 mol/L)

NaCl	12,0 g
Distilled H_2O to	1000,0 mL

Preparation of cyanhaemoglobin

- Collect blood from a healthy donor into 3,2% citrate solution (1:5 by volume).

NOTE: the blood must be tested for HIV and HBV. Only noninfected blood should be processed.

- Centrifuge the blood and decant the supernatant plasma.
- Wash cells twice with NaCl (0,154 mol/L) (1:9 by volume) and again twice with NaCl (0,2 mol/L) (1:9 by volume).
- Add an equal volume of distilled water to the packed cell volume and add 0,4 volume of toluene.
- Stir the cell suspension thoroughly and place it in the refrigerator for 12 hours.

- Centrifuge the mixture at high speed. Remove the toluene layer by suction, decant the red haemoglobin layer from the precipitated cell debris.
- Filter the haemoglobin solution through a Jena glass G-3 filter.
- Determine the haemoglobin concentration by the cyanhaemoglobin method. (It is usually about 150 g/L.)
- Dilute the haemoglobin with reagent solution so that the desired concentration is 0,55 to 0,6 g/L.
- For sterilization, the cyanhaemoglobin solution is filtered through a 0.22 µm membrane filter under aseptic conditions and filled into 10 mL ampoules of brown borosilicate glass.

The cyanhaemoglobin solution should be stable for 1 to 2 years.

III. Haemoglobin solution

The procedure describes the preparation of a haemoglobin solution from haemolysed red blood cells.

Reagents and solutions

a) Anticoagulant solution (glucose, 0,104 mol/L; NaCl, 71,5 mmol/L; citrate, 19,6 mmol/L)

Glucose	18,66 g
NaCl	4,18 g
Citric acid, trisodium salt	5,76 g
Distilled H_2O to	1000,0 mL

- Dissolve the chemical components in distilled water and filter through a 0.22 µm membrane filter.

b) Sterile saline (0,154 mol/L)

- Dissolve 9 g of NaCl per 1 litre of distilled water and filter through a 0,6 µm glass filter.

c) Sterile water

- Filter distilled water through a 0,6 µm glass filter.

Preparation of the haemolysate

- Collect 300 mL of blood from a healthy donor in a 500 mL bottle containing 150 mL of the anticoagulant solution. Shake the blood continuously but gently during the collection.

NOTE: Test the collected blood for HBV and HIV.

- Mix the collected blood well and divide into 5 portions of about 90 mL in 5 bottles of 250 mL volume.
- Wash the red cells 4 times with 100 mL of sterile NaCl solution to remove the plasma, leukocytes and platelets. Mix well each time after the addition of the NaCl solution. Centrifuge each time at 1600 *g* for 5 minutes. Remove the supernatant by suction using a Pasteur pipette.
- Mix the red cells vigorously with distilled sterile water (2 volumes red blood cells + 1 volume sterile water). Mix twice more within 30 minutes.
- Add 0,5 part of toluene to the mixture and place it for 2 hours in a shaker.
- Place the haemolysate in the refrigerator overnight. Thereafter centrifuge the haemolysate at 1600 *g*, for 5 minutes.
- Transfer the haemolysate (under the toluene layer) into 2 sterile bottles using a blood collecting system and a Pasteur pipette.
- Filter the haemolysate through a coarse and fine filter (0,22 µm) and test the resulting solution for sterility.
- Divide the haemolysate into 3 to 5 mL portions in 10 mL bottles and label the containers.
- Store the bottles in the refrigerator at 4°C.

Under these conditions a sterile haemolysate is stable for more than 2 years.

5.2.3 References

[1] de Cediel N, Déom A, Hill PG, Sarkar AK et al. Haemoglobin method: haemoglobincyanide. *Methods recommended for esssential clinical chemical and haematological tests for intermediate hospitals laboratories,* LAB/86.3, Geneva, World Health Organization, 1986.

[2] van Assendelft OW, Holtz AM and Lewis SM. *Recommended method for the determination of haemoglobin concentration of blood,* WHO/LAB/84.10, Geneva, World Health Organization, 1984.

5.3 Reagents for cell counting

5.3.1 Quality control material: fresh red blood cells

Materials for quality control in haematology are difficult to prepare if they are to serve for cell counting as well as for haemoglobin measurement. Blood cells, when they

are stabilized, have different shelf-lives from one cell group to another. On the other hand, their size and rigidity may change during stabilization which will affect their properties during centrifugation when measuring the packed cell volume. Furthermore, they behave differently during haemolysis which will affect haemoglobin measurement.

The following method of preparation has been shown to give a red blood cell concentrate suitable for distribution in external quality control schemes. The material prepared by the procedure described below has been used for the External Quality Assessment Scheme of the German Society for Clinical Chemistry, for control of red cell count, PCV and haemoglobin.

Preparation

- Collect blood from healthy blood donors who have the same blood group. The blood must be collected into blood bags containing CPD stabilizer. Check for the absence of infective agents HIV, HBV and HCV. Leave the blood for 4 to 5 days at 4°C.
- Centrifuge the blood for 20 minutes at 3000 *g*. Separate the plasma and the buffy coat and store the plasma. Transfer the red blood cell concentrate into 500 mL glass bottles.
- Mix 300 mL of red blood cell concentrate carefully with 150 mL of NaCl (0,154 mol/L).
- Centrifuge the cell suspension for 20 min at 3000 *g*.
- Remove the supernatant and the upper layer of the erythrocyte layer by suction.
- Repeat steps 3 to 5.
- Dilute the plasma that was separated in step 2 with NaCl (0,154 mol/L) at a ratio 2,5:1. Add streptomycin and penicillin (1 g streptomycin base and 1 mega unit penicillin per 150 mL plasma) for preservation.
- Mix the erythrocyte concentrate, as obtained in the final step 6 with the diluted plasma, as obtained in step 7, at a desired ratio to obtain specimens for external quality control. Dispense the suspension in aliquot volumes into clean vials, cap the vials with a rubber stopper so that they can be mailed for testing.

Stability

The red blood cell preparation is stable for at least 2 weeks under moderate temperatures. The preparation can be used for red blood cell counting, PCV determination and haemoglobin measurement.

5.3.2 Quality control material: fixed red blood cells

The preparation of a calibrator for blood cell counting poses considerable problems and depends on its intended purpose, since the stability varies from one type of blood cell to another. A major handicap is that cell calibrators quickly deteriorate and debris may accumulate during storage of the material which will give rise to a high background count. The high background count may interfere particularly in platelet counting and to a lesser extent in red blood cell and white blood cell counting.

The procedure described below has been developed for calibrator material for semiautomated or automated red blood cell counting. It has been thoroughly tested at the National Institute of Public Health (RIVM), Bilthoven, The Netherlands [1].

Materials

Blood bags or bottles
Glass bottles 250 mL
Jena glass filter 26 D1 (Jena, Germany)
Jena glass filter G 4 (Jena, Germany)
Glass vials 5 mL
Silicone-coated rubber stoppers
Flip-off caps

Equipment

Centrifuge
Mechanical shaker
Equipment for sterile filtration

Solutions:

I. Enriched acid citrate dextrose (ACD) solution, pH 8

Citric acid, trisodium salt	5,76 g
NaCl	4,18 g
Glucose	18,66 g
Adenine	0,24 g
Inosine	3,60 g
Distilled H_2O to	1000 mL
adjust to pH 8	

II. Formaldehyde fixative

Na-acetate (anhydrous)	19,0 g
Formaldehyde (38%, reagent grade)	50 mL
Distilled H_2O to	100 mL

Preparation

- Collect blood from healthy donors into blood bags or bottles containing enriched ACD solution to give a final volume ratio 4,5:1.
- Test the collected blood for HIV, HBV and HCV. In the meantime store the blood at 4°C.
- Dispense 100 mL aliquots of the collected blood into glass bottles of 250 mL.
- Centrifuge the bottles at 4°C. Remove the supernatant. Suspend the cells in cold saline and wash the cells 4 times by the procedure described above.
- After final washing resuspend the cells in the fixative solution at a volume ratio 1:9 (1 part cells, 9 parts fixative). Let the bottles gently shake for 3 hours at room temperature by a mechanical shaker.
- Let the cells settle and decant the supernatant.
- Add the same volume of fresh fixative solution and leave the cells overnight on the shaker.
- Renew the fixative solution on the second day and thereafter twice a week under continuation of slight shaking over a 2-week period.
- Finally let the cells settle, remove the supernatant and resuspend the packed cells in an equal volume of fixative solution.
- Add 50 mL glycerol per 1 litre of resuspended cells. (Glycerol facilitates the resuspension of cells).
- Store the preparation at room temperature for 6 months.
- After 6 months storage, filter the fixed cells through a Jena glass filter to remove aggregates and adjust the cell suspension with freshly prepared fixative solution to approximately 4 x 10^{12} cells/L.
- Dispense approximately 2,3 mL of the suspension in glass vials of 5 mL. Close the vial with a silicone-coated rubber stopper and fix the stopper with a flip off cap.

Storage

The vials can be stored at room temperature. The suspension has a stability of more than 10 years.

In this preparation the red blood cells have a mean corpuscular volume (MCV) of about 70 fL.

5.3.3 Quality control material for platelet counting

- Collect blood from donors with the same blood group and under the same precautions as described for the preparation of red blood cell concentrates (Section 5.3.1).
- Isolate the platelets using a cell separator (250 mL of platelet concentrate having 0,75 x 10^{12} cells per litre).
- Add 50 mL NaCl 0,154 mol/L containing 0,5 g K_2-EDTA and 100 U vitamin E to each 250 mL of platelet concentrate. Mix for 3 minutes at 25°C.
- Prepare a relevant dilution by mixing the platelet concentrate with plasma from the same donors prepared as described in step 7 for the preparation of red blood cells. Dispense aliquot volumes of the platelet preparation into glass tubes, stopper properly so that they can be distributed by mail.

5.3.4 Preparation of pseudo leukocytes

Leukocytes from human blood are very labile and cannot be used for the preparation of quality control material for cell counting. For automated leukocyte counting, avian red blood cells which are nucleated can be used. Glutaraldehyde fixed chicken red blood cells are about the same size as human lymphocytes when counted with automated cell counters.

Reagents

1. Orthophosphoric acid, monosodium salt, dihydrate, $NaH_2PO_4.2H_2O$, Mr 156,01
2. Orthophosphoric acid, disodium salt, anhydrous, Na_2HPO_4, Mr 141,96
3. Glutaraldehyde 50% (w/w), $C_5H_8O_2$, Mr 100,12

Preparation of phosphate buffer (0,15 mol/L)

Stock solutions

A.	$NaH_2PO_4 . 2H_2O$	23,4 g
	Distilled H_2O to	1000,0 mL
B.	Na_2HPO_4	21,3 g
	Distilled H_2O to	1000,0 mL

Keep stock solutions A and B in a refrigerator.

Phosphate buffer

Mix 18 mL of solution A with 82 mL of solution B.
The pH should be 7,4.

Glutaraldehyde fixative

50% glutaraldehyde solution	5 mL
Phosphate buffer	1000 mL

The fixative solution must be used immediately after preparation.

Preparation

1. Centrifuge chicken blood at 150 *g* for 15 minutes. Remove platelet and leukocyte-rich plasma.
2. Add an excess of phosphate buffer to the chicken red cells. Mix, recentrifuge and discard the supernatant.
3. Repeat step 2 twice.
4. Suspend the washed chicken red cells in the same volume of glutaraldehyde fixative. Rotate slowly on a mechanical mixer for 1 hour. Leave the suspension for a further 2 hours.
5. Centrifuge the fixed cells at 100 *g* for 20 minutes and discard supernatant.
6. Wash the cells with distilled water, centrifuge and discard the supernatant.
7. Repeat step 6 twice.
8. Resuspend the washed, fixed cells to approximately 30% concentration in 0,1% sodium azide in saline.
9. Autoclave at 121°C for 15 minutes.
10. For use mix vigorously by hand shaking, followed by roller mixing for at least 20 minutes.

5.3.5 Diluent for red blood cell and platelet counting in automated cell counters

The following reagent is suitable as a diluent for blood for cell counting with a counter working on the impedance principle.

NaCl	9,3 g
$CaCl_2$	0,011g
Na_2HPO_4	1,0 g
Glucose	1,0 g
Distilled H_2O to	1000,0 mL
pH: 7,5 (to be adjusted with 1 mol/L HCl)	

The water used for dissolving the salts must be particle-free for automated cell counting. The final salt solution must be filtered through a 0,45 µm micropore filter to remove any particles. The filtered solution should not have more than 50 particles per

millilitre when passed through the counter at the correct setting for red cell counting. If necessary, the solution must be filtered again through a 0,22 μm filter.

5.3.6 References

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[2] Lewis SM, Verwilghen RL eds. *Quality assurance in haematology*, London, Philadelphia, Toronto, Tokyo, Bailliere Tindall, 1988.

[3] de Boroviczeny CH ed. Standardization, documentation and normal values in haematology. *Bibl. Haemat.,* 1965, 21:75-78.

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5.4 Reagents for coagulation tests

5.4.1 The activated partial thromboplastin time (APTT)

The APTT is the main test for screening for intrinsic clotting defects, including haemophilia, and for laboratory monitoring of heparin administration. The test system includes an incomplete (partial) thromboplastin phospholipid extract and an activator. An activated PTT gives more consistent and shorter clotting times than the non-activated test.

A reliable APTT reagent should be sufficiently sensitive to record an abnormal result when the level of any single or combined intrinsic clotting factor deficiency is reduced to a level which may be clinically important, i.e. which causes spontaneous bleeding or haemorrhage following a haemostatic challenge. The APTT should also be able to detect the presence of low concentrations of heparin whilst giving a linear response to graded concentrations of heparin, spanning a clinically relevant range.

5.4.1.1 PREPARATION OF AN EXTRACT OF PARTIAL THROMBOPLASTIN FOR THE ACTIVATED THROMBOPLASTIN TIME (APTT) TEST

Partial thromboplastin may be prepared from animal tissue or from vegetable sources, e.g. soya bean.

Solutions

Owren's buffer, pH 7,35	
Na diethylbarbiturate	11,75 g
NaCl	14,67 g

Distilled H_2O	1570 mL
HCl (0,1 mol/L)	430 mL

The method described is based on animal brain tissue.

- Strip the meninges from freshly obtained brain tissue. Cut the tissue into small pieces.
- Place in a large mortar, cover well with cold acetone. Pound with a pestle for 2 minutes then filter through Whatman GP filter paper. Pour on fresh, cold acetone and pound again for 2 minutes. Repeat the process until the material has a 'sandy' consistency (up to 10 times). The neutral lipids and water are removed by the acetone, leaving the phospholipids.
- Leave overnight, on filter paper, in a fume cupboard to complete the drying process, or preferably vacuum desiccate for several hours.
- To 10 g dried material add 250 mL ether. These measurements may have to be varied according to the source. Leave overnight in a tightly stoppered flask or reagent bottle with a ground glass stopper, in a fume cupboard.
- Filter through Whatman GP filter paper. Discard the residue.
- Evaporate the filtrate to dryness at 37°C. Use a Buchner flask connected to a filter pump to facilitate the process. Rotary evaporation is the method of choice.
- Resuspend the dried filtrate in 100 mL Owren's buffer containing 2% trehalose, at 45°C. Add buffer gradually, shaking between each addition (glass beads may facilitate the process). Shake continuously on a mechanical shaker until the precipitate is completely broken up and a fine suspension is obtained.
- Store in small aliquots in plastic containers at -40°C or below.

5.4.1.2 STANDARDIZATION OF THE EXTRACT

Prepare dilutions (1:500 to 1:10,000*) from an aliquot of the deep-frozen extract in Owren's buffer and test against fresh normal plasmas to determine the dilution which gives the desired normal range. Further testing against different levels of many known coagulation defects and inhibitors are required to establish the range of sensitivity of the extract.

The reliability of the test in the detection of abnormalities of intrinsic clotting depends not only on the composition of the phospholipid extract but also on the choice of activator. Light kaolin (2,5 g/L) of high grade is recommended, with an incubation time of 10 minutes with the plasma and PTT extract, prior to recalcification (see below).

(*) The range of dilutions will depend on the source of phospholipid extract and the potency of the extraction.

5.4.1.3 TEST METHOD (MANUAL TECHNIQUE)*

Into a glass test-tube, prewarmed in a water-bath at 37°C add the following, in the order indicated, without delay:

Test plasma	0,1 mL
Phospholipid extract	0,1 mL
Pre-warmed kaolin (2,5 g/L in Owren's buffer)	0,1 mL

- Start stopwatch.
- Tilt gently three times to mix and subsequently at approximately one minute intervals to maintain the kaolin in suspension.
- At exactly 10 minutes add:
  Calcium chloride (0,025 mol/L) 0,1 mL
- Tilt gently until a solid clot forms.

5.4.2 The prothrombin time test (PT)

The PT is the screening test for the extrinsic (tissue) clotting system. Its responsiveness to depression of the extrinsic clotting factors depends on the source and type of tissue thromboplastin extract. The most common practical use of the test is in the laboratory control of the dosage of oral anticoagulant treatment. The majority of commercially available thromboplastin extracts is prepared from rabbit brain. Locally prepared extracts from human brain tissue are no longer advised on account of the possibility of transmission of viruses, particularly HIV, and animal tissue extracts are therefore recommended. A wide variety of animal tissues (especially brain and lung) are rich in thromboplastin. However, those from some animals are less sensitive to deficiencies of vitamin K-dependent clotting factors in man. This may be due to a 'species' effect when tested against human plasma owing to the poor reactivity of the animal tissue with human clotting factors.

5.4.2.1 PREPARATION OF A SALINE EXTRACT OF TISSUE THROMBOPLASTIN

The method described is based on experience in the preparation of human brain extract but modified for animal tissue preparations [1,2].

*The above method can be adapted for many types of coagulometer, although a fixed, shorter incubation time on some models will reduce the sensitivity of the test. For coagulometers requiring low opacity reagents, kaolin should be replaced by an alternative activator but there may be some loss of sensitivity to coagulation deficiencies and inhibitors.

- Obtain fresh tissue (within 24 to 48 hours of death). Remove the cerebellum and peel off the meninges under running tap water. Cut the remaining tissue into slices.
- Large blood vessels and clots should be removed from the surface of the brain.
- Macerate the sliced tissue with a mortar and pestle to a coarse, lumpy consistency. The use at a slow speed of an electric mixer with a whisk attachment for a few seconds will simplify and expedite this stage.
- Add warm phenol saline (between 0,5 to 2,0% of phenol in normal saline) and continue to macerate the tissue until an even, consistent suspension is produced. The length of time of the maceration process is dependent on the type of tissue. Incubate the suspension at 37°C for a period of 30 minutes to 4 hours. The length of time of incubation will vary according to the source of tissue extract and degree of homogenization.
- Decant into a sterile glass container and leave at 4°C for 24 hours.
- Centrifuge at 2-8°C. The speed and duration will depend upon the degree of homogenization. Following centrifugation, the material should form clearly visible layers of sediment. Remove the supernatant and measure its volume. Remove the top layer of the sediment ('cream') and add one part of this to nine parts of supernatant. Discard the remainder of the sediment.
- Store the extract at 2-8°C in sterile containers. It is also advisable to add an antibiotic and an antifungal preparation to prevent contamination.

5.4.2.2 CALIBRATION OF THE THROMBOPLASTIN

The overall sensitivity of a thromboplastin reagent to the depression of factors II, VII and X during oral anticoagulant treatment is quantified numerically as an International Sensitivity Index (ISI), according to the WHO international system of prothrombin time standardization [1,3]. Determination of the ISI is achieved by calibration of the local thromboplastin reagent against the WHO International Reference Preparation (IRP) for thromboplastin or a secondary IRP calibrator in terms of the primary IRP. Before use, each production batch must be calibrated against an IRP, or house standard which has previously been calibrated in terms of an IRP, to determine its ISI. Full instructions for ISI calibration are provided elsewhere [4,5,6].

5.4.3 References

[1] Hermans J, van den Besselaar AMPH, Leoliger EA, Van der Velde EA. A collaborative calibration study of reference materials for thromboplastin. *Thromb. Haemost.*, 1984, 52:336-42.

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[4] Tomenson JA and Thomson JM. Standardisation of the prothrombin time. Thomson JM ed. *Blood Coagulation and Haemostatis: a practical guide.* Edinburgh, Churchill Livingstone, 1985, p. 370-409.

[5] WHO Expert Committee on Biological Standardization, 1984, 34th report. WHO Technical Report Series 700, Geneva, World Health Organization, 1984, p. 18-19.

6

STAINS FOR HAEMATOLOGY, MICROBIOLOGY AND PARASITOLOGY

6. Stains for haematology, microbiology and parasitology

This section describes the preparation of some convenient solutions used for staining of specimens for light microscopy. Since different names may be used for stains, the chemical index (CI) numbers have been added in brackets for information. The names of dyes and indicators, some of their synonyms and their chemical index number (CI), that are mentioned in the description of reagents for haematology, microbiology and parasitology, are given in Annex 5.

6.1 Buffered methylene blue solution (BMB)

Buffered methylene blue is used for the detection of parasites in wet mounts. The pH of the stain solution is important for bringing out the morphological details of the nuclei of protozoan trophozoites. In practice, a small quantity of methylene blue in acetate buffer at pH 3,8 has given satisfactory results. Other buffers, e.g. phthalate buffer or those made out of commercially available buffer tablets, can also be used.

Composition

Methylene blue (CI 52051)	0,06 g
Sodium acetate (anhydrous)	0,894 g
Glacial acetic acid	5,35 mL
Distilled H_2O to	1000,0 mL

6.2 Field stains

Field stains are water-soluble Romanovsky stains. These stains contain methylene blue and eosin. They are used for staining thin films for malaria parasites.

Field stain A

Composition

Methylene blue (CI 52051)	1,6 g
Azur I (CI 52010)	1,0 g
Na_2HPO_4, anhydrous	10,0 g (if $Na_2HPO_4.12H_2O$ is used, then 25,2 g).

KH_2PO_4, anhydrous	12,5 g (if $KH_2PO_4.2H_2O$ is used, then 16,0 g).
Distilled H_2O to	1000,0 mL

- Dissolve the two phosphates in the water.
- Pour about half of the phosphate solution into a litre bottle containing a few glass beads.
- Add the stain powders and mix well.
- Add the remainder of the phosphate solution. Mix well and filter.

Field stain B

Composition

Eosin (yellow, water-soluble) (CI 45380)	2,0 g
Na_2HPO_4, anhydrous	10,0g (if $Na_2HPO_4.12H_2O$ is used, then 25,2 g).
KH_2PO_4, anhydrous	12,5g (if $KH_2PO_4.2H_2O$ is used, then 16,0 g).
Distilled H_2O to	1000,0 mL

Dissolve the two phosphates in the water. Pour into a one litre bottle. Add the eosin. Mix until dissolved. Filter.

Field stains A and B can also be prepared from commercial powders according to the manufacturer's instructions.

NOTE: Field stain solutions, in contrast to other Romanovsky stains, must be kept separate because the methylene blue forms a precipitate with eosin when mixed together in an aqueous solution. This does not occur with Romanovsky stains with an alcohol as solvent in which no precipitating salt is formed.

6.3 Giemsa stain

Giemsa is the standard Romanovsky stain for staining blood films for malaria diagnosis. The quality of this stain, in ready-made solution or powder form, varies according to its source of supply and it is advisable to obtain it from a reputable manufacturer. Even so, stain quality needs to be established by testing each batch after it has been made up and prior to routine staining of large numbers of blood films.

Composition

Giemsa powder	0,5 g
Methanol	100 mL
Glycerol	250 mL

The stain should be stored in a dark bottle with 50 solid glass beads of about 5 mm in diameter.

6.4 Gram's stain

Gram's stain is used for the microscopical differentiation of bacteria according to their ability to absorb crystal violet.

1. Crystal violet

Crystal violet (CI 42555)	0,5 g
Distilled H_2O	100 mL

2. Gram's iodine solution

Iodine	1,0 g
Potassium iodide	2,0 g
Distilled H_2O	300 mL

Dissolve the potassium iodide in a small volume of water; add the iodine and dissolve. Then add the remainder of the distilled water to make 300 mL.

3. Decolorizer

   a Acetone-alcohol decolorizer

Acetone	1 volume
Ethanol (95%)	1 volume

   or:

   b Pure acetone can also be used by itself. Differentiation is better but great care must be taken not to allow the acetone to act for too long (1-2 seconds is all that is needed).

4. Counter stains

   a

Neutral red (IC 50040)	1,0 g
Acetic acid (1% v/v)	2,0 mL
Distilled H_2O	1000,0 mL

   or:

   b

Safranine O (CI 50240)	1,0 g
Distilled H_2O	100 mL

6.5 Leishman stain

Leishman stain is a Romanowsky stain which is used for staining parasites.

Composition

Leishman powder	1,5 g
Methanol (absolute), acetone-free	1000,0 mL

Grind the stain powder with a mortar with a little of the methanol. Allow the undissolved stain to settle; then decant the fluid into a bottle. Treat the residue in the mortar with a little more methanol and repeat the process until all the stain is in solution.

The stain is ready for use the following day. When preparing an alcohol Romanowsky stain such as Leishman stain, it is important not to allow moisture to enter the stain during its preparation or storage, otherwise the dyes may precipitate as a salt.

7

QUALITY ASSURANCE AND QUALITY CONTROL OF INGREDIENTS FOR MICROBIOLOGICAL CULTURE MEDIA

7. Quality assurance and quality control of ingredients for microbiological culture media

7.1 Introduction

Ingredients for microbiological culture media can be classified as chemically defined and chemically undefined. They are further subdivided according to their role in media, e.g. solvents, gelling agents, nutrients, reactive substances, selective agents, indicators, redox potential regulating agents, pH buffers, etc.

Quality control includes the following stages:

- control of reagents for the preparation of media;
- in-process control;
- control of the final product.

In-process control and master formula

An essential part of in-process control is a master formula, setting out in writing the starting materials (quality and quantity), as well as a detailed manufacturing procedure. Checkpoints during the preparation should be also stated. These could be pH measurement, measurement of electrical conductivity or osmotic pressure, or gel strength. A master form should contain spaces to fill in with:

- date of production
- batch number and brand
- initials of the operator
- results of control measurement
- signature of the person responsible for the manufacturing process

Whenever possible, the master formula should be prepared for standard batch sizes and should not contain comments or amendments. When changes are necessary, the master formula should be rewritten. A person experienced in the manufacturing process should be responsible for the content of the master formula, while the technicians should know the importance of carefully following the stated instructions. An example of a master formula is shown in Table 7.1.

Table 7.1 Quality control protocol for agar-agar

Substance: agar-agar **Chemical formula: undefined** **Code: 1613**

Batch: _____ **Sample no.** _____ **Sterile sampling: YES/NO**

Characteristic	Sample Limit/Standard
Physical	
Granulation	95% > 315 nm
Water content	<12%
Clarity after autoclaving in distilled water:	
(% transmission at 560 nm and 60°C)	>95%
Endo broth	>93%
Caso broth	>90%
Challenge broth	>88%
Gel strength (gelomat, 1,2% in Caso broth)	50-60 g
Setting point (water-bath, 1,2% Caso broth)	<40°C
Concentration of gelling limit	<0,25%
Melting point (Gel, 1,2%)	>85°C
Diffusion rate (Safranine, 24 hrs)	>30 mm
Chemical	**Sample Limit/Standard**
Identity	specific for Agar-agar
Oxidizing substances	absent
Ash (total)	<5%
Acid insoluble ash	<0,5%
Starch	absent
Gelatin	absent
Heavy metals as Pb	<0,0005%
Calcium (Ca)	<0,05%
Magnesium (Mg)	<0,01%
Phosphate (P)	<0,5%
Biological	**Sample Limit/Standard**
Antimicrobial activity (disk, medium)	absent

Continued

Characteristic	Sample Limit/Standard
Antagonistic activity (Mueller-Hinton agar)	absent
Bioburden	USP Limits
Heat resistant microorganisms	absent
Thermophilic microorganisms	absent
Performance in:	
Blood agar base	according to Standard
Caso agar	according to Standard
Wort agar	according to Standard
Baird Parker agar	according to Standard

A minimal programme of quality control must be effective, rapid and practical. For this, three principles are of paramount importance:

- The sample must be representative.
- The examination should start with the simplest eliminatory test.
- Biological tests should have priority over chemical tests.

The workload in controlling the ingredients can be minimized if large batches of the ingredients are purchased. Their expiry dates should be taken into account when deciding upon the quantities that shall be procured.

Control procedures include:

- record of identity of batch and the sampling of the batch;
- physical testing (solubility, clarity, colour, compatibility with other ingredients, gel strength, diffusion rate, etc.);
- chemical testing (check on identity; pH; presence and level of organic and inorganic contaminants;
- biological testing.

In order to carry out effective biological testing, a laboratory must possess a good collection of representative microorganisms, including freshly isolated strains, and correct microbiological techniques must be employed. Biological quality control includes testing for presence of inhibitory or antagonistic activity; determination of numbers of microbes in the unsterilized ingredient; quantitative determination of growth-promoting properties for a wide variety of organisms; investigation of biological reactivity and performance in complex media.

7.2 Classification of ingredients

The fact that a substance is chemically defined does not automatically mean that this poses no problems as a microbiological ingredient. However, the problems raised by chemically defined ingredients are minor in comparison with the multitude of difficulties presented by undefined ingredients. The classification of ingredients is given in Table 7.2.

Table 7.2 Classification of ingredients for culture media

Chemically defined substances	Chemically undefined materials
Water	
Gelling agents (silica gel, organic polymers)	Gelling agents (agar, gelatin, agarose, carrageenin, etc.)
Nutrients (salts, carbohydrates, amino acids, etc.)	Nutrients (extracts, hydrolysates, biologicals)
Reactive substances for test reactions: (salts, carbohydrates, fats, amino acids)	Reactive substances (casein, egg yolk, starch, blood, etc.)
Selective agents (dyes, selenite, azide, antibiotics etc.)	Selective agents (ox bile, etc.)
Redox potential regulating substances (cysteine, thioglycollate, etc.)	Redox materials (haemoglobin, liver powder /extract, etc.)
Indicators (aniline blue, bromothymol blue, phenol red, bromocresol purple, resazurin, etc.)	
pH-adjusting substances (solid, liquid)	

7.3 Principles of quality control of microbiological ingredients

Adequacy of sample

An adequate sample is one which is representative of a well defined batch. When necessary, the sampling should be made under sterile conditions. Criteria for batch definition, homogeneity, and aseptic sampling are found in the U.S. Pharmacopoeia, 1975.

Procedure for elimination of inappropriate materials

Experience has shown that it always pays to begin the examination with the simplest test that has an eliminatory value. These are, in general, physical criteria. If, for example, a given peptone sample is not clearly soluble, there is no reason to proceed further to chemical and biological tests. Biological criteria have priority over physical and chemical tests. If a biological test gives aberrant results, although physical and chemical tests are satisfactory, the ingredient must be rejected. If the biological test is passed but some chemical tests fail, acceptance of the ingredient is still possible.

7.4 Methods for quality control of ingredients

7.4.1 Physical examination of ingredients

Colour, odour, structure, granulation, water content

Most ingredients which enter into the composition of media are dry materials, powders or granulates. The colour must lie within an accepted range; dark powders yield dark solutions and are not acceptable. The structure of the dry material must be homogeneous and within a certain particle size range. Large particles are not readily soluble. The water content must be as low as possible. For peptones the value is 3-6% w/w, and for agar it is usually under 12%. If the moisture is too high, there is an increased danger of biological deterioration. No conglomerates, foreign materials, structure modification or abnormal odours are permitted.

Solubility, clarity, and colour of ingredient solutions

These are simple physical eliminatory criteria. In general, concentrations of 0,5, 1,0 and 2,0 g per 100 mL of deionized water are used for the first screening. The cold, boiled and autoclaved solutions are measured photometrically against standard controls and distilled water. For each concentration, the colour corrected absorbance must not exceed a given value. Optically these solutions must be clear, without deposits or insoluble particles and the colour must remain clear after boiling and autoclaving and after several days standing at room temperature and in the refrigerator. Clarity of gelling agents is measured at 60-70°C and only in autoclaved solutions.

Compatibility with other ingredients

Microbiological ingredients are mostly used in complex formulae and therefore their physical compatibility must be tested with other frequently used ingredients. It is not possible to test a given ingredient against all possible microbiological ingredients and in practice a limited spectrum of formulations is selected. Peptones and other extracts are

tested in 3 to 5 common fluid formulations (such as tryptic-soy-broth, nutrient broth, MacConkey broth, peptone water, etc.).

Gel strength, setting point, melting point

The gel strength and the setting temperature are eliminatory criteria for agar; the melting temperature is not so critical. There are many methods for measuring the gel strength of agar. As the gel strength values are strongly influenced by the concentration of agar, temperature, pH value, composition of the solvent, surface humidity, and many other factors, a standardized procedure is recommended. In most cases testing with different seeding procedures might indicate if a satisfactory gel strength has been obtained.

The setting temperature is best determined in a water-bath with accuracy of ±0,2°C, with at least two agar concentrations (1,2 and 1,5%) and in volumes of not less than 500 mL. A good agar for microbiology must remain perfectly fluid at 40°C for at least 12 hours. As the composition of the solvent has an important effect on the setting temperature, it is advisable to test this with different liquids as well as deionized water. Material to be mixed with biological fluids (milk, serum, blood, egg yolk) must also be regularly tested.

Diffusion rate

Agar-containing media are widely used for diffusion tests (such as antibiotic sensitivity testing of clinical isolates). Therefore, the diffusion characteristics of the agar preparations should be known. A useful test is based on the diffusion of safranin in 1% aqueous solution; the diffusion is recorded at 25° and 37°C after 4 hours and 20 hours. Agar batches which show a diffusion zone of less than 25 mm after 20 hours at 25°C are not acceptable for diffusion media.

7.4.2 Chemical examination of ingredients

Chemical analysis of ingredients' constituents

Chemical examination includes tests for identity, inorganic components, phosphate, Na, Ca, Mn, Cu, As, S, chloride, nitrite, K, Mg, Fe, Pb, Zn and organic components: total N, proteose N, peptone N, amino N, urea, ammonia, carbohydrates, amino acids, peptides (carnosine, anserine, etc.), guanidines (creatine, creatinine, etc.), purines (hypoxanthine), organic acids (lactic, glycolic, fatty acids), vitamins, etc. For chemically defined ingredients reagent grade quality is required. Major problems arise with the chemically undefined ingredients. The methods used for the chemical examination of the ingredients are those of general analytical chemistry.

It is difficult to establish values for limits of acceptance (which would fit all purposes) for chemicals because microbiological ingredients are used in an infinite number of

combinations. Standard values and limits for defined microbiological media may be taken from pharmacopoeias, e.g. the U.S. Pharmacopoeia 1975 and 1980.

The quality of the water used for solution of the ingredients might influence the properties of the media. If problems with the quality of water occur, chemical analysis and tests for pyrogens in the water should be made. Another water supply may also be considered. Testing for presence of chlorine and osmolarity should be done on a weekly basis. Testing for absence of heavy metals such as copper and lead should also be done.

7.4.3 Biological examination of ingredients

Absence of inhibitory and/or antagonistic activity

Tests for inhibitors and antagonists are applied theoretically to all ingredients with the exception of the selective agents but the chemically undefined ingredients, particularly the gelling agents and the nutrients, are the main field of application. There are many substances which may occur during production or sterilization and which may have antimicrobial activity. The principal substances in this category are para-aminobenzoic acid (PAB), which interferes with the sulfonamides, and thymidine, which interferes principally with trimethoprim in the sensitivity testing of *Streptococcus faecalis* and other bacteria. Excess agaropectin (acid sulfate groups) interferes with polymyxin B and aminoglycoside antibiotics. Table 7.3 shows some of these so-called 'natural' inhibitors.

The testing for inhibitory activity of microbiological ingredients is performed mainly by two methods:

The diffusion method. Paper discs impregnated with different concentrations of the substance to be tested are placed on nutrient agar plates seeded with different sensitive microorganisms, such as *Bacillus subtilis, Bacillus stearothermophilus, Micrococcus luteus*, or other specific cultures. The development of inhibition zones is recorded.

The dilution method. The determination of the MIC (minimal inhibitory concentration) against the same sensitive microorganisms as shown above.

The substitutional method. The tested ingredient is substituted in a complex formulation for a standard ingredient known to be devoid of inhibitors and compared with the noninhibitory complex medium.

Testing for antagonists is performed using quantitative sensitivity tests against known standard media devoid of such antagonists, usually by the substitution method. Testing for inhibitory activity is very important and must be performed routinely for all undefined and

Table 7.3 Common natural bacterial inhibitors in microbiological ingredients and media

Substance	present in	appears during	avoided through	neutralizing agent
unsaturated fatty acids	peptone,agar	production	control and purification	activated charcoal starch, serum albumin, blood
colloidal sulfur and sulfides	peptones, cystine	autoclaving	sterile filtration	starch, serum albumin, blood
H_2O_2 & organic peroxides	complex media	autoclaving	sterile filtration aerobic bacterial growth	haematin, catalase, blood
serine, valine cysteine,	peptones and other hydro-lysates	production (normal hydro-lysis)	addition of amino acids	glutamic acid, alanine, aspartic acid
histones	organ extracts	production	selection of raw materials	serum albumin, plasma
heavy metals (Cu, Pb, Zn, As)	peptones, extracts, gelatin, agar, other ingredients	production	control and better production technique	EDTA,other chelating agents, serum albumin, blood

for some chemically defined microbiological ingredients. Even the most sophisticated chemical methods are sometimes not able to reveal the presence of some inhibitors.

Microbial 'bioburden'

This term is explained in the U.S. Pharmacopoeia, 1980. It means the qualitative and quantitative microbial load of a material. The microbiological ingredients are not sterile products and even if they are sterilized before use, the microbial load is important from at least two points of view. First, the microbiological ingredients must be microbiologically safe; they must not contain pathogenic microorganisms. Secondly, the number and type of microorganisms present must not interfere with the quality and stability of the product. A large number of bacteria and fungi in a hydrolysate, for instance, could make this ingredient unsuitable for analytical purposes (e.g. vitamin assay) because the substances contained in the microbial cells can interfere with the test. Also, heavily contaminated ingredients are not suitable for the production of dehydrated media because they reduce the stability of these media. The methods used to determine the microbial bioburden of the microbiological ingredients are those generally used in

food and pharmaceutical microbiology. The quantitative and qualitative limits are essentially those for pharmaceutical raw materials: see U.S. Pharmacopoeia, 1975.

Growth-promoting properties

Examination of growth-promoting properties is an important step in the evaluation of undefined nutrients. There are two routine methods for the testing of this parameter:

Microbial multiplication in single nutrient solution. Solutions of different concentrations (0,5-2,5% w/v) of the substance to be tested in water are inoculated with known numbers of bacterial cells belonging to several species and the intensity of multiplication is then evaluated from the rate of increase in turbidity or dry weight, or from the generation time. The choice of microbial species and other conditions of testing depend on the product tested.

Microbial multiplication on solidified single nutrient solutions. In this test, the number, size and other characteristics of the colonies are evaluated on agar-solidified solutions in plates. In both these methods a set of noninoculated and inoculated standard media are included as controls.

Biological reactivity

Certain properties of chemically undefined nutrients are important if they are to be accepted for microbiological media. These are:

Absence of fermentable carbohydrates. Some peptones and extracts intended to be used to test carbohydrates (fermentation studies) should not contain such substances in their composition. The intrinsic presence of carbohydrates would falsify the result of such tests.

Indole produced by tryptophanase-forming bacteria. This property is affected by the tryptophan content of the tested nutrient.

Production of H_2S during growth of H_2S-producing bacteria. This ability is related to the content of sulfur-containing aminoacids.

There are other effects on biological properties such as:
- influence on bacterial haemolysin production;
- influence on culture pigmentation;
- protective action against chemical disinfection, etc.,

which can be determined in special circumstances in microbiological ingredients.

Biological performance in complex media

Examination of the performance of an ingredient in a complex medium is the decisive biological test of its suitability for microbiological purposes. This test applies to all

categories of microbiological ingredients and is in principle very simple. A classical complex medium (such as trypticase soy broth, desoxycholate citrate agar or violet red bile agar) is prepared with known, pretested ingredients, omitting the ingredient to be tested. Several portions of this incomplete medium are prepared; to some of these portions, the missing ingredient is added in different concentrations (above and below the usual ones). To other portions, one or more standard ingredients are added as controls. By seeding all these media with appropriate cultures, the performance of the tested ingredient is evaluated by comparison with the control and an optimal concentration can be determined. For the selective media, a convenient evaluation system is the determination of the 'relative growth index' (RGI). Table 7.4 shows a set of media in which microbiological ingredients can be tested.

Table 7.4 Testing of microbiological ingredients in complex media

Ingredient to be tested	Complex medium used for the test
Agar	Mueller-Hinton agar, Endo agar, etc.
Gelatin	nutrient gelatin, special nutrient gelatin for microcolony counting
Heart extract	brain heart broth,Columbia agar
Casein peptone	Caso broth, Endo agar, mannitol salt agar, phenol red agar
Starch	Mueller-Hinton agar, GSP agar
Bile	Brila broth, Brolacin agar, EMB agar
Sodium selenite	Selenite enrichment broth according to Leifson selenite cysteine enrichment broth

Control of the final product

The final product should be tested for sterility and microbiological performance. An adequate sample of each prepared batch should be placed in the incubator and examined for the presence of microbiological contaminants. If growth occurs, measurements should be taken to eliminate the sources during production. For each media preparation at least two, and eventually more, reference microorganisms should be included for testing. The appropriate microorganisms should be indicated on each recipe. Examples of quality control tests for liquid and solid media used for the differentiation of *streptococci* are listed in Tables 7.5 and 7.6.

Table 7.5 Quality control tests for liquid media* used in the differentiation of *Streptococci*

Medium	Test organism	Reaction
Sodium hippurate broth**	*Streptococcus group A*	no colour with ninhydrin
	Streptococcus group B	colour with ninhydrin
Inulin broth	*Streptococcus sanguis*	yellow colour (acid)
	Streptococcus group D	no change of colour (no acid)
Lactose broth	*Streptococcus faecalis*	yellow colour (acid)
	Streptococcus equinus	no change of colour (no acid)
Litmus milk	*Streptococcus group D*	acid and clotting
	Streptococcus equinus	no change of colour (no acid)
Mannitol broth	*Streptococcus faecalis*	yellow colour (acid)
	Streptococcus sanguis	no change of colour (no acid)
NaCl (6,5%) broth	*Streptococcus faecalis*	growth
	Streptococcus bovis	no growth
Raffinose broth	*Streptococcus bovis*	yellow colour (acid)
	Streptococcus faecalis	no change of colour (no acid)
Sorbitol broth	*Streptococcus faecalis*	yellow colour (acid)
	Streptococcus bovis	no change of colour (no acid)

* All carbon sources at 1% w/v
**Na hippurate at 1%.

Table 7.6 Quality control tests for solid media used in the differentiation of *Streptococci*

Medium	Test organism	Reaction
Starch agar (nutrient agar +0,3 soluble starch)	*Streptococcus bovis*	hydrolysis of starch
	Streptococcus faecalis	no hydrolysis
Sucrose agar (5% sucrose)	*Streptococcus faecalis*	yellow colour (acid)
	Streptococcus viridans	no change of colour (no acid)
	Streptococcus durans	
TTC agar	*Streptococcus faecalis*	colonies with brick red centre,
	Streptococcus bovis	no colourless colonies

Continued

Medium	Test organism	Reaction
Blood agar	*Streptococcus pyogenes*	ß-haemolysis
TKT agar	*Streptococcus agalactiae*	typical colonies with ß-haemolysis
Tellurite agar (glucose yeast extract agar + 0,04% K tellurite)	*Streptococcus faecalis*	black colonies
Camp test on blood agar	*Streptococcus agalactiae*	clear zone of haemolysis
Todd Hewitt agar with dextrose	*Streptococcus group A*	growth
Todd Hewitt agar without dextrose	*Streptococcus viridans*	growth

7.5 Technical aspects in the preparation of media for microbiology

The basic components from commercial suppliers are dehydrated and hygroscopic. They must be stored in a dry and dark place to avoid destruction by light and at a temperature recommended by the manufacturer.

Only distilled or deionized, sterile water with a conductivity < 3 µS/cm must be used for the preparation of media. If distilled water is prepared from chlorinated water, the chlorine must be neutralized prior to distillation (see also Section 3.16.2.3). Demineralized water prepared by ion exchange may have a high content of microorganisms. Therefore, it must be filtered through a 0,22 µm filter for further use.

The water should be stored in inert containers; glass or polyethylene, not metal.

Sterilization

Culture media and reagents can be sterilized by different techniques, but most often they are sterilized by moist heat at 121°C for 15 minutes. After sterilization the pH, sterility and efficiency to support bacterial growth of the media must be controlled at regular short-term intervals.

Labelling and storage

The bottles, tubes and Petri dishes containing reagents or prepared media must have the following labels:

- batch number
- name for identification
- date of preparation and/or expiry

They must be stored at 4°C for a maximum of 3 months, or between 18°C and 23°C for 1 month under conditions that do not cause decomposition. Agar media in Petri dishes must be stored in plastic bags or cellophane until required. Ideally all plates should be stored in the dark. Reference should be made to suppliers' technical specifications to determine storage conditions.

Melting of agar culture media

Agar culture media are melted at boiling water temperature. Heating over a longer period of time must be avoided by withdrawing the media as soon as they have solubilized. The molten media should be kept in a thermostatically controlled water-bath at 47° ±2°C until further use but not longer than 8 hours. Never use culture media at a temperature higher than 50°C. Never remelt a medium.

8

REAGENTS FOR MICROBIOLOGY

8. Reagents for microbiology

In this section the preparation of media for transportation and culture of microorganisms is described. Although a great number of media exist, many of which are commercially available, mainly of those media which are mentioned in the WHO publication *Basic laboratory procedures in clinical bacteriology* are discussed in alphabetical order. It is important to remember that different names may be used by different suppliers for one medium. Therefore, it may be necessary to compare the composition of the media when purchased from different suppliers. Sometimes it may also occur that media prepared for the same purpose may differ slightly in their composition. This does not necessarily imply that their quality for culture of micro-organisms will be different. However, it is advisable to use standardized media for semiquantitative tests such as the susceptibility testing of bacteria towards antibiotics.

Some of the media described in this chapter contain ingredients that are prepared from extraction or digestion of material from animal or plant tissue. These ingredients are sometimes difficult to prepare. They are all commercially available. The composition of the products is not standardized and may differ rather extensively. Moreover, the names given to the commercial products vary. This **must** be remembered when comparing products of different origin.

8.1 Ingredients for culture media

The following ingredients are mentioned for the preparation of media discussed below:

1. Casein peptone, acid hydrolysate

Commercial synonyms:	Acid hydrolysate of casein
	Peptone 5 (acid)
	Peptamine
	Casamino acid
	Acidicase peptone

The material is prepared from hydrolysis of casein by hydrochloric acid. It is partially neutralized with sodium hydroxide and dried. It is deficient in cystine and tryptophan and lacks vitamins. pH 5,0 ±0,5 for 5% solution.

2. Casein peptone

Commercial synonyms:
- Pancreatic digest of casein
- Peptone C
- Trypticase peptone
- Tryptone
- Pepticase
- Casitone
- Peptone 50, Peptone 140

The material is prepared from degradation of casein by pancreatic enzymes. It has a balanced amino acid content but is carbohydrate free. pH 7,0 ±0,5 for 5% solution.

3. Casein/meat peptone (50/50)

Commercial synonyms:
- Dipeptone
- Polypeptone
- Peptone
- Tryptose
- Proteose peptone Nr. 3
- Neopeptone
- Pantone

The material is a mixture of equal amounts of a pancreatic digest of casein and of a peptic digest of animal tissue. pH 7,0 ±0,5 for 5% solution.

4. Gelatin peptone

Commercial synonyms:
- Pancreatic digest of gelatin
- Peptone G
- Gelysate
- Bacto-peptone

The material is prepared by digestion of gelatin with pancreatic enzymes. It is deficient in cystine and tryptophan and carbohydrate free. pH 7,0 ±0,5 for 5% solution.

5. Heart peptone

Commercial synonyms:
- Pancreatic digest of heart muscle
- Proteose peptone
- Myosate
- Tryptic digest of heart muscle

The material is prepared from digestion of heart muscle tissue by pancreatic enzymes. It is highly nutritive. pH 7,0 ±0,5 for 0,5% solution.

6. Meat peptone

Commercial synonyms: Pancreatic digest of animal tissue
Beef peptone
Tryptose
Proteose peptone
Peptone P

The material is prepared from digest of animal tissue by peptic enzymes. It is rich in sulfur-containing components. pH 6,5 ±0,5 for 5% solution.

7. Soy peptone

Commercial synonyms: Peptone soya
Papain digest of soy bean meal
Phytone peptone
Peptone S
Soyotone

The material is prepared by enzymic degradation of soy bean meal with papain. It is rich in carbohydrates, essential amino acids and vitamins. pH 7,0 ±0,5 for 5% solution.

8. Meat extract

Commercial synonym: Beef extract
LAB LEMCO

The material is prepared from extraction of enzymatically digested beef muscle tissue. It is a good nutrient base with a concentration of 3-5%. pH 6,5 ±0,5 for 5% solution.

Meat extract preparation

Put 500 g of lean minced meat (beef heart or fat-free meat) into a pan and add 1 litre of water. Place it in the refrigerator (4°C) overnight. After 14 hours bring it to boiling point and simmer for 15 minutes, while stirring with a glass rod. Filter through a wet paper filter to remove fat. Add water to make 1 litre (to replace the loss in boiling).

Meat infusion broth preparation

Heat 1 litre of 0,05 molar aqueous NaOH to boiling and add 1000 g of fat-free minced fresh meat. Mix thoroughly, bring to boiling point and allow to simmer for 20 minutes stirring frequently. The mixture should have a pH of about 7,5. Strain through several layers of cheesecloth, squeeze out excess liquid, adjust the volume to 1000 mL with distilled water and use the liquid immediately for making up media as specified in the formulations.

Brain infusion broth

Heat 1 litre of 0,05 molar aqueous NaOH to boiling and add 1000 g of calf brain. Mix thoroughly, bring to boiling point and allow to simmer for 20 minutes stirring frequently. The mixture should have a pH of about 7,5. Strain through several layers of cheesecloth, squeeze out excess liquid, adjust the volume to 1000 mL with distilled water and use the liquid immediately for making up media as specified in the formulations.

Egg yolk emulsion

- Break eggs and separate whites from yolks aseptically.
- Break yolks and mix with an equal volume of sterile physiological saline.
- Filter through sterile gauze and distribute in sterile bottles.
- Heat at 60°C for 30 minutes.
- Store at 4°C.

9. Yeast extract

The material is an aqueous extract of autolysed brewer's or baker's yeast (*Saccharomyces cervisiae*). It is rich in carbohydrates and vitamin B complex. The concentration is 3 to 5%; pH 7,0 ±0,5.

8.2 Acetate agar

Acetate agar is used for the differentiation of *Shigella* sp. from *E. coli*. The medium is similar to Simmons citrate agar in which sodium acetate is substituted for sodium citrate. Typical cultures of *Shigella* are unable to utilize acetate and fail to grow on it, while *E. coli* cultures grow well. The blue colour of the bromothymol blue, which has been added as indicator, is due to the production of alkaline products from the utilization of sodium acetate.

Composition

Sodium acetate	2,0 g
$MgSO_4 . 7 H_2O$	0,1 g
NaCl	5,0 g
$(NH_4)H_2PO_4$	1,0 g
K_2HPO_4	1,0 g
Agar	20,0 g
Distilled H_2O	1000 mL

Preparation

- Heat 20 g of agar in 1 litre distilled water and add the salt ingredients while hot;
- Add 10 mL of a bromothymol blue solution (0,8% w/v);
- Cool to 50°C; adjust to pH 6,5-7,0;
- Autoclave at 121°C for 15 minutes.

Quality control

Growing test organism:	*Escherichia coli*
non-growing test organism:	*Shigella flexneri*

8.3 Amies transport medium

Medium containing charcoal for conservation and transport of pathogenic microorganisms with lasting preservation of the microbial flora. The absence of a source of nitrogen prevents growth, while preserving live bacteria.

Composition

NaCl	3,0 g
KCl	0,2 g
Na_2HPO_4, anhydrous	1,15 g
KH_2PO_4	0,2 g
Sodium thioglycollate	1,0 g
$CaCl_2$, 1% aqueous solution freshly prepared	10,0 mL
$MgCl_2 . 6 H_2O$, 1% solution	10,0 mL
Agar	4,0 g
Charcoal	10,0 g
Distilled H_2O	1000 mL

Preparation

- Heat 4 g of agar in 1 litre of distilled water until dissolved by boiling and add, while hot but not boiling, the other ingredients.
- Stir until dissolved and add 100 g charcoal (pharmaceutical neutral grade).
- Mix thoroughly so that the charcoal is evenly distributed, and distribute in 1/4 oz (7 mL) Bijou bottles, filling them almost to the brim. Apply screw-cap and screw down tightly.
- Autoclave at 121°C for 15 minutes.

- Invert the bottles during cooling to distribute the charcoal evenly, making sure that the caps are tightened securely. Final pH, 7,2 ±0,2.
- Store in the dark in a cool place.

Old medium should be freshly steamed and the charcoal resuspended before use.

Quality control

growing test organisms: *Staphylococcus aureus*
Escherichia coli

8.4 Andrade's peptone water carbohydrate medium

Andrade's peptone water carbohydrate medium is peptone water (see 8.25) supplemented with a carbohydrate and an indicator (Andrade's indicator, Unipath). It is used for the investigation of microorganisms fermenting sugars and producing CO_2 or acidic metabolites. The indicator is acid fuchsin titrated with sodium hydroxide until the colour changes from pink to yellow. The peptone water is colourless after the addition of the indicator, but turns pink during metabolic acidification and yellow in alkaline pH.

Andrade's indicator

NaOH (1 mol/L)	15,0 mL
Acid fuchsin (CI 42685)	0,5 g
Distilled H_2O	100 mL

Preparation

- Dissolve the acid fuchsin in the water and initially add 15 mL of 1 mol/L NaOH per 100 mL of indicator solution.
- Mix and allow to stand at room temperature for 24 hours. Shake frequently during the 24-hour period. The colour should change from pink to brownish-red.
- If the dye has not been sufficiently decolorized, add another 1 mL of 1 mol/L NaOH per 100 mL, mix thoroughly and leave for a further 24 hours.
- If further additions of NaOH are required, mix thoroughly and leave for a further 24 hours, but only the minimum of alkali should be added to achieve the desired final straw-yellow colour.

Andrade's peptone water

Andrade's indicator solution	10 mL
Peptone water medium	1000 mL

Preparation

- Add 10 mL of Andrade's indicator to peptone water medium (see Section 8.25).
- Check the pH and adjust to 7,4 if necessary.
- Sterilize in the autoclave at 121°C for 15 minutes.

The medium is pink when hot but loses its colour on cooling.

Quality control

Growing test organisms: *Pseudomonas aeruginosa*
Escherichia coli

Carbohydrate solutions (glucose, maltose, sucrose, trehalose starch)

Preparation

- Dissolve 10 g of the appropriate carbohydrate in 90 mL of distilled water and steam for 30 minutes in a steam sterilizer to sterilize. Alternatively the solutions may be filtered. Appropriate carbohydrates can be mono-, di-, tri- and polysaccharides, depending on the microorganism to be investigated.
- Add one part of carbohydrate solution to nine parts of Andrade's peptone water to give a final concentration of 1% carbohydrate.
- Aseptically distribute the final medium into small screw-capped bottles or tubes (e.g. about 3 mL volumes). Some carbohydrates may cause an acid reaction, i.e. the medium turns pink, therefore before distribution, to restore to the original colour, carefully add sterile 0,1 mol/L NaOH.

Starch peptone water is unstable. It is recommended that starch solution be added to Andrade's peptone water at the time of use. This is done by preparing a 2,5% solution of soluble starch in distilled or deionized water. Sterilize in the autoclave at 115°C for 10 minutes. Add 0,6 mL of this to 3 mL of base medium (final concentration 0,4%). Not all batches of soluble starch are satisfactory and should be pretested in made up form, with known starch fermenting and non-starch fermenting *Corynebacteria*. The 2,5% starch solution is usually stable for about three months at +4°C.

It is recommended that known starch positive and negative *Corynebacteria* be included with each test or batch of tests.

8.5 Bile aesculin azide agar

Bile aesculin azide agar is a selective medium used to isolate and enumerate faecal *Streptococci* (Lancefield group D) in food and pharmaceutical products.

The growth of bile tolerant *Enterococci* is favoured, while that of contaminating gram-negative bacteria is inhibited by sodium azide and that of gram-positive bacteria is inhibited by bacteriological bile. The *Enterococci* hydrolyse aesculin to glucose and aesculetin. The aesculetin produced forms an olive green to black complex in the presence of ferric ions arising from ferric citrate in the medium.

Composition

Casein peptone	17,0 g
Meat peptone	3,0 g
Yeast extract	5,0 g
Bovine bile	10,0 g
NaCl	5,0 g
Sodium citrate	1,0 g
Aesculin	1,0 g
Ammonium ferric citrate	0,5 g
Sodium azide	0,25 g
Agar base	15,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 6,6 ±0,2.

Preparation

- Dissolve 15 g of agar in 400 mL distilled water while heating.
- Dissolve 10 g of bovine bile in 400 mL distilled water.
- Dissolve 1 g aesculin in 100 mL distilled water under gentle heating and sterilize by millipore filtration.
- Dissolve the other ingredients in 100 mL distilled water.
- Mix the agar solution with the bile solution, add the other ingredients' solution and adjust to pH 6,6.
- Autoclave at 121°C for 15 minutes and cool to 50°C.
- Add the sterile aesculin solution to the base medium; mix well and dispense into screw-capped tubes.

An additive of 5% (v/v) of horse serum optimizes the growth of *Enterococci.*

Quality control

Growing test organisms:	*Staphylococcus faecalis*
	Staphylococcus aureus
Non-growing test organisms:	*Streptococcus agalactiae*
	Streptococcus viridans

8.6 Blood agar (BA)

For the preparation of blood agar plates to be used for the isolation of pathogenic microorganisms and their ability of haemolysis. The following dehydrated base media have been found satisfactory.

(a) Bacto-brain-veal agar

(b) Columbia agar

(c) Blood agar base

Composition of blood agar base

Heart peptone	2,0 g
Casein peptone	13,0 g
Yeast extract	5,0 g
NaCl	5,0 g
Agar	15,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 6,8 ±0,2.

Preparation

- Dissolve the ingredients in distilled hot water.
- Autoclave at 121°C for 15 minutes.
- Cool to 50-60°C and add defibrinated sheep, horse or bovine blood to give 5 to 8% final concentration. The pH must be kept at 6,8 ±0,2 for adequate preservation of blood cells.
- Mix well without shaking and pour plates (12-15 mL medium per 9 cm Petri dish).
- Dry the surface before use and store at 4°C in a sealed plastic bag to prevent dehydration.

Stability: 3 months.

Quality control

Growing test organisms: *Staphylococcus* sp.
Streptococcus sp.

8.7 Blood culture broth

Blood culture media are used for the identification of microorganisms in blood. Therefore, the media are free of blood and may contain a number of additives to provide

optimal conditions for growth of bacteria. Successful blood culture media must rapidly grow all organisms of medical significance.

1. Glucose broth

Composition

Meat extract broth	1000 mL
NaCl	5,0 g
Glucose	5,0 g
Peptone	10,0 g

Preparation

a. Meat extract broth
   - Put 500 g of lean minced meat (beef heart or fat-free meat) into a pan and add 1 litre of water.
   - Place it in the refrigerator (4°C) overnight.
   - Next morning, bring to boiling point and simmer for 15 minutes, while stirring with a glass rod.
   - Filter through a wet paper filter, to remove fat.
   - Add water to make 1,0 litre (to replace that lost in boiling).

b. Glucose broth
   - Maintain the meat extract at 80°C and add peptone, NaCl, and glucose.
   - Adjust pH to 7,6 (with 0,1 mol/L solution of NaOH). Distribute in 100 mL volumes in vials or tubes with screw-caps or cotton-wool plugs.
   - Sterilize at 121°C for 15 minutes and store at 4°C.

2. Bile-salt broth can be prepared by replacing the glucose in glucose broth by 5 g of sodium taurocholate.

3. Bile-salt streptokinase broth can be prepared by adding aseptically 100 000 units of streptokinase (Calbiochem Ltd) to 1 litre of bile-salt broth after autoclaving and cooling.

4. Liquoid broth can be prepared by adding 10 mL of liquoid solution in normal saline to 1 litre of nutrient broth. Liquoid (sodium polyanethol sulfonate) is obtainable, e.g. from Hoffmann La Roche. It can neutralize the antibacterial property of blood besides being an anticoagulant.

Sterility test

Incubate all bottles for 48 hours at 37°C; look for turbidity and if it appears, discard bottles.

8.8 Brolacin agar (CLED agar)

CLED (cystine lactose electrolyte deficient) agar is used for the enumeration and differentiation of bacteria from urine. This medium favours the growth of all microorganisms in urine while cultures of *Proteus* are prevented from spreading as a result of the low concentration of electrolytes. Lactose is added to the medium to differentiate between lactose fermenting microorganisms and non-lactose fermenting organisms. Lactose degradation causes the bromothymol blue to turn yellow.

Composition

Gelatin peptone	4,0 g
Casein peptone	4,0 g
Meat extract	3,0 g
L-cystine	0,128 g
Lactose	10,0 g
Agar	15,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,3 ±0,1.

Preparation

- Suspend the ingredient in the water.
- Bring to a boil to dissolve.
- Add 10 mL of bromothymol blue solution (0,2% w/v).
- Sterilize for 15 minutes at 121°C, and mix well before pouring into plates.

Quality control

Growing test organisms:	*Proteus mirabilis*
	Staphylococcus aureus
Non-growing test organisms:	*Shigella*

8.9 Cary-Blair transport medium

Cary-Blair medium is a transport medium with low nutrient content, similar to Amies transport medium but with a pH at 8,4. The low oxidation-reduction potential of the medium ensures bacterial survival over long periods.

Composition

Sodium thioglycollate	1,5 g
Na_2HPO_4	1,1 g

NaCl	5,0 g
Agar	5,6 g
Distilled H_2O	1000 mL

Preparation

- Dissolve the ingredients in the water while heating in a boiling water-bath until the solution is clear. Do not allow to boil.
- After cooling to 50°C, add 9 mL of freshly prepared 1% calcium chloride solution and adjust the pH to 8,4 ±0,2 (with 0,1 mol/L sodium hydroxide).
- It is important to dispense 7 mL amounts into 9 mL screw-capped clean and sterilized bottles (e.g. Bijou bottles) leaving a small air space at the top and the caps loosened.
- Sterilize by steaming at 100°C or in a boiling water-bath for 15 minutes, and tighten the caps after sterilization.
- Record batch date, label, and store in a cool, dark place.

This transport medium can be used for 18 months or longer under proper conditions of storage during which there is no loss of volume and no evidence of contamination or colour change.

Quality control

Growing test organisms:	*Shigella sonnei*
	Vibrio parahaemolyticus
	Salmonella typhi
	Escherichia coli

8.10 Chocolate agar enriched

Chocolate agar is used for the isolation and cultivation of fastidious bacteria like *Neisseria* and *Haemophilus* spp. Chocolate agar consists of agar base, haemoglobin and IsoVitaleX or CVA enrichment.

Composition

(a) Agar base

Casein/meat peptone (50/50)	15,0 g
Corn starch	1,0 g
K_2HPO_4	4,0 g
KH_2PO_4	1,0 g

NaCl	5,0 g
Agar	10,0 g
Distilled H_2O	500 mL

(b) Haemoglobin

a dried powder of bovine haemoglobin	10,0 g
Distilled H_2O	500 mL

(c) IsoVitaleX (= CVA enrichment; = Thayer Martin's supplement II) provides components of *Haemophilus* and *Neisseria* spp.

Nicotinamide-adenine dinucleotide (NAD)	0,25 g
Thiaminopyrophosphate	0,1 g
p-aminobenzoic acid	0,013 g
Thiamine-HCl	0,003 g
Vitamin B_{12}	0,1 g
L-glutamine	10,0 g
L-cystine	1,1 g
L-cystine-HCl	25,9 g
Adenine	1,0 g
Guanine-HCl	0,03 g
Ferric nitrate x 9 H_2O	0,02 g
Glucose	100,0 g
Distilled H_2O	500 mL

Preparation of 1 litre medium

- Suspend the ingredients of the agar base in 500 mL of distilled water; mix; heat to boiling point and cool to 50°C.
- Suspend 10 g haemoglobin in 500 mL of distilled water, autoclave and cool to 50°C.
- Mix the agar base with the haemoglobin solution, add 10 mL of the enrichment solution (IsoVitaleX) to the medium; mix and pour in Petri dishes.

NOTE 1. If haemoglobin solution is not available, add 5 mL of defibrinated blood from, rabbit, guinea pig or horse to 100 mL of autoclaved agar base at 50°C. Place the mixture in a hot water-bath at 80°C for 15 minutes or until chocolate colour has developed. Cool to 50°C and add the supplement.

NOTE 2: The agar base can be replaced by tryptone soy agar (TSA).

Quality control

Growing test organisms: *Neisseria meningitidis*
Neisseria gonorrhoeae
Haemophilus influenzae

8.11 Columbia agar base

Columbia agar base is used as a versatile medium for the cultivation of fastidious microorganisms. It is also used for the preparation of various special growth media, as well as for blood agar and chocolate agar.

Composition

Casein peptone	20,0 g
Meat peptone	3,0 g
Corn starch	1,0 g
NaCl	5,0 g
Agar	13,0 g
Distilled H_2O	1000,0 mL

Preparation

- Dissolve the ingredients in 1 litre of boiling distilled water.
- Dispense the medium in flasks and sterilize at 121°C for 15 minutes.

For the preparation of special media containing supplements, melt the base medium, cool to 50°C and add the supplements.

(a) for blood agar:

- Mix 1 litre of base medium with 50 mL of sterile sheep or horse blood.

(b) for chocolate agar:

- Mix 100 mL of blood with 900 mL of base medium.
- Warm up to 80°C for 10 minutes until the solution turns a brown colour.

A simpler procedure may be:

- Once agar is melted, it is cooled to 80°C.
- Add 100 mL of horse blood to 100 mL of base medium.
- With continous gentle mixing, hold at 80°C for 5 minutes until solution turns brown.
- Cool to 50°C and pour on plates.

Quality control

Growing test organisms: *Streptococcus aureus*
Streptococcus pyogenes

8.12 Desoxycholate citrate agar (DCA) (modified)

The medium contains high concentrations of desoxycholate and citrate which prevent growth of gram-positive bacteria and also more or less inhibit growth of coliform bacteria. The growth of *Salmonella* is not impaired, while some *Shigellae* (e.g. *Shigellae shigae*) may be slightly inhibited. The bacteria metabolize lactose which acidifies the medium. The acidification is detected by the formation of a red halo from the pH indicator neutral red. Thiosulfate is reduced to sulfide which reacts with iron ions to black iron sulfide. gram-positive and some gram-negative bacteria are inhibited in their proliferation by the presence of desoxycholate and citrate.

Composition

Base medium:

Meat extract broth	1000 mL
Meat peptone	10,0 g
Lactose	10,0 g
Neutral red (CI 50040) 1% solution	2,5 mL
Agar	17,0 g

Solution 1:

Sodium citrate	17,0 g
Sodium thiosulfate ($Na_2S_2O_3$. 5 H_2O)	17,0 g
Ammonium ferric citrate	2,0 g
Distilled H_2O	100,0 mL

Solution 2:

Sodium desoxycholate	10,0 g
Distilled H_2O	100,0 mL

pH at 25°C: 7,9 ±0,1.

Preparation of base medium:

- Adjust the reaction of the broth to pH 8,0-8,4 and dissolve the agar by heating in a boiling water-bath or in steam at 100°C.
- Filter the molten agar immediately upon removal from heating through multi-layer surgical gauze; adjust to pH 7,4.

- Add 2,5 mL of freshly prepared 1% solution of neutral red and 10 g of lactose and 10 g of proteose peptone.
- Mix well and distribute in 200 mL amounts in screw-capped bottles.
- Sterilize by heating in steam at 100°C for 1 hour, followed by autoclaving at 110°C for 10 minutes.
- Tighten the caps, record batch, label, and store at 4°C.

Preparation of plates:

- Melt 200 mL of the base medium and cool to about 80°C.
- Add aseptically 10 mL of solution 1 and the appropriate volume of solution 2 (as indicated in the following procedure on titration) using two different pipettes, mix well after each dilution.
- Distribute into sterile Petri dishes. The medium must cool rapidly, otherwise it may become too soft for good isolation.

Titration of sodium desoxycholate:

- Melt 7 bottles of the base medium and label from 6 to 12.
- Add 10 mL of solution 1 to each bottle.
- Add respectively 6, 7, 8, 9, 10, 11, and 12 mL of solution 2 to the bottles 6, 7, 8, 9, 10, 11 and 12.
- Mix well and pour plates (label plates with the same numbers as the bottles).
- Select the plates that give the best growth of *Salmonella* and *Shigella*. Record the volume of solution 2 used.

NOTE: - pH is critical. If pH is lower than 7,0, bile salts will begin to precipitate.

Store medium in the dark.

This medium is selective for *Salmonella* and *Shigella*. *Salmonella* organisms produce raised colourless or translucent colonies. *Shigella* organisms produce opaque ground-glass colonies. It must be remembered that other non-lactose-fermenting organisms will grow on DCA, and these have to be differentiated from *Salmonella* and *Shigella* by biochemical tests.

Quality control

Growing test organisms:	*Salmonella typhimurium*
	Shigella sonnei
Non-growing test organism:	*Enterococcus faecalis*

8.13 DNAse agar

The medium is used for the identification of DNAse positive microorganisms, and in particular *Staphylococcus aureus* and *Serratia marcescens*. These microorganisms hydrolyse DNA that has been added as supplement. The hydrolysis of DNA can be detected as a clear zone surrounding the colonies compared to the opaque appearance of the plate. The detection is improved by flooding the plate after incubation with 1,0 mol/L HCl.

Composition

Casein peptone	20,0 g
Deoxyribonucleic acid	2,0 g
NaCl	5,0 g
Agar	12,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,3 ±0,1.

Preparation

- Dissolve12 g of agar in distilled water while heating.
- When the medium has cooled to 50-55°C, add the other ingredients and autoclave at 121°C for 15 minutes.
- Dispense in sterile Petri dishes.

Store the plates at 2 8°C in sealed plastic bags to prevent loss of moisture.
Shelf-life: 3-4 weeks when stored in plastic bags providing there is no change in the appearance of the medium to suggest contamination or deterioration.

Quality control

Growing test organisms:	*Staphylococcus aureus*
	Serratia marcescens
Non-growing test organisms:	*Staphylococcus epidermidis*
	Klebsiella pneumoniae

8.14 Elek's base medium

Elek's medium is used for toxigenicity testing of *Corynebacterium diphtheriae* by the Elek's test.

The medium is prepared in two parts, A and B.

Composition and preparation

Part A

Meat peptone	20,0 g
Maltose	3,0 g
Lactic acid	0,7 mL
Distilled H_2O	500 mL

- Mix the ingredients in 500 mL distilled water.
- Add 3,25 mL of 40% sodium hydroxide.
- Heat to boiling to precipitate the phosphates, which is essential for the medium to work satisfactorily.
- Cool and filter through Whatman No. 113 v filter paper.
- Adjust pH to 7,8 with 1 molar hydrochloric acid.

Part B

Agar	10,0 g
NaCl	5,0 g
Distilled H_2O	500 mL

- Dissolve the agar and NaCl in 500 mL of boiling water, cool and adjust to pH 7,8.

Equal volumes of part A and B are mixed and filtered while molten through Whatman No. 113 v filter paper. Distribute in 15 mL volumes in screw-capped tubes or bottles and sterilize by autoclaving at 115°C for 10 minutes.

For use, melt 15 mL of the agar, cool to 50°C and add 3,5 mL of sterile horse or calf serum from a batch periodically checked in the Elek test. Pour into a 9 cm Petri dish and allow to solidify.

8.15 Eosin methylene blue lactose sucrose agar

Eosin methylene blue agar is recommended for the isolation of gram-negative enteric bacteria. The ingredients lactose and sucrose allow the differentiation of lactose and sucrose negative *Salmonellae* and *Shigellae* from lactose positive coliform bacteria and from lactose negative, but sucrose positive commensal bacteria (*Proteus, Citrobacter, Aeromonas*). The growth of unwanted microbes, such as gram-positive bacteria, is inhibited by the stains.

Composition of base

Peptone	10,0 g
K_2HPO_4	2,0 g
Lactose	5,0 g
Sucrose	5,0 g
Agar	13,5 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,2 ±0,2

Preparation

- Dissolve 13,5 g of agar in distilled water while heating.
- Add the other dry base ingredients.
- Add 10 mL of 4% (w/v) of eosin Y (CI 15380) and 10 mL of 0,7% (w/v) methylene blue (CI 52015).
- Autoclave at 121°C for 15 minutes.

Quality control

Growing test organisms:	*Staphylococcus aureus*
	Pseudomonas aeruginosa
	Escherichia coli

8.16 Kligler's iron agar

This is a differential medium for the identification of the gram-negative flora from stool cultures. Similarly as in triple sugar iron agar, carbohydrates are metabolized and acidify the medium. The acidification is indicated by the colour change of phenol red from red-orange to yellow, while alkalinization is indicated by a colour change to dark red. Thiosulfate is reduced by some bacteria to sulfide which precipitates iron salts as black iron sulfide.

Composition

Meat extract	3,0 g
Meat peptone	5,0 g
Yeast extract	3,0 g
Casein peptone	15,0 g
NaCl	5,0 g
Lactose	10,0 g

Glucose	1,0 g
Ferrous sulfate ($FeSO_4 . 7 H_2O$)	0,2 g
Sodium thiosulfate ($Na_2S_2O_3 . 5 H_2O$)	0,3 g
Agar	12,0 g
Distilled H_2O	1000 mL

Preparation

- Dissolve 12 g of agar in 1 litre of distilled water while heating.
- Cool to 60°C and add the other ingredients; mix well.
- Add 5 mL of a phenol red solution (0,5%,w/v).
- Distribute in screw-cap tubes, in 5-6 mL amounts and sterilize by autoclaving at 121°C for 15 minutes.
- Allow the medium to cool and set with a slant of 2,5 cm and a butt 2,5 cm deep.
- Record batch, label, and store at room temperature not exceeding 25°C.

Quality control

Growing test organisms:

glucose fermenters:	*Salmonella typhi*
	Proteus vulgaris
	Shigella flexneri
glucose and lactose fermenters:	*Escherichia coli*
	Enterobacter aerogenes
	Klebsiella pneumoniae
neither glucose nor lactose fermenters:	*Pseudomonas aeruginosa*

8.17 Löwenstein-Jensen medium (base)

Löwenstein-Jensen medium is used for the growth, isolation, transfer and storage of strains of *Mycobacterium tuberculosis* and other *Mycobacteria*. The medium is based on eggs in its formula. When it is heated it becomes sufficiently solid to be inoculated. Secondary contaminating flora are inhibited by malachite green.

Composition

L-asparagine	6,0 g
Potato starch	50,0 g
KH_2PO_4	4,2 g
$MgSO_4 . 7 H_2O$	0,4 g
Magnesium citrate	1,0 g

Distilled H_2O	1000 mL
	pH: 7,0+/- 0,2 at 25°C.

Preparation

- Dissolve 50 g of potato starch in 1 litre of distilled water.
- Cool to 60°C and dissolve the other ingredients.
- add 33 mL of 2% (w/v) malachite green (CI 42000) solution.
- Add 20 mL of glycerol. (This step is omitted for a medium to cultivate bovine bacilli).
- Slowly bring to boiling with stirring.
- Continue boiling for 2 minutes.
- Sterilize in an autoclave at 121°C for 15 minutes.

Instructions for use

- Cool to 48°C.
- Aseptically add 1,6 litre of a homogeneous mixture of egg whites and yolks, without introducing air.
- Slowly add the mixture to the base and homogenize perfectly.
- Distribute in sterile screw-capped tubes. Slant the tubes on an appropriate support.
- Allow the medium to coagulate at 85°C for 45 minutes. Cool in the inclined position.
- Inoculate by streaking.
- Incubate at 35°C for 2 to 12 weeks with the caps not tightly closed.

NOTE:

1. The colour of the medium should be pale green.
2. The presence of egg yolk particles is characteristic.
3. The presence of yellow or blue zones around these particles is a sign of contamination. Tubes with this feature are to be discarded.
4. The complete medium can be stored at 4°C in darkness for several days. It must be warmed to room temperature before inoculation.

Quality control

Growing test organisms:	*Escherichia coli*
	Mycobacterium tuberculosis
Non-growing test organism:	*Staphylococcus epidermidis*

Agar base	13,5 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,1 ±0,2.

Preparation

- Follow the same procedure as described above .
- Add 10 mL of neutral red (CI 50040) solution (0,5% w/v) and 10 mL of crystal violet (CI 42555) solution (0,01% w/v) in distilled water.
- Slowly bring to boiling for 30 minutes, until complete dissolution.
- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.

c) MacConkey agar without electrolytes and without crystal violet

Selective agar on which the swarming of *Proteus* species is suppressed. The medium is recommended for urine examination.

Composition

Gelatin peptone	17,0 g
Casein/meat peptone (50/50)	3,0 g
Lactose	10,0 g
Bile salts	5.0 g
Agar	13,5 g
Distilled H_2O	1000 mL

Preparation

- Follow the same procedure as described above.
- Add 10 mL of neutral red (CI 50040) solution (0,5% w/v).
- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.

Quality control

Growing test organisms:	*Enterococcus faecalis*
	Staphylococcus aureus

8.20 Mannitol salt agar

Mannitol salt agar is a selective medium for the detection and enumeration of pathogenic *Staphylococci*. The high sodium chloride concentration inhibits the growth

of most other bacteria. Mannitol fermentation, characterized by the colour change of phenol red to yellow, is used for differential diagnosis. Pathogenic *Staphylococci* form pigmented colonies, surrounded by a yellow halo resulting from the fermentation of mannitol. Non-pathogenic *Staphylococci* generally form small red colonies that do not change the colour of the medium.

Several strains of *Staphylococcus epidermidis* can ferment mannitol.

Composition

Casein peptone	5,0 g
Meat peptone	5,0 g
Meat extract	1,0 g
Mannitol	10,0 g
NaCl	75,0 g
Agar	15,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,4 ±0,2.

Preparation

- Dissolve 15 g of agar in 1 litre of distilled water while heating.
- Add 10 mL of water-soluble phenol red solution (0,25% w/v).
- Add the other ingredients under stirring until complete dissolution.
- Dispense in tubes or flasks. Sterilize in an autoclave at 121°C for 15 minutes

Quality control

Growing test organisms:

Mannitol fermenting:	*Staphylococcus aureus*
Mannitol non-fermenting:	*Staphylococcus epidermidis*
Non-growing test organisms:	*Escherichia coli*
	Streptococcus pyogenes

8.21 Modified Thayer-Martin medium

Thayer-Martin medium is used for the selective isolation of *Neisseria gonorrhoea* and *Neisseria meningitides*. It consists of a special basal medium enriched with haemoglobin and yeast supplement, and made selective by the addition of antibiotic cocktail, to suppress growth of saprophytic *Neisseria* spp. and other contaminating bacteria. The original medium of Thayer-Martin contains vancomycin, colistin and nystatin. Although widely used, this medium has been criticised because 3% to 10% of gonococcal

strains are inhibited by vancomycin. A modified Thayer-Martin medium using lincomycin in place of vancomycin avoids the problem of sensitivity to vancomycin.

Composition

Base medium:

Casein/meat peptone (50/50)	15,0 g	
Glucose		1,0 g
Corn starch		1,0 g
KH_2PO_4		4,0 g
K_2HPO_4		1,0 g
NaCl		5,0 g
Agar		12,0 g
Distilled H_2O		900 mL

Alternative base medium:

Columbia agar base (available from Unipath)

Selective supplements:

A variety of supplements can be used depending upon local conditions. These supplements can be home made or are available commercially (Becton Dickenson, Difco, Unipath).

VCNT supplement		VCAT supplement	
Vancomycin	1,0 mg	Vancomycin	1,0 mg
Colistin	300 000 U	Colistin	300 000 U
Nystatin	12,5 U	Amphotericin B	0,5 mg
Trimethoprim	5,0 mg	Trimethoprim	5,0 mg

LCAT supplement	
Lincomycin	0,5 mg
Colistin	300 000 U
Amphotericin B	0,5 mg
Trimethoprim	5,0 mg

All antibiotic concentrations are expressed per millilitre of water. The addition of 1 mL of each antibiotic concentration of the appropriate supplement to 1000 mL of base medium will produce the correct final concentration.

Lysed blood

Commercially available lysed blood can be used. If commercial lysed blood is not available, lysed defibrinated blood by heating at 55 to 56°C for at least one hour.

Preparation of final medium

- Dissolve base ingredients of the base medium in 900 mL of distilled water. Sterilize at 121°C for 15 minutes.
- Cool to 60°C and add 100 mL of lysed blood.
- Add 1 mL of each antibiotic in the chosen supplement.
- Mix well and pour into Petri dishes.

Quality control

Growing test organisms:	*Neisseria gonorrhoeae*
	Neisseria meningitidis
Partially non-growing test organism:	*Staphylococcus epidermidis*

8.22 Motility indole urea (MIU) medium

MIU semi-solid medium is used to differentiate *Enterobacteriaceae* by their motility, urease, and indole reactions.

Composition

Casein peptone	30,0 g
KH_2PO_4	1,0 g
NaCl	5,0 g
Agar	4,0 g
Phenol red (0,25%) solution	2,0 mL
Distilled H_2O	1000 mL

Preparation of the phenol red solution:

- To prepare 0,25% (w/v) solution of phenol red, dissolve 0,25 g of phenol red in 50 mL of absolute ethanol and add 50 mL of distilled water; mix well.

Preparation of complete medium:

- Mix the dry ingredients in 1 litre of water and heat to 100°C to dissolve (place the flask in a container of boiling water).
  Allow to cool to 50-55°C and then add 2 mL of a 0,25% phenol red solution. Mix well.
- Dispense in 95 mL amounts in screw-cap bottles. Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes. When the medium has cooled, tighten the bottle caps.

- Date the medium and give it a batch number. Store in a cool, dark place. The medium can be stored for 6 months or longer, provided there is no change in the appearance to suggest contamination or alteration of pH.

To make 20 tubes of MIU medium:

- Melt 95 mL of the base medium by placing the bottle in a container of water and heating to 100°C.
- Cool to 50-55°C. Add aseptically 5 mL of sterile 40% w/v urea solution. Mix well.
- Dispense aseptically in 5 mL volumes in sterile screw-cap tubes. Allow to cool in an upright position. For use, place an indole detection paper strip in the neck of the tube. Use the bottle cap to hold the paper in place.

Preparation of urea solution

Dissolve 20 g of pure urea in 50 mL of sterile distilled water and filter to sterilize. pH at room temperature: 7,1 ±0,2.

Performance:

- Test the medium for its urease reaction by inoculating with a known urease producing organism e.g. *Proteus* species, and a known non-urease producing organism e.g. *Salmonella* species.
- Test for motility by inoculating the medium with a *Salmonella* sp. (motile) and with a *Shigella* sp. (non-motile).
- Store the tubes in a cool dark place or at 2-8°C with the caps tightly screwed.

Shelf-life: Up to 6 weeks or more providing there is no change in the volume or appearance of the medium to suggest contamination or alteration of pH.

8.23 Mueller-Hinton broth

The broth is used for determination of the mean inhibitory concentration (MIC) of antibiotics in a dilution series.

Composition

Meat extract	2,0 g
Casein peptone, acid digest	17,5 g
Soluble starch	1,5 g
Distilled water	1000 mL
	pH at 25°C: 7,4 ± 0,2.

Preparation

- Dissolve the ingredients in 1 litre of distilled water while heating.
- Mix and dispense 2 mL in small tubes.
- Sterilize at 121°C for 15 minutes.

Quality control

Growing test organisms: *Escherichia coli*
Pseudomonas aeruginosa
Staphylococcus aureus
Enterococcus faecalis

8.24 Mueller-Hinton agar

Mueller-Hinton agar is the widely used culture medium for antibiotic susceptibility testing by the Kirby-Bauer diffusion method as recommended by the NCCLS. The medium can also be modified to Mueller-Hinton blood or Mueller-Hinton chocolate agar.

Composition

Mueller-Hinton base:	
Meat infusion extract	2,0 g
Casein peptone, acid digest	17,5 g
Cornstarch	1,5 g
Agar	13,0 g
Distilled water	1000 mL
	pH at 25°C: 7.3 ± 0.2.

Preparation

- Add the ingredients of the Mueller-Hinton base to 1 litre of hot distilled water. Sterilize at 115°C for 15 minutes.

NOTE:

1. The corn starch acts as a protective agent against toxic materials present in the medium.
2. The agar should contain low levels of thymine and thymidine that inhibit sulfonamide activity.
3. The concentration of Ca^{++} and Mg^{++} should be 50-100 mg/L and 20-35 mg/L, respectively, to obtain appropriate aminoglycoside activities.

These components must be controlled in the raw materials.

Quality control

Growing test organisms: *Staphylococcus aureus*
Streptococcus pyogenes
Pseudomonas aeruginosa
Escherichia coli

Preparation of Mueller-Hinton chocolate agar

- Add the ingredients of the Mueller-Hinton base to 1 litre of hot distilled water. Sterilize at 115°C for 15 minutes.
- Cool to 80°C and add 50 mL of sterile horse blood. Mix thoroughly.
- Mix well and pour into plates.

The prepared culture plates can be stored at 4°C in sealed plastic bags for 3 weeks.

8.25 Peptone water medium

This is used for non-selective enrichment of bacteria, particularly of pathogenic *Enterobacteriaceae*. The medium provides optimal conditions for intensive growth of bacteria even when they are partially changed.

Composition

Meat peptone	10,0 g
NaCl	5,0 g
Distilled H_2O	1000 mL

Preparation

- Dissolve the meat peptone and NaCl by heating in the water. Adjust to pH 8,0-8,4 and boil for 10 minutes.
- Filter through Whatman No. 1 filter paper. Adjust to pH 7,2-7,4.
- Distribute in volumes as required, in screw-capped bottles or tubes.
- Sterilize in the autoclave at 121°C for 15 minutes.

Alkaline peptone water:

- Add ingredients to the water and adjust pH to 9,0-9,2 with a concentrated sodium hydroxide solution.
- Distribute and autoclave at 121°C for 15 minutes. Store the alkaline medium in bottles with tightly screwed caps to prevent a drop in pH.

Buffered peptone water:

- Add 9,0 g disodium hydrogen phosphate and 1,5 g monopotassium dihydrogen phosphate to the above formula for peptone water; pH: 7,2 ± 0,2.

Reduced peptone water:

- Add sodium thioglycollate 0,5 g and L-cystine 0,25 g to the above formula for peptone water.

NOTE: See also Andrade's peptone water carbohydrate medium.

Quality control

Growing test organisms: *Escherichia coli*
Salmonella typhimurium
Aeromonas hydrophila

8.26 Purple lactose serum agar

Purple lactose serum agar is used for the isolation and culture of *Staphylococci*, *Enterococci* and *Enterobacteriaceae*. Lactose fermentation causes the bromocresol purple to turn yellow.

Composition of base medium

Meat peptone	20,0 g
NaCl	5,0 g
Agar	15,0 g
Distilled H_2O	1000 mL

Preparation

- Dissolve the meat peptone, NaCl and agar in distilled water by boiling. Allow to cool to 80°C.
- Add 20 g lactose, mix to dissolve.
- Add 10 mL of 0,3% (w/v) bromocresol purple and 5 mL of 0,5% (w/v) phenol red solution.
- Cool to 50°C and adjust to pH 7,4-7,8.
- Add 70 mL of sterile horse serum, mix well and pour on plates.

Quality control

Growing test organisms:	
lactose fermenters:	*Escherichia coli*
	Staphylococcus aureus
	Streptococcus faecalis
lactose non-fermenters:	*Pseudomonas aeruginosa*

8.27 Sabouraud dextrose broth

Sabouraud dextrose broth is recommended for the detection of yeasts and moulds, as well as for sterility tests in pharmaceutical preparations. The broth provides optimal conditions for growth of yeasts with its high concentration of carbohydrates. The low pH 5,6 prevents growth of bacteria other than acidophilic.

Composition

Casein peptone	5,0 g
Meat peptone	5,0 g
Glucose	20,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 5,6 ±0,2.

Preparation

- Dissolve the ingredients in 1 litre of distilled water.
- Slowly stir until complete dissolution.
- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.

Quality control

Growing test organisms:	*Candida albicans*
	Aspergillus niger
Non growing test organism:	*Escherichia coli*
	Staphylococcus aureus

8.28 Selenite faecal broth

This is used for selective enrichment of *Salmonella* in stool and urine. Selenite inhibits growth of gram-positive bacteria, coliform bacteria and *Enterococci* during the first 12 hours of culture. Growth of *Salmonella*, *Proteus* and *Pseudomonas* is not inhibited.

Composition

Sodium hydrogen selenite[1]	4,0 g
Casein/meat peptone (50/50)	5,0 g
Lactose	4,0 g
Na_2HPO_4	9,5 g
NaH_2PO_4	0,5 g
Distilled H_2O	1000 mL

[1]**NOTE:** Sodium hydrogen selenite is toxic and teratogenic. Care must be taken not to inhale the powder.

Preparation

- Dissolve 4 g of sodium hydrogen selenite in 1000 mL deionized water in a flask at 80°C.
- Add the other ingredients in the order given. The pH should be 7,1 ±0,1; the correct reaction may be obtained by adjustment of the phosphates; a pH near neutral is necessary for effective inhibition of *E. coli*.
- Distribute in 10 mL amounts into test-tubes (16 x 150 mm) or screw-capped bottles.
- Sterilize by heating to boiling or steam at 100°C for no longer than 20 minutes. Do not autoclave.
- Tighten screw caps after boiling, label and store in a cool place.

The final medium is a pale yellow colour and may contain a slight red precipitate. If a brown precipitate is present, it is indicative of overheating. This brown precipitate may develop on storage. Medium showing brown precipitate may be toxic and should be discarded.

Quality control

Growing test organism:	*Salmonella typhimurium*
Non-growing test organism:	*Escherichia coli*

8.29 Simmons citrate agar

Test agar for the identification of *Enterobacteriaceae* and certain yeasts with citrate as the only energy source. The consumption of citrate by the microorganisms shifts the pH of the medium into the alkaline range. This is indicated by the change of colour of the pH indicator, bromothymol blue, to deep blue.

Composition

$(NH_4)H_2PO_4$	1,0 g
K_2HPO_4	1,0 g
NaCl	5,0 g
$MgSO_4 . 7 H_2O$	0,2 g
Agar	13 g
Distilled H_2O	1000 mL
	pH at 25°C: 6,6 ±0,1.

Preparation

- Dissolve 13 g of agar in 1 litre distilled water.
- Add the salt ingredients under stirring.
- Add 10 mL of bromothymol blue solution (0,8% w/v) in distilled water.
- Sterilize at 121°C for 15 minutes.
- Pour into culture plates or prepare slopes in bottles or tubes.

NOTE: The addition of 10 g/L inositol prior to autoclaving provides a medium for the culture of *Klebsiella* spp.

8.30 Stuart's transport medium

This medium is used for conservation and transport of pathogenic microorganisms like *Neisseria*, *Trichomonas*, *Haemophilus*, *Pneumococci*, *Streptococci*, *Salmonella*, *Shigella* and others, without supporting their growth but sustaining their life (see also Amies transport medium, Section 8.3).

Composition

Sodium thioglycollate	0,5 g
Sodium glycerophosphate	10,0 g
$CaCl_2 . 2H_2O$	0,1 g
Cysteine hydrochloride	0,5 g
Agar	5,0 g
Distilled water	1000 mL
	pH at 25°C: 7,4 ±0,2.

Preparation

- Dissolve 5 g of agar in 1 litre distilled water while heating.
- Cool to 50°C and dissolve the other ingredients under stirring.

- Add 1 mL of the methylene blue solution (0.2% w/v).
- Add 10 g of neutral charcoal powder under stirring.
- Dispense 5 to 6 mL into vials with screw cover.
- Autoclave at 121°C for 20 minutes.
- Invert the tubes prior to solidification in order to distribute the charcoal uniformly.
- Store in the refrigerator.

A blue coloration of the stored medium indicates that it is aerated and unfit for use. The air present may be removed by resteaming with the screw cap loosened.

Quality control

Growing test organisms: *Streptococcus pyogenes*
Staphylococcus aureus
Escherichia coli

8.31 Tellurite blood agar

A. Tellurite blood agar, Hoyle's (TBA)

Tellurite blood agar is a highly selective medium for the cultivation of *Corynebacterium diphtheriae*. In this medium tellurite reducing microorganisms appear as grey-to-black colonies while growth of commensal organisms is at least partially inhibited.

Composition

(a) Base medium

Casein/meat peptone (50/50)	20,0 g
Glucose	2,0 g
NaCl	5,0 g
Agar	15,0 g
Distilled water	1000 mL

- Dissolve the ingredients by boiling, cool and adjust to pH 7,8 ±0,1.
- Distribute in screw-capped bottles in amounts suitable for local requirements and sterilize at 121°C for 15 minutes.

(b) Potassium tellurite solution

Potassium tellurite	35,0 g
Distilled H_2O	1000 mL

- Dissolve the potassium tellurite in sterile distilled or deionized water.

The solution is self-sterilizing and is stable for up to 6 months at room temperature in temperate climates but store at 4°C in higher temperatures.

(c) Lysed horse, bovine or sheep blood

- Make up a 10% solution of saponin in distilled or deionized water.
- Sterilize in the autoclave at 121°C for 15 minutes.
- Add 0,5 mL of the saponin solution to 10 mL of whole blood, mixing gently to avoid formation of air bubbles.
- Incubate at 35-36°C for 30 minutes. Store at +4°C.

Preparation

- Melt 1 litre of the base medium, cool to 50-55°C.
- Add 10 mL of the 3,5% potassium tellurite solution (final concentration 0,035%). Mix well without shaking.
- Add 50 mL of the lysed blood, mix well without shaking.
- Pour plates (about 12-15 mL per 9 cm Petri dish). Dry the surface before use.

The medium is stable and can be stored at room temperature in temperate climates but at +4°C in higher temperatures. Plates should be stored in containers or plastic bags to prevent excessive drying. The ability of the medium to support growth of *C. diphtheriae* and to produce typical colony morphology should be checked by plating out the *C. diphtheriae* biotypes on each batch prepared. Blood lysed with saponin may become toxic on storage. Therefore, once lysed it should be used within 1 to 2 hours. Unused lysed blood should be discarded.

B. Tellurite blood agar, Downie (TBA)

Composition

- Columbia agar base medium
  Alternatively Hoyle's base medium as described above can be used.
  2,0% potassium tellurite solution
- Dissolve 20 g of potassium tellurite in 1 litre sterile distilled water.

The solution is self-sterilizing and is stable for up to six months at room temperature in temperate climates or at +4°C in higher temperatures.

- Whole blood

Defibrinated sheep blood is recommended but horse or bovine blood may also be used. Best results are obtained by using rabbit or guinea pig blood, but it is most unlikely that these would be obtainable in large enough quantities.

Preparation

- Melt 1 litre of the base medium, cool to 50-55°C.
- Add 20 mL of the 2% potassium tellurite solution. Mix well without shaking.
- Add 50 mL of whole blood. Mix well without shaking.
- Pour plates (about 12-15 mL per 9 cm Petri dish). Dry the surface before use.

Plates should be stored for not more than seven days at +4°C.

Quality control

Growing test organisms: *Corynebacterium diphtheriae*
Escherichia coli

8.32 Tellurite taurocholate gelatin agar (TTGA: Monsur's)

Tellurite taurocholate gelatin agar is used for the isolation and culture of *Vibrio cholerae* and other enteropathogenic *Vibrio* spp. The strong alkalinity inhibits growth of *Enterobacteriaceae*. Taurocholate prevents growth of *Enterococci*.

Composition

Casein peptone	10,0 g
NaCl	10,0 g
Sodium taurocholate	5,0 g
Na_2CO_3	1,0 g
Gelatin	30,0 g
Agar	15,0 g
Distilled H_2O	1000 mL

Preparation

- Dissolve all ingredients in water with heating. Adjust the pH to 8,5.
- Dispense the measured volumes in screw-capped bottles and sterilize at 121°C for 15 minutes.
- Before use, add 0,5 to 1 mL of a filter-sterilized 1% aqueous solution of potassium tellurite. Mix well and pour on to plates.

Quality control

Growing test organisms: *Vibrio cholerae*
Vibrio parahaemolyticus

8.33 Thioglycollate broth

Thioglycollate broth is used to culture anaerobes in blood. The reducing agents thioglycollate and cysteine inactivate heavy metal ions, which might otherwise inhibit growth, and provide good anaerobic conditions for fastidious anaerobes. The indicator resazurin (1mg/L) is added to indicate a possible increase of oxygen concentration by a change of colour to red.

Composition

Yeast extract	5,0 g
Casein peptone	15,0 g
Glucose	5,5 g
Sodium thioglycollate	0,5 g
NaCl	2,5 g
L-cystine	0,5 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,1 ±0,2.

Preparation

- Dissolve the ingredients in distilled water while heating.
- Dispense the well mixed medium in 50 mL amounts in bottles fitted with screw-caps that have a central hole and rubber liner.
- Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes.
- When cool, tighten the bottle caps. Cover each bottle top with a foil cap, or other protective covering (previously soaked in 70% v/v ethanol). Label the bottles. Date the medium and give it a batch number.
- Store in a cool dark place.

Shelf-life: Up to 2 years, providing there is no change in the volume or appearance of the medium to suggest oxidation or contamination.

If at the time of use more than a narrow band at the surface of the medium appears pink, this indicates oxidation and the broth should not be used. It should be reheated by placing the bottle in a container of boiling water for about 15 minutes (with bottle cap loosened) to drive off the dissolved oxygen.

Quality control

Growing test organisms:	*Staphylococcus aureus*
	Streptococcus pyogenes
	Candida albicans

8.34 Thiosulfate citrate bile salts sucrose agar (TCBS)

TCBS agar is used for the isolation and selective culture of *Vibrio cholerae* and other enteropathogenic *Vibrio* spp. High concentrations of thiosulfate and citrate as well as the alkalinity of the medium, inhibit growth of *Enterobacteriaceae* extensively. Ox bile and cholate particularly inhibit growth of *Enterobacteriaceae*. Coliform bacteria that may possibly grow do not metabolize sucrose. However, some sucrose positive *Proteus* spp. can form vibrio-like colonies. The indicator mixture of bromothymol blue and thymol blue distinctly changes to yellow even in alkaline pH when acids are formed from sucrose metabolism. The TCBS agar is superior to other tellurite media used for culture of *V. cholerae.*

Composition

Yeast extract	5,0 g
Casein/meat peptone (50/50)	10,0 g
Sucrose	20,0 g
Sodium thiosulfate ($Na_2S_2O_3$. 5 H_2O)	10,0 g
Sodium citrate	10,0 g
Ox bile	8,0 g
Ferric citrate	1,0 g
NaCl	10,0 g
Bromothymol blue (0,2% solution)	20,0 mL
Thymol blue (1%) solution	4,0 mL
Agar	15,0 g
Distilled H_2O	1000 mL

Bromothymol blue solution:

- Add 2,5 mL of 0,1 mol/L NaOH to 47,5 mL water. Then add 0,1 g of bromothymol blue.

Thymol blue solution:

- Add 2,2 mL of 0,1 mol/L NaOH to 7,8 mL of water. Then add 0,1 g of thymol blue.

Preparation

- Add all ingredients to 980 mL of distilled water.
- Heat to boiling with agitation to obtain complete solution. Do not autoclave.
- Cool to 45-40°C, adjust pH to 8,6 ±0,2 and pour out 15-20 mL volumes on to Petri plates.

Quality control

growing test organism: *Vibrio cholerae*

8.35 Triple sugar iron agar (TSI)

TSI agar is used to identify *Enterobacteriaceae*. It rapidly detects the fermentation of lactose, of glucose (with or without gas production), of sucrose and the production of hydrogen sulfide. Alkalinization is shown by a deep red colour. Hydrogen sulfide production from thiosulfate is detected by a black colour due to iron sulfide, formed in the presence of ferric citrate. TSI agar provides the following information:

(1) Glucose fermentation:

- Butt red: glucose not fermented (glucose -)
- Butt yellow: glucose fermented (glucose +)

(2) Fermentation of lactose and/or sucrose:

- Slant red: lactose and sucrose not fermented (lactose -, sucrose -)
- Slant yellow: lactose and/or sucrose fermented (lactose and/or sucrose +)

(3) Gas production:

- Gas bubbles in the butt.

(4) H_2S formation:

- Formation of a black colour between the butt and the slant or along the stab.

Composition

Casein peptone	14,0 g
Yeast extract	3,0 g
Meat extract	3,0 g
Glucose	1,0 g
Lactose	10,0 g
Sucrose	10,0 g
NaCl	5,0 g
Sodium thiosulfate ($Na_2S_2O_3$. 5 H_2O)	0,3 g
Ammonium ferric citrate	0,2 g
Agar	12,5 g
Distilled H_2O	1000 mL
	pH: at 25°C. 7,4 ± 0,2.

Preparation

- Suspend the ingredients in 1 litre of distilled or deionized water.
- Slowly bring to boiling, stirring until complete dissolution.
- Add 10 mL of water-soluble phenol red solution (0,25% w/v).
- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes. Incline the tubes to obtain a 3 cm high butt and a slant.

NOTE: If the medium is not used within one week of its preparation, it is recommended to regenerate it in a boiling water-bath and let it solidify again in the correct position.

Quality control

	Slant	Butt	H_2S
Escherichia coli	acid	acid	-
Proteus mirabilis	acid	acid	+
Shigella sonnei	alkaline	acid	-
Salmonella enteridis	alkaline	acid	+

8.36 Tryptone soy agar (CASO agar; TSA)

Tryptone soy agar is a universal nutrient medium. As a result of its excellent nutritive value, the medium can be used to grow and isolate aerobic and anaerobic bacteria and also to grow very fastidious bacteria. It furnishes excellent results for determining the antibiotic sensitivity of bacteria. It is used in the cosmetics industry for enumerating bacteria by adding 5 g/L of Tween and 0,7 g/L of lecithin. The medium may also be used as a blood agar base and for the preparation of 'chocolate agar'.

Composition

Casein peptone	15,0 g
Soya peptone	5,0 g
NaCl	5,0 g
Agar	15,0 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,3 ±0,2.

Preparation

- Suspend the ingredients in 1 litre of distilled water.
- Slowly bring to boiling, stirring until complete dissolution.

- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.

NOTE: TSA supplemented with blood for the determination of hacmolysis may show differences in reaction depending on the origin of the blood (e.g. horse blood or sheep blood).

8.37 Tryptic soy broth (Caso broth; TSB)

This is used as a general purpose medium for growth of fastidious bacteria and fungi. TSB may be used for the cultivation of aerobic and facultative anaerobic microorganisms. It is particularly useful for the isolation of organisms from blood.

Composition

Casein peptone	17,0 g
Soya peptone	3,0 g
NaCl	5,0 g
K_2HPO_4	2,5 g
Glucose	2,5 g
Distilled H_2O	1000 mL
	pH at 25°C: 7,3 ±0,1.

Preparation

- Suspend the ingredients in 1 litre of distilled water.
- Mix well to dissolve and distribute in tubes. Sterilize at 121°C for 15 minutes.

8.38 Viral transport medium

Composition

Viral transport medium consists of Hank's balanced salt solution (HBSS) supplemented with:

10% (w/v) bovine serum albumin
1-2% sodium bicarbonate (7,5% solution) to adjust pH to 7,3.
10 mg% vancomycin
10 mg% gentamicin
40 mg% amphotericin B
(The composition is given in terms of final concentrations)

HANK's balanced salt solution (HBSS)

Composition:	
NaCl	8,0 g
KCl	0,4 g
$MgSO_4 . 7 H_2O$	0,1 g
Na_2HPO_4	0,048 g
$CaCl_2 . 2 H_2O$	0,185 g
$NaHCO_3$	0,35 g
KH_2PO_4	0,06 g
$MgCl_2 . 6 H_2O$	0,1 g
Glucose	1,0 g
Phenol red water soluble solution (1% w/v)	10 mL
Distilled H_2O	1000 mL

8.39 Wilkins-Chalgren agar

Wilkins-Chalgren agar is used for susceptibility testing of anaerobic bacteria. The advantage of this medium is that blood is not required to obtain satisfactory growth of anaerobic bacteria of clinical interest. The medium and the technique are described in the protocol used in "NCCLS Collaborative Study of the Proposed Reference Dilution Method of Antimicrobial Susceptibility Testing of Anaerobic Bacteria".

Composition

Casein peptone	10,0 g
Gelatin peptone	10,0 g
Yeast extract	5,0 g
NaCl	5,0 g
Glucose	1,0 g
L-arginine	1,0 g
Sodium pyruvate	1,0 g
Haemin	5,0 mg
Menadione	0,5 mg
Agar base	10,0 g

pH at 25°C: 7,1 ±0,2.

Preparation

- Dissolve 10 g of agar in 1 litre distilled water while heating.
- Cool to 60°C and add the other ingredients under continuous stirring.

- Dispense in tubes or flasks.
- Sterilize in an autoclave at 121°C for 15 minutes.

8.40 Antibiotic discs for susceptibility testing

Antibiotic discs are filter paper discs impregnated with an antimicrobial agent for bacterial susceptibility testing by the Kirby-Bauer diffusion test on agar medium. To obtain results that are comparable from day to day and from laboratory to laboratory the discs must be standardized, i.e. they must have a standardized size and be impregnated with a definitive amount of a single antibiotic. The antibiotic must retain its antimicrobial activity during the preparation and the subsequent storage of the discs. The quality of the antibiotic discs can be controlled and monitored by means of culture of reference strains of appropriate bacteria.

Materials
- Filter paper*
- Punch, diameter 6 mm
- Stoppered glass containers with silica

Equipment
- Stainless steel tray
- Micropipettes for 10 µL and 20 µL
- Pipette tips
- Incubator

Reagents
- Antibiotics
- Phosphate buffer (pH 6,5)
- Sodium hydroxide 0,01 mmol/L, pH 9
- Distilled water

Preparation and testing of filter paper discs

The determination of the absorption capacity of the filter discs must be known for the antibiotic solutions to be used for impregnation of the discs.

- Work under sterile conditions.
- Punch out discs with a diameter of 6 mm from a good quality filter paper or blotting paper or use already coded discs.

* Coded filter paper discs with the codes mentioned in Table 8.1 for susceptibility testing are available from Schleicher & Schuell GmbH, Germany.

- Determine the average dry weight of the discs by weighing a series of 20 discs on an analytical balance, and calculate the mean dry weight of the discs.
- Dip the disc into distilled water and weigh again.
- Subtract the dry weight from the wet weight of each disk to obtain the amount of absorbed water.

The average amount of absorbed water should be about 25 µL, if the discs are to be impregnated with 20 µL.

Preparation of antibiotic solutions

The antibiotics to be used for impregnation are normally industrial products which may vary in their antimicrobial activity. They can be supplied as chemicals with a 100 % purity or less. The degree of purity of the antibiotic must be indicated on the container or stated in the attached certificate of analysis. If the activity is less than 100%, the amount to be weighed in must be adjusted according to the given value. If a product labelled with a specific activity cannot be obtained as powder, an ampoule of the antibiotic can be used. In this case the total content of the ampoule must be used. It is important to remember that the content of ampoules can vary within certain limits.

Most antibiotics can be dissolved in distilled water. Beta-lactam antibiotics are preferably solubilized in phosphate buffer, pH 6,5. Nalidixic acid and some sulfonamides are dissolved at alkaline pH to obtain a clear solution. Table 8.1 lists the essential antibiotics that are recommended by WHO.

The preparation of a solution for impregnation of filter paper discs with a defined amount of an antibiotic must take into account the absorption capacity of the filter disc. The discs can be impregnated with 30-80% of the volume of distilled water that can be absorbed. Usual volumes are 10 or 20 µL of the antibiotic solution.

Example: The label of an antibiotic is given with an activity of 610 mg/g substance. This means that 1,64 g (= 1000 mg/610 (mg/g)) of the product has an antibiotic activity of 1 g pure substance. Thus if the disc is to be impregnated with 10 µg of pure substance, this corresponds to 16,4 µg of the available product. This quantity is to be applied in 20 µL of antibiotic solution for impregnation. Therefore the solution to be prepared must have an amount of 8,2 mg of the available product in 10 mL to obtain a concentration of 0,82 mg/mL.

Preparation of the antibiotic discs:

- Sterilize the coded filter paper discs for 1 hour at 160°C.
- Prepare the antibiotic solution at the appropriate concentration using distilled water, phosphate buffer or sodium hydroxide solution.

The final amount of the antibiotic to be impregnated in the disc is given in Table 8.1.

- Place the sterile discs in a stainless steel tray and dispense the appropriate volume of the prepared antibiotic solution with a micropipette onto each disc. Work under sterile conditions.

Table 8.1 Zone diameter limits for control strains [7]

Antibiotic	Code[1]	Disc Potency		Diameter of zone inhibition(mm)		
				S.aureus (ATCC 25923)*	*E.coli* (ATCC 25922)*	*P.aeruginosa* (ATCC 27853)*
Amikacin	AN	30	µg	20 - 26	19 - 26	18 - 26
Ampicillin	AM	10	µg	27 - 35	16 - 22	--
Benzylpenicillin	P	10	IU	26 - 37	--	--
Cefalotin	CF	30	µg	29 - 37	15 - 22	--
Cefalozin	CFZ	30	µg	29 - 35	23 - 29	--
Cefotaxime	CTX	30	µg	25 - 31	29 - 35	--
Ceftriaxone	CRO	30	µg	22 - 28	29 - 35	17 - 23
Cefuroxime	CXM	30	µg	27 - 35	20 - 26	--
Chloramphenicol	C	30	µg	19 - 26	21 - 27	--
Ciprofloxacin	CIP	5	µg	22 - 30	30 - 40	25 - 33
Clindamycin	CM	2	µg	24 - 30	--	--
Erythromycin	E	15	µg	22 - 30	--	--
Gentamicin	GM	10	µg	19 - 27	19 - 26	16 - 21
Nalidixic acid	NA	30	µg	--	22 - 28	--
Nitrofurantoin	FT	300	µg	18 - 22	20 - 25	--
Norfloxacin	NOR	10	µg	17 - 28	28 - 35	22 - 29
Oxacillin	OX	1	µg	18 - 24	--	--
Piperacillin	PIP	100	µg	--	24 - 30	25 - 33
Sulfafurazole[2]	I	300	µg	24 - 34	18 - 26	--
Tetracycline	TE	30	µg	19 - 28	18 - 25	--
Tobramycin	TM	10	µg	19 - 29	18 - 26	19 - 25
Trimethoprim	TMP	5	µg	19 - 26	21 - 28	--
Vancomycin	VA	30	µg	15 - 19	--	-

[1] Non-standardized codes

[2] As sulfonamide

*The reference stains can be obtained from:
American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md 20852-1776, USA.

- Dry the impregnated discs at 40°C. It is important that air should circulate freely across all surfaces of each disc to minimize the uneven precipitation of the antibiotic in the filter paper.
- After drying transfer the antibiotic discs to containers that can be tightly stoppered and contain a suitable desiccant with a moisture indicator such as silica gel.

Storage and stability of antibiotic discs

The stability of the discs will depend on the conditions of storage. Discs kept in a tightly closed container with a desiccant at -20°C are stable for at least 18 months, and at +4°C for 1 month. Working supplies can be kept in the refrigerator for up to 1 week.

NOTE: Remove the container from the freezer or refrigerator 1 to 2 hours before opening. The equilibration to room temperature before opening will minimize the water absorption on the discs.

Disc production control

The quality of antibiotic discs is most commonly controlled in an agar diffusion test system, i.e. as a relative assay. In the assay a newly prepared disc (X) is compared with carefully prepared standard discs. The content of the standard discs can be:

3 times the labelled content of the test disc (A)
1,5 times the labelled content of the test disc (B)
0,75 times the labelled content of the test disc (C)
0,375 times the labelled content of the test disc (D)

Testing

- Inoculate an agar plate with a control strain of a suitable microorganism. Control strains are listed in Table 8.1.
- Place the standard discs and test discs on each of 6 agar plates.
- Incubate the plates at 35°C for 16 hours.
- Measure the diameters of the zones of inhibition after incubation.
- Calculate the values for (A),(B),(C),(D) and (X).
- Plot the values on semilogarithmic paper and draw the standard curve from the values (A),(B),(C), and (D). Use the x-axis for the zone diameter and the y-axis (logarithmic scale) for the antibiotic content of the disc.

The content of antibiotic of the test disc (X) is determined from the standard curve. The control strains can be obtained from the American Type Culture Collection. They are treated like other pure cultures. When the procedure is correctly performed, zone

diameters obtained with the control organisms will fall within the range of diameters given in the table.

NOTE: WHO has recommended 75-135% of the labelled strength as acceptable limits of the disc content.

Uniformity test

The uniformity of discs is determined by placing 6 discs on each of 6 agar plates inoculated with a suitable microorganism. Incubate overnight and measure the zone diameters. The difference between the largest and smallest zone diameter must not exceed 2,5 mm.

8.41 References

[1] MacFaddin JF. *Media for isolation-cultivation-identification-maintenance of medical bacteria,* Baltimore, London, Williams & Wilkins, 1985.

[2] *Difco Manual,* 10th edition, Detroit, Difco laboratories, 1984.

[3] *The Oxoid Manual,* 6th edition, Basingstoke, Unipath Ltd., 1990.

[4] *Manual of BBC Products and Laboratory Procedures,* Cockeysville, MD, Becton Dickinson Microbiology Systems, 1988.

[5] *Bench level manual for sexually transmitted diseases,* WHO/VDT/89.443, Geneva, World Health Organization, 1989.

[6] *Manual for laboratory investigations of acute enteric diseases,* WHO/CDD/83.1 rev. 1, Geneva, World Health Organization, 1987.

[7] National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disc susceptibility tests. Approved standard, document M2-4A, Villanova, PA, NCCLS, 1990.

[8] National Committee for Clinical Laboratory Standards. Evaluating production lots of dehydrated Mueller-Hinton agar; proposed standard, document M 6 P, Villanova, PA, NCCLS, 1986.

9

REAGENTS FOR IMMUNOLOGY

9. Reagents for immunology

9.1 Introduction

Reagents for immunological tests are of biological origin, i.e. antibodies and antigens. The preparation of these cannot be described in a schematic way, since there is no standardized method: rather the preparation of each biological reagent raises its own problems. The preparation of specific biological reagents therefore implies ample experience. The basic techniques in immunology have been described in two Bench Manuals from WHO [1,2]. This section describes the preparation of latex and erythrocyte reagents and tests for the following analytes:

rheumatoid factor (RF)
human choriogonadotropic hormone (hCG)
antistreptolysin O (ASLO)
whole complement activity (C_{100})

In addition, some details on nitrocellulose membrane techniques for enzyme immunoassays on discs, strips and dip sticks are described.

9.2 Preparation of latex particle immunoreagents

Antigen or antibodies bound to a latex carrier agglutinate upon interaction with antibodies or antigens and thus serve to indicate their presence. Both agglutination and agglutination inhibition tests may be used.

A number of latex agglutination tests are commercially available such as the test for the detection of autoantibodies in rheumatoid arthritis, which is one of the most widely used. Patients with rheumatoid arthritis often develop antibodies against their own immunoglobulins. This anti-globulin antibody, known as rheumatoid factor (RF), is readily detected by its ability to agglutinate latex particles coated with IgG.

Further common applications of direct latex agglutination include tests for anti-nuclear factors and C-reactive protein. Inhibition testing for soluble antigen can be performed with antigen-coated latex-antibody systems, e.g. latex particles coated with hCG. Anti-hCG antibodies are used for testing urine containing hCG in case of pregnancy.

The spherical latex particle is a negatively charged polymer of styrene. In aqueous solution it behaves like a lyophobic colloid. It forms a relatively stable, chemically inert, colloidal suspension, which can be easily stored. The routine latex fixation test employs

particles between 0,5 and 10 μm, but preferably 0,5 to 1 μm in diameter. Latex particles are available from various companies (see Annex 4).

9.2.1 Synthesis of polystyrene latex

Reagents

Potassium persulfate, $K_2S_2O_8$, Mr 270,33
Sodium polyphosphate (sodium metaphosphate), $NaPO_3$, Mr. 101.96
Dodecylbenzene sulfonate (SDBS), $C_{19}H_{31}O_3S$ Na, Mr 362
Styrene, C_8H_8, Mr. 104,14

Preparation

- Boil distilled or deionized water for ten minutes to remove the dissolved oxygen and CO_2.
- Measure 1620 mL of the boiled water while it is hot and dissolve 1,8 g of potassium persulfate, 1,8 g of sodium polyphosphate and 10 mL of 0,1% SDBS.
- Pour the hot solution into a four-necked flask, and immediately add 207 mL of styrene. Keep the solution at 85°C under flowing nitrogen for 8 hours while constantly stirring and refluxing.
  Within 30 minutes the solution should rapidly change from colourless to turbid and milky colloid, indicating the beginning of polymerization.
- At the end of the eight hour period keep the polystyrene latex overnight at room temperature and then filter through Whatman No. 1 filter paper.

The filtrate contains the polystyrene particles with diameter about 0,8 μm. The dry solid amounts to about 10% (w/v). The latex can be stored at room temperature, but preferably at 4°C.

9.2.2 Synthesis of carboxylate-modified latex (CML)

Carboxylate-modified latex (CML) is a styrene-acrylic acid copolymer. It provides carboxylate residues at the particle surface which can be used for chemical binding or better adsorption of an immunoreagent. The procedure for preparation of CML is the same as described above except that 2,7 mL of acrylic acid is added to the reaction mixture after the addition of the styrene.

Standardization of latex particles:

The concentrated latex suspension must be diluted and standardized to serve as a stock suspension. The semi-solid latex is diluted in water to the point where 0,1 mL

mixed with 9,9 mL of water will match the light transmission of a standard barium sulfate solution[1] when examined in a spectrophotometer at 650 nm. The suspension then contains ca. 4,5 x 10^8 latex particles per millilitre. Alternatively, commercially prepared standard latex suspension can simply be diluted according to the manufacturer's instructions. The standardized latex stock suspension can be stored for several months at 4°C until used.

[1]**NOTE:** The standard barium sulfate solution is made by adding 3 mL of 0,05 mol/L barium chloride to 3 mL of a 0,335 mol/L sulfuric acid solution and heating at 56°C for 1 minute.

Polystyrene latex particles are unstable and tend to agglutinate between pH 5 and 8. Glycine buffer pH 8,2 is suitable for coating (see Section 9.2.4).

9.2.3. Factors influencing the preparation of immunoreagent latex particles

The preparation of latex particles includes anionic detergents, such as SDBS, in the reaction mixture. The relative amount of detergent remaining with the particles increases with decreasing particle diameter. Other impurities include salts and soluble monomers and polymers. Such impurities may interfere with ligand attachments. This is why latex particles must be cleaned of impurities. Methods for latex particle clean up include ion exchange cleaning, repeated centrifugation and washing, dialysis and ultrafiltration.

Latex particles stripped of detergent tend to agglutinate spontaneously due to the generally hydrophobic character of latex. Surface modified latex particles, such as CML particles, are more stable than plain polystyrene particles. Also, smaller particles are less stable than larger ones of the same surface type. The addition of small amounts of protein to cleaned latex suspensions can result in agglutination due to lowering of the particle surface charge potential. Adding ligand/buffer solution to latex suspensions immediately after cleaning will minimize problems due to spontaneous agglutination.

9.2.4 Preparation of latex reagent by adsorption of the biological reagent

SOLUTIONS

1. **Glycine buffer, 0,27 mol/L, containing 0,154 mol/L NaCl**

Glycine	20,25 g
NaOH (1 mol/L)	6,75 mL
NaCl	9 g

Distilled H_2O to 1000 mL
Adjust pH to 8,2.

2. **NaCl (0,154 mol/L)**

3. **Glycine buffer, 0,054 mol/L**
   Dilute 1 volume of glycine buffer (0.27 mol/L) with 4 volumes of NaCl (0,154 mol/L)

4. **Antigen or antibody solution**

Preparation

- Wash 800 µL latex suspension twice with 40 mL 0,054 mol/L glycine containing 0,154 mol/L NaCl, pH 8,2; mix and centrifuge at 12500 *xg* for 15 minutes.
- Resuspend the latex in 20 mL 0,054 mol/L glycine containing 0,154 mol/L NaCl and add 300 µL of a 10 mg/mL solution of antigen or antibody.
- Mix the suspension for 30 minutes at room temperature.
- Wash the latex two times by adding 40 mL 0,054 mol/L glycine containing 0,154 mol/L NaCl; centrifuge the latex 12500 *xg* for 15 minutes.
- Resuspend the latex in 20 mL 0,27 mol/L glycine containing 0,154 mol/L NaCl and 0,1% of an irrelevant protein such as bovine serum albumin to block any remaining protein-binding sites; store at 4°C.

NOTE: Different substances are adsorbed to the latex particles at different degrees. The amount of adsorbed material influences the stability of the latex system. Some proteins are not adsorbed and require covalent coupling with carbodiimide (see Section 9.2.5).

An example of a latex particle reagent with adsorbed antibodies for a rheumatoid factor (RF) reagent is described in Section 9.2.7.

Nonspecific agglutination of coated latex particles

Nonspecific agglutination is also a common problem in working with coated latex particles. If a ligand does not cover the particle surface, exposed hydrophobic areas of the latex particles can cause nonspecific agglutination reactions. The effect is especially pronounced in protein-rich samples, such as serum.

Nonspecific particle agglutination can be prevented by blocking the remaining areas on the particle surface after ligand binding with an inert protein, such as bovine serum albumin, gelatine, ovalbumin or casein, or the cautious use of surfactants. If surfactants are used for preventing nonspecific agglutination, they must be added in optimal amounts,

since excessive amounts may also inhibit the specific agglutination reaction. Nonionic surfactants are particularly useful for preventing nonspecific agglutination.

9.2.5 Preparation of latex reagents with chemically bound agent

The preparation of chemically bound biological agents may be preferred if the reacting agent cannot be firmly attached to latex particles by physical adsorption. Chemically bound antigens do not desorb from the particles unless their chemical attachment is disrupted. This can be avoided or at least be minimized by appropriate storage conditions. The biological agents must be chemically attached in such a way that they are neither denatured nor lose their immunological properties. This implies mild coupling conditions in an aqueous solution. Water soluble carbodiimides fulfil this requirement.

Carbodiimide activation of latex particles

Carbodiimides convert carboxyl groups to acylurea groups which readily bind to amino groups of proteins or other compounds. Proteins have free carboxyl groups which can react with any excess carbodiimide. This can lead to crossbinding (ligand-ligand or particle-ligand-particle) unless the carbodiimide is removed after activation of the latex. Carbodiimide activated carboxyl groups undergo hydrolysis and may only be able to react with proteins for 1 to 2 hours after activation. For this reason, immediate coupling of the ligand after carbodiimide activation of the latex is important.

Surface adsorbed ligand

Surface adsorption of the ligand plays a significant role in the preparation of lattices with covalently coupled ligand. Passive adsorption of the ligand occurs quite rapidly followed by the covalent coupling reaction. However, not all adsorbed ligand may be covalently coupled. In some immunolatex preparations the portion of adsorbed ligand may be up to 20%. This must be considered in the evaluation of the reagent stability and reagent performance which may decrease because of slow desorption of the adsorbed ligand. If desorption is a problem, the freshly prepared immunolatex reagent should be washed with a detergent and treated with a blocking agent to inactivate residual activated but unreacted acylurea groups on the latex.

Blocking of activated groups

After covalent coupling of a ligand there may be unreacted activated groups left on the surface of the latex particle. These groups can also cause a nonspecific agglutination. Reaction with an excess of an inert compound will prevent this interference. Inert amines

are used for blocking activated carboxyl groups. The most commonly used compounds are:

- ethanolamine
- ethylenediamine
- glucosamine
- glycine
- lysine

Proteins mentioned in Section 9.2.4, such as bovine serum albumin may also be used.

An example for the preparation of a latex reagent with chemically bound antibodies is discussed in Section 9.2.8.

9.2.6 Slide agglutination

Reagents

Antigen-coated latex
Specific antiserum
Glycine buffer, 0,054 mol/L, pH 8,2, containing 0,154 mol/L NaCl

Preparation

- Prepare doubling dilutions of the specific antiserum.
- Mix 0,025 mL of each antiserum dilution with 0,025 mL coated latex on a glass slide.
- Rock gently for 2 minutes and read agglutination visually, illuminating the slide from the side, against a dark background.

It is advisable to dilute the sera for use, as a prozone effect (where no agglutination is seen at the highest concentrations of serum) can easily occur.

9.2.7 Reagent for detection of rheumatoid factor (RF) in serum by latex agglutination

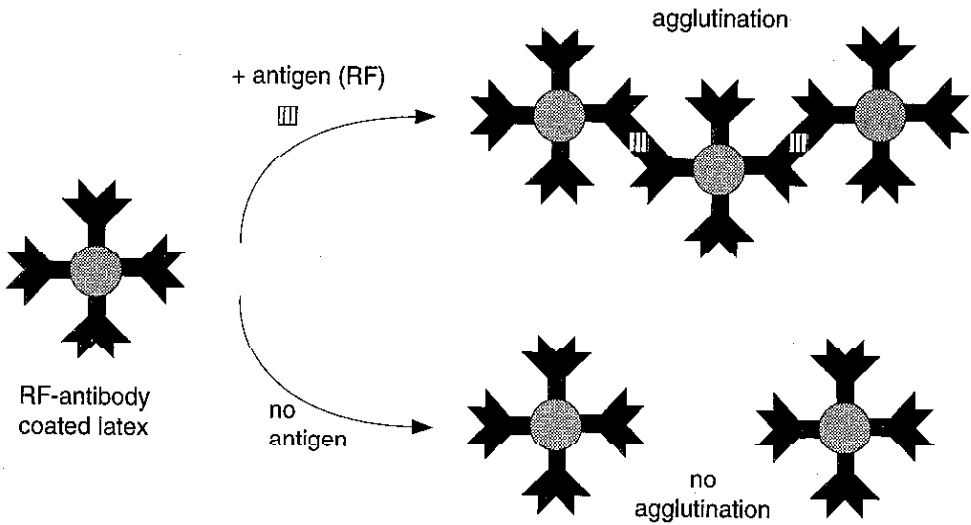
Principle of test

I. Isolation of human serum IgG

Reagents

Ammonium sulfate
NaCl, 0,154 mol/L

Figure 9.1 Latex agglutination for rheumatoid factor



Preparation

- Treat fresh human serum twice with saturated ammonium sulfate 1:1 (v/v).
- Dissolve the second precipitate in physiological saline (to original serum volume) and mix with saturated ammonium sulfate 1:1 (v/v).
- Dissolve the final precipitate in the minimum volume of saline.
- Dialyse against saline at 4°C; change the saline three times each after 24 hrs.
- Centrifuge the dialysed IgG solution to remove any precipitate.
- Determine the protein concentration of the supernatant and dispense the solution in small vials; store below -20°C.

II. Preparation of human IgG coated latex reagent

- Dilute one part of a 10% polystyrene latex suspension with five parts of 0,054 mol/L glycine buffer containing 0,154 mol/L NaCl, pH 8,2.
- Dilute the stored IgG solution to 1,25 mg/mL with the same buffer.
- Mix diluted polystyrene latex suspension and IgG solution together in equal volumes, and stir for two hours at room temperature.
- Centrifuge the reacted mixture and wash twice with 0.054 mol/L glycine buffer, pH 8,2, containing 0,154 mol/L NaCl.
- Resuspend the final precipitate with the same buffer containing 0,1% of sodium azide to the original volume of the mixture.
- After assessing that the reagent is of good quality (see below) dispense the reagent in glass vials.

III. Protocol for detection of RF in serum by latex agglutination

- Dilute serum to 1:20 with 0,154 mol/L NaCl.
- Add one drop (about 0,05 mL) of the diluted serum into the block or circle of a glass or plastic slide.
- Add same amount of latex reagent into the test sample and rock the slide for three minutes at ambient temperature.
- Put the slide on the bench and interpret the result.

If the mixture still has a milky appearance, no reaction has occurred and the result is negative; if any visible aggregates appear, a reaction has occurred and the result is positive. The bigger the aggregates, the higher the titre of RF in the test serum. Dilute the RF positive serum from 1:20 serially and test for good performance as above. The greatest dilution of a test sample showing the definitive positive reaction is its RF titre.

IV. Test for nonspecific agglutination

- Test the latex reagent using saline as the sample and the protocol described above; *no agglutination should be observed in the mixture.*
- Test 10 RF negative control sera; all of them should show negative reaction.
- Test 10 RF positive control sera; all sera should show positive reaction, and the reactivity of reagent should not be beyond ±0,5 of the titre intervals of the control sera.

9.2.8 Preparation of latex reagent for detection of human choriogonadotropic hormone (hCG) in urine by agglutination inhibition

Principle of test

I. Isolation and purification of hCG

- Precipitate hCG from urine of pregnant women by treating with benzoic acid.
- Purify the crude hCG by fractionated precipitation with different concentrations of ethanol solution.

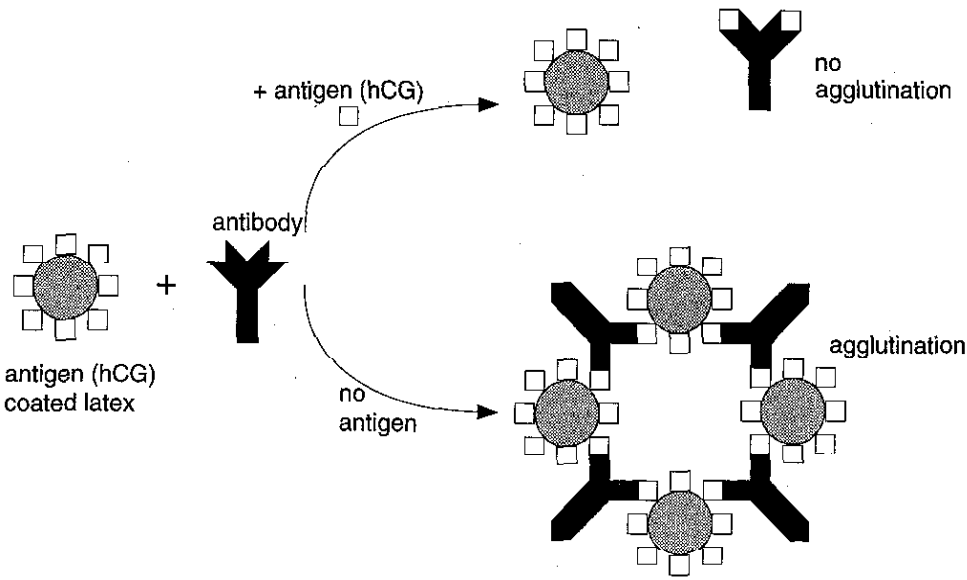
A biological titre of purified hCG above 1400 IU/mL can be used to immunize rabbits to produce anti-hCG antibodies and to prepare the hCG coated latex reagent.

II. Production of rabbit anti-hCG antibodies

- Dissolve two milligrams of hCG with specific activity of at least 2500 IU/mg in 1,0 mL of saline.

- Emulsify the hCG solution in Freund's complete adjuvant.
- Immunize New Zealand rabbits, weight of 2-3 kg with the emulsified hCG antigen by multiple-site subcutaneous injection.
- Boost the animals with a progressively increasing dose of antigen every three weeks.
- Take a blood sample from the ear vein after the fourth booster injection and measure its anti-hCG titre.
- Collect blood by cardiac puncture when the anti-hCG titre has risen to 1:1200 or more.
- Separate the anti-serum, containing polyclonal antibodies, add sodium azide (1 mg/mL) as a preservative; keep in refrigerator until further use.

Figure 9.2 Latex agglutination for hCG



III. Preparation of hCG coated latex reagent

a) Linking the arm spacer to the CML

Reagents and solutions

10% of cleaned CML	400 mL
6-Amino-caproic acid (0,025 mol/L)	800 mL
Phosphate buffer (0,2 mol/L)(pH 7,6)	400 mL
Water soluble carbodiimide	2, 4 g

- Mix the components and let react at 30°C for one hour under constant stirring.

- Centrifuge the mixture at 6000 rpm, and resuspend the precipitate in 1600 mL of 0,05 mol/L phosphate buffer, pH 7,6, to give a final concentration of 2,5% of the arm spacer linked CML in suspension.

b) Coupling hCG with the arm spacer linked CML

Reagents and solutions

2,5% of the arm spacer linked CML	1600 mL
hCG solution	160 mL
water soluble carbodiimide	4,0 g

- Dissolve 400 mg of hCG in 160 mL of 0,05 mol/L phosphate buffer pH 7,6.
- Mix the components together and let react at 30°C for two hours under constant stirring.
- Add 0,05 mL of 0,1 mol/L glycine solution to block excessive carbodiimide activated spacer groups.
- Centrifuge the mixture and wash twice with 0,05 mol/L phosphate buffer, pH 7,6.
- Resuspend the final precipitate in 4 L of the same buffer containing sodium azide (1 mg/mL) as preservative.

IV. Testing and matching of rabbit anti-hCG serum

- Dilute the anti-hCG serum with 0,05 mol/L phosphate buffer, pH 7,6, to 1:200, 1:400, ...
- Add 0,05 mL of the diluted anti-hCG solution onto the block or circle or glass or plastic slide.
- Add 0,05 mL of 2,5 IU/mL hCG standard solution to the diluted anti-hCG solution, mix well, and rock the slide for two minutes.
- Add 0,05 mL of hCG coated latex reagent to the above mixture; rock the slide for 3 minutes at ambient temperature. Put the slide on the bench and interpret the result.

The hCG latex reagents should show a very clear agglutination inhibition until six minutes. An optimal titre or dilution of the anti-HCG serum, showing strong agglutination inhibition, is chosen for further routine investigations. The titrated anti-hCG serum should have a clear appearance and be without any suspended microparticles visible to the naked eye.

V. Protocol for the detection of hCG in urine by latex agglutination inhibition

The hCG latex reagent is tested analogously as described in Section 9.2.7, but with urine instead of serum. Use the optimal dilution of anti-hCG serum. The results are positive when no agglutination is observed, while they are negative when latex agglutination is visualized.

Ten urine samples with 2,5 to 3,5 IU/mL of hCG:	10/10 (i.e. 10 positive)
Ten urine samples with 1,5 to 2,5 IU/mL of hCG:	> 4/10 (i.e. at least 4 positive)
Ten urine samples without hCG:	0/10 (none positive)

9.2.9 Preparation of latex reagent for detection of the anti-streptolysin O (ASLO) in serum

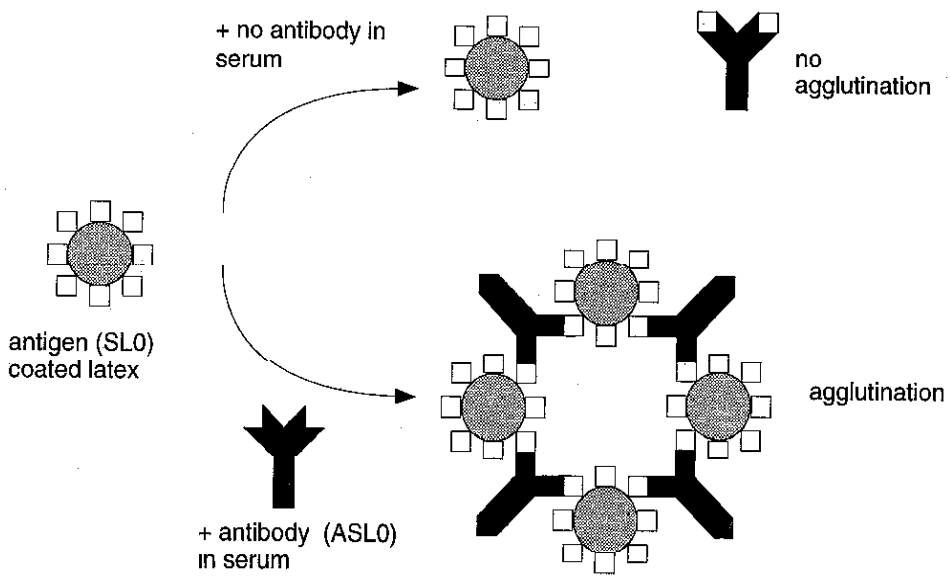
Streptolysin is a complete antigen which initiates the formation of a specific antibody, antistreptolysin, in man. The antibody inhibits the haemolysis induced by streptolysin. This reaction can be used for the detection of ASLO in serum of patients with streptococcal infection.

Principle of test

Preparation of streptolysin O

Bacterium strain. Haemolytic *Streptococci* of Groups A, C or G produce streptolysin. The ability to produce streptolysin seems to be distributed quite fortuitously within these groups. Most *Streptococci* form streptolysin of only moderate strength. It is important that the chosen *Streptococcus* forms a powerful streptolysin. Therefore a suitable strain

Figure 9.3 ASLO agglutination



must be carefully seeded. Freshly isolated *Group A Streptococci* are best for the preparation of streptolysin [5,6]. The strain may be selected from streptococci freshly isolated from throat swabs of scarlatina patients. *Streptococcus* Richard strain is commonly used for commercial production.

The *Streptococcus* strain can be stored at 4°C as a stab culture in meat extract agar. In that case sub-culture should be made about once a month. The best way, however, is to store the strain in the desiccated state in ampoules. By a combination of refrigeration and evacuation almost the total water content in the culture is removed. The ampoules are stored in a refrigerator at 4°C.

Isolation of streptolysin O (SLO)

Reagents

Calcium phosphate (powder)
Ammonium sulfate, crystalline and 1 mol/L

Oxserumbroth:

5% ox serum
0,1% glucose
0,05% haemoglobin

Saltsolution:

Glucose	5,0 g
$NaHCO_3$	5,0 g
NaCl	5,0 g
$Na_2HPO_4 . 12 H_2O$	2,5 g
Distilled H_2O to	100 mL

- Prepare the solution and filter through a Berkefeld filter.

Todd-Hewittbroth:

fat-free beef heart or horse meat	450.0 g
Neopeptone (Difco)	20,0 g
NaCl	2,0 g
$NaHCO_3$	2,0 g
$Na_2HPO_4 . 12 H_2O$	0,4 g
Glucose	2.0 g
Distilled H_2O	1050 mL

- Grind 450 g of beef heart or horse meat and mix with 1050 mL distilled water.
- Keep overnight at 4°C.

- Stir the mixture gently at 85°C for 90 minutes.
- Filter through cotton wool.
- Add 20 g of Neopeptone (Difco) and adjust to pH 8,0 with 1 mol/L NaOH.
- Add the rest of the ingredients and boil for 15 minutes.
- Filter through filter paper and sterilize for 20 minutes.
  The final pH should be 7,8.
- Preheat the Todd - Hewitt broth for 1 hour at 37°C prior to inoculation with the *Streptococcus* strain.
- Prepare the salt solution just before use.
- Add 40mL of salt solution to 1L preheated Todd-Hewitt broth.
- Filter through a Berkefeld filter.

Isolation of SLO

- Open the ampoule of dried culture of a streptococcus strain with appropriate haemolysin properties and suspend the contents in one tube of filtered ox serum broth.
- Incubate overnight at 37°C.
- Seed from this culture into 1% glucose broth [2 drops of culture to each tube (5 mL)]. Incubate for 6 hours at 37°C.
- Add the contents of two preculture tubes (= 10 mL) to 1 L of the preheated Todd-Hewitt broth.
- Incubate for 18 hours at 37°C.
- Add calcium phosphate to adsorb the SLO secreted by the bacteria into the medium.
- Filter the calcium phosphate with the adsorbed SLO with a Büchner funnel.
- Desorb the SLO from the calcium phosphate with 1 mol/L ammonium sulfate solution.
- Add crystalline ammonium sulfate to the SLO containing solution up to 80% saturation.
- Separate the precipitating crude SLO by filtration through a Büchner funnel and dry in a vacuum desiccator over a desiccant. The dried SLO/ammonium sulfate powder can be kept at low temperature.

For coating latex particles with the SLO, the crude powder is dissolved in distilled water and dialysed for removing the ammonium sulfate.

Preparation of the arm spacer linked CML

Carboxylate modified latex particles are prepared as described in Section 9.2.5.

Preparation of the SLO coated latex reagent

Reagents

2,5% of the arm spacer linked CML	2250 mL
Dialysed SLO solution	300 mL
Water soluble carbodiimide	4,0 g

- Mix the components and let react at 30°C for one hour with constant stirring.
- Add 50 mL of 0,4 mol/L glycine solution to the mixture to block excessive carbodiimide activated spacer groups.
- Wash the mixture twice with 0,05 mol/L phosphate buffer pH 7,6 and centrifuge to isolate the latex precipitate.
- Resuspend the latex precipitate in 5600 mL phosphate buffer containing sodium azide 1 mg/mL as preservative.

Preparation of the ASLO positive control serum

A serum with high titre of ASLO can be obtained by immunization of rabbits with *streptococcus* Richard strain or by screening of the population. The strong ASLO positive serum is diluted with the buffer (stabilized with 15% ethylene glycol) to the extent of titre 1:500 to 1:625 to serve as positive control serum.

Quality assessment of latex reagent for detection of ASLO in serum

Follow the protocols as described in Section 9.2.7.

The positive control serum should show clear agglutination reaction by the standardized test protocol; the negative one should still keep the milky appearance.

Stability and storage of the reagent kit

The reagent is stable for at least one year when stored at 2-8°C. Do not freeze.

9.3 Agglutination tests with erythrocytes

Apart from latex particles and other microspheres, soluble antigens can also be attached to erythrocytes. The antigen-labelled erythrocytes can be agglutinated by antisera. The attachment of antigen may be achieved by physical or chemical means.

(a) Erythrocytes may spontaneously adsorb polysaccharides to their surface during incubation. Although this is a non-covalent binding, there is very little leaching off of the antigen during the assay.

(b) Coupling to chemically modified erythrocytes. Antigens can be coupled to erythrocytes by various coupling reagents such as bisdiazotized benzidine, glutaraldehyde and chromic chloride.

The following procedure describes the chemical coupling of human serum albumin as antigen to sheep erythrocytes stabilized with tannic acid. However, the method can be used for many other protein antigens.

9.3.1 Preparation of tanned erythrocytes

Reagents and solutions

1. Alsever's solution

NaCl	4,2 g
Citric acid	0,55 g
Citric acid, trisodium salt	8,0 g
Glucose	20,5 g
Distilled H_2O to	1000 mL

- Dissolve the chemicals and steam three times for 30 minutes without pressure.

2. Borate succinate buffer (0.15 mol/L, pH. 7,5)

solution A:	Na_2 tetraborate	19,0 g/L
solution B:	Succinic acid	5,9 g/L
NaCl (crystalline)		
Horse serum		

Preparation:

- Add solution B to 1000 mL of solution A until the pH is 7,5.
- Add crystalline NaCl so that the concentration is 0,14 mol/L.
- Inactivate horse serum by heating to 56°C for 45 minutes.
- Add inactivated horse serum to a final concentration of 1%.

***Phosphate - saline buffer* (pH 7,2):**

Potassium di-hydrogen phosphate (KH_2PO_4)	12,2g
Di-soduim hydrogen phoshpate (Na_2HPO_4)	40,4g
Sodium chloride	36,0g
Dissolve in 5L of distilled water	

Sheep erythrocytes, (SRBC), in Alsever's solution
Phosphate - saline buffer (pH 7,2)
Borate - succinate buffer (0,15 mol/L)(pH 7,5)
NaCl, 0,154 mol/L
Tannic acid
Human serum albumin, HSA
37% aqueous formaldehyde (formalin)

Preparation:

- Wash sheep red blood cells (SRBC) three times with 40 volumes of saline by centrifugation (300 *g* for 10 min).
- Adjust SRBC suspension to 40% v/v in phosphate - saline buffer, pH 7,5.
- Add 2.5 mg of tannic acid to 50 mL of phosphate - saline buffer and mix with 50 mL of 4% SRBC suspension.
- Incubate at 37°C for 15 minutes.
- Spin down cells very gently (100 *g* for 20 min). If the cells are pelleted too quickly they will agglutinate.
- Divide the cells into two aliquot portions, and wash each with 50 mL phosphate-saline buffer by centrifugation (100 *g* for 20 min). One aliquot will be used for antigen coating and the other as control cells.
- Resuspend 1 aliquot of cells in 50 mL phosphate - saline buffer and add 50 mL of human serum albumin (HSA) solution (2 mg/mL initial concentration).
- Incubate at 37°C for 30 minutes.
- Wash in phosphate - saline buffer by gentle centrifugation and resuspend in 100 mL of borate - succinate buffer.
- Resuspend the second aliquot of cells in 100 mL of borate - succinate buffer. The control cells are not coated and are used both to adsorb the test antisera and as control cells in the assay.
- Add 10 mL of formalin to both cell suspensions while stirring. The formalin must be added dropwise over the period of 20-30 minutes.
- Leave overnight at 4°C and add a further 10 mL of formalin to both suspensions.
- Leave the cells to settle (24 hrs) and pour off the supernatant.
- Add a large volume of borate-succinate buffer and resuspend the cells by vigorous shaking.
- Allow cells to settle (24 hrs) and wash again by sedimentation in borate-succinate buffer.
- Adjust both cell suspensions to 1% v/v and add 0,2% formalin (final concentration) as a preservative.

The cells can be stored at 4°C for up to 2 years.

9.3.2 Assay procedures

Direct agglutination

- Titrate the antiserum as described in Section 9.2.8 but use an initial dilution of 1:5.
- Prepare serum dilutions in duplicates.
- Add 0,025 mL of the 1% suspension of HSA-coated SRBC to one dilution series.
- Add 0,025 mL of 1% control (uncoated but tanned) SRBC to the other dilution series.
- Place 0,025 mL of coated and uncoated cells on separate glass slides or in separate empty wells of a tile plate to test for spontaneous agglutination. (Add 0,025 mL of buffer to each of the control cells.)
- Gently shake and leave to stand for 1 hour.
  Positive agglutination is seen when the cells form a continuous carpet on the slide or the bottom of the wells.

NOTE 1. Nonspecific agglutination with control cells may occur if the antiserum contains heterophile antibodies. In this case the antiserum must be adsorbed to remove heterophile antibodies as follows:

- Add 0,1 mL of serum to 1 mL of packed control cells.
- Incubate at 37°C for 10 minutes and spin off the erythrocytes.
- Repeat the agglutination assay and readsorb if necessary.

NOTE 2. If the coated or control cells agglutinate spontaneously, add 1% normal serum to the buffers used in the assay.

Indirect agglutination

IgG antibodies are less efficient at agglutinating red cells. Addition of 0,025 mL of 1% bovine serum albumin to the reaction mixture can sometimes enhance the agglutination. Otherwise, the addition of a second antibody can be used:

- Centrifuge red cells gently.
- Remove the supernatant.
- Resuspend the red cells gently in 0,050 mL of suitably diluted anti-IgG for agglutination.

9.4 Tests for serum complement

Sensitized red cells can be lysed by antibodies in the presence of complement. This reaction can be used for the investigation of the human serum complement system. This system is similar to the plasma clotting system and comprises a number of complement factors.

Complement activity is usually determined either by functional measurement of the whole complement system, which is clinically indicated only in patients with a suspected genetic complement deficiency, or by immunochemical measurement of individual components, (e.g. C_3, C_4), which may be helpful in patients with glomerulonephritis, in immune complex disease, [e.g. systemic lupus erythematosis (SLE)], in some forms of vasculitis and other conditions such as dengue haemorrhagic fever. A test for the determination of whole complement activity (C_{100}), is described below.

Estimation of total lytic activity of complement (C_{100})

Antibody-sensitized sheep red cells are incorporated into molten agarose. Complement, from human serum added into wells punched into the agarose, diffuses into the agarose, binds to the antibodies coated to red cells and initiates a lytic reaction.The appearance of circles caused by the haemolysis of the sheep red cells is taken as a measure of the activity of the human serum complement system.

Reagents and solutions

Barbital-saline buffer (0,15 mol/L), pH 7,5

stock solution A:	NaCl	95 g
	Diethylbarbituric acid, sodium salt	2,75 g
	Distilled H_2O	1000 mL
stock solution B:	Diethylbarbituric acid	5,75 g
	Hot distilled H_2O	500 mL
stock solution C:	$MgCl_2 . 6 H_2O$	20,3 g
	Distilled H_2O	50 mL
	Dissolve;	
	$CaCl_2$ (1 mol/L)	30 mL
	Mix;	
	Distilled H_2O	to 100 mL

- Mix solutions A and B, then cool to room termperature.
- Add 5 mL of solution C.
- Adjust volume to 2 L with distilled water. Store at 4°C.

NOTE:

This solution must be diluted before use (1 volume buffer + 4 volumes of distilled water.

Ammonia solution (0,04%).
Sensitized sheep red cells (SRBC) (see below).
Agarose 2% (w/v) in barbital buffered saline (see below).
Haemolysed horse blood.

Standardization of sensitized sheep red cells

- Check the barbital buffer containing 0.154 mol/L NaCl for turbidity, which may be caused by bacterial contamination. Microbes interfere with the test for total complement activity.
- Wash 4 mL of the erythrocyte suspension (supplied at about 25% (v/v) in Alsever's solution (see Section 8.3)) three times in barbital buffer containing 0,154 mol/L NaCl (200 g for 3 minutes).
- Resuspend the washed erythrocytes in 15 mL of barbital buffer containing 0,154 mol/L NaCl (use a measuring cylinder).
- Mix 1 mL of erythrocyte with 25 mL of ammonia solution to lyse the cells and read the absorbance at 541 nm. For a 6% SRBC suspension, in a 1 cm cuvette, the absorbance should be 0,48-0,50. Adjust the suspension as required.
- Mix 15 mL barbital buffer containing 0,154 mol/L NaCl, 0,1 mL of horse haemolytic serum (= antierythrocyte antibody from horse) and 15 mL of 6% SRBC. Strictly, the anticrythrocyte serum should be titred until the highest dilution still giving full complement fixation is reached; however, for most purposes it is sufficient to use a 1:150 dilution.
- Incubate at 37°C for 15 minutes.

The sensitized cells have a limited stability and must be used within 24 hours.

Measurement of C_{100} activity

- Warm 1,5 mL of the barbital buffer to 56°C in a water-bath.
- Cool 1,2 mL of molten 2% agarose to 56°C and add to the barbital buffer.
- Mix and cool to 45°C in a water-bath.
- Add 0,2 mL of sensitized sheep red-cell suspension and mix gently.
- Place the glass plate on a level surface, use a spirit level to check.
- Pour the mixture quickly onto the plate to form a smooth, even surface.
- When set, place the plate in a box containing moist filter paper and chill to 4°C for a few minutes to harden the agarose.

- Cut two rows of five wells, approximately 3 mm across using an Ouchterlony gel cutter and remove the agarose plugs with a Pasteur pipette.
- Dispense 8 µL of each serum to be investigated into separate wells. To establish a standard curve, similarly, add four doubling dilutions of a standard serum to a series of wells. (For accurate research studies, a large plate can be used to permit replicate determinations.)
- Incubate the plate in a moist box, overnight at 4°C.
- Warm the plate, still in the box, to 37°C for 2 hours, to allow cell lysis to occur.
- Measure two diameters at right angles across each well and calculate their mean.
- Plot the value of the areas (πr^2) of the standard serum dilutions (linear scale) against the log dilution.
- Determine the total complement activity of the specimens by extrapolation from the standard curve.

9.5 Nitrocellulose membrane techniques

9.5.1 Principle, materials and reagents

Nitrocellulose membrane (NCM) immuno dot tests are simple and economical means for qualitative immunological investigations. The principle of investigation is an ELISA test using horseradish peroxidase conjugates for the indicator reaction. Many versions of ELISA using NCM have been published, especially for advanced testing such as Western blots [3,4].

Materials

The NCM most often used for these tests, are:

- Nitrocellulose membranes, 0,2 µm pore size (Ba 83, Schleicher & Schuell Co, Germany);
- Nitrocellulose membranes, 0,22 µm pore size (Millipore Inc., USA).

The NCM must always be handled with gloves or tweezers to prevent contamination. For easy handling, membranes backed with plastic have been recently developed for the dipstick version of tests:

- Mylar-backed nitrocellulose sheets, 0,45 µm pore size (Ba 85, Schleicher & Schuell Co., Germany).

The incubation and washing procedures for NCM tests can be performed in tubes, plastic boxes, and for discs, in microplates. In some procedures the washing steps can be replaced by rinsing with clean tap water.

The coating of NCM is done by "dots" of very small amounts (1 μL) of reagents. Special micropipettes are needed for preparation, e.g. Hamilton microsyringes, Microcapillary displacement pipette (Cole-Parmer Instr. Co., USA); Microdispensers (Socorex ISBA, SA, Switzerland).

Reagents

The reagents for NCM tests are buffer solutions, horseradish peroxidase antibody labelled conjugates and enzyme substrate. The preparation of the reagents is as follows:

Horseradish peroxidase antibody labelled conjugate

Chemicals and reagents for conjugation

Acetic Acid
Borax
Boric acid
Dithiothreitol (DTT)
Ethanol - absolute
Glycerol
Horseradish peroxidase (HRP) Sigma Type VI(EC 1.11.17) No.P8375
Hydrochloric acid
Hydrogen peroxide
Methyl-D-mannoside
Merthiolate preservative
N-succinimidyl 3-(2-pyridyldithio)-propionate, (SPDP), Pharmacia
Sodium acetate
Sodium borohydride
Sodium carbonate
Sodium chloride
Sodium hydrogen carbonate
Sodium periodate
Tween-20
Standard human immunoglobulin antigens IgG, IgM
Antibodies to human immunoglobulins as IgG fractions (rabbit, sheep, goat antisera) to:
IgG (Fc)-specific
IgM (Fc)-specific
Mixed human immunoglobulins (anti Ig)

Bovine serum albumin - highly purified fraction

Buffers

1) Phosphate buffered saline (PBS; pH 7,4) (also used as incubation buffer)

NaCl	8,0 g
KH_2PO_4	0,2 g
$Na_2HPO_4 . 12H_2O$	2,8 g
KCl	0,2 g

- Dissolve the salts in 1 litre of distilled water.

2) Sodium acetate buffer, 1,0 mmol/L, pH 4.4

Solution A

Sodium acetate (anhydrous)	8,24 g
or hydrated sodium acetate	13,61 g

- Dissolve the salt in 1 litre of distilled water.

Solution B

Acetic acid	6,00 g (= 6,00 mL)

- Add 6,00 g of glacial acetic acid to distilled water to make 1 litre.
- Add 1 part of solution A to 2 parts of solution B.
- Dilute 1:100 in distilled water to give a 1,0 mmol/L buffer.

3) Sodium bicarbonate buffer 0,2 mol/L, pH 9,5

Solution A

Na_2CO_3 (anhydrous)	0,2 mol/L	21,2 g/litre

Solution B

$NaHCO_3$	0,2 mol/L	16,802 g/litre

- Add A to B until pH 9,5 is achieved.

4) Sodium carbonate/bicarbonate buffer, 0,05 mol/L, pH 9,5

Na_2CO_3 (anhydrous)	1,59 g
$NaHCO_3$	2,93 g

- Dissolve in 1 litre of distilled water and adjust pH if necessary.

5) Borate buffer, 0,1 mol/L, pH 7,4

Boric acid	24,732 g

- Dissolve in 4 litres distilled water.

Borax	19,07 g

- Dissolve in 500 mL distilled water.
- Add approximately 115 mL of borax solution to 4 litres boric acid solution and adjust to pH 7,4 by further small additions.

6) Carbonate coating buffer 0,05 mol/L, pH 9,6

Na_2CO_3 (anhydrous)	1,59 g
$NaHCO_3$	2,93 g

- Dissolve both salts in 1 litre distilled water containing merthiolate (0.02% w/v).
- Adjust to pH 9,6 by addition of one or other salt at the same molarity.

Conjugation procedures

Two procedures are known to produce high quality conjugates of HRP to antibody. These are:

(i) the periodate method which can give a good recovery of the enzyme in the conjugate. It involves the oxidation of peroxidase with periodate and generates some proportion of conjugate in polymeric form. The conjugation procedure is uncomplicated.

(ii) the use of heterobifunctional linking reagents such as N-succinimidyl-3-2/2-pyridyldithio)-propionate (SPDP), or N-(4-carboxycyclo-hexylmethyl)-maleimide. The method, although substantially more complex to perform, offers more control over the size of the conjugates produced and can yield a predominance of monomeric couplets of antibody and enzyme.

Conjugation with sodium periodate

- Take 4-8 mg HRP (P8375 Sigma - activity: 285 purpurogallin units/mg solid) and dissolve in 1 mL distilled water.
- Prepare a fresh solution of 0,1 mol/L sodium periodate in distilled water.
- Add 0,2 mL to the HRP solution which immediately turns green.
- Stir gently for 20 minutes at room temperature.
- Dialyse the reaction mixture overnight against a large excess of sodium acetate buffer 1,0 mol/L, pH 4,4 at 4°C.
- Raise the pH to 9,0-9,5 by addition of 20 µl of sodium bicarbonate buffer 0,2 mol/L, pH 9,5.
- Add immediately 10 mg of immunoglobulin (antibody) solution in 1,0 mL, previously dialysed against 0,05 mol/L sodium carbonate/bicarbonate buffer, pH 9,5.
- Stir the mixture gently for 2 hours at room temperature in the rotary wheel mixer.
- Add 0,1 mL of freshly prepared sodium borohydride solution (4 mg/mL) in distilled water to reduce the free enzyme.

Leave the solution standing for 2 hours at 4°C.

- Dialyse the conjugated mixture overnight at 4°C against 0,1 mol/L borate buffer, pH 7,4.
- Separate the enzyme-antibody conjugate from uncoupled materials by passing the reaction mixture down a Sephadex G-200 column (1.6 x 35 cm) in borate buffer.
- Collect the first (enzyme active) peak.
- Dilute the conjugated material 1:2 in 60% glycerol in borate buffer.
- Add merthiolate to a final concentration of 0.02%, divide into small (100 µL) samples in glass bottles at 4°C, or freeze-dry larger volumes in buffer in glass vials.

Conjugates prepared in this way and stored thus, maintain their activity for at least 1-2 years.

Conjugation with SPDP

- Dissolve 10,0 mg HRP (as above) in 2,0 mL sodium phosphate buffer 0,1 mol/L, pH 7,5 containing 0,1 mol/L NaCl.
- Add 400 µg of SPDP dissolved in 0,5 mL absolute ethanol, dropwise under stirring.
- Allow the mixture to react for 30 minutes at room temperature with occasional further stirring.
- Remove the excess of SPDP and the reaction product N-hydroxysuccinimide from the 2-pyridyl-disulfide-substituted enzyme by gel filtration through a 1,6 x 35 cm Sephadex G-25 column, eluting the substituted HRP in the first PBS peak.
- Perform an identical reaction with SPDP and the IgG (antibody) preparation, using 10 mg of IgG and in this case only 10-15 µg of SPDP in 0,2 mL ethanol.
- Harvest the substituted antibody from a Sephadex G-25 column in PBS.
- Generate reactive thiol groups on the substituted HRP molecules by reduction of the 2-pyridyl disulphide groups with dithiothreitol (DTT) using 2,5 mM per mg substituted enzyme, (or a final concentration of 0,05 mol/L), at room temperature.
- Remove the excess of reducing agent and pyridine-2-thione by gel filtration, by Sephadex G-25 gel filtration.
- Mix the thiol-containing peroxidase and the 2-pyridyl-disulphide-containing antibody solutions 1:1 (w/w) and leave the solution at 4°C for 18 hours.
- Apply the reaction mixture to a freshly-prepared Con-A-Sepharose column in PBS.
- Wash the column thoroughly with PBS, and elute the concanavalin A-bound HRP-antibody conjugate in PBS containing methyl-D-mannoside (10 mmol/L).

- Separate the eluted peak, which is antibody and enzyme-active, into conjugate and uncoupled enzyme by passage through a Sephacryl S-200 column (1,6 x 95 cm) at a flow rate of 6-20 mL/cm²/hr using PBS.
  The first enzyme-active peak contains the conjugate which is predominantly of molecular weight about 200 000 daltons.
- Dilute the conjugate 1:2 in 60% glycerol in borate buffer.
- Add merthiolate and store small samples in the cold as above, or freeze-dry and seal in glass vials.

Substrate mixture

(prepare the mixture freshly before use):

Phosphate buffered saline (PBS) (pH 7,4)	15 mL
4-Chloro-1-naphthol, 0,3%	0,9 mL
H_2O_2, 3%	0,12 mL

- Dissolve 1 tablet of 30 mg 4-chloro-1-naphthol (Sigma, C.6788) in 10 mL of methanol to obtain a 0,3% solution.
- Store the tablets wrapped in aluminium foil in a refrigerator.

Coating buffer:

Although for some tests carbonate buffer (pH 9,6) can be used, others recommend PBS pH 7,8 to 8,2, which is prepared by adding a few drops of 1 mol/L NaOH to PBS to obtain the appropriate pH.

Blocking solution:

TRIS base	2,4 g
NaCl	29,2 g
Distilled H_2O to	1000 mL
pH adjusted with HCl to 7,5	
Gelatin	30 g

NOTE: Depending on the test to be prepared, other blocking solutions are also suitable (see Section 9.2.5).

9.5.2 Procedures

With some tests antigens can be directly detected whereas for other antigens the indirect or sandwich version is preferable. Only the basic procedures will be described here. The criteria for the choice of reagents (antigens, antibodies, conjugates), are the same criteria as for microplate assays (i.e. optimal dilutions, etc.) [1.2]. Three tests using NCM are described below.

NCM - disc tests

- Cut NCM into 5 mm diameter discs (handle with forceps).
- Dot each of the discs in the centre with 1 µL of the immunoreagent at predetermined dilution in coating buffer (i.e. antigen for the detection of antibody or vice versa), using microdispensers.
- Leave the discs to dry for 10 minutes.
- Submerge the dried discs into blocking solution for 30 minutes.
- Place the discs on tissue paper to dry.

  The coated discs can be stored under desiccation at -20°C for 1 year.

Testing of specimens with NCM discs:

- Place the appropriately prepared discs in wells of microplates.
- Incubate the discs with 0,1 mL of the specimen solution to be investigated for at least 30 minutes.
- Wash the discs with distilled water for 30 seconds.
- Incubate the discs with the antibody horseradish peroxidase conjugate for 30 minutes.
- Wash the discs with distilled water for 30 seconds.
- Incubate the discs with substrate solution for 15 to 30 minutes.
- Wash with distilled water and allow to dry.

The results are read visually and interpreted by comparing the reaction of test samples with positive and negative controls.

NCM - strip test

- Cut NCM strips of convenient size (depending on number of tests on 1 strip), e.g. 10,0 cm x 0,6 cm, which is suitable for 10 dots.
- Mark the strips for identification with waterproof ink.
- Soak the strips in (PBS; pH 7,4) and leave on tissue paper to dry.
- Dot the appropriate reagents in coating buffer (1 µL) on to each strip (up to 10 dots for the size mentioned above) and leave to dry for 10 minutes.
- Place the strips in blocking solution for 30 minutes, thereafter on tissue paper to dry.

  The strips can be stored in the same way as discs.

Testing of specimens with NCM strips

For testing, the strips are treated similarly as described for the discs above. The incubations are best done in 15 mL screw-cap tubes with 5 mL solutions. The strips can be washed in Petri dishes or plastic boxes. After incubation with the substrate mixture

the strips are washed and read (or photographed while wet; the colour fades during drying).

A modification of this technique has been widely used for the determination of immunoglobulin classes and subclasses of mouse monoclonal antibodies: test samples of monoclonal antibodies are dotted on several strips, one strip is incubated with conjugate to all Ig classes, other strips with conjugates to subclasses (e.g. IgG, IgG_{2a}, IgG_{2b}, IgG_3, IgM).

NCM - dipstick test

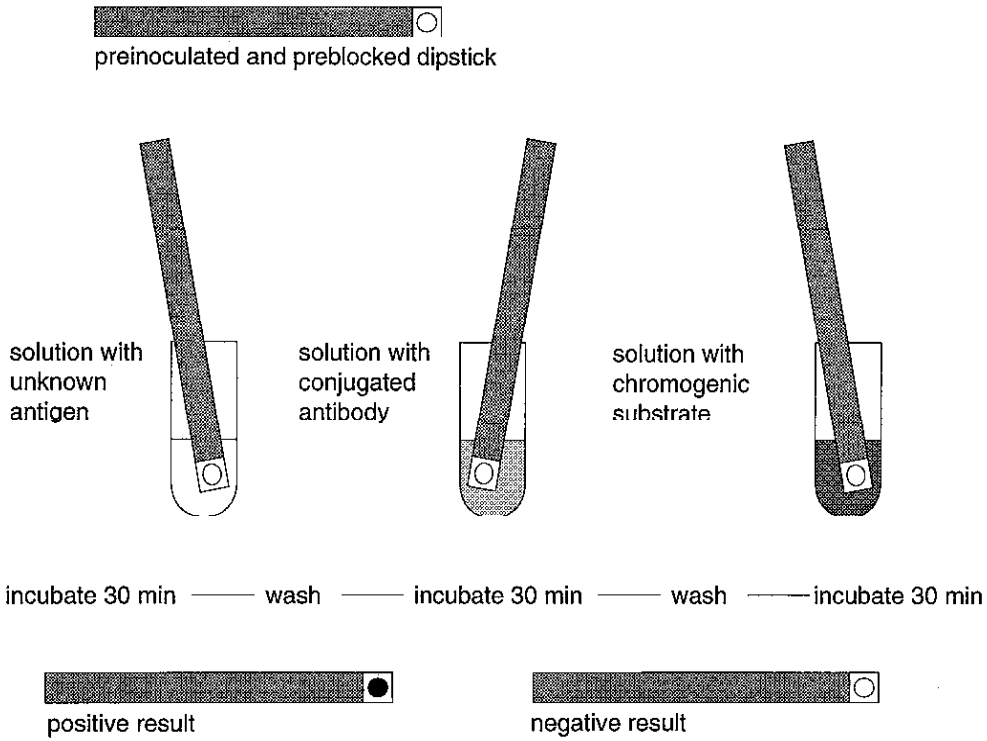
- Cut NCM sheets into strips to produce dipsticks of the desired size (e.g. 15 dipsticks 3,75 cm long and 0,5 cm wide). Mylar-backed NCM BA-85 are recommended for easy handling.
  Mark the design of dipsticks with a waterproof pen but do not cut them yet.
- Draw a line about 0,5 cm from one end to mark the reactive place on the dipstick. The opposite end is used for identification numbers, etc.
- Soak the sheets in distilled water for 15 minutes and remove the excess moisture by light blotting with a paper towel.
- Add 1 µL of antibody, e.g. goat anti-human IgG, (using microdispenser) in coating buffer (PBS pH 7,8 to 8,2) in the middle of each of the marked reactive places of the wet dipsticks.
- Allow to dry 5 to 10 minutes and submerge the sheets in a blocking solution for 30 minutes.
- Remove and allow to air dry, then cut into strips.

Dipsticks can be used immediately or stored desiccated over silica gel at 4°C for up to 1 year. The precoated dipsticks can be air-mailed in plastic envelopes with silica-gel worldwide without loss of activity.

Testing of specimens with NCM dipsticks:

- Label each dipstick for permanent record with a waterproof pen, cut the desired number of dipsticks from the blocks and submerge the reactive site of the dipstick in a tube containing test sample (e.g. human serum at different dilutions). Duplicates can be incubated in the same tube.
- Incubate 30 minutes, wash the dipsticks individually under flowing distilled water (or clean tap water) for 30 seconds; shake or blot the excess water from dipsticks.
- Place dipsticks into a tube containing a solution of conjugate (.e.g. goat anti-human IgG, horseradish peroxidase-labelled) and incubate 30 minutes.

Figure 9.4 The "dipstick" assay



- Wash and blot as before.
- Place dipsticks in freshly prepared substrate solution and incubate for 15-30 minutes, wash under running water and leave to air dry.
- Evaluate visually.

Dry dipsticks can be kept for permanent record. The results are interpreted by comparing the reaction of test samples with the positive and negative controls which should always be included.

The example shown in Fig. 9.4 is used for antigen detection by sandwich ELISA, but the same arrangement can be used for antibody detection. As in microplate tests, the indirect tests have the advantage of using a single conjugate; also, the sandwich version is usually more sensitive than direct tests.

The pre-labelled blocked dipsticks are quite stable when kept under desiccation. Therefore, they are suitable for preparation of kits for rapid testing and do not require electricity or complicated instruments. Kits, containing dipsticks with several reactive

places (for the detection of several substances on the same dipstick) have been commercially produced (e.g. Line- Immunoassay-Strips by Innogenetics, SA, Belgium; Staller-sticks by CMG, Switzerland). These types of dipsticks are suitable for individual testing. For routine tests the kits using NCM-plates (backed with filter paper and plastic) are more economical (e.g. kits produced by Biokema, Crissier, Switzerland = 40 tests performed at the same time on 1 plate).

9.6 References

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ANNEXES

ANNEX 1
Manufacturing recipe

EXAMPLE: ALBUMIN REAGENT

40 litres batch

1. Preparation (2 hours)

1.1. Materials and equipment

PE beaker	1 L
PE beaker	10 L
Pyrex glass beaker	1 L
graduated flask	50 mL
magnetic stirrer (with heating device)	
PE barrel (with stirrer)	50 L
pH-meter	
Filtration barrel 40 L, filter 0,6 µm, diameter 14 cm	
Ready-made: 40 PE bottles 1L, 40 labels	

1.2. Reagents

For the quality of reagents see Section 3.2.2.1

1.2.1 224 g succinic acid is weighed in a 1 L PE beaker.

1.2.2 25 g Brij 35 is weighed in a 1 L Pyrex glass beaker; add approximately 1 L of distilled water and dissolve by heating at 60°C on a magnetic stirrer.

1.2.3 2,32 g bromocresol green is weighed in an aluminium foil and dissolved with 10 L distilled water in a 10 L PE beaker.

1.2.4 4 g NaN_3 is weighed on a parafilm foil.

1.2.4a 20 mL formol are added in a graduated cylinder.*

1.2.5 0, 25 mol/L NaOH solution.

1.2.6 1 g succinic acid is dissolved in 20 mL distilled water.

2. Manufacture of the reagent solution (12 hours)

2.1 Approximately 10 L distilled water are filled into the 10 L PE barrel.

2.2 Succinic acid (1.2.1) is added and dissolved.

---

* For larger volumes it is safer to use formol as preservative.

2.3 Brij 35 (1.2.2), dissolved in hot water, is added.
2.4 15 L distilled water is added and the solution is mixed.
2.5 NaN_3 (1.2.4), is added.*
2.6 The bromocresol green solution (1.2.3) is added and mixed.
2.7 Adjust the pH to 4.20 ±0,05 with NaOH (1.2.5) or succinic acid (1.2.6).
2.8 Adjust the final volume with distilled water to 40 L.
2.9 Fill the solution into the filtration barrel and filter through a 0,6 µm filter under nitrogen pressure. The filtrate is pooled in a clean PE barrel.
2.10 500 mL of the reagent solution are taken for quality assurance.
Wait for the quality assurance report.
2.11 Distribute aliquot 1,005 L of the solution into PE bottles and label.
2.12 Store at 2 - 8°C.

NOTE: The solution requires careful preparation.

3. Quality Assurance (4 hours)

3.1 Appearance: colour
3.2 Viscosity (cSt):
3.3 Surface tension (dyn/cm):
3.4 Density (g/L, 20°C):
3.5 pH: 4,15 - 4,25
3.6 Photometric absorbance: see Section 3.2.2.3
3.7 Functional test: this reagent is used according to the method mentioned in Section 3.2.2.4
3.8 Quality control comparison: control serum with assigned values.

* For larger volumes it is safer to use formol as preservative

ANNEX 2
Abbreviations

A	Absorbance
ADP	Adenosine-5'-diphosphate
ALAT, ALT	Alanine aminotransferase (glutamate pyruvate transaminase, GPT)
AMP	Adenosine-5'-monophosphate
ASAT, AST	Aspartate aminotransferase (glutamate oxalate transaminase, GOT)
Bl	Blank
ΔA	Difference of two absorbances
EDTA	Ethylene diamino tetra-acetic acid (ethylene dinitrilo tetra-acetic acid)
kat	katal, derived SI unit (see also SI) for the expression of the catalytic activity of enzymes (= mol/s) for practical reasons: μkat = 10^6 kat or nkat = 10^9 kat
min	Minute
NAD, NAD^+	Nicotinamide-adenine dinucleotide, oxidized form
NADH	Nicotinamide-adenine dinucleotide, reduced form
NADP, $NADP^+$	Nicotinamide-adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced form
s	second
S	specimen
SI unit	International system of units (système internationale d'unités)
St	Standard
TRIS	Tris(hydroxy methyl) amino methane
U (IU)	International unit for the expression of the catalytic activity of enzymes (= μmol/min); sometimes for practical reasons: kU = 10^3 U = 10^3 μmol/min

ANNEX 3
Selected organizations of relevance to laboratory reagents

ICSH International Committee for Standardization in Haematology
Royal Postgraduate Medical School
Du Cane Road
London W12 0NN, UK

IFCC International Federation of Clinical Chemistry
IFCC Technical Secretariat
Centre du Médicament, Université de Nancy 1
30 rue Lionnois
54000 Nancy, France

IUPAC International Union of Pure and Applied Chemistry
Bank Court Chambers
2-3 Pound Way, Templar Square
Cowley
Oxford OX4 3YF, UK

IUIS International Union of Immunological Societies
Institute of Immunology and Rheumatology
Rikshospitalet University Hospital
N-0172 Oslo 1, Norway

NBS Office of Standard Reference Materials
National Bureau of Standards
Washington, DC 20234, USA

NCCLS National Committee for Clinical Laboratory Standards
National Office
771 East Lancaster Avenue
Villanova, PA 19085, USA

SFBC Société Française de Biologie Clinique
Faculté de Pharmacie
B.P. 403
54001 Nancy, France

WASP World Association of Societies of Pathology
Department of Clinical Pathology
Jichi Medical School
Minami - Kawao
Tochigi
32904 Japan

WHO World Health Organization
CH-1211 Geneva 27, Switzerland

WHO Collaborating Centre for Research and Reference Services in Clinical Chemistry
Wolfson Research Laboratories
Queen Elizabeth Hospital
Birmingham B15 2TH, UK

ANNEX 4
Selected of companies providing chemicals and biochemicals*

Bachem Feinchemikalien AG Hauptstrasse 144 CH-4416 Bubendorf, Switzerland Tel: +41 61 9312333	Chemicals
J.T. Baker Inc. 222 Red School Lane Phillipsburg, NJ 08865, USA Tel: +1 908 859 2151	Chemicals
Bangs Laboratories Inc. 979 Jeystone Way Carmel, IN 46032, USA	Latex
BDH Diagnostics, Broom Road, Poole, BH12 4NN UK	Chemicals
Becton Dickinson P.O. Box 243 Cockeysville, MD 21030, USA Tel: +1 800 638 8663 Immengasse 7 CH 4056 Basel, Switzerland Tel: +41 61 3225830	Diagnostics
Behringwerke AG P.O. Box 1140 D-3550 Marburg, Germany Tel: +49 6421/394959; fax: +49 6421/66064	Immunochemicals Diagnostics

* The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned.

Biokar 29 rue Delizy 93698 Pantin Cedex, France Tel: +33 1 48 91 02 32	Media
Biokema, Crissier Lausanne, Switzerland	Nitrocellulose membrane dipsticks
Biomérieux Chemin de l'Orme 69280 Marcy l'Etoile, France Tel: +33 78 87 20 00	Media
Boehringer-Mannheim GmbH P.O. Box 310120 D-6800 Mannheim 31, Germany Tel: +49 621 7591	Diagnostics Biochemicals
CMG Fribourg, Switzerland	Nitrocellulose membrane dipsticks
Cole-Parmer Instr. Co. Chicago, Illinois, USA	Microcapillary displacement pipettes; microsyringes
Difco Laboratories Ltd P.O. Box 14B Central Avenue East Mosley, Surrey KI8 0SE, UK Tel: +44 181 979 9951	Media
Dyna Particles AS 18th Floor West St Alphage House, 2 Fore Street London EC27 5OA, UK	Latex
Gibco Ltd P.O. Box 35 Trident House Renfrew Road Paisley PA3 4EF Renfrewshire, Scotland Tel: +44 141 889 6100	Media

Hughes and Hughes Ltd,
Romford, Essex RM3 0HR, UK
Fermcozyme 653

ICN Corp.
K & K Laboratories Inc.
121 Express Street
Plain View, NY 11803, USA
Tel: +1 516 433 6262
Chemicals

Innogenetics, SA
Antwerp, Belgium
Nitrocellulose membrane dipsticks

E. Merck
P.O. Box 4119
Frankfurterstrasse 250
D-6100 Darmstadt 1, Germany
Tel: +49 615 1720
Chemicals
Biochemicals
Media

Millipore, Inc.,
Bedford, Ma., USA
Filtration equipment

Nordic Immunological Laboratories
55-61 Langestraat 5038SC Tilburg
P.O. Box 22
5000A Tilburg, The Netherlands
Tel: +31 13 35 10 85 or 42 11 70; tlx 52 293 (NILAB NL)
Immunochemicals

Rhône-Poulenc
Spécialités chimiques
Les Miroirs
Défense 3
92097 Paris La Défense, France
Tel: +33 1 409 16123
Latex

Riedel-De Hën AG
Wunstorfer Strasse 40
D-3016 Seelze 1, Germany
Tel: +49 5137 7070; tlx 921 295 rdhs d
Fax: +49 (5137) 91979; +49 (5137) 707123
Chemicals

Sanofi-Pasteur
3 boulevard Raymond Poincaré
92430 Marnes-la-Coquette, France
Tel: +33 1 47 95 60 00
Immunochemicals

Schleicher &Schuell GmbH P O Box 4 D-3354 Dassel, Germany tel: +49 5561 7910; fax: +49 5564 4309	Nitrocellulose sheets
Sekisni Chemical Ltd Center for Diagnostic Products Inc. 800 Huntington Avenue Boston, MA 02115, USA	Latex
Seradyn Inc. P.O. Box 1210 Indianapolis, IN 46209-9853, USA Tel: +1 317 266 2915	Latex
Sigma Chemical Company P.O. Box 14508 St. Louis, MO 63178, USA Tel: +1 314 771 5750	Chemicals Biochemicals
Soconex ISBA, SA Renens Switzerland	Microdispensers
Unipath Ltd Wade Road Basingstoke Hampshire RG24 OPW, UK Tel: +44 1256 841 144	Latex
Glaxo Foundation Ltd 189 Euston Road London NW1 2BP, UK Tel: +44 171 387 4477; fax: +44 171 388 3530	Diagnostics Biochemicals

ANNEX 5
Dyes and indicators and their synonyms

CI number	Name		Synonyms	
CI42685	Acid fuchsin	Acid Rubin	Acid Roseine	Acid Magenda
	Aesculin	Polychrome	Escosyl	
CI42755	Aniline blue	mixture of sulfonated triphenyl methanes		
	Azur I	mixture of oxidized methylene blue containing Azur A and Azur B		
	Dibromo-o-cresol sulphonphtalein		Bromocresol Purple	
	Dibromothymol sulphonphtalein		Bromothymol Blue	
CI42555	Crystal Violet	Basic Violet 3	Gentiana Violet	Aniline Violet
CI45380	Tetrabromofluorescein		Eosin Y	Acid Red 87
CI42000	Malachite Green	Fast Green	Aniline Green	
CI50040	Neutral Red	Basic Red 5	Nuclear Fast Red	Kernecht Rot
	Phenol sulphonphtalein		Phenol red	
	Resazurin	Resazoin	Diazoresorcinol	
CI50240	Safranine O			
CI52015	Tetramethylthionium chloride		Methylene Blue	Basic Blue 9 Swiss Blue

PRODUCTION OF BASIC DIAGNOSTIC LABORATORY REAGENTS

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