Laboratory Investigation of Intestinal Infectious Diseases

Quality Assurance Manual

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საქართველოს შრომის, ჯანმრთელობისა და სოციალური დაცვის სამინისტრო

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PATH currently works in more than 70 countries in the areas of health technologies; maternal and child health; reproductive health; vaccines and immunization; and emerging and epidemic diseases. Staff provide expertise in public health, epidemiology, technology development and transfer, technology introduction, biotechnology and vaccine development, vaccine distribution, business development, education and training, communication, advocacy, and procurement.

Headquartered in Seattle, Washington, since its inception in 1977, PATH operates 34 offices in 19 countries. PATH's 2008 budget is US$218 million, which is provided by foundations, the US government, other governments, multilateral agencies, corporations, and individuals. PATH's 11-member board of directors consists of representatives from countries where PATH works, including the United States.

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Curatio International Foundation (CIF) is a not-for-profit, nongovernmental organization founded in 1994 in the Republic of Georgia with the mandate to support reforms in the health and social sectors in developing countries and transitional economies.

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CIF coverage areas are Southern Caucasus, Central Asia, Eastern Europe, North Africa and Middle East.

For more information about CIF, visit **www.curatiofoundation.org**.

About this Document

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Acronyms

A–G	A9 and 20 echoviruses
AP	Alkaline Phosphatase
API	Analytical profile index
APW	Alkaline peptone water
ART	Arterial
AST	Antimicrobial susceptibility testing
ASTGN card	Antimicrobial susceptibility testing Gram Negative card
ATB	Antibiotic
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guerin (BCG) Vaccine
BSC	Biosafety Cabinet
BSL	Biosafety Level
CA	Coxsackie A viruses
CASFM	Comité de l'Antibiogramme de la Société Française de Microbiologie
CB	Coxsackie B viruses
CLSI	Clinical and Laboratory Standards Institute
CP	Coxsackievirus B1-6 pool
CPE	Enterovirus Cytopathic Effect
CPH	Center for Public Health
CSF	Cerebral spinal fluid
CVA	Campylobacter selective medium containing cefoperazone, vancomycin and amphotericin B (CVA)
DCA	Desoxycholate citrate agar
DD	Diarrhoeal Diseases
DIN	Document Identification Number
DNA	Deoxyribonucleic Acid
DoB	Date of Births
DP	Document Procedures
EDTA	Ethylene Diamine Triacetic Acid
EHEC	Escherichia coli Enterohaemorrhagic
EIA	Enzyme immuno assay
ELB	Equipment Log Book
ELISA	Enzyme-linked immunosorbent assay
EQC	External quality control
F/T	Freeze/Thaw
FBS	Fetal Bovine Serum
GN card	Gram Negative card
HAV	Hepatitis A virus
HE	Hektoen enteric agar
HEPA	High-Efficiency Power Amplifier
HUS	hemolytic-uremic syndrome
ICD	International Classification of Diseases
IDEIA	Rotavirus Test
IgM	Immunoglobulin M
IHR	International Health Regulations
IQC logbook	Internal quality control logbook
ISO	International Organization for Standardization
ISO standard	International Organization for Standardization
KIA, LIA	Kligler iron agar, Lysine iron agar
LIMS	Laboratory Information Management System
LIS	Laboratory Information Systems

MAC	MacConckey agar
MIC	Minimum Inhibitory Concentration
MoLHSA	Ministry of Labor, Health and Social affairs
NaDCC	Sodium dichloroisocyanurate
NaOCl	Sodium Hypochlorite
NB	Nota Bene
NCDC	National Center for Disease Control and Public Health
OD	Optical density
PATH	Program for Appropriate Technology in Health
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	positive hydrogen ion concentration; measure of acidity
PHRplus	Partners for Health Reformplus
PP	Pooled polio antiserum
PSIpettes	Pipettes
QA	Quality Assurance
QC	Quality Control
QAM	Quality Assurance Manual
RD	Rhabdomyosarcoma
RF	Rheumatoid Factor
RNA	Ribonucleic acid
RT-PCR	Reverse type Polymerase Chain Reaction
S/I/R	Susceptible / Intermediate / Resistant
SE	Selenite Broth
SIRS	sepsis – inflammatory response syndrome
SMAC	Sorbitol Mac Conkey
SOP	Standard Operating Procedures
SS	Salmonella-Shigella agar
TB	Tuberculosis
TC	Tissue Culture
TCBS	Thiosulfate citrate bile salts sucrose agar
TSI Agar	Triple Sugar Iron Agar
VTM	Viral Transport Medium
WHO	World Health Organization
XLD	Xylose lysine desoxycholate

Contributors

The manual was prepared by the multi-agency task force headed by Dr. Paata Imnadze, Director of the National Center for Disease Control and Public Health with technical assistance of Dr. Antoine Pierson[1] (International Laboratory Consultant).

The working group composition was as follows:

Paata Imnadze	NCDC, General Director
Antoine Pierson	International Laboratory Consultant
Shota Tsanava	NCDC, Deputy Director
Guram Katsitadze	NCDC, Head of the Research and Information Technologies Department
Nino Macharashvili	Infectious Disease, AIDS and Clinical Immunology Research Centre, Head of the Bacteriological Laboratory
Tamar Ghaniashvili	NCDC, senior specialist of cholera and other intestinal infection laboratory
Lela Urushadze	NCDC, Senior specialist of polio and enteric viruses laboratory
Marika Karchava	Infectious Disease, AIDS and Clinical Immunology Research Centre, senior specialist of clinical virology laboratory

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[1] www.antoinepierson.net

Purpose and Objective of this manual

This QAM concerns laboratory diagnosis of selected Intestinal Infectious Diseases such as Salmonellosis, Shigellosis, Escherichiosis, Campylobacteriosis, Cholera, Adenovirus, Non-Polio Enterovirus, Rotavirus, Norovirus infections, Hepatitis A, Amebiasis.

The QAM contains the following parts:

- Introduction and general facts
- Biosafety considerations
- Equipment management
- Pre-analytical procedures (sampling, sample transportation, sample preparation)
- Analytical procedures (sample analysis)
- Post-analytical procedures (reporting, archiving, backup)
- QA measures (for all 3 different steps)

When suspecting an intestinal infectious disease, a clinician can orient the analysis using clinical and epidemiological criteria. Once this orientation is available, the laboratory specialist should first refer to pre-analytical procedures in order to make sure the specimen is sampled, transported and preserved in the best possible way. Do not forget that transport media need to be prepared and stored in advance (it will be too late the day a huge number of specimen will require to be handled).

Then, the laboratory specialist needs to follow the analytical procedures, which have been split into different groups. Media and reagent preparation are also handled in this analytical procedure group.

Once analysis is over, post-analytical procedures need to be followed in order to make sure that the result will reach its correct recipient(s) in the shortest amount of time.

In order to get the best possible results, quality assurance measures need to be followed scrupulously.

This guideline is organized in different chapters, sorted out by the analytical process chronological order. Each procedure can be read independently, although numerous references to other procedures are being made. Refer to the table of content in order to get a global overview of all aspects developed.

NCDC recommends the use of these guidelines and procedures by public and private laboratory specialists throughout the country.

Dr. Paata Imnadze
NCDC Director

Tbilisi
January 1st, 2009

Address book

Area	First/last name	Institution	Phone/@mail
		NCDC	+(995 32) 39 89 46; ncdc@ncdc.ge
Emergencies	Nikoloz Tsertsvadze	NCDC	+(995 99) 23 59 64; niko@ncdc.ge
Biosafety	Lela Bakanidze	NCDC	+(995 99) 57 24 84; threatred@ncdc.ge
Bacteriology	Nino Macharashvili	Infectious Disease, AIDS and Clinical Immunology Centre	+(995 99) 45 48 54; ninomachar@yahoo.com
	Lia Tevsadze	NCDC	+(995 32) 36 45 31; +(995 99) 98 75 7
	Tamar Ghaniashvili	NCDC	+(995 99) 98 80 91; Ghaniashvili@yahoo.com
Virology	Lela Urushadze	NCDC	+(995 99) 24 54 34; lelincdc@gmail.com
	Marika Karchava	Infectious Disease, AIDS and Clinical Immunology Centre	+(995 32) 39 91 43; Kmarika11@hotmail.com

1. Introduction

1.1. Terminology and definitions

- Quality assurance

A set of predefined and systematic actions set up in the framework of the quality strategy as demonstrated needs, in order to provide appropriate confidence that a process will comply with quality requirements. In the context of medical laboratories, quality assurance allows the organization of tasks leading to quality, and refers to pre-analytical, analytical and post-analytical steps of the diagnostic test.

- Quality control/ proficiency testing

Control by an independent external body of the quality of results produced by a single laboratory and by a group of laboratories.

- Documented procedure

Specific manner to carry out any activity. This is a written rule for organization, which describes the modalities and steps to be performed in order to obtain the expected result. This is a set of written instructions specific for each laboratory, describing every operation to be performed, precautions to be taken, rules to be applied in the laboratory.

- SOP

Document describing the way the process should be performed and all necessary means for its achievement.

- Pre-analytical steps

The sum of all steps carried out before the analysis itself. This is usually related to sampling, sample transportation, sample conservation and sample labeling.

- Post-analytical steps

The sum of all steps carried out after the analysis itself. This is usually related to the validation of results, printing/copying of results, transmission of analysis (to clinicians and public health authorities), storage of specimens and materials after analysis (serology) and other interactions with health actors.

- Quality manual

Document describing the general measures set up to ensure quality assurance.

- Validation

Process of checking technical conditions during the processing of laboratory analysis in order for the result to be secured by safe and reliable procedures and is in concordance with other medical characteristics of the patient. Validation has two components: one analytical and the other - biological. Analytical validation refers to compliance of analysis realization with official procedures, and includes validity of results obtained through internal controls. Biological validation is, on the other hand, a control of coherence in data from all analyses performed for the same patient, depending on its clinical status, personal conditions, ongoing treatments and previous analyses.

- Certification

Procedure by which an authoritative body gives formal recognition insuring that an entire institution is complying with quality management processes. Certification is granted for an entire institution only. The ISO standard to be used is the generic ISO9000 standard (same whatever activity is considered).

- Accreditation

Procedure by which an authoritative body gives formal recognition insuring that a body or person is competent to carry out one or more specific tasks. Accreditation is granted separately for each of the tasks. In the field of health laboratories, the ISO standard used for accreditation is ISO15189, when ISO17025 is used for non-medical laboratories (food, research, veterinarian, etc.).

1.2. The Quality Manual

This manual was developed to implement a quality management system for intestinal infectious diseases aimed primarily at achieving customer satisfaction by meeting customer requirements through the application of the system, continuous improvement of the system and prevention of the occurrence of nonconformities. The quality management system proposed by this manual was based on and demonstrates throughout its wording good quality principles, such as leadership, customer focus, continual improvement, involvement of the staff, mutually beneficial supplier relationships, process focus, management by a system of processes and ultimately decisions that are based on good quality data.

This quality manual describes the Quality Management System of the Unit of Clinical Microbiology**.** Its purpose is for:

- Internal use - to communicate to the staff the laboratory's Quality Policy and quality objectives, to make the staff familiar with the methods, which are used to achieve compliance with quality requirements, and facilitate the implementation and maintenance of the Quality Management System as well as to ensure its continuity and required updates under altering circumstances, to provide effective communication and quality control related activities and a documented base for quality system audits.
- External use **-** to inform the Clinical Microbiology Laboratory's customers and other interested external partners about its Quality Policy as well as the Clinical Microbiology Laboratory's implemented Quality Management System and measures of compliance with quality.

The Quality Management Committee maintains this manual for the Quality Management System and for its use by the laboratory staff.

Requests for changes should be submitted to the Quality Management Committee. Manual updates are issued as required. The computerized version of this quality manual is available at NCDC web page (**www.ncdc.ge**). The computerized version is to be considered the most current and takes precedence over any printed copy.

It is the responsibility of the Director of the Laboratory to ensure that all staff is familiar with the manual's content related to their work and responsibilities and that they are kept informed of any changes and updates.

Effective date of this Quality Manual: 01/01/09

1.3. Decrees and Official texts that Regulate Laboratories

Decrees

- "*Sanitary norms for working with pathogenic biological agents (pathogenic microorganisms)*. *Decree No 317/N, December 6, 2005, Tbilisi, MoLHSA.* The decree defines the following: risk assessment principles and bio-safety levels; classification of pathogenic biological agents; laboratory equipment requirements, general working principles, requirements on health personnel medical follow-up, good microbiological practices for basic (I and II biosafety levels) and high (III) and highest (IV) biosafety level laboratories; requirements for individual protective equipment; control measures during accidents. The decree in a separate attachment contains the guidelines for Anthrax diagnosis and prophylaxis.
- Rules of urgent notification of communicable diseases are established in this decree. All institutions and providers rendering health care services regardless of their subordination and forms of ownership including laboratories must notify the local public health service whenever they diagnose, suspect, or even receive positive lab results. The decree endorses lab notification form *"Management and submitting of medical statistical information" – Decree No. 101/n April 5, 2005, Tbilisi, MoLHSA.*
- Reagents and diagnostics for laboratory testing of infectious diseases in limited amounts are provided by the *"State Program on Coordination of Epidemiological Surveillance, Control of Quarantined (Plague, Cholera, Hemorrhagic Contagious Viral Fevers), Particularly Dangerous and Equated to them Infectious Disease and Prevention of Other Communicable Diseases", 2003, MoLHSA and DPH.* The goal of the program is to gradually reduce the spread of infectious diseases through their liquidation, elimination and control. The program is financed by the state budget.
- Vaccination of personnel working with live cultures is recommended in *"Concerning Approval of the Preventive Vaccination Calendar" – Decree No 122/N June 4, 2003;*
- Instructions for handling medical waste, their storage, decontamination, and disposal are in *"Concerning Storage and Disposal of Medical Waste of Health Care Facilities" – Decree No. 300/n August 16, 2001, Tbilisi, MoLHSA.*
- Requirements for handling the materials suspected or containing poliovirus are described in "*Concerning Containment of Poliomyelitis Virus in Georgia" – Decree No. 97/o June 6, 2000, Tbilisi, MoLHSA.*
- A decree provides indicators for effectiveness of laboratory performance, sample passport (form) for laboratory testing, and instructions for sample collection and transportation. *"Concerning additional activities for poliomyelitis eradication and improvement of AFP surveillance" – Decree No 243/o July 2, 1997, Ministry of Labor, Health and Social Affairs (MoLHSA).*

Note: All the decrees are available at NCDC and in regional CPHs.

Laws of Georgia

- **Public Health Law of Georgia - 27.06.2007.** Regulates the rights and responsibilities of individuals and legal entities with regard to public health. The **Chapter V - "Providing Biosecurity/ Biosafety"** outlines the state obligations with regard to biological security and safety. The following clauses are specifically dedicated to this issues:
    - **Cl.16 - Providing Biosecurity / Biosafety**
    - **Cl.17 - Limitation of Possession, Use, Transfer, Transportation and Destruction of Causative Agents of Especially Dangerous Infections**
    - **Cl.18 - Destruction of Causative Agents of Especially Dangerous Infections**

    - **Cl.19 – Import and Export of Causative Agents of Especially Dangerous Infections**
    - **Cl.20 – Functions of Sufficient Institutions Working on Biosafety/Biosecurity**
    - **Cl.21 – Establishing Unique Laboratory System for Detection, Surveillance and Response to Causative Agents of Especially Dangerous Infections.**
- **Law on Licensing and Permits – 24 June, 2005.** According to this law, a license is required only for those institutions, who are working on especially dangerous pathogens.
- **Law on Medical Activity – June 8, 2001.** The law determines the mandatory qualification of medical workers in Georgia.
- **Law on Patient Rights– May 5, 2000.** The law protects patient rights.
- **Law on Health Care – December 12, 1997.** The law regulates relations between the state and legal institutions and physical bodies in the field of health care throughout Georgia.

2. Document management

2.1. SOP management

Responsibilities

A Quality Assurance Manager and a Biosafety Officer should be designated to each laboratory. A Quality Assurance Manager is responsible for the management of quality and different SOPs.

Methodology

Managing Documented Procedure and Standard Operating Procedure (DP and SOP, see definitions) documents is a critical responsibility. DP and SOP documents provide definitive guidance for the laboratory's staff as they collect field data and execute different analysis. These procedures need to be written, reviewed, approved, published, distributed and archived.

Operating mode

Managing DP and SOP documents is a cooperative process between all users of the DP and SOP document set.

Creation/Editing

The documents, such as DPs and SOPs are produced by an author who often starts with using the template developed by the laboratory or an existing document. Each section of the template is completed or deleted if not applicable. Once a draft of the document is ready for review, it should be sent to the National Quality Management Committee for reading, reviewing and commenting. This Quality Management Committee is detailed in DP "Quality Management". A copy should be sent to the Biosafety Committee for the biosafety parts when included. The Biosafety Committee is detailed in DP "Biosafety Management ".

Review

The Quality Management Committee and Biosafety Committee use either a hardcopy or a word processor's electronic annotation feature to make notes and comments on the draft. In most cases, the draft version is sent back to the author for revision. The document may circulate many times between the author(s) and the committee before an acceptable version is completed.

Approval

The finalized version should be sent to the Quality Management Committee and, if needed, to the Biosafety Committee, for revision and final review and authorization. The committee may deny approval, or reject further implementation of the document and send it back to the author for additional revision. Once approved, it is sent to the Document Manager for hardcopies to be printed and the action of approval is noted by physical handwritten signatures on the hardcopy.

Publishing

Documents that have been approved should be placed in the area determined for document production. Hardcopies of signed documents are stored in file cabinets. Electronic versions are often stored in file server directories with access controlled by user authorization.

Distribution

The Quality Manager should ensure that finalized and approved documents are distributed to the staff that uses them to guide regulated processes. The Quality Manager will keep track of who needs specific documents, and will determine who has actually read the documents as will the

Biosafety Officer. The staff members are supposed to confirm by a signature, that they have acquainted themselves with each SOP.

Revision

Decisions that modify procedures are not casual decisions, and should only be recommended after thorough consideration of all possible consequences. If possible, changes to procedures should be reviewed by the author(s) and the Quality Management Committee and/or the Biosafety Committee. If no coordination of changes by the original author of the procedures is possible, the proposed changes should be discussed by the committee. After a decision to modify a procedure has been reached, the committee will serve as the point of contact responsible for ensuring that the new procedure is properly recorded by the Quality Manager for further use.

Once a DP or SOP has been revised, the date of the revision, the person making the revision, the changes made, the reasons for the revision, and the new version number, shall be identified on the front page of the document in the appropriate boxes of the template. The reasons for the revision and the changes shall be sufficiently documented to ensure that the background for the revision is clear.

During use, field personnel and committee staff may determine that a SOP requires modification. If the need is urgent, and must be done in the field, the modified procedures should be thoroughly documented in the existing SOP document. At the earliest possible time following field modifications, the need for modification should be communicated to the quality committee, which will "check" the current version of the DP or SOP pending submittal of a revised 'electronic' copy of the DP or SOP.

Regular assessments of the effectiveness of the follow-up of DP and SOPs will be carried out by the Quality Manager according to DP "DP and SOP auditing".

Archiving

Once a published and distributed document has been revised, the previous version should be stored in an archive for 7 years and later disposed. This includes both the hardcopy and any electronic copies of the document.

2.2. Test Report

The final test report is the way to formalize the analytical results into a user-friendly, well summarized document. Please, keep in mind that most of the patients will never understand all the complicated analytical steps the laboratory staff had to go through in order to produce a good result. Badly printed or handwritten test report will not leave a good image to a patient, thus, a standardized (printed or well handwritten) report should be promoted.

A standardized test report should include:

- Temporal data: date and hour of sampling, date of the report
- Patient data: first and last name, DoB, sex, address (or district)
- Prescriber/clinician data: name/institution
- Tests data: value, normal value (if any), units (international units recommended), technique used (especially for serology or automated systems)

- Validation data: name and signature of the laboratory specialist, eventual comments.

Test report transmission is also very important, and is being addressed in the chapters 12.3 and 12.4.

Example of a Test Report form:

Name of Institution _____

Date _of sampling __/____/____ / time _____hour

Date _of report __/____/____ / time_____ hour

Patient Name _____ ☐ Male ☐Female

Address: _____

Age ____years or Date of birth___/____/____

Microbiological Identification Test

Biochemical tests		**Vitek 2 Compact**	
API system		**Serological typing**	

Sample ID_____

Test result:_____ XXXXXXX (normal value) XXXX units

Test performed by (name & signature) _____

Head of Laboratory (name & signature)

Address _____ _____ Tel. _____Fax

2.3. Laboratory logbooks

All received and tested samples should be registered in the laboratory logbooks. Below is an example of the logbook:

Number	Sample ID	Patient first and last name	Age	Gender	Address	Place of work / study	Facility that sent patient / sample	Person under investigation (patient, contact, risk group)	Sample	Date of sampling	Sample received	Test result	Date of releasing test result	Signature	Comment

Completed logbooks should be kept at the facilities for five years.

3. Safety and Hygiene

3.1. Biosafety policy and management

According to the Public Health Law, the MoLHSA is responsible for defining a national policy to ensure biosafety to the population. The MoLHSA identifies the list of Especially Dangerous Pathogens to be updated every three years and upon the necessity. The Government establishes the Integrated Laboratory System of Detecting, Epidemiological Surveillance and Response of Especially Dangerous Pathogens. The management of the Integrated Laboratory System is performed by the Coordination Council, headed by the Prime Minister. The structure and staffing of the council is defined by the Government of Georgia resolution. The Laboratory System consists of the network of different biosafety level laboratories.

Specific biosafety procedures for different biosafety level laboratories are defined by MoLHSA Decree No 317/N, December 6, 2005 on “Sanitary Norms for Working with Pathogenic Biological Agents (pathogenic microorganisms).

3.2. Safe manipulation

In many laboratories and national laboratory programs, a code of Safe Manipulation Procedure may be given the status of “rules" for laboratory operation.
Good microbiological technique is fundamental to laboratory safety and cannot be replaced by specialized equipment, which can only supplement it. Some requirements are the same as sampling requirements:

1. The international biohazard sign (see Illustration 1) should be displayed on the doors of the rooms where Risk Group 2 micro-organisms are handled.

2. Mouth pipetting should be prohibited.

3. Eating, drinking, smoking, storing food, and applying cosmetics must not be permitted in the laboratory work areas.

4. Labels must not be licked but sticked; materials must not be placed in the mouth.
5. The laboratory should be kept neat, clean, and free of materials that are not pertinent to the work.

6. Work surfaces must be decontaminated after any spill of potentially dangerous material and by the end of a working day.

7. Members of the staff must wash their hands after handling infectious materials and animals, and before they leave the laboratory.

8. All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets.

9. All contaminated materials, specimens, and cultures must be decontaminated before disposal or cleaning for reuse. They should be placed in leak-proof, color-coded plastic bags for autoclaving or incineration on the premises. These bags should be supported in rigid containers. If it is necessary to move the bags to another site for decontamination, they should be placed in leak-proof (i.e. solid-bottomed) containers, which can be closed before they are removed from the laboratory.

10. Laboratory coveralls, gowns, or uniforms must be worn for work in the laboratory. This clothing should not be worn in non-laboratory areas such as offices, libraries, staff rooms, and canteens. Contaminated clothing must be decontaminated through appropriate methods.

11. Open-toed footwear should not be worn.

12. Protective laboratory clothing should not be stored in the same lockers or cupboards as street clothing.

13. Safety glasses, face shields (visors), or other protective devices must be worn when it is necessary to protect the eyes and face from splashes and impacting objects.

14. Only persons, who have been advised of the potential hazards and who meet specific entry requirements (e.g. immunization), should be allowed to enter the laboratory working areas. Laboratory doors should be kept closed when the work is in progress; access to animal cages/kennels should be restricted to authorized persons; children should be excluded from laboratory working areas.

15. There should be an arthropod and rodent control program.

16. Animals not involved in the work of the laboratory should not be permitted in or near the lab.

17. The use of hypodermic needles and syringes should be restricted to parenteral injection and aspiration of fluids from laboratory animals. Their use for removing the contents of diaphragm bottles should be limited (opening devices are available, permitting the use of pipettes). Hypodermic needles and syringes should not be used as substitutes for pipetting devices in the manipulation of infectious fluids. Cannulas should be used instead of needles whenever possible.

18. Gloves appropriate for the work must be worn for all procedures that may involve accidental direct contact with blood, infectious materials, or infected animals. After use, gloves should be removed aseptically and autoclaved with other laboratory wastes before disposal. Hands must then be washed. Reusable gloves must be washed while on the hands and, after the gloves are removed, they must be cleaned and disinfected before reuse.

19. All spills, accidents, and overt or potential exposures to infectious materials must be reported immediately to the laboratory supervisor. A written record of such accidents and incidents should be maintained.

20. Appropriate medical evaluation, surveillance, and treatment should be provided.

21. Baseline serum samples may be collected from laboratory staff and other persons at risk. These should be stored as appropriate. Additional specimens should be collected periodically depending on the organisms handled and the function of the laboratory.

22. The laboratory supervisor should ensure that training in laboratory safety is provided. A safety or operation manual that identifies known and potential hazards and that specifies practices and procedures to minimize or eliminate such hazards should be adopted. Personnel should be advised of special hazards and required to read and follow the standard practices and procedures. The supervisor should make sure that the personnel understand these.

Illustration 1. Biohazard label

3.3. Personal protective clothing and equipment required in the laboratory

Personal protective clothing and equipment may act as a barrier to minimize the risk of exposure to aerosols, splashes, and accidental inoculation. Clothing and equipment selected is dependent on the nature of the work performed. Protective clothing should be worn when working in the laboratory.

Before leaving the laboratory, protective clothing should be removed, and hands should be washed. **Table 1** summarizes some personal protective equipment used in laboratories and the protection afforded.

Table 1. Personal protective equipment

Equipment	Hazard corrected	Safety features
Laboratory coats, gowns, coveralls	Contamination of clothing	Back opening Cover street clothing
Plastic aprons	Contamination of clothing	Waterproof
Footwear	Impact and splash	Closed toe
Goggles or safety spectacles	Impact and splash	Impact-resistant lenses (must be optically correct/worn over corrective eye glasses) Side shields
Face shields	Impact and splash	Shield entire face Easily removable in case of accident
Respirators	Inhalation of aerosols	Designs available include hoods, full-face or half face masks
Gloves	Accidental direct contact Accidental punctures or cuts	Disposable latex or vinyl Hand protection

Laboratory coats, gowns, coveralls, aprons

Laboratory coats should preferably be fully buttoned. However, long-sleeved, back-opening gowns or coveralls give better protection than laboratory coats and are preferred in microbiology laboratories and when working in the biological safety cabinet. Aprons should be worn over laboratory coats or gowns where necessary to give further protection against spillage of chemicals or biological materials such as blood or culture fluids.

Goggles, safety spectacles, face shields

The choice of equipment to protect the eyes and face from splashes and impacting objects will depend on the activity performed. Prescription or plain eye glasses can be manufactured with special frames that allow lenses to be placed in frame from the front, using shatterproof material either curved or fitted with side shields (safety glasses). Goggles should be worn over normal prescription eye glasses and contact lenses, which do not provide protection against biological hazards. Face shields (visors) are made of shatterproof plastic, fit over the face, and are held in place by head straps or caps.

Respirators

Respiratory protection may be used when carrying out high-hazard procedures (e.g. cleaning up a spill of infectious material). The choice between mask and respirator, and type of respirator will depend on the type of hazard. Respirators are available with interchangeable filters for protection against gases, vapors, particulates, and microorganisms. Note that no filter other than a HEPA filter will provide protection against microorganisms, and it is imperative that the filter be fitted in the correct type of respirator. To achieve optimal protection, respirators should be individually fitted to the operator's face and tested. Fully self-contained respirators with an integral air supply provide full protection. Advice should be sought from a suitably qualified person for selection of the correct respirator.

Gloves

Contamination of hands may occur when laboratory procedures are performed. Hands are also vulnerable to "sharps" injuries. Disposable latex or vinyl surgical-type gloves are used widely for general laboratory work, and for handling infectious agents and blood and body fluids. Reusable gloves may also be used, but attention must be paid to their correct washing, removal, cleaning,

and disinfection. Gloves should be removed and hands thoroughly washed after handling infectious materials and working in a biological safety cabinet, and before leaving the laboratory. Used disposable gloves should be discarded with infected laboratory wastes. Allergic reactions such as dermatitis and immediate hypersensitivity have been reported in laboratory and other workers wearing latex gloves, particularly those with powder. Alternatives such as powder-free latex or vinyl gloves should be used if problems occur. Stainless steel mesh gloves should be worn, when there is a potential exposure to sharp instruments e.g. during postmortem examinations. However, such gloves protect against slicing motion, but do not protect against needle injury.

3.4. Biosafety equipment

Responsibilities

The laboratory director will, after consultation with the Biosafety Officer and Biosafety Committee, ensure that adequate equipment is provided and that it is used properly.

Operating mode

Equipment is selected to take account of certain general principles:

- Designed to prevent or limit contact between the operator and the infectious material
- Constructed of materials that are impermeable to liquids, resistant to corrosion material
- Fabricated to be free of burrs, sharp edges and unguarded moving parts
- Designed, constructed and installed to facilitate simple operation and provide for ease of maintenance, cleaning, decontamination and certification testing.

The Biosafety Officer will check that the following essential biosafety equipment is present in the laboratory, working correctly, used appropriately and regularly maintained:

- Pipetting aids: to avoid mouth pipetting
- Biological safety cabinets, to be used whenever:
    - Infectious materials are handled
    - There is an increased risk of airborne infection
    - Procedures with high potential for producing aerosols are used.
- Screw-capped tubes and bottles
- Autoclaves or other appropriate means to decontaminate infectious materials
- Plastic disposable Pasteur pipettes, whenever available, to avoid glass.

3.5. Disinfection and chemical cold sterilization

Chemical germicides

Many types of chemicals can be used as disinfectants and antiseptics and there is an ever increasing number and variety of commercial products. Formulations must therefore be carefully selected for specific needs, and stored, used, and disposed of as directed by the manufacturer. The germicidal activity of many chemicals is faster and better at higher temperatures. At the same time, higher temperatures can accelerate their evaporation and also degrade them faster. Particular care is needed in the use and storage of such chemicals in tropical regions, where their shelf-life may be reduced because of high ambient temperatures.

Many germicides can be harmful to humans and the environment. They should therefore be selected, handled, and disposed of with care. For personal safety, gloves, aprons and eye protection are recommended when preparing use-dilutions of chemical germicides. Chemical germicides are therefore not required for regular and general cleaning of floors, walls, equipment, and furniture except in case of outbreak control.

Equipment

Temperature sensitive

Disinfectants, Disinfection

Important note: before any disinfection activities, materials and equipment have to be regularly "pre-cleaned," for two main reasons:

- Decreasing the number of micro-organisms
- Avoiding the precipitation of proteins by highly effective disinfectant agents.

In conclusion, only "clean" material will be disinfected.

Pre-cleaning and cleaning laboratory materials

In practical terms, cleaning is the removal of visible dirt and stains. This is generally achieved either by (a) brushing, vacuuming, or dry dusting; or (b) washing or damp mopping with water containing a soap or detergent. Where the risk of human or animal contact with pathogen contaminated materials is high and subsequent decontamination is needed, pre-cleaning is routinely carried out. This is necessary because dirt and soil can shield microorganisms and can also interfere with the killing action of chemical germicides. In such cases, pre-cleaning is essential to achieve proper disinfections or sterilization. Also, many germicidal products claim activity only on pre-cleaned items. Pre-cleaning must be carried out with care to avoid exposure to infectious agents, and materials chemically compatible with the germicides to be applied later must be used. It is quite common to use the same chemical germicide for pre-cleaning and disinfections.

Main chemical disinfectants

- Chlorine
- Sodium dichloroisocyanurate
- Chloramines
- Chlorine dioxide
- Formaldehyde
- Glutaraldehyde
- Phenolic compounds
- Quaternary ammonium compounds
- Alcohols
- Iodine
- Hydrogen peroxide and peracids

Chlorine (sodium hypochlorite)

Chlorine, a fast-acting oxidant, is a widely available and broad-spectrum germicide. It is normally sold as bleach, an aqueous solution of sodium hypochlorite (NaOCl), which can be diluted with water to provide various concentrations of available chlorine. Chlorine, especially as bleach, is highly alkaline and can be corrosive to metal. Its activity is considerably reduced by organic matter (protein). Storage of stock or working solutions of bleach in open containers, particularly at high temperatures, releases chlorine gas, thus weakening their germicidal potential. The frequency with which working solutions of bleach should be changed depends on their starting strength, the type (e.g., with or without a lid) and size of their containers, the frequency and nature of use, and ambient conditions. As a general guide, solutions receiving materials with high levels of organic matter several times a day should be changed at least daily, while those with less frequent use may last for as long as a week.

A general all-purpose laboratory disinfectant should have a concentration of 1g/l available chlorine. A stronger solution, containing 5g/l available chlorine, is recommended for dealing with biohazardous spillage and in the presence of large amounts of organic matter. Sodium hypochlorite containing 5g/l available chlorine is recommended as the disinfectant of choice in emergency situations involving viruses such as Hantavirus, and Lassa and Ebola viruses.

Sodium hypochlorite solutions such as domestic bleach contain 50g/l available chlorine and should therefore be diluted 1:50 or 1:10 to obtain final concentrations of 1g/l and 5g/l, respectively. Industrial solutions of bleach have a sodium hypochlorite concentration of nearly 120g/l and must be diluted accordingly to obtain the necessary levels.

Granules or tablets of calcium hypochlorite (Ca(ClO)2) generally contain about 70% available chlorine. Solutions prepared with granules or tablets, containing 1.4g/l and 7.0g/l, will then contain 1.0g/l and 5g/l available chlorine, respectively.
Bleach is not recommended as an antiseptic, but may be used as a general-purpose disinfectant and for soaking contaminated metal-free materials. In emergencies, bleach can also be used to disinfect drinking water with a final concentration of 1–2 mg/l available chlorine.

Note: Chlorine gas is highly toxic. Bleach must therefore be stored and used in well-ventilated areas only. Also, bleach must not be mixed with acids in order to avoid the rapid release of chlorine gas. Many by-products of chlorine can be harmful to humans and the environment, so that indiscriminate use of chlorine-based disinfectants, and in particular bleach, should be avoided.

Sodium dichloroisocyanurate
Sodium dichloroisocyanurate (NaDCC) in powder form contains 60% available chlorine. Solutions prepared with NaDCC powder at 1.7g/l and 8.5g/l will contain 1g/l or 5g/l available chlorine, respectively. Tablets of NaDCC generally contain the equivalent of 1.5g available chlorine per tablet. One or four tablets dissolved in 1 liter of water will give the required concentrations of 1 g/l or 5 g/l, respectively. NaDCC as powder or tablets is easy and safe to store. Solid NaDCC can be applied on spills of blood or other biohazardous liquids and left for at least 10 min before removal.

Further cleaning of the affected area can then take place.

Chloramines
Chloramines are available as powders containing about 25% available chlorine. Chloramines release chlorine at a slower rate than hypochlorites. Higher initial concentrations are therefore required for efficiencies equivalent to those of hypochlorites. On the other hand, chloramine solutions are not inactivated by organic matter to the same extent as hypochlorite solutions, and concentrations of 20g/l are recommended for both “clean” and “dirty” situations. Chloramine solutions are virtually odor-free. However, items soaked in them must be thoroughly rinsed to remove any residue of the bulking agents added to chloramine-T (sodium tosylchloramide) powders. Chloramines can also be used to disinfect drinking water when used at a final concentration of 1–2 mg/l available chlorine.

Chlorine dioxide
Chlorine dioxide is a strong and fast-acting germicide, often reported to be active at levels lower than those needed by chlorine as bleach. To obtain an active solution for laboratory use, it is generally necessary to mix two separate components, hydrochloric acid (HCl) and sodium chlorite (NaClO2).

Stability can be an important issue with this germicide, and materials compatibility and corrosiveness must also be considered when selecting products based on it.

Formaldehyde
Formaldehyde (HCHO) is a gas that kills all microorganisms and their spores at temperatures above 20°C. Formaldehyde is not active against prions. It is relatively slow-acting and needs a relative humidity level of about 70%. It is marketed as the solid polymer, paraformaldehyde, in flakes or tablets, or as formalin, a solution of the gas in water of about 370g/l (37%), containing methanol (100ml/l) as a stabilizer. Both formulations are heated to liberate the gas, which is used for decontamination and disinfection of enclosed volumes such as safety cabinets and rooms.

Formaldehyde (5% formalin in water) may be used as a liquid disinfectant.
Note: Formaldehyde is a suspected carcinogen. It has a pungent smell and its fumes can irritate eyes and mucous membranes. It must therefore be stored and used in a fume-hood or well-ventilated areas. Applicable regulations on chemical safety must be consulted prior to its use.

Glutaraldehyde
Like formaldehyde, glutaraldehyde (OHC(CH2)3CHO) is also active against vegetative bacteria, spores, fungi and lipid- and non lipid-containing viruses. It is non-corrosive and faster acting than formaldehyde. However, it takes several hours to kill bacterial spores. It is generally supplied as a solution with a concentration of about 20g/l (2%) and most products need to be "activated" (made alkaline) before use by the addition of a bicarbonate compound supplied with the product. The activated solution can be reused for 1–4 weeks depending on the formulation and type and frequency of its use. Dipsticks supplied with some products give only a rough indication of the levels of active glutaraldehyde available in solutions under use. Glutaraldehyde solutions should be discarded, if they become turbid.

Note: Glutaraldehyde is toxic and an irritant to skin and mucous membranes, and contact with it must be avoided. It must be used in fume-hood or in well-ventilated areas. It is not recommended as a spray or solution for the decontamination of environmental surfaces. Applicable regulations on chemical safety must be consulted prior to its use.

Phenolic compounds
Phenolic compounds, a broad group of agents, were among the earliest germicides. However, results of more recent safety concerns restrict their use. They are active against vegetative bacteria and lipid-containing viruses and, when properly formulated, also show activity against mycobacteria.

They are not active against spores and their activity against non-lipid viruses is variable. Many phenolic products are used for the decontamination of environmental surfaces and some (e.g., triclosan and chloroxylenol) are among the more commonly used antiseptics. Triclosan is common in products for hand-washing. It is active mainly against vegetative bacteria and safe for skin and mucous membranes. However, in laboratory-based studies, bacteria made resistant to low concentrations of triclosan also show resistance to certain types of antibiotics. The significance of this finding in the field remains unknown.

Note: Phenolic compounds are not recommended for use on food contact surfaces and in the areas with young children. They may be absorbed by rubber and can also penetrate the skin.

Quaternary ammonium compounds
Many types of quaternary ammonium compounds are used as mixtures and often in combination with other germicides, such as alcohols. They have good activity against vegetative bacteria and lipid containing viruses. Certain types (e.g. benzalkonium chloride) are used as antiseptics.

Note: The germicidal activity of certain types of quaternary ammonium compounds is considerably reduced by organic matter, water hardness, and anionic detergents. Care is therefore needed in selecting agents for pre-cleaning when quaternary ammonium compounds are to be used for disinfection. Potentially harmful bacteria can grow in quaternary ammonium compound solutions. Owing to low biodegradability, these compounds may also accumulate in the environment.

Alcohols
Ethanol (ethyl alcohol, C2H5OH) and 2-propanol (isopropyl alcohol, (CH3)2CHOH) have similar disinfectant properties. They are active against vegetative bacteria, fungi and lipid-containing viruses but not against spores. Their action on non-lipid viruses is variable. For highest effectiveness they should be used at concentrations of approximately 70% (v/v) in water: higher or lower concentrations may not be as germicidal. A major advantage of aqueous solutions of alcohols is that they do not leave any residue on treated items. Mixtures with other agents are more effective than alcohol alone, e.g., 70% (v/v) alcohol with 100g/l formaldehyde, and alcohol containing 2g/l available chlorine. A 70% (v/v) aqueous solution of ethanol can be used on skin, work surfaces of laboratory benches and biosafety cabinets, and to soak small pieces of surgical instruments. The contact time on skin should be no less than 10 seconds, and that on environmental surfaces - no less than 3 minutes. Since ethanol can dry the skin, it is often mixed with emollients. Alcohol-based handrubs are recommended for the decontamination of lightly soiled hands in the proper situations.

Hand-washing is inconvenient or not possible. However, it must be remembered that ethanol is ineffective against spores and may not kill all types of non-lipid viruses.

Note: Alcohols are volatile and flammable and must not be used near open flames. Working solutions should be stored in proper containers to avoid the evaporation of alcohols. Alcohols may harden rubber and dissolve certain types of glue. Proper inventory and storage of ethanol in the laboratory is very important to avoid its use for purposes other than disinfection.

Iodine and iodophors
The action of these disinfectants is similar to that of chlorine, although they may be slightly less inhibited by organic matter. Iodine can stain fabrics and environmental surfaces and is generally unsuitable for use as a disinfectant. On the other hand, iodophors and tinctures of iodine are good antiseptics. Polyvidone-iodine is a reliable and safe surgical scrub and preoperative skin antiseptic.

Antiseptics based on iodine are generally unsuitable for use on medical/dental devices. Iodine should not be used on aluminum or copper.

Note: Iodine can be toxic. Organic iodine-based products must be stored at 4–10 °C to avoid the growth of potentially harmful bacteria in them.

Hydrogen peroxide and peracids
Like chlorine, hydrogen peroxide (H2O2) and peracids are strong oxidants and can be potent broad-spectrum germicides. They are also safer than chlorine to humans and the environment.

Hydrogen peroxide is supplied either as a ready-to-use 3% solution or as a 30% aqueous solution to be diluted to 5–10 times its volume with sterilized water. However, such 3–6% solutions of hydrogen peroxide alone are relatively slow and limited as germicides. Products now available have other ingredients to stabilize the hydrogen peroxide content, to accelerate its germicidal action and to make it less corrosive. Hydrogen peroxide can be used for the decontamination of work surfaces of laboratory benches and biosafety cabinets, and stronger solutions may be suitable for disinfecting heat-sensitive medical/dental devices. The use of vaporized hydrogen peroxide or peracetic acid (CH3COOOH) for the decontamination of heat-sensitive medical/surgical devices requires specialized equipment.

Note: Hydrogen peroxide and peracids can be corrosive to metals such as aluminum, copper, brass and zinc, and can also decolorize fabrics, hair, skin, and mucous membranes. Articles treated with them must be thoroughly rinsed before contact with eyes and mucous membranes. They should always be stored away from heat and protected from light.

3.6. Dry Heat Sterilization

Dry heat sterilization is the most common among the physical agents used for the decontamination of pathogens. “Dry” heat, which is totally non-corrosive, is used to process many items of laboratory ware which can withstand temperatures of 160°C or higher, for 2–4 hours.

Sterilization

An oven is used to sterilize. Usually, a table of time and temperature is provided by the manufacturer in order to help the practical implementation (160°C for two hours is usually recommended). Material usually dry-heated:

- All water-sensitive devices
- Glassware
- Large parts (not fitting into autoclave)

Control of the sterilization

Temperature sensitive strips have to be used during **each** sterilization process. The change in their color (time and temperature dependant) signifies the quality of the

sterilization. All indicators have to be put into a dry heat sterilization logbook, in order to track each sterilization activity, as shown on illustration 1. Sterilization logbook (Bulgaria, 2003)

Incineration

Incineration is useful for disposing of animal carcasses as well as anatomical pieces and other laboratory waste with (recommended) or without prior decontamination. Incineration of infectious materials is an alternative to autoclaving only if the incinerator is under laboratory control, and it is provided with an efficient means of temperature control and a secondary burning chamber. Many incinerators, especially those with a single combustion chamber, are unsatisfactory for dealing with infectious materials, animal carcasses, and plastics. Such materials may not be completely destroyed and the effluent from the chimney may pollute the atmosphere with microorganisms, toxic chemicals, and smoke. However, there are many satisfactory configurations for combustion chambers. Ideally the temperature in the primary chamber should be at least 800°C and that in the secondary chamber at least 1000°C. Materials for incineration, even with prior decontamination, should be transported to the incinerator in bags, preferably plastic. Incinerator attendants should receive proper instructions about loading and temperature control. It should also be noted that the efficient operation of an incinerator depends heavily on the right mix of materials in the waste being treated.

3.7. Wet Heat Sterilization

Introduction

“Wet” or “moist” heat is most effective when used in the form of autoclaving. Boiling does not necessarily kill all microorganisms and/or pathogens, but it may be used as minimum processing for disinfections, where other methods (chemical disinfections or decontamination, autoclaving) are not applicable or available. Sterilized items must be handled and stored so that they remain uncontaminated until used.

Autoclaving

Saturated steam under pressure (autoclaving) is the most effective and reliable means of sterilizing laboratory materials. For most purposes, the following cycles will ensure sterilization of correctly loaded autoclaves:

- 3 min holding time at 134 °C
- 10 min holding time at 126 °C
- 15 min holding time at 121 °C
- 25 min holding time at 115 °C.

Each autoclave in technical manual has its best regime of sterilization. Examples of different autoclaves include the following.

***Gravity displacement autoclaves*:** In gravity displacement autoclaves, steam enters the chamber under pressure and displaces the heavier air downwards and through the valve in the chamber drain, fitted with a HEPA filter.

***Pre-vacuum autoclaves*:** Pre-vacuum autoclaves allow the removal of air from the chamber before steam is admitted. The exhaust air is evacuated through a valve fitted with a HEPA filter. At the end of the cycle, the steam is automatically exhausted. These autoclaves can operate at 134 °C and the sterilization cycle can therefore be reduced to 3 minutes. They are ideal for porous loads, but cannot be used to process liquids because of the vacuum.

***Fuel-heated pressure cooker autoclaves*:** Fuel-heated pressure cooker autoclaves should be used only if a gravity displacement autoclave is not available. They are loaded from the top and heated by gas, electricity, or other types of fuels. Steam is generated by heating water in the base of the vessel and air is displaced upwards through a relief vent. When all the air has been removed, the valve on the relief vent is closed and the heat reduced. The pressure and temperature rise until

the safety valve operates at a preset level. This is the start of the holding time. At the end of the cycle the heat is turned off and the temperature allowed to fall to 80°C or below before the lid is opened.

***Loading autoclaves*:** Materials should be loosely packed in the chamber of a loading autoclave for easy steam penetration and air removal. Bags should allow the steam to reach their contents.

Precautions for the use of autoclaves

The following rules can minimize the hazards inherent in operating pressurized vessels:

Responsibility for operation and routine care should be assigned to trained individuals and a preventive maintenance program should include yearly inspection of the chamber, door seals, and all gauges and controls by qualified personnel.

The steam should be saturated and free from corrosion inhibitors or other chemicals, which could contaminate the items being sterilized.

All materials to be autoclaved should be in containers that allow ready removal of air and permit good heat penetration; the chamber should not be tightly packed or steam will not reach the load evenly.

For autoclaves without an interlocking safety device that prevents the door being opened when the chamber is pressurized, the main steam valve should be closed and the temperature allowed to fall below 80°C before the door is opened.

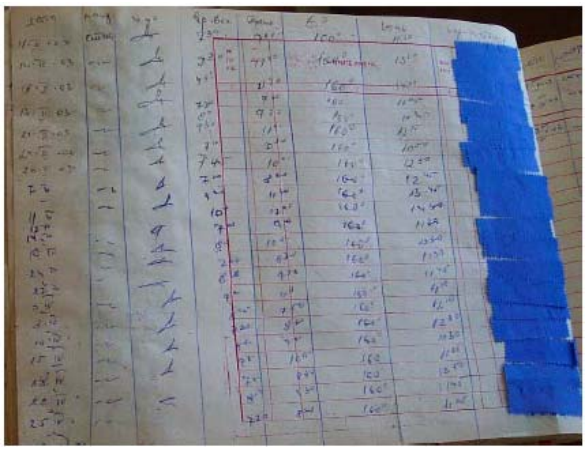
Operators should wear suitable gloves and visors for protection when opening the autoclave, even when the temperature has fallen below 80°C.

In any routine monitoring of autoclave performance, biological indicators or thermocouples should be placed at the center of each load. Regular monitoring with thermocouples and recording devices in a “worst case” load is highly desirable to determine proper operating cycles.

The drain screen filter of the chamber (if available) should be removed and cleaned daily.

Care should be taken to ensure that the relief valves of pressure cooker autoclaves do not become blocked by paper, etc. in the load.

Control of the process



As for dry heat, temperature sensitive strips have to be used during each sterilization process, as shown on the illustration (sterilization logbook).

Change in their color (time and temperature dependant) signs the quality of sterilization. All indicators have to be put into a wet heat sterilization logbook in order to track each sterilization activity.

Proper work and precision of manometer should be regularly controlled by the specialist.

3.8. Washing of tissue, lab coat

Reusable laboratory clothes cannot be washed as regular clothes. Some rules apply:

- Pre-disinfection of clothes used in dirty areas is recommended by immersing them in disinfectants (see 3.5). Contaminated areas of clothing also must be processed this way before washing.
- Laboratory clothes cannot be washed at home.
- Laboratory clothes must be washed only with other laboratory clothes.

Frequency of washing:

- Napkins and tissues: daily
- Labcoat: weekly

Once contaminated, clothes have to be washed immediately.

3.9. Hand washing

Whenever possible, suitable gloves should be worn when handling biohazardous materials. However, this does not replace the need for regular and proper hand-washing by laboratory personnel.

Hands must be washed

- Before and after sampling
- After microbiological manipulation
- Before and after lunch and coffee breaks
- Before and after going to the restroom
- At the end of the day, after washing the laboratory, just before leaving.

Usually, a liquid soap is recommended. It should contain an antiseptic such as chlorhexidine or iodine.

Note: Take care not to mix several different antiseptics, such as hypochlorite and chlorhexidine, or iodine and mercurochrome

Procedure for hand-washing

1. Open the tap.

2. Wet hands and wrist.

3. Take a few milliliters of soap.

4. Carefully wash both hands for 2–3 minutes. Focus on areas between fingers, nails, and palm. Never use a brush.

5. Rinse hands with clean water. This operation is very important, because it will flush the dirt away. If needed, soap and rinse a second time.

6. Dry hands preferably using paper tissue. Close the tap with a tissue in the hands. If no paper tissue is available, use a napkin that will be changed and disinfected daily.

3.10. Lab washing floor, benches

See section 3.5 for materials to use in pre-cleaning and cleaning the laboratory.

3.11. Biohazard injury

Accidental injection, cuts, and abrasions

The affected individual should remove protective clothing, wash the hands and the affected part, apply an appropriate skin disinfectant, go to the first-aid room, and inform the person in charge about the cause of the wound and the organisms involved. If considered necessary, a physician should be consulted and his or her advice followed. Appropriate and complete medical records should be kept.

Accidental ingestion of potentially hazardous material

Protective clothing should be removed and the individual taken to the first-aid room. A physician should be informed of the material ingested and his or her advice followed. Appropriate and complete medical records should be kept.

Potentially hazardous aerosol release (other than in a biological safety cabinet)

All persons should immediately vacate the affected area and any exposed persons should be referred for medical advice. The laboratory supervisor and the biosafety officer should be informed at once. No one should enter the room for at least 1 hour, to allow aerosols to disperse and heavier particles to settle. If the laboratory does not have a central air exhaust system, entrance should be delayed for 24 hours. Signs should be posted indicating that entry is forbidden. After the appropriate time, decontamination should proceed, supervised by the biosafety officer. Appropriate protective clothing and respiratory protection should be worn for this.

Broken and spilled infectious substances, including cultures

Broken objects contaminated with infectious substances, including vials or containers, or spilled infectious substances, including cultures, should be covered with cloth or paper towels. Disinfectant should then be poured over these and left for at least 30 minutes. The cloth or paper towels and the broken material may then be cleared away; glass fragments should be handled with forceps. The contaminated area should then be swabbed with disinfectant. If dustpans are used to clear away the broken material, they should be autoclaved or placed in an effective disinfectant for 24 hours. Cloths, paper towels, and swabs used for cleaning up should be placed in a contaminated-waste container.

Gloves should be worn for all these procedures. If laboratory forms or other printed or written matters are contaminated, the information should be copied onto another form and the original discarded into the contaminated-waste container.

3.12. Emergency procedures

Fire, flood, and natural disaster

Fire and other services should be involved in the development of emergency plans. They should be told in advance which rooms contain potentially infectious materials. It is beneficial to arrange for these services to visit the laboratory to become acquainted with its layout and contents if possible.

After a flood or other natural disaster (including earthquake), local or national emergency services should be warned of the potential hazards within and/or near laboratory buildings. They should enter only when accompanied by a trained laboratory worker. Cultures and infectious materials should be collected in leak-proof boxes or strong disposable bags. Salvage or final disposal should be determined by safety staff on the basis of local knowledge.

Vandalism

Vandalism is usually selective (e.g., aimed at animal cages/kennels). Suitable defenses are strong, heavy doors, good locks, and restricted entry. Screened windows and intruder alarms are desirable. Action after vandalism is the same as that for other emergencies.

Emergency services: whom to contact

The telephone numbers and addresses of the following should be prominently displayed near all telephones:

- Institution or laboratory itself (the address and location may not be known in detail by the caller or the services called)
- Director of the institution or laboratory
- Laboratory supervisor
- Biosafety officer
- Fire services
- Hospital/ambulance service (if a particular hospital has arranged to accept casualties, e.g. high-risk personnel, the names of individual departments and doctors)
- Police
- Medical officer
- Responsible technician
- Water, gas, and electricity services

Emergency equipment

The following emergency equipment must be available:

- First-aid kit, including universal and special antidotes
- Stretcher
- Appropriate fire extinguishers, fire blankets

The following are also suggested but may be varied according to local circumstances:

- Full-face respirators with appropriate chemical and particulate filter canisters
- Room disinfections apparatus, e.g., sprays and formaldehyde vaporizers
- Tools, e.g., hammers, axes, spanners, screwdrivers, ladders, ropes
- Hazard area demarcation equipment and notices

3.13. Waste disposal and legislation

The disposal of laboratory and medical waste must be carried out according to ‘‘Sanitary Norms of Waste Gathering, Keeping and Decontamination in Medical and Prophylactic Institutions’’ (Sanitary Rules and Norms from 2.1.7.000-00, Ministry of Labor, Health and Social Affairs of Georgia, Tbilisi, 2000).

Waste handling and disposal procedures

Identification and separation system for infectious materials and their containers should be adopted. Categories should include the following:

1. Non-contaminated (non-infectious) waste that can be reused or recycled or disposed of as general, “household” waste

2. Contaminated (infectious) “sharps” – hypodermic needles, scalpels, knives and broken glass; these should always be collected in puncture-proof containers fitted with covers and treated as infectious

3. Contaminated material for decontamination by autoclaving and thereafter washing and reuse or recycling

4. Contaminated material for autoclaving and disposal

5. Contaminated material for direct incineration

Sharps

After use, hypodermic needles should not be recapped, clipped or removed from disposable syringes. The complete assembly should be placed in a sharps container. Sharps containers must be puncture-proof and not be filled to capacity. When they are three-quarters full they should be placed in "infectious waste" containers and incinerated, with prior autoclaving if laboratory practice requires it. Sharps containers must not be disposed of in landfills.

Disposable syringes, used alone or with needles, should be placed in containers and incinerated, with prior autoclaving if required.

Contaminated (infectious) materials for autoclaving and reuse

No pre-cleaning should be attempted of any contaminated (infectious) materials to be autoclaved and reused. Any necessary cleaning or repair must be done only after autoclaving or disinfection.

Contaminated (infectious) materials for disposal

Apart from sharps, which are dealt with above, all contaminated (potentially infectious) materials should be autoclaved in leak-proof containers, e.g., autoclavable, color-coded plastic bags, before disposal. After autoclaving, the material may be placed in transfer containers for transport to the incinerator. If possible, the materials deriving from health care activities should not be discarded in landfills even after decontamination. If an incinerator is available on the laboratory site, autoclaving may be omitted: the contaminated waste should be placed in designated containers (e.g., color-coded bags) and transported directly to the autoclave or incinerator. Reusable transfer containers should be leak-proof and have tight-fitting covers. They should be disinfected and cleaned before they are returned to the laboratory for further use.

Discard pots, pans or jars, preferably unbreakable (e.g., plastic), and containing a suitable disinfectant (see 3.5), freshly prepared each day, should be placed at every work station. Waste materials should remain in intimate contact with the disinfectant (i.e., not protected by air bubbles) for the appropriate time, according to the disinfectant used. The disinfectant should then be poured into a container for autoclaving or incineration. The discard pots should be autoclaved and washed before reuse.

Incineration is the method of choice for the final disposal of contaminated waste, including carcasses of laboratory animals. Incineration of contaminated waste must meet with the approval of the public health and air pollution authorities, as well as that of the laboratory biosafety officer.

3.14. Cold chain procedures

Cold chain control and monitoring is a very important and effective tool for maintaining QA at the highest possible level:

- All reagents must be stored according to manufacturer recommendations.
- For instances of electrical power outage, some cooling devices (refrigerators, freezers) have to be protected with a generator. The main critical (and/or expensive) reagents have to be stored in these protected devices. An automated system should allow the launch of the generator in case of power outage.
- Problems that might happen at night or on weekends should be planned for in advance; clearly written standardized procedures to deal with such problems must be developed.
- For cases of major electrical problems that affect generators, an alternative strategy for transport of goods and reagents to another location also must be planned for.
- Daily temperature monitoring should be available through a recording system using weekly disks (one week recorded on one disk).

- Alarms should be installed on the main refrigerators to warn the technical staff that temperature rises above a predetermined threshold.
- When reagents are received at night or on weekends, they should be directly transported to the main cold chamber available at the NCDC.

Regular temperature control should be performed for the following devices:

- Refrigerator
- Freezer (regular and deep freezers)
- Waterbath
- Cooled centrifuge
- Incubators (30°, 37°, 42°, 55°)

It is recommended to perform the measures twice a day:

- Early morning: basal temperature, providing information about the equipment itself
- Mid afternoon (end of routine period): working temperature, providing information about the way the equipment is being used

Temperature is being written on a temperature chart (see **Attachment 1**). If the laboratory owns a basic computer, some specific software such as Temperature© can be used in order to collect and follow-up all thermal devices.

3.15. Signs access to laboratory

The laboratory is a restricted area.

- **"No entrance to outside staff"** sign should be posted immediately after the reception room. The area where samples are received should be strictly separated from the other rooms of the laboratory.
- A **bell** is recommended at the entrance. The best way to accept the sample would be through a small opening built in a wall or in a door, so only the staff would have to ring to enter the laboratory.
- A **separate room** for coffee and smoking should be also clearly defined and labeled.

The **international biohazard** sign (see Illustration 1) should be displayed on the doors of the rooms, where Risk Group 2 micro-organisms are handled.

3.16. Thermal area procedures

Air conditioning

Air conditioning is recommended for the rooms containing incubators, spectrophotometer (including ELISA reader), and all other temperature-sensitive equipment.

Ventilation and illumination

In the planning of new facilities, however, consideration should be given to the provision of mechanical ventilation systems that provide an inward flow of air without recirculation. If there is no mechanical ventilation, windows should be openable and preferably fitted with arthropod-proof screens. Skylights should be avoided.

Cold rooms

Refrigerators and freezers (especially minus-80 freezers) should not be placed in corridors; such placement can dramatically decrease their lifespan.

4. Equipment management

4.1. Equipment logbook

Empty equipment logbook

Below is an example of the equipment logbook chapters that should be developed for any equipment available in the laboratory:

1. Identity of the equipment

2. Manufacturer's/Distributor's name, type identification and serial number or other unique identification

3. Manufacturer's/Distributor's contact person and telephone number, as appropriate

4. Date of receiving and date of putting into service

5. Current location, where appropriate

6. Condition when received (e.g. new, used or reconditioned)

7. Manufacturer's instructions if available

8. Table for the confirmation of the equipment's performance and suitability for use

9. Maintenance plan or reference to it

10. Damage to, or malfunction, modification or repair, of the equipment

11. Predicted replacement date, if possible

4.2. Equipment responsibilities and organization

Each laboratory needs to designate an "Equipment Responsible" (under the responsibility of the QA manager). Ideally this should be a person with some biomedical engineering background, but a well motivated medical person or laboratory technician can also be promoted to such position. The roles and activities of this person are numerous and critical:

- Links with equipment manufacturers and/or resellers
- Links with national metrology units
- Cold chain responsible
- Preparation of equipment logbooks
- Preparation of the simplified user manuals
- Preparation of the maintenance schemes
- Securization of a minimal set of spare parts
- Staff training about preventive and curative maintenance
- Decision about old equipment retirement

Note: Several laboratories do have a maintenance contract with an engineering company. Such contracts are extremely important in order to insure the maximum quality of the equipment. Nevertheless, this contract will not replace the role of an Equipment Responsible, for the daily preventive maintenance and follow up of the equipment.

4.3. Calibration/maintenance policy

Currently, there are no national calibration and maintenance policies available in Georgia, although some specific agencies do have a national agreement for metrological purposes. Thus, each laboratory needs to develop its own schemes for calibration and maintenance. The following chapters provide details about the common preventive maintenance and maintenance demands for

the equipment to be used in the focus of intestinal infectious diseases. It can be used as a basis for laboratory - specific maintenance schemes.

Each maintenance scheme contains “activities” (such as emptying a waste bottle, checking a wavelength or rinsing some plastic tubes). Each activity is characterized by its rhythm (daily, weekly, monthly…) and should be written, once achieved, in the maintenance logbook as described in chapter 4.1.

The calibration of equipment and other subjects related to metrology should be undertaken by appropriate services approved by the Georgian official agencies.

Note: For the laboratories owning a basic computer, preventive maintenance software such as “Maintenance Scheduler©” can be used.

4.4. Centrifuge

General

- Satisfactory mechanical performance is a prerequisite of microbiological safety in the use of laboratory centrifuges.
- Centrifuges should be operated according to the manufacturer’s instructions.
- Full user manual should be readily available. A Georgian-language summary of this manual must be available at a bench level.
- Centrifuges should be placed at a level allowing all users to see into the bowl to place trunnions and buckets correctly.
- Centrifuge tubes and specimen containers for use in the centrifuge should be made of thick-walled glass or preferably of plastic and should be inspected for defects before use.
- When using angle head centrifuge rotors, care must be taken to ensure that the tube is not overloaded as it might leak.
- After use, buckets should be stored in an inverted position to drain the balancing fluid.
- Never try to open a centrifuge while it is running and/or stop the rotor in order to save some time. The centrifuge produces aerosols of potentially contaminated liquids that may injure hands and fingers.

Biosafety

- Tubes and specimen containers should always be securely capped (screw-capped if possible) for centrifugation.
- Buckets and trunnions should be paired by weight and, with tubes in place, correctly balanced. Distilled water or alcohol (propanol, 70%) should be used for balancing empty buckets. Saline or hypochlorite solutions should not be used, as they corrode metals.
- A sealed bucket is recommended to avoid manipulators contamination. If manipulating micro-organisms of Risk Groups 3 and 4, sealable centrifuge buckets must absolutely be used.
- If possible, the manipulation of tubes and buckets has to be done in a biological safety cabinet.
- Infectious airborne particles may be ejected when centrifuges are used. These particles travel at speeds too high to be retained by the eventual safety cabinet air flow, in the case that the centrifuge is placed in a traditional open- fronted Class I or Class II biological safety cabinet.
- Enclosing centrifuges in Class III safety cabinets prevents emitted aerosols from dispersing widely. However, good centrifuge technique and securely capped tubes offer adequate protection against infectious aerosols and dispersed particles.

Preventive maintenance

- The interior of the centrifuge bowl should be inspected daily for staining or soiling at the level of the rotor. If staining or soiling is evident then the centrifugation protocols should be re-evaluated.

- Centrifuge rotors and buckets should be inspected daily for signs of corrosion and for hair-line cracks.
- Buckets, rotors, and centrifuge bowls should be decontaminated weekly or after any hazardous manipulation.
- Carbons have to be checked every 3 months, and the dust should be cleaned. They should be changed yearly.

Breakage of tubes containing potentially hazardous material in centrifuges not having sealable buckets

If a breakage occurs or is suspected while the machine is running, the motor should be switched off and the machine left closed for 30 minutes. If a breakage is discovered after the machine has stopped, the lid should be replaced immediately and left closed for 30 minutes. In both instances, the biosafety officer should be informed. Strong (e.g. thick rubber) gloves, covered if necessary with suitable disposable gloves, should be worn for all subsequent operations. Forceps, or cotton held in the forceps, should be used to retrieve glass debris. All broken tubes, glass fragments, buckets, trunnions, and the rotor should be placed in non-corrosive disinfectant known to be active against the organisms concerned and left for 24 hours and/or autoclaved. Unbroken, capped tubes may be placed in disinfectant in a separate container and recovered after 60 minutes.

The centrifuge bowl should be swabbed with the same disinfectant, at the appropriate dilution, left overnight, and then swabbed again, washed with water and dried. All materials used in the cleanup should be treated as infectious waste.

Breakage of tubes inside sealable buckets (safety cups)

All sealed centrifuge buckets should be loaded and unloaded in a biological safety cabinet. If a breakage is suspected, the cap should be opened and left loose, before autoclaving the bucket.

4.5. Microscope

General use:

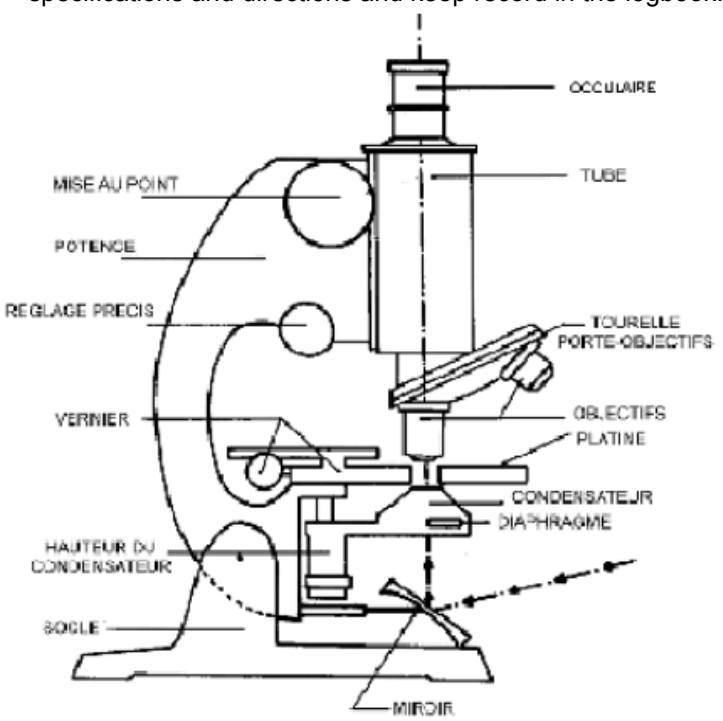
- Microscope will be operated according to the manufacturer's instructions.
- The microscope will have a sticker affixed to it that indicates:
- The qualification date,
- The re-qualification date
- The SOP reference
- The logbook reference
- Don't change lenses from one microscope to the other, they may not be compatible, even if they are from the same trademark.
- After use, clean objective lenses and eyepieces with lens cleaner and lens paper.
- Cover the microscope after work.
- If the microscope has stopped or is not working correctly, inform head technician or quality manager or his/her assistant for repair and notify in the logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair following the DP Decontamination of laboratory equipment.

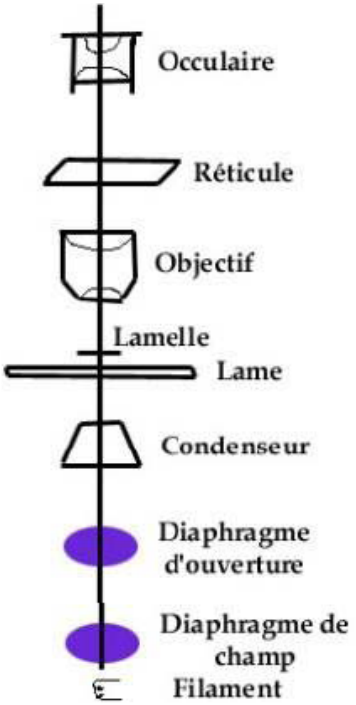
If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Preventive maintenance:

- When not in use, always keep microscopes covered
- Do not let oil magnification lens in the down position when not in use.

- Turn off light when not in use.
- Always hide lens plugs with the correct cap. If no cap is available, hide the plug with adhesive, in order to avoid the accumulation of dust.
- The person assigned to a bench is responsible for daily cleaning and inspection of the microscope.
- After each work, clean objective lenses and eyepieces with lens cleaner and lens paper.
- After each day, clean slide stage with alcohol to remove dust and oil. If a certain amount has gotten on stage, remove with a SMALL drop of xylene. Be careful, xylene is carcinogen.
- Each month, check the Kohler centering of the lenses/ diaphragm/condenser, if not correct, inform the head technician or quality manager or his/her assistant so that to call the maintenance service.
- Annually, microscopes are cleaned and inspected by an outside contractor, and repaired and calibrated as needed. Keep all maintenance recorded in the logbook.
- Always plan extra bulb and extra fuse in order to be able to perform any change without any delay.
- To replace a light bulb if necessary, refer to the individual microscope manual for bulb specifications and directions and keep record in the logbook.





Biosafety consideration:

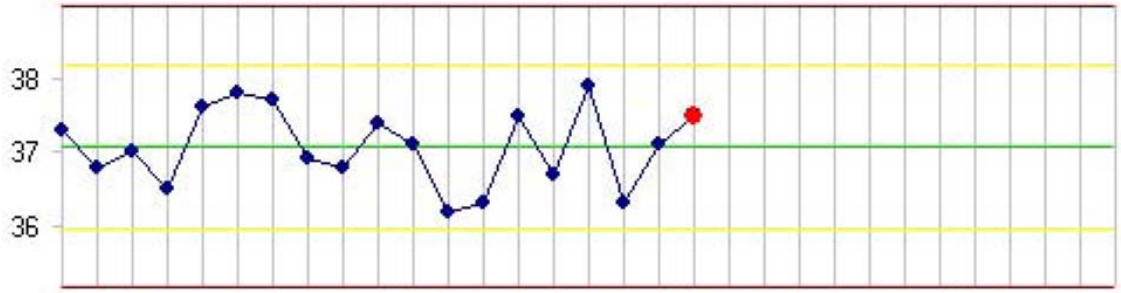
If any biosafety problem occurs, inform the Biosafety Officer, who will refer to DP Minor occurrence to report the problem and find an eventual solution if needed with the Biosafety Committee.

4.6. Incubator

General

- Full user manual should be readily available. A Georgian-language summary of this manual must be available at a bench level.
- Always keep the incubators doors closed, even when a double door system is available.

- Incubator has to be filled with specific devices in order to hold tubes (racks) and Petri dishes (metal holders).
- The space between the trays should let a candle jar to be inserted.
- Each incubator has to be equipped with a thermometer.
- Incubator temperature is monitored twice a day, once in the morning (basal temperature) and once in the afternoon (working temperature), and temperature chart is filled in. This action is the main IQC activity for the incubator.



- Linkage with metrology unit (see 4.3) must be carried out, in order to check thermometer on a yearly basis.

Biosafety

- Incubator has to be cleaned inside and outside once a week and fully disinfected once a month. Usually, a specific anionic disinfectant is used (see 3.5 for more details).
- If leakage or breakage occurs inside the incubator, it should be cleaned immediately after the problem occurs.

4.7. Biological safety cabinet

General use:

- Biological safety cabinet will be operated according to the manufacturer's instructions.
- Biological safety cabinet full user manual should be easily available (usually in the English language) along with a Georgian language summary
- The Biosafety Cabinet will have a sticker affixed to it that indicates:
- The qualification date
- The requalification date
- This SOP reference
- The logbook reference
- Turn on the cabinets at least 5 minutes before beginning the work to allow the cabinet time for contaminated air to be removed from the cabinet environment or at the beginning of the day and wait 5 min before the first use.
- Laboratory coats will be worn whenever using a biological safety cabinet.
- Masks, back-closing laboratory gown and safety glasses will be used.
- Put on gloves. Gloves should be pulled over the wrists of the gown.
- Spray gloved hands and sleeves thoroughly with the disinfectant solution.
- Decontaminate the interior surface.
- Manipulations of materials within biological safety cabinets should be delayed for about 1 minute after placing hands and arms inside to allow the cabinet to adjust and to „air sweep" the surface of the hands and arms.
- Place all necessary items into the cabinet before beginning manipulations so that to minimize the movements across the front opening.
- Materials to be placed inside the cabinet should be surface-decontaminated with 70% alcohol. Work may be performed on disinfectant-soaked absorbent towels to capture splatters and splashes.

- Place all materials as far back in the cabinet, towards the rear edge of the work surface, as practical without blocking the rear grill.
- Operator needs to be careful to maintain the integrity of the front opening air inflow when moving their arms into and out of cabinets. Arms should be moved in and out slowly, perpendicular to the front opening.
- Bulky items such as biohazard bags, discard pipette trays and suction collection flasks will be placed to one side of the interior of the cabinet. Active work should flow from clean to contaminated areas across the work surface.
- When the work is finished, all items within the cabinet including equipment, should be surface-decontaminated and removed from the cabinet.
- Decontaminate the interior surface after the work.
- At the end of the day, the work surface and interior walls will be wiped with a solution of bleach or 70% alcohol. A second wiping with sterile water is needed when a corrosive disinfectant, such as bleach, is used.
- Take the gloves off.
- Turn off cabinets at least 5 minutes after finishing the day's work to allow the cabinet time for contaminated air to be removed from the cabinet environment.
- If the Biosafety cabinet has stopped or is not working correctly, inform head technician or quality manager or his/her assistant for repair and notify in the adequate logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair following the DP Decontamination of laboratory equipment.
- If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Preventive maintenance:

- Biological safety cabinets must be decontaminated before filter changes and before being moved. The decontamination will be effected by fumigation with formaldehyde gas. This decontamination will be performed by the Biosafety Officer. It will be recorded in the logbook.
- All repairs will be made by a qualified technician and notified in the logbook.
- If ultraviolet lights are used, they must be cleaned weekly to remove any dust and dirt that may block the germicidal effectiveness of the light. This weekly cleaning must be notified in the logbook.
- Ultraviolet light intensity must be checked when the cabinet is recertified to ensure that light emission is appropriate and notified in the logbook.

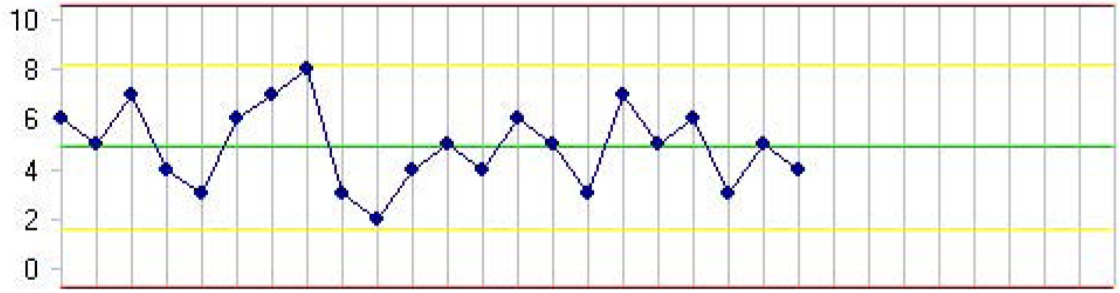
Biosafety consideration:

- When a spill of biohazardous material occurs within a biological safety cabinet, clean up should begin immediately, while the cabinet continues to operate. An effective disinfectant should be used and applied in a manner that minimizes the generation of aerosols. All materials that come into contact with the spilled agent should be disinfected and/or autoclaved.
- Airflow alarms indicate a disruption in the cabinet's normal airflow pattern. This represents an immediate danger to the operator or product. When an airflow alarm sounds, the work should cease immediately.
- If any biosafety problem occurs, inform the Biosafety Officer, who will report the problem and find an eventual solution if needed with the Biosafety Committee. This will be recorded in the logbook.

4.8. Fridge or freezer

General facts

- No food, drink, or other personal items should be placed in laboratory refrigerators or freezers.
- Refrigerator and freezer temperature is monitored twice a day, once in the morning (basal temperature) and once in the afternoon (working temperature), and temperature chart is filled in. This action is the main IQC activity for the incubator.



- An inventory of the contents of the device must be maintained and printed. It is then posted to the door.
- Flammable solutions must not be stored in a refrigerator unless they are explosion proof. Notices to this effect should be placed on refrigerator doors.

Maintenance of the cooling devices

- Refrigerators, deep-freezers, and solid carbon dioxide (dry-ice) packs should be defrosted and cleaned periodically, and any ampoules, tubes, etc. that have broken during storage removed. Face protection and heavy-duty rubber gloves should be worn during cleaning. After cleaning, the inner surfaces of the cabinet should be disinfected.
- All containers stored in freezers and eventually in refrigerators should be clearly labeled with the scientific name of the contents, the date stored, and the name of the individual, who stored them. Unlabelled and obsolete materials should be autoclaved and discarded.
- Refrigerators - Clean, defrost (if appropriate), and disinfect every 3 months, more often if needed, and check for expired reagents every 3 months. Monitor twice a day.
- Freezers - Monitor twice a day. Remove excess frost at -70°C and clean out and organize at -70° and -20° every 6 months. Disinfect each time you defrost. Do not completely defrost at –70° more than once a year.

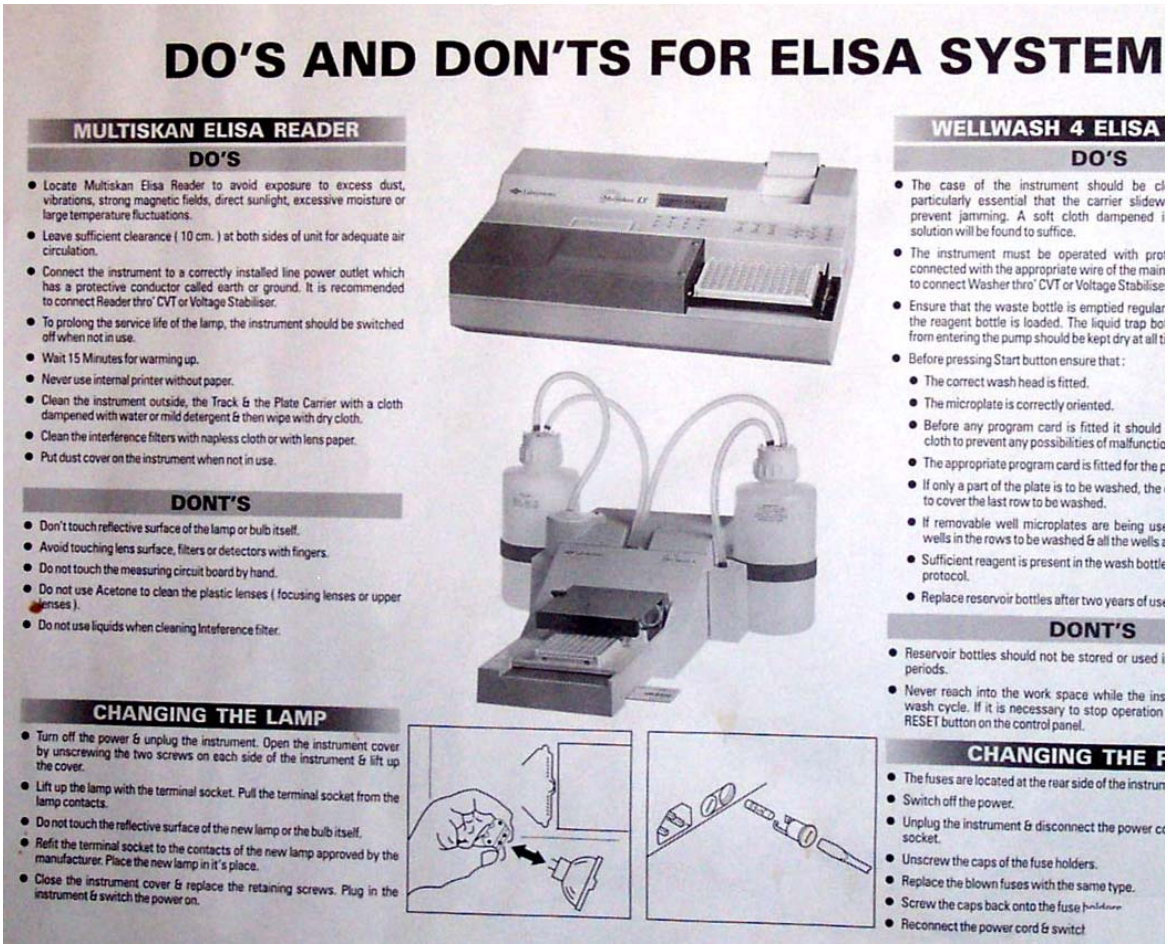
4.9. Scale

Control operation should be performed by the metrology unit.
Manipulator has to refer to the user manual and to a summary written in the Georgian language.

4.10. ELISA chain

Use of ELISA equipment

Example of LabSystem SOP: “Do’s and don’ts”:



Control of the equipment itself

This control is usually done by a specialist, with a contract planned by the laboratory. Metrology unit can also be involved (see 4.3). These operations can be summarized:

For the reader

- Control of the precision of the wavelength
- Control of the repeatability of the wavelength
- Control of the precision of the absorbance
- Control of the repeatability of the absorbance
- Control of the linearity of the response
- Control of the presence of parasitic light
- Control of the stabilization time
- Control of decreasing the light with time

For the washer

- Check the tubes (water tightness, formation of angles, porosity)
- Check the eventual precipitation of minerals in the circuit

For the incubator

- Check the temperature

Control of the biological performance

- Positive and negative control with each series
- External quality control (EQC) on a regular basis (at least twice a year)

Maintenance

- Daily rinsing of the washer (including tanks) with distilled water
- Change the tubes as specified by the manufacturer (usually yearly)
- Change the lamp of the reader, when its performances are under the level

4.11. Autoclave and oven

Refer to procedures that specify how to use and control these pieces of equipment, discussed above in:

- 3.6. Dry heat sterilization
- 3.7. Wet heat sterilization
- 4.3. Calibration/maintenance policy
- 4.2. Equipment responsibilities and organization

In addition, for the autoclave, all rubber parts should be checked monthly, as they ensure the water-tightness of the autoclave.

4.12. Water distiller

Use of the water distiller equipment

Follow the user manual.
Note: A summary in the Georgian language should be available at a bench level.

Physico-chemical control

A pH-meter (2 decimals) and a conductimeter (units in mS/cm) should be available:

- Check pH, should be at 7,00 +/- 0,01
- Check conductance, should be at +/- 0,001 mS/cm

Note: Before checking the both, the final container and the container used for the measure should be rinsed with HCl 0,1 N, in order to decrease the amount of Ca++ and HCO3- ions, which may interfere with the measure. A final rinse with distillated water will be done before the measures.

Sterilization control

Pour 10 ml of water into a nutritive broth, incubate 24 hours at 37°C, then subculture the mix on a normal agar, let stand 48 hours at 37°C. No micro-organism should grow.

Flow control

Measure the volume of water produced in one hour and compare to the volume specified by the manufacturer. Conclude.

Maintenance of water distiller

- Follow the instruction specified by the manufacturer.
- Check the quality of the rubber parts and change them if you detect water leaks.
- Rinse the distiller monthly with first HCl 0,1 N, and then with pure distilled water.

4.13. Micropipette

General use:

Micropipette will be operated according to the manufacturer's instructions.
The Micropipette will have a sticker affixed to it that indicates:

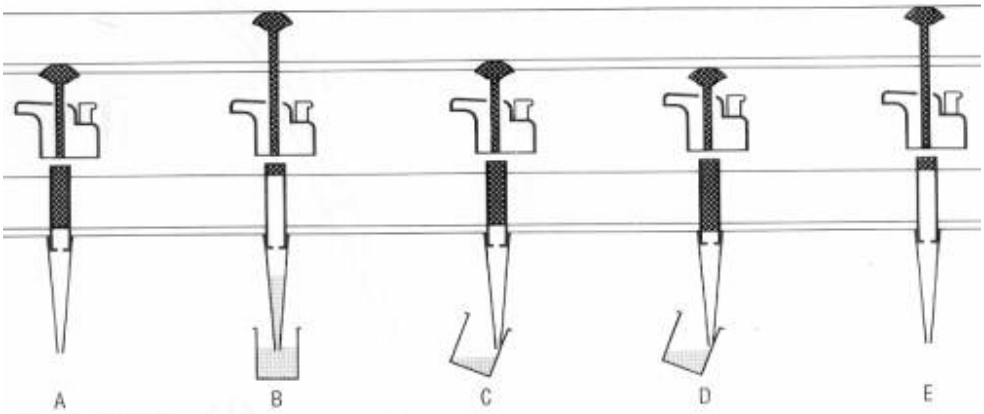
- The qualification date
- The re-qualification date
- This SOP reference
- The logbook reference

Adjust the volume (if not a fixed volume pipette). Adjust the tip which correctly fits the micropipette.
Aspiration:

- Pre-rinse the tip
- Press till the first step (A)
- Hold pipette vertically
- Immerge the end of the tip in the solution (2-4 mm for blue tips)
- Let gently and regularly the pump go back to initial position (B)
- Wait a second
- Gently absorb extra liquid outside the tip without touching the end

▪ Distribution

- Hold pipette 10 to 40° angle to the tube
- Push the pump till the first step (C)
- Wait a second
- Push till the second step (D)
- Take the pipette back
- Let the pump go back (E)
- Eject tip



If the micropipette has stopped or is not working correctly inform the head technician or quality manager or his/her assistant for repair and notify in the logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair following the DP decontamination of laboratory equipment.
If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Control of the pipette:

Different classes of micropipettes are existing, depending on their accuracy, reliability, uncertainty … and price. Their control depends also on their class. In this table you can find all differences and the rhythm, you should control them.

Table 2. Characteristics of micropipettes

Pipette volume µl	Class	Check freq. in month	Accuracy in µl	Reliability in %	Uncertainty in µl
1000	A	3	20	2	+/-20
1000	B	3	40	4	+/-40
1000	C	6	80	8	+/-80
500	A	3	10	2	+/-10
500	B	3	20	4	+/-20
500	C	6	35	8	+/-40
200	B	3	8	4	+/-8
200	C	6	16	8	+/-16
100	B	3	4	4	+/-4
100	C	6	8	8	+/-8
50	B	3	2	4	+/-2
50	C	6	4	8	+/-4
10	B	3	0,4	4	+/-0,4
10	C	6	0,8	8	+/-0,8

Accuracy and reliability:

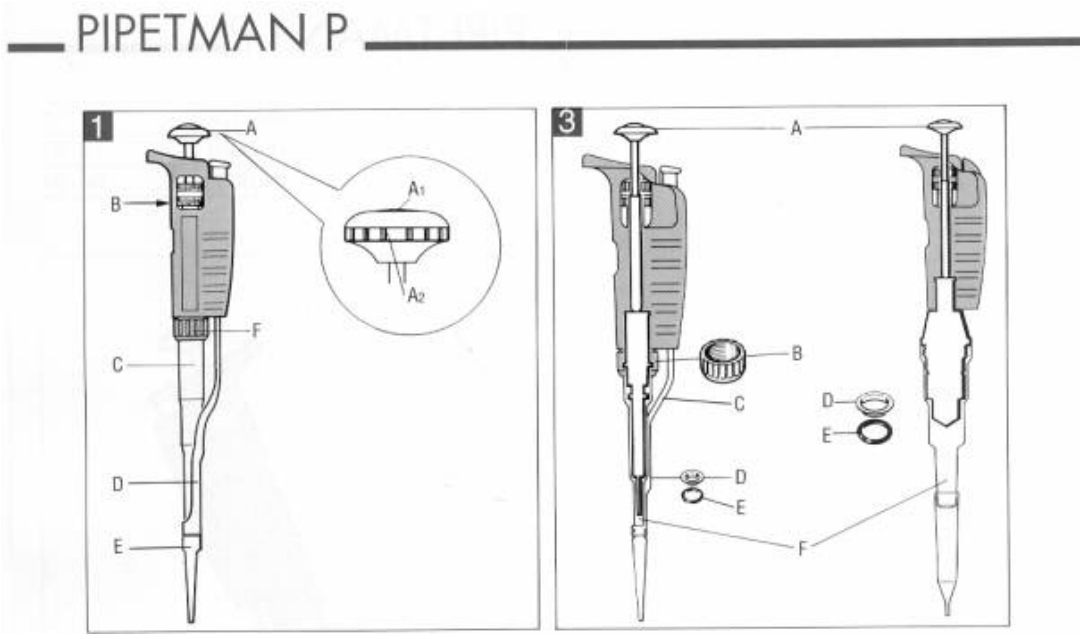
A 1/10 mg precise scale is needed. The local conditions of atmosphere pressure, temperature and hygrometry may interfere.

- Place a container on the scale
- Perform zero position of the scale
- Aspirate the chosen volume (use volume, minimum volume, maximum volume) of distilled water
- Dispense in the bin
- Aspirate
- Dispense in the container
- Note the measure
- Perform zero position of the scale
- Aspirate
- Dispense in the container
- Note the measure
- … till you have 10 measures
- Report all measures on the control form (Attachment 1) present in the logbook.
- Calculate
- Conclude

Preventive maintenance:

- Check water-tightness monthly:
  - Aspirate the maximum volume of water the pipette can hold
  - Hold the pipette vertically during one minute
  - No drop should appear
  - If the micropipette is leaking, inform head technician or quality manager or his/her assistant for repair and notify in the logbook. For repair change the toric rubber (E on picture).
- External cleaning of the tip-carrier every day:
  - With a paper wet with water or alcohol
- Clean the entire micropipette every month:
  - Unscrew the tip-ejector and the tip-carrier (see picture)
  - Clean all parts
  - Clean and eventually change the pump
  - Clean and eventually change the toric rubber

- In any case, see also the user manual of the pipette. Record in the logbook when done.



Biosafety consideration:

- When a contamination of biohazardous material occurs, clean the entire micropipette immediately as indicated in the preventive maintenance above. An effective disinfectant should be used and applied. All materials that come into contact with the spilled agent should be disinfected and/or autoclaved.
- Whenever a biosafety problem occurs, inform the Biosafety Officer, who will refer to DP Minor occurrence to report the problem and find an eventual solution if needed with the Biosafety Committee.

4.14. Agitator and shaker

General use:

- Agitator will be operated according to the manufacturer's instructions.
- Agitator full user manual should be easily available (usually in the English language) along with a Georgian language summary.
- The agitator will have a sticker affixed to it that indicates:
  - The qualification date
  - The requalification date
  - This SOP reference
  - The logbook reference
- Detail the use of the agitator.
- Clean the agitator after use with the product recommended by the manufacturer.
- If the agitator has stopped or is not working correctly inform head technician or quality manager or his/her assistant for repair and notify in the logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair.
- If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Preventive maintenance:

Refer to the specific recommendations of the agitator producer and write a short maintenance scheme accordingly.

Biosafety consideration:

If any biosafety problem occurs, inform the Biosafety Officer, who will refer to DP Minor occurrence to report the problem and find an eventual solution if needed with the Biosafety Committee. This will be recorded in the logbook.

4.15. Spectrophotometer

General use:

- Spectrophotometer will be operated according to the manufacturer's instructions.
- Spectrophotometer full user manual should be easily available (usually in the English language) along with a Georgian language summary.
- The spectrophotometer will have a sticker affixed to it that indicates:
  - The qualification date
  - The requalification date
  - This SOP reference
  - The logbook reference
- Detail the use of your specific spectrophotometer in a short summary in the Georgian language.
- If the spectrophotometer has stopped or is not working correctly, inform head technician or quality manager or his/her assistant for repair and notify in the adequate logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair.
- If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and

makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Preventive maintenance:

Refer to the specific recommendations of the spectrophotometer producer and write a short maintenance scheme accordingly.

Biosafety consideration:

If any biosafety problem occurs, inform the Biosafety Officer, who will refer to DP Minor occurrence to report the problem and find an eventual solution if needed with the Biosafety Committee. This will be recorded in the logbook.

4.16. Water bath

General use:

- Water bath will be operated according to the manufacturer's instructions.
- Water bath full user manual should be easily available (usually in the English language) along with a Georgian language summary.
- The water bath will have a sticker affixed to it that indicates:
  - The qualification date
  - The requalification date
  - This SOP reference
  - The logbook reference
- Detail the use of your specific water bath in a short summary in the Georgian language.
- If the water bath has stopped or is not working correctly, inform head technician or quality manager or his/her assistant for repair and notify in the logbook. The Biosafety Officer will be informed and will proceed to the equipment decontamination, if needed, and affix the release/certification tag for service or repair.
- If the equipment is not working correctly or its qualification has expired, label it "OUT OF CALIBRATION-DO NOT USE." Such label takes the instrument "out of the inspection," and makes sure that no work is performed, or has been performed on that instrument during the time that it is out of calibration.

Preventive maintenance:

Refer to the specific recommendations of the water bath producer and write a short maintenance scheme accordingly.

Biosafety consideration:

If any biosafety problem occurs, inform the Biosafety Officer, who will refer to DP Minor occurrence to report the problem and find an eventual solution if needed with the Biosafety Committee. This will be recorded in the logbook.

5. Pre – analytical procedures

5.1. Sampling room

Improper collection, internal transport, and receipt of specimens in the laboratory carry a risk of infection to the personnel involved. Specimens have to be collected in a separate room. In laboratories, it is strictly forbidden to:

- Eat
- Drink
- Chew gum
- Use cosmetics
- Smoke
- Put hands into the mouth (e.g., bite nails!)

If a separate room cannot be used, a clearly delimited place inside a bigger room has to be secured. Any other activity cannot be carried out in this specific area.
Sampling rooms are usually used for blood, skin, eye, nose, ear, vaginal and urethral samples; when toilets are usually kept for urine, stool and sputum samples (if not performed at home).

Ideally, dedicated toilets should be used for patients only (not being shared by the staff) and the wall of the toilets should clearly indicate how the sample should be performed.

5.2. General sampling conditions (including disinfection)

Sampling and sample handling is perhaps one of the most important analytical steps, but also one of the most bio-unsafe manipulations, thus a great care has to be taken for this initial step.

We have to keep in mind that a bad sample will never lead to a good and quality assured result, and in most of the cases, it will not be possible to perform another sample.

Different components can be distinguished in the sampling room:

- Protective equipment: gloves, lab coat, goggles
- Sampling and labeling equipment: needles, tubes, swabs, slides, tourniquet, labels, pens, etc.
- Disinfection equipment: cotton, skin disinfectant, general disinfectant (in case of leaking or spilling)
- Sampling furniture: chair (blood), gynecological bed (vaginal, urethral), light mounted on a flexible
- Hand washing equipment: sink, soap, tissues
- Waste equipment: general waste container (regular non contaminant waste), contaminated waste, sharp container

Note: Some little toys or teddy bears available in the sampling room will always make children sampling easier.

5.3. Sample labeling and numbering

Laboratory tracking is an important component of Quality Control because it ensures proper sample identification and accuracy in test results reporting. To retain sample identity and maintain quality assurance throughout the laboratory testing, the following procedures should be performed:

All collected samples are labeled and recorded at the time of collection. A nurse or laboratory assistant (sample collector) is usually responsible for that.

Sample Label should contain the following elements:

- Unique number, so called sample ID. The sample receives unique identification number (ID) upon receipt into the laboratory or at the time of sample collection. In some cases (if patient medical record is maintained) a medical record number can be used. If more than one sample is obtained from the same patient, additional data (e.g. order number) should be used to distinguish the samples. The sample ID is recorded on the sample itself and all subsequent paperwork generated in the laboratory. Labeling of all materials is necessary.
- Name (in some cases indication of the patient name may be inappropriate) / gender
- Date / hour

Below is given an example of the Sample Label.

Patent name _____☐ female ☐ male ____Identification number_____
Specimen collected on ___/____/_____(date) at _____(time)

5.4. Blood sampling

Equipment needed

- Disposable syringes and needles or all-in-one systems such as Vaccutainer
- Tubes, with and without anticoagulant
- Tourniquet
- Cotton
- Disinfectants (alcohol, iodine, etc.)

Sampling

From an INTESTINAL INFECTIOUS DISEASE perspective, blood sampling collection is done mostly for serology or for haemoculture in case of Salmonella septicemia. Haemoculture is being addressed in the procedure number 5.5

1. Collect a minimum of 5 ml (at least 3 ml in newborns) of blood in any collection tube without anticoagulant.

2. Allow blood to clot.

3. Centrifuge blood at low speed long enough to obtain cell-free serum (10 minutes at 1000 rpm).

4. Remove the serum by pipette to a sterile vial or a small test tube.

Whole blood may be sent if specimen is shipped and received on the day of collection.

Note: Special Vaccutainer tubes containing a gel (yellow cap) may be used for serological purpose. After centrifugation, no separation of the serum is needed; the gel making an interface between cells and serum.

Never freeze whole blood.
Serum or plasma may be used in the test. The common anticoagulants are acceptable for blood collection. Always ensure that serum is aliquoted from completely clotted blood. Store separated serum or plasma for up to 5 days at 4°C and thereafter, freeze it at –20°C if testing is delayed. If specimen shows evidence of precipitation or particulate matter, clarify the sample by centrifugation at 100 x g for 10 to 15 minutes prior to testing. Avoid repeated freezing and thawing.

Note: Universal precautions should be used when collecting any blood specimen, and all blood products (e.g., serum and plasma) should be considered potentially infectious in this testing procedure. Refer to Chapter 3.2.

Whole blood can be sent on the same day that it is collected.

5.5. Haemoculture sampling

Blood samples for culture must be obtained at screening (up to 24 hours prior to initiation of study, drug is acceptable). Repeat blood cultures approximately every 24 to 48 hours until 2 consecutive cultures obtained on separate days are negative. Blood cultures should be performed at any time the signs/symptoms of sepsis are present. When blood cultures are indicated, obtain 2 sets (each set including aerobic and anaerobic bottles) from 2 separate blood draws.

The criteria for the inclusion in the study will include the definitions for sepsis and related syndromes that was proposed by the Society of Critical Care Medicine Consensus Conference[2]:

General guidelines for proper specimen collection

- Collect specimen before administrating antimicrobial agents when possible.
- Collect specimen with as little contamination from indigenous microbiota as possible to ensure that the sample will be representative of the infected site.
- Collect an adequate amount of specimen. Inadequate amounts of specimen may yield false – negative results.

General considerations

- Number and timing:
- Obtain two separate blood cultures at least 1 h apart.
  Volume of blood
- Adults: 10 to 30 ml of blood per venipuncture
- Collection of blood cultures:
- Note: Wear gloves while handling any body fluids

[2] American College of Chest Physicians/Society of Critical Care Medicine. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Crit Care Med 1992; 20:864–874

- Disinfect the venipuncture site and the stoppers of culture bottles, as well as collection tubes prior to blood collection (note: do not use iodine to disinfect the stoppers of BACTEC bottles.)
- Clean the site with 70% isopropyl or ethyl alcohol.
- Swab concentrically, starting at the center with 1 to 2 % tincture of iodine solution (if the patient is hypersensitive to iodine, prepare the skin by using a double application of 70% alcohol).
- Allow the disinfectant to dry (note: do not palpate the vein after disinfecting skin prior to inserting needle.)
- Draw blood through a syringe and needle, and deliver through transfer set into a sterile collection bottle or tube (a new needle or transfer set should be used for each venipuncture.)
- After venipuncture and after inoculation of culture collection bottle, wipe residual iodine from the skin with alcohol to prevent skin irritation. Dispose of collection system in accordance with universal precautions.

Do not forget:

- Clearly label the specimen container with the patient's name and identification number and with the date and time of collection.
- Identify the specimen source and /or specific site correctly so that proper culture media will be selected during processing in the laboratory.
- Transport all specimens to the laboratory promptly.
- If impossible to bring the bottles immediately to the laboratory, they must absolutely be kept at 35-37°C prior to delivery.
- Ideally all specimen containers should be transported in sealed plastic bags with the request form securely attached outside the bag.

5.6. Stool and rectal swab sampling

Material needed

- Sterile container
- Transport media for viruses

Note: A sampling room is not a reception room: a sampling room contains a "dirty" area; a reception room is ONLY administrative. The samples received from outside cannot be opened at the reception.
Location of the collection: preferably in the laboratory, otherwise at home. Bring the collection to the laboratory as soon as possible, preferably immediately after obtaining.

Collection of fecal specimens for bacterial cultures

General considerations:
Keep stool specimen cool (can be refrigerated at 4-8^{o}C); do not incubate it. If a stool specimen cannot be plated within 1 hour of collection, it should be mixed with transport medium (for example, Cary-Blair transport or buffered glycerol saline). See 5.8.

Do not use toilet paper to collect stool. Toilet paper may be impregnated with barium salts, which are inhibitory for some fecal pathogens.

Have patient obtain stool specimen by one of the following methods:
Pass stool directly into a sterile, wide-mouth, leak-proof container with tight-fitting lid. Pass stool into a clean, dry bedpan, and transfer stool into a sterile, leak-proof container with a tight-fitting lid.

Collection of fecal specimens for virology

1. Place 2–4 g of the stool into a sterile container (e.g., penicillin vial, washing of which is not necessary).

2. Add 8–10 ml of VTM (Viral Transport Medium) to prevent drying if transport to laboratory is not immediate. Order, quality control, management, and prepositioning of the VTM at peripheral level are done by the World Health Organization (WHO) polio eradication program.

3. Remember to label and regularly pack the stool for shipment.

4. Ship the parcel to NCDC's enteric viruses department.

Rectal swabs

Submitted primarily for the detection of Shigella species. Pass the tip of a sterile swab approximately 2-3 cm. beyond the anal sphincter. Carefully rotate the swab to sample the anal crypts, and withdraw the swab. Send the swab in a swab transport system.

5.7. Test request or order

The test request or order must include the following.

- Complete name of patient
- Hospital number
- Hospital location
- Complete and specific description of specimen source
- Name of requesting physician
- Date and time of collection
- Diagnosis or clinical impression

Below is given an example of the Laboratory Request Order, recommended by the VPD QA manual[3]:

UNIVERSAL LABORATORY TEST REQUEST ORDER

Date ___/____/____ Patient Name _____Ref. Number___ ☐ Male ☐Female

Address: _____

Age ____years or Date of birth___/____/____ (for children under 5)

Preliminary clinical diagnosis_____(if specimen is taken from a contact, state so here)

Date of disease (rash in case of measles) onset ___/____/____

Date of the last dose of vaccine specific for the disease in question ___/____/____(for VPDs only)

Type of specimen (e.g., feces, blood) _____Date collected ___/____/____

Date shipped___/____/____

Name of the person to whom laboratory test results should be sent _____

Facility name and address _____ _____ Tel._____Fax _____

This part should be filled by the Laboratory

Date specimen received by the laboratory /------/-------/------/

Name of the person who received specimen_____

Is the specimen in good condition Yes No

[3] Laboratory Reference Manual for Surveillance and Control of Vaccine-Preventable Diseases in Georgia, Health Information and Disease Surveillance Reform Program, 2005

5.8. Cary Blair transport media

Introduction

Cary-Blair transport medium can be used to transport many enteric pathogens, including *Shigella*, *Vibrio cholerae*, and *Escherichia coli* O157:H7. Cary-Blair's semisolid consistency provides for ease of transport, and the prepared medium can be stored after preparation of room temperature for up to one year. Because of its high pH (8.4), it is the medium of choice for transport and preservation of *V*. *cholerae*. Cary Blair Transport Medium is a semi-solid medium used for maintenance of various pathogens and enteric pathogens bacteria as *Campylobacter*, *Salmonella* and *Shigella* in stools specimens.

Formula

For one liter of purified filtered water:

- Sodium Thioglycollate 1,5g
- Sodium Chloride 5,0g
- Sodium Phosphate, dibasic 1,1g
- Agar 5,0g

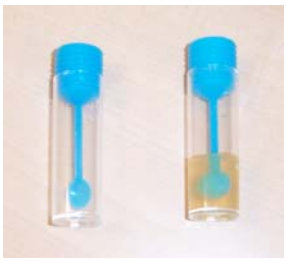
Adjust pH to 8,0 +/- 0,2 at 25º C.

Preparation

- Suspend 12,6 g of dehydrated media into 991 ml of purified filtered water.
- Heat with frequent agitation and boil for one minute.
- Add 9 ml of a 1% Calcium Chloride Solution. DO NOT AUTOCLAVE.
- Dispense into sterile culture tubes and steam for 15 minutes.

Or prepare according to manufacturer's instructions.

Note: There are several commercially available dehydrated formulations of Cary-Blair. Some require the addition of calcium chloride and some do not. Cary-Blair can also be prepared from individual ingredients.



Aliquoting

When Cary-Blair is prepared, it should be dispensed into containers in a sufficient volume so that the swabs are covered by at least 4 cm of medium. For example, 5- to 6-ml amounts may be dispensed into 13 x 100- mm screw cap tubes. With the caps loosened, sterilize by steaming (do not autoclave) at 100°C for 15 minutes. Tighten the caps after sterilization only.

Quality Control

Results after 24 hrs at 25ºC:

Table 3. Results after 24 hrs at 25ºC

Organisms	ATCC number	GROWTH
Streptococcus pyogenes	19615	+
Haemophilus influenzae	10211	+
Bacteroides fragilis	25285	+
Escherichia coli	25922	+
Salmonella typhimurium	14028	+
Shigella flexneri	12022	+

Storage

Store prepared media ideally at 2-8° C, protected from direct light. It can usually be kept at room temperature for 1 or 2 years as long as there is no loss of volume, contamination, or color change.

Dehydrated powder should be kept in a dry place, in tightly-sealed containers at 2-25° C.

User Quality

Media should not be used if the expiry date has passed. Prepared media should not be used if there are signs of contamination or deterioration (shrinking, cracking, evaporation or discoloration). Do not use dehydrated media if it is caked.

Inoculation procedure

Prior to inoculate, the prepared media should be brought to room temperature.

Dip swabs and break into culture media. Inoculate the culture media as soon as possible and streak to obtain isolated colonies. Incubate as required.

5.9. Viral transport media

VTM should be used if the sample cannot reach the recipient laboratory **within 24 hours** following the sampling.

Preparation of the VTM:

- To a 500ml bottle add:
  - 10 g veal infusion broth (2.5% final concentration)
  - 2.0 g of BSA* (0.5% final concentration)
  - 0.8 ml gentamicin 50mg/ml (100 μg/ml final conc.)
  - 3.2 ml of Amphotericin B 250 μg/ml (2 μg/ml final conc.)
- Distillated de-ionized water up to 400 ml.
- Swirl gently to dissolve or let stand at 4°C for 1 hour.
- Sterile filtration
- Label as “virus transport medium”, with the name of the preparator, the date of preparation
- VTM can be used 3 months after preparation if stored at 4°C.

*BSA: Bovine Serum Albumin

Use of the VTM

- In a 2 ml cryovial, pour 1 ml of VTM
- Put 0.5ml (2-4 g) of the pathogenic material in the vial
- The vial can be kept 2 days at +4°C. If more, freeze at -70°C
- VTM can be used for **any type of viruses**

5.10. Campylobacter transport media

Notes:
Some specific transport media for Campylobacter can be used, such as Campy-Thio medium. Even if not Campylobacter-specific, Cary-Blair transport media can also be a very good alternative for Campylobacter preservation.

In order to avoid the multiplication of transport media at a laboratory level, we are recommending the use of Cary Blair transport media for all non fastidious bacteria, including Campylobacter.

We nevertheless provide some information about Campy-Thio transport media, which can be used if a low number of bacteria are expected in the sample (such as in carriers, post-symptomatic

cases, or patients treated with antibiotics). See chapter 9.4 about Campylobacter diagnosis in order to get more precisions about them)

- **Campy-Thio medium**

Campylobacter Thioglycollate Medium is a selective holding medium recommended for the isolation of *Campylobacter* from clinical specimens, when immediate inoculation of CVA agar cannot be performed. The incorporation of antimicrobial agents, i.e., amphotericin B, cephalothin, polymyxin B, trimethoprim and vancomycin associated to refrigeration, inhibits further multiplication of normal microbial flora in fecal specimens, thus facilitating isolation of *C. jejuni*. Cary-Blair transport media can be used for isolation of Campylobacter from rectal swabs.

Preparation

- Suspend 39.5 g of dehydrated medium in 1 L of purified water: mix thoroughly.
- Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
- Autoclave at 121□C for 15 minutes.
- Store fluid Thioglycollate media at 15-30□C. If more than 30% of the medium is pink prior to use, reheat once (100□C) to drive off absorbed oxygen.

Adjust to pH 7.0 +/- 0,2 à 25º C, store media at 2-8ºC protected from direct light.

Quality Control
Results after 48 hrs at 42ºC:

Table 4. Results after 42 hrs at 42ºC

Organisms	ATCC	Growth
Campylobacter jejuni	33291	+
Escherichia coli	25922	- or partial
Proteus mirabilis	12453	- or partial
Streptococcus faecalis	29212	- or partial

Colonies of C. jejuni will appear on Campylobacter Blaser Agar as small, mucoid, flat or slightly raised, non-hemolytic translucent and gray. Some strains may vary from brown to tan.

Inoculation procedure
Prior to inoculate, the prepared media should be brought to room temperature.

1. **Swab:** Place the swab 2 cm into medium and twirl the swab. Then, lower it to the bottom of the tube. **Diarrhoeal Stools:** Place 3-5 drops into the Campy-Thio Medium. **Solid Stools:** Place 3-5 drops of a saline suspension into the Campy-Thio Medium.
2. Refrigerate overnight (Incubate at 2-10°C overnight)
3. Subculture by placing the tip of a sterile pipette about 2 cm below the surface of the broth and placing 3 drops on a Campylobacter CVA Agar. Streak the plate to obtain isolated colonies.
4. Incubate at 42º C under a reduced O_2 atmosphere for 48 hours.

5.11. Sample acceptance criteria

Specimen reception

Maintain registration book:

- Document the time the specimen was received.
- Verify that the patient identification on the request form matches the one on the specimen.
- Assign an accession number to be used as specimen identification in the laboratory.
- Examine the specimen visually.

- Carefully review and evaluate the specimen request for appropriateness of orders.
- Determine the appropriateness of the container, including the following:
    a. Holding medium or preservative
    b. Intact transport void of leaks and cracks

Specimen acceptance and rejection

Unlabeled or mislabeled specimens:

- An unlabeled, mislabeled, or mismatched specimen is unacceptable.
- If an unacceptable specimen can be replaced, notify the ward and request another specimen.
- Do not discard the specimen until the patient's physician or nurse has confirmed that a repeat specimen can be collected.
- Document the reason for the specimen unacceptability and request for a repeat specimen.
- If the patient has already been started on an antimicrobial therapy or if a repeat specimen cannot be collected, this must also be documented.

Duplicate specimens:

- Most duplicate specimens received on the same day should not be processed; exceptions include blood cultures, CSF (cerebral spinal fluid), tissue, and sterile body fluid excluding urine.
- If it has been verified by the person collecting the specimen that two specimens received at the same time are the same, these specimen may be combined and processed as one specimen.
- If duplicate specimens are received at different times on the same day, notify the patient's physician or nurse, and document. If it is acceptable not to process the specimen, report "Duplicate specimen: test not performed," and refer to the number of the specimen that was processed for smear and/or culture results.
- Do not process more than three duplicate specimens on consecutive days.

Leaky containers:

- A specimen is unacceptable when the outside of the container is grossly contaminated with the specimen. Follow the steps outlined above.
- If the container is leaking, set up the specimen only if it is possible to process it without contaminating the processor.

Contaminated specimens:

Do not contaminate the specimen with another type of specimen. For example, a urine specimen should not contain stool, and vice versa.

Unacceptable specimen sources:

- Do not process saliva in place of sputum.
- 24-hour urine specimens are unacceptable for routine bacterial cultures.
- Request a proper specimen for the test requested.

Delayed transport time and specimen processing:

Ideally, all other specimens should be less than 2 hours old when received. Appropriate transport media and detailed instructions should be available for specimens transported to reference laboratories (see 5.8 and 5.9).

Request a new specimen as outlined above. Note that the specimen was received after prolonged delay.

Test request or order, sample identification:

The test request or order must include the following:

- Complete name of a patient
- Hospital number
- Hospital location
- Complete and specific description of a specimen source
- Name of requesting physician
- Date and **time** of collection
- Diagnosis or clinical impression
- Guess this is good enough

5.12. Sample pretreatment for laboratory dispatching and storing

Refer to each pathogen specific procedures (chapter 9).

6. Analytical procedures – reagents and supplies

6.1. Review manufacturer's data policy

The objectives of the review of manufacturer's data policy are:
To test the material and equipment before purchasing to check the data are correct.
Review these data on an annual base.

Commitment to the review of manufacturers' data policy:
It is the responsibility of the Quality Manager to ensure all kits, equipment and reagents are tested before purchase to ensure the manufacturers' data are in compliance with its use in the laboratory. These studies will be documented and filed with each respective logbook.

Note: This review and these studies can be achieved at a central level (NCDC) for the entire country, before the commercial authorization needed for each kit.

6.2. Culture media preparation for bacteriology

Media and reagent preparation and control:

Test each new lot number of dehydrated medium, reagent, or ingredient in parallel with an approved lot, before it is released for use. This means that orders have to be done enough in advance in order to allow little overlapping period between old and new reagents.

Maintain a logbook containing:

- Preparation date
- Name of preparator
- Amount of media or reagent prepared
- Source(s) of raw media
- Lot number
- Sterilization method and number of sterilization logbook for media
- Expiration date

If needed, other information as pH, osmolarity, resistivity …

Note on each Petri dish/tube/slants the name of the media (or a code), the date of expiration and the lot number.
Note all reagents with their date of reception and/or date of preparation. Further, the date of opening or reconstitution (if needed) will also be specified along with the initials of the manipulator.

Quality control of the home made media:

Note aspect, color, sterility in the same logbook as the one used for preparation.

Check each lot for contamination by incubating a 5 % sample for batches of 100 or less or 10 randomly selected units from larger batches.
Incubate medium for 48h at the temperature at which it will be used and then for another 48h at room temperature.
A weekly growth test can be performed using a regular registered strain; ideally it is recommended to test different inocula, especially for selective media.

Storage of prepared media and reagent:

Store media at 4°C. Note that media can only be used after a minimum period of 2 days (waiting for sterility results). Outdated reagent or media will be discarded immediately, or kept for training purposes (clearly labeled).

Main culture media used for enteric pathogens

Note: We are only providing short description and way of use for each media. For full details, please refer to your own documentation, or to the documentation provided along with your culture media.

- **Hektoen media**

Hektoen enteric agar is used for the isolation and differentiation of enteric pathogens.
Compared to other enteric differentiating media commonly used in clinical laboratories, Hektoen Enteric Agar increases the isolation rate of *Salmonella* spp. and *Shigella* spp.

Hektoen Enteric Agar is used to isolate and differentiate *Salmonella* spp. and *Shigella* spp., both of which cause a variety of serious human gastrointestinal illnesses.

Preparation

- Suspend 75 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Do not overheat. DO NOT AUTOCLAVE.
- Cool in water bath.
- Pour 20 ml portions into sterile Petri dishes
- Let dry 2 h with lids partially removed.

Final pH: 7.5 ± 0.2.

Quality Control:
Dehydrated Appearance: Powder is homogeneous, free flowing, and light green-beige.
Prepared Appearance: Prepared medium is trace to slightly hazy and light to dark green.
Expected Cultural Response: Cultural response on Hektoen Enteric Agar at 35 ± 0.2°C after 18 - 24 hours incubation:

Table 5. Cultural response on Hektoen Enteric Agar at 35 ± 0.2°C after 18 - 24 hours incubation

Microorganism	Response	Reactions
Escherichia coli ATCC 25922	partial to complete inhibition	yellow to salmon-orange colonies
Enterococcus faecalis ATCC 29212	inhibited	---
Shigella flexneri ATCC 12022	fair to good growth	green colonies
Salmonella typhimurium ATCC 14028	fair to good growth	green colonies with black centers

- **Kligler media**

(See chapter 8.2, tube identification gallery for preparation and use).

Quality Control:
Dehydrated Appearance: Powder is homogeneous, free flowing, and light beige.
Prepared Appearance: Prepared medium is reddish-orange to red, trace to slightly hazy.
Expected Cultural Response: Cultural response on Kligler Iron Agar at 35°C after 18 - 24 hours incubation:

Table 6. Cultural response on Kligler Iron Agar at 35°C after 18 - 24 hours incubation

Microorganism	Response	Slant	Butt	Gas	H_2S
Escherichia coli ATCC 25922	growth	yellow	yellow	+	--
Proteus mirabilis ATCC 12453	growth	pink	yellow	--	+
Pseudomonas aeruginosa ATCC 27853	growth	pink	pink	--	--
Salmonella typhimurium ATCC 14028	growth	pink	yellow	+/-	+
Shigella flexneri ATCC 12022	growth	pink	yellow	--	--

The organisms listed are the minimum that should be used for quality control testing.
A: acid- yellow, K: alkaline- pink, +: positive, -: negative, +/-: usually positive

- **Lysine Iron Agar**

Lysine Iron Agar is used for the differentiation of microorganisms on the basis of lysine decarboxylase and hydrogen sulfide production.

Preparation:
- Suspend 33 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Dispense into test tubes and autoclave at 121°C for 15 minutes.
- After autoclaving, allow medium to solidify in a slanted position.

Final pH: 6.7 ± 0.2 at 25°C

Test Results:
- A positive lysine decarboxylase reaction shows purple (alkaline) butt, purple slant.
- A negative reaction shows a yellow (acid) butt, purple (alkaline) slant.
- A positive lysine deaminase reaction is a red slant.
- A negative reaction is a purple slant. (Proteus spp. and Providencia spp. produce a red slant over a yellow [acid] butt.)
- A positive hydrogen sulfide reaction is blackened medium at the apex of the slant.

Quality Control:
Dehydrated Appearance: Powder is homogeneous, free flowing, and gray to grayish beige.
Prepared Appearance: Prepared medium is red-purple and trace to slightly hazy.
Expected Cultural Response: Cultural response on Lysine Iron Agar at 35°C after 24 hours:

Table 7. Cultural response on Lysine Iron Agar at 35°C after 24 hours incubation

Microorganism	Response	Slant	Butt	H2S
Citrobacter freundii ATCC 8090	growth	K	A	+
Escherichia coli ATCC 25922	growth	K	K	-
Proteus mirabilis ATCC 12453	growth	R	A	+
Salmonella typhimurium ATCC 14028	growth	K	K	-

K: alkaline, R: red (oxidative deamination), A: acid, +: H2S produced, ---: H2S not produced

- **MacConkey Agar**

MacConkey Agar is used for the isolation and differentiation of Gram-negative enteric bacilli. MacConkey Agar is recommended for the detection and isolation of Gram-negative organisms from clinical, dairy, food, water, pharmaceutical, and industrial sources.

Preparation

- Suspend 50 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Autoclave at 121°C for 15 minutes.
- Cool to 45 - 50°C and dispense into sterile Petri dishes.

Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non-lactose fermenting organisms grow as colorless or clear colonies.

Quality Control
Dehydrated Appearance: Powder is homogeneous, free flowing, and pink-beige.
Prepared Appearance: Prepared medium is red-purple and slightly opalescent.
Expected Cultural Response: Cultural response on MacConkey Agar at 37°C after 24 hours:

Table 8. Cultural response on MacConkey Agar at 37°C after 24 hours incubation

Microorganism	Response	Reactions
Escherichia coli ATCC® 25922	growth	pink colonies
Proteus mirabilis ATCC® 12453	growth	colorless colonies, swarming inhibited
Salmonella typhimurium ATCC® 14028	growth	colorless colonies
Staphylococcus aureus ATCC® 25923	inhibited	---

- **Sorbitol MacConkey Agar**

MacConkey Agar W/ Sorbitol is used for the isolation of pathogenic Escherichia coli.

MacConkey Agar W/ Sorbitol contains sorbitol instead of lactose for differentiating enteropathogenic E. coli serotypes; these strains are typically sorbitol negative. MacConkey Agar W/ Sorbitol is recommended for clinical and food testing.

Preparation

- Suspend 50 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Autoclave at 121°C for 15 minutes.
- Cool to 45 - 50°C and dispense into sterile Petri dishes.

Final pH: 7.1 ± 0.2 at 25°C

Quality Control:
Dehydrated Appearance: Powder is homogeneous, free flowing, and pink-beige.
Prepared Appearance: Prepared medium is red-purple, slightly opalescent.
Expected Cultural Response: Cultural response on SMA Agar at 37°C after 18 - 24 hours incubation:

Table 9. Cultural response on SMA Agar at 37°C after 18 - 24 hours incubation

Microorganism	Response
Escherichia coli ATCC 25922	good growth, pink colonies are sorbitol positive
Escherichia coli ATCC 35150	good growth, colorless colonies are sorbitol negative

Staphylococcus aureus ATCC 25923	inhibited

The organisms listed are the minimum that should be used for quality control testing.

Results *E. coli* O157:H7, and other organisms that do not ferment sorbitol, are colorless on MacConkey Agar W/ Sorbitol. Sorbitol-fermenting organisms produce pink colonies. Confirmatory biochemical and serological testing should be performed on suspected colonies.

Limitations of the Procedure

- Due to nutritional variation, some strains may be encountered that grow poorly or fail to grow on this medium.
- Colonies that are sorbitol positive can revert, and can be mistaken for sorbitol negative.
- E. coli O157:H7 can ferment sorbitol after prolonged incubation (more than 72 hours).

• Endo agar

Endo Agar is used for the enumeration of coliforms, especially *Escherichia coli.*

Preparation

- Suspend 51 g of the medium in 1 liter of purified water containing 20 ml of non-denatured Ethanol.
- Heat with frequent agitation and boil to completely dissolve the medium.
- Avoid overheating. DO NOT AUTOCLAVE.

Final pH: 7.2 ± 0.2 at 25°C

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free flowing, and light purple.
Prepared Appearance: Prepared medium is red to purple and none to trace hazy.
Expected Cultural Response: Cultural response in m-Endo Agar at 35 ± 2°C and examined for growth after 18 - 24 hours incubation.

Table 10. Cultural response on m-Endo Agar at 35 ± 2°C and examined for growth after 18 - 24 hours incubation

Microorganism	Response	Reaction
Escherichia coli ATCC 23226	good to excellent growth	green, metallic sheen
Enterobacter aerogenes ATCC 13048	good to excellent growth	green, metallic sheen
Salmonella typhimurium ATCC 14028	good to excellent growth	pink to red
Staphylococcus aureus ATCC 25923	inhibited	---

• Salmonella Shigella media

Salmonella Shigella Agar (SS Agar) is a differentially selective medium for the isolation of pathogenic enteric bacilli, especially those belonging to the genus ***Salmonella***. Salmonella Shigella Agar is used for the isolation of *Salmonella* spp. and some strains of *Shigella* spp.

Enteric organisms are differentiated by their ability to ferment lactose. *Salmonella* spp. and *Shigella* spp. are non-lactose fermenters and form colorless colonies on Salmonella Shigella Agar. H_2S positive *Salmonella* spp. produce black-center colonies. Some *Shigella* spp. are inhibited on Salmonella Shigella Agar. *E. coli* produces pink to red colonies and may have some bile precipitation.

Preparation

- Suspend 60 g of the medium in one liter of purified water.

- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- DO NOT AUTOCLAVE.

Final pH: 7.0 ± 0.2 at 25°C

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free-flowing, and light to medium pinkish-beige.
Prepared Appearance: Prepared medium is light to medium reddish-orange to peach, trace to slightly hazy.
Expected Cultural Response: Cultural response at 35°C after 18 - 24 hours incubation.

Table 11. Cultural response on SS Agar at 35°C after 18 - 24 hours incubation

Microorganism	Response	Reactions
Enterococcus faecalis ATCC 29212	complete inhibition	---
Escherichia coli ATCC 25922	partial to complete inhibition	pink to rose-red colonies with precipitate
Salmonella typhimurium ATCC 14028	growth	colorless colonies with black centers
Shigella flexneri ATCC 12022	growth	colorless colonies

- ## Campylobacter CVA media

Campy CVA Agar is a selective medium used in the primary isolation and cultivation of *Campylobacter jejuni* from human fecal specimens.

This medium consists of defibrinated sheep blood that provides additional nutrients. Antimicrobial agents are incorporated to suppress the growth of normal fecal flora that could mask the presence of *C. jejuni*. Cefoperazone is a cephalosporin antibiotic that suppresses the growth of gram-negative enteric bacilli and some gram-positive species. Vancomycin is a glycopeptide antibiotic that inhibits many species of gram-positive bacteria. Amphotericin B is an antifungal agent.

Preparation
- Dissolve 44.4 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Autoclave at 121°C for 15 minutes.
- Cool medium to 50°C and aseptically add 10 mL of a filtered sterilized solution containing 3 mg of Cefoperazone, 2 mg of Vancomycin, 0.4 mg of Amphotericin and 5% of sterile laked sheep blood.
- Mix well and pour into Petri dishes.

Final pH: 7.0 ± 0.2 at 25°C

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free flowing, and light to medium beige.
Prepared Appearance: Campy CVA Agar should appear opaque and cherry red in color.
Expected Cultural Response: The cultural response of *Campylobacter* spp test strains on Campy CVA Agar at 35 ± 2°C in a microaerophilic atmosphere and examined for recovery after 24 - 96 hours incubation. All other test strains were incubated at 35 ± 2°C in an aerobic atmosphere and read for inhibition over 24 - 96 hours.

Table 12. Cultural response on Campy CVA Agar at 35 ± 2°C after 24 – 96 hours incubation

Microorganism	Response
Escherichia coli ATCC 25922	inhibited

Enterococcus faecalis ATCC 29212	inhibited
Proteus mirabilis ATCC 12453	inhibited
Campylobacter coli ATCC 33559	growth
Campylobacter fetus ATCC 33246	growth
Campylobacter jejuni ATCC 29428	growth
Campylobacter jejuni ATCC 33291	growth

Left:
Campylobacter jejuni (ATCC33291) colonies growing on Campy CVA Agar. Incubated under microaerophilic conditions for 48 hours at 35°C.
Right:
Escherichia coli (ATCC 25922) growth inhibited. Incubated aerobically for 24 hours at 35°C.

Campylobacter colonies may appear as small, mucoid, grayish, flat colonies with irregular edges and no hemolytic patterns at 24–48 hours. Colonies may also appear pink or yellow-grey. Depending on the species, colonies may also appear as round, convex, entire, glistening colonies 1–2 mm in diameter. Certain strains of C. jejuni may appear lightly pink or tan in color.

- ## Selenite broth

Selenite broth is used for the selective enrichment of *Salmonella* species. 1 ml of sample (stool) are added to 10 ml Selenite broth and incubated at 35°C during 24 hours.

Preparation

- Dissolve 23 g of the medium in one liter of purified water.
- Heat to boiling to completely dissolve the medium.
- DO NOT AUTOCLAVE. Use immediately.

Final pH: 7.0 ± 0.2 at 25°C

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free flowing, and off-white.
Prepared Appearance: Prepared medium is clear, very pale yellow with no or very slight precipitation.
Expected Cultural Response: Cultural response is exhibited on MacConkey Agar after enrichment in Selenite Cystine Broth at 35°C for 24 - 48 hours.

Table 13. Cultural response on MacConkey Agar at 35°C after 24-48 hours incubation

Microorganism	**Response**	**Reactions** (if recovered)
Escherichia coli ATCC 11775	partial to complete inhibition	pink with bile precipitate
Escherichia coli ATCC 25922	partial to complete inhibition	pink with bile precipitate
Salmonella typhi ATCC 19430	good growth	colorless colony
Salmonella typhimurium ATCC 14028	good growth	colorless colony
Shigella sonnei ATCC 25931	fair to good growth	colorless colony

- ## Urea media

Urea broth and Urea Agar Base is used for the differentiation of microorganisms on the basis of urease production. Using an inoculating loop, collect 2–3 non-lactose-fermenting colonies from the primary plates and transfer them to the tube containing urea. Incubate the tubes for 2–4 hours at 35°C and observe for a change in color to pink (urease-positive).

Preparation

- Dissolve ingredients in distilled water.
- Do not heat.
- Sterilize by filtration through 0.45 µm membrane.
- Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes.

Final pH: 6.8 ± 0.2.

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free flowing, and off-white.
Prepared Appearance: Prepared medium is light to medium yellow-orange and trace to slight hazy.
Expected Cultural Response: Cultural response on Urea Agar at 35°C after 18 - 24 hours:

Table 14. Cultural response on Urea Agar at 35°C after 18 - 24 hours incubation

Microorganism	Reactions
Escherichia coli ATCC 25922	negative
Klebsiella pneumoniae ATCC 13883	weak positive
Proteus vulgaris ATCC 13315	positive
Salmonella typhimurium ATCC 14028	negative

- **Mueller Hinton media**

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to the recommendations of the Clinical Laboratory Standards Institute (CLSI).

Preparation

- Suspend 38 g of the medium in one liter of purified water.
- Heat with frequent agitation and boil for one minute to completely dissolve the medium.
- Autoclave at 121°C for 15 minutes. Cool to room temperature.
- OPTIONAL: Supplement as appropriate. Pour cooled Mueller Hinton Agar into sterile Petri dishes on a level, horizontal surface to give uniform depth. Allow to cool to room temperature.
- Check prepared Mueller Hinton Agar to ensure the final pH is 7.3 ± 0.1 at 25°C.

Quality Control Specifications
Dehydrated Appearance: Powder is homogeneous, free flowing, and beige.
Prepared Appearance: Prepared medium is slightly opalescent with no significant precipitation, and light to medium amber.
Expected Cultural Response: Prepare, inoculate and dispense antibiotic disks following the procedure described by CLSI. The cultures listed should have middle range zone sizes of the concentration tested.

Table 15. Cultural response on Mueller Hinton Agar

Microorganism	Response & Reactions
Enterococcus faecalis ATCC 29212	growth; zone diameters within published specifications
Escherichia coli ATCC 25922	growth; zone diameters within published specifications
Escherichia coli ATCC 35218	growth; zone diameters within published specifications
Pseudomonas aeruginosa ATCC 27853	growth; zone diameters within published specifications

Staphylococcus aureus ATCC 25923	growth; zone diameters within published specifications
Staphylococcus aureus ATCC 43300	growth; zone diameters within published specifications

Also refer to chapter 8.4 about antibiotic susceptibility testing tests.

6.3. Biochemistry tests for bacteriology

Biochemistry tests for bacteriology can be divided into two groups:

- Specific tests
- Sugar assimilation tests

For more details about these tests, please refer to your documentation.

Specific tests

- **Catalase test**

Catalase is a common enzyme found in nearly all living organisms. Its functions include catalyzing the decomposition of hydrogen peroxide to water and oxygen. Catalase test is useful in the presumptive identification and differentiation of many bacteria.

Preparation and use

- Hydrogen Peroxide: 3% and Acetophenetidine.
- Protect from light, excessive heat and freezing.

Organisms to be tested for the Catalase Test must be taken from an 18-24 hour old culture as organisms lose their catalase activity with age.

It is recommended that the colonies subject to testing be taken from non-blood containing media due to the endogenous catalase activity present in animal red blood cells.

Table 16. Quality Control Specifications

Test Organisms	Reaction
Staphylococcus aureus ATCC 25923	Positive, bubbles seen
Streptococcus pyogenes ATCC 19615	Negative, no bubbles seen

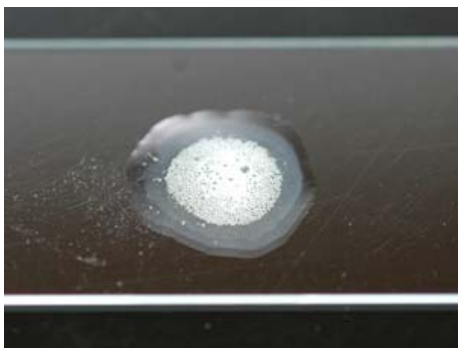
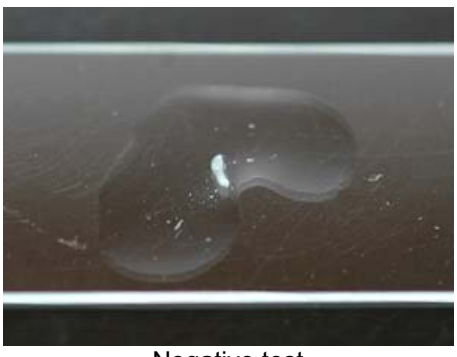


Illustration 2 Positive test



Negative test

- **Hippurate test**

Hippurate Test is to be used in the presumptive identification of *Gardnerella vaginalis*, *Campylobacter jejuni, Listeria monocytogenes* and group B *Streptococcus*, by detecting the ability of the organism to hydrolyze hippurate.

Using a heavy inoculum from an 18-24 hour culture, make a heavy suspension of the organism in the Hippurate Reagent with a standard inoculating loop. Incubate the tube for two hours at 35-37°C. After the incubation period, add two drops of the Ninhydrin Indicator Solution to the tube. Reincubate at 35-37°C for 30 minutes. Observe the tubes at 10 minute intervals for the appearance of a deep blue color, which is a positive test.

Table 17. Quality Control Specifications

Test Organisms	Reaction
Streptococcus agalactiae ATCC 12386	Positive: Dark blue color change
Streptococcus pyogenes ATCC 19615	Negative: No color change or a faint blue seen

- **Indole test**

The Indole test is a qualitative procedure for determining the ability of bacteria to produce Indole by deamination of tryptophan. Three different types of reactions are being used:

Kovacs tube reagents, with incubation

Indole spot reagent (on a filter paper)

Kovacs tube reagents, used as a spot reagent.

Depending on the reagent available, use any of these 3 methods, the first one being the most sensitive.

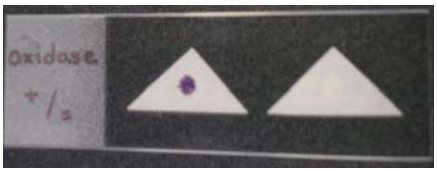
Interpretation of Results:
A positive Kovacs tube test reaction is denoted by the appearance of a pink to red color in the top alcohol layer. Negative reactions remain colorless or light yellow.

A positive spot reagent reaction is denoted by the appearance of a blue to blue-green color change, or red-violet in the case of *Providencia alcalifaciens*, within 10 seconds. Negative reactions remain colorless or light pink.

Table 18. Quality Control Specifications

Test Organisms	Indole Kovacs	Indole Spot
Escherichia coli ATCC 25922	Positive; red color change	Positive; blue to blue-green color change
Pseudomonas aeruginosa ATCC 27853	Negative; no color change or light yellow	Negative; no color change or light pink

- **Oxidase Test**



Oxidase test is a test used to determine if a bacterium produces certain cytochrome C oxidases. Typically the Pseudomonadaceae are OX+ and Enterobacteriaceae are OX-. This test is especially helpful in identifying organisms belonging to the genera Campylobacter, Pseudomonas and Aeromonas.

Procedure
Use only 18-24 hour old colonies from non-selective, non-differential media. Place a piece of filter paper in a sterile, plastic, disposable Petri dish. Moisten the piece of filter paper with a few drops of oxidase reagent. Rub a small amount of the colony to be tested onto the paper using a platinum loop or a wooden applicator stick. Observe for purple or blue-black color development. Any color change after 20 seconds should be disregarded.

Table 19. Quality Control Specifications

Test Organisms	Reaction within 10-20 seconds
Pseudomonas aeruginosa ATCC 27853	Oxidase-positive; blue/purple color develops
Escherichia coli ATCC 25922	Oxidase-negative; no color development

- **Citrate test**

Citrate Agar is used for the differentiation of gram negative bacteria on the basis of citrate utilization. A positive reaction is indicated by growth with an intense blue color in the slant. A negative reaction is evidenced by no growth to trace growth with no change in color (medium remains dark green).

Preparation:

- Suspend 24.2 g of the powder in 1 L of purified water.
- Mix thoroughly.
- Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
- Dispense and autoclave at 121□C for 15 minutes.
- Allow to cool in a slanted position for use as slants. The agar also may be used as plating medium.
- Test samples of the finished product for performance using stable, typical control cultures.

Quality Control Specifications

Dehydrated Appearance: Fine, homogeneous, free of extraneous material, may contain many dark and gray flecks.

Prepared Appearance: Medium to dark, green, clear to slightly hazy, with a small amount of precipitation and as many as a large amount of insolubles.

Table 20. Citrate test

ORGANISM	ATCC	RECOVERY	REACTION
Enterobacter aerogenes	13048	Good	Alkaline (blue)
Escherichia coli	25922	Partial to complete inhibition	–
Klebsiella pneumoniae	33495	Good	Alkaline (blue)
Shigella flexneri	9199	Complete inhibition	–

Enterobacter aerogenes ATCC 13048
Positive

Escherichia coli ATCC 25922,
Negative

- **Motility Test Medium**

Motility Test Medium is used for the detection of motility of gram-negative enteric bacilli. Bacterial motility can be observed directly from examination of the tubes following incubation.

Preparation:

- Suspend 22 g of the powder in 1 L of purified water. Mix thoroughly.
- Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

- Dispense and autoclave at 121°C for 15 minutes.
- Test samples of the finished product for performance using stable, typical control cultures.

Final pH: 7.3.

Test Procedure
Inoculate tubes with a pure culture by stabbing the center of the column of medium to greater than half the depth. Incubate tubes for 24-48 hours at 35 ± 2°C in an aerobic atmosphere.

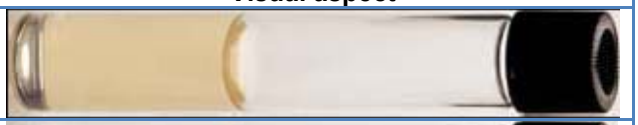
Test Result:
After incubation, observe the tubes for growth in relation to the stab line. Non-motile organisms grow only along the line of inoculation, while motile organisms spread out from the line of inoculation and may even grow throughout the medium.

Negative tubes can be reincubated at 25 ± 2°C for additional 5 days, if desired.

Quality control:
Dehydrated Appearance: Fine, homogeneous, free of extraneous material
Prepared Appearance: Pale to light, yellow to tan, clear to slightly hazy after 28-24 hours at 35°C

Table 21. Motility test

ORGANISM	ATCC	Recovery	Motility
Enterobacter aerogenes	13048	Good	+
Escherichia coli	25922	Good	+
Klebsiella pneumoniae	33495	Good	-
Salmonella choleraesuis, choleraesuis serotype Typhimurium	14028	Good	+
Shigella flexneri	9199	Good	-
Proteus vulgaris	8427	Good	+

Organism	ATCC	Visual aspect
Uninoculated	/	
Escherichia coli	25922	
Klebsiella pneumoniae	13883	

Sugar assimilation tests

Sugar assimilation testing can be achieved through manual tube method, with all different sugars included in a regular agar. Nevertheless, it is much more convenient to use ready prepared kits such as API gallery. Refer to chapter 8.3 for more details.

Quality control of these tests

As specified for each reagent, quality control with reference strains needs to be done, at least once a week. The results of these QC processes need to be written and

archived, as in the document provided in Attachment 2.

6.4. Buffers for virology

Wash buffers

Allow all reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the Wash buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until crystals dissolve. Dilute the stock wash buffer, usually **1 to 20** with distilled or deionized water (in any case, follow manufacturer's instructions). Use only clean vessels to dilute the Wash buffer. Write the date of preparation on the vial.

Diluted wash buffer is stable at the room temperature up to two weeks.

HRP-Conjugate

Allow all reagents and samples to reach room temperature (18-30°C) for at least 15-30minutes. Conjugate needs to be usually diluted at 1/50 in the buffer prior to addition to the wells (in any case, follow manufacturer's instructions). The diluted conjugate is stable for 3 hours at room temperature.

Chromogen

Depending on the kits used, chromogen can be either ready to use or to be prepared.
Usually, chromogen solution A needs to be added to the Chromogen solution B at 1:1 proportion prior to addition to the wells and are stable for 3 hours at room temperature. In any case, follow manufacturer's instructions.

Negative control, positive control as well as stopping solutions are ready to use and should not be pretreated before the addition to the wells.

Note: The components of the kit will remain stable through the expiration date indicated on the label and package when stored between 2-8°C; **do not freeze**. To assure maximum performance of ELISA kit, protect the reagents from contamination with microorganism or chemicals.

6.5. Other reagents for bacteriology and virology

Please refer to each disease specific procedure (chapters 9 and 10).

7. Analytical procedures – staining procedures

7.1. Preparation and QC of staining methods

In order to control the stains and staining procedure (differential staining of gram-positive and gram-negative organisms), use local strains of *E. coli* for Gram negative bacteria and *Staphylococcus spp.* for Gram positive bacteria.
When to perform such control?

- When preparing new Gram-stain reagents
- When changing batch number or stain kits
- In case of problems while staining
- In any case, regularly on a weekly basis

7.2. Smears, films, slides performing

Labeling of slides

Frosted ends are desirable for accurate labeling and convenient handling. If not, the edge of another slide can be used for labeling. Special markers can be used.

Smear preparation

Proper preparation should produce a monolayer of organisms sufficiently dense for easy visualization but sparse enough to reveal characteristic arrangements. Use clean glass slides. Best results are obtained with slides kept in an alcohol container and removed just prior to use. They may be air dried or flamed.

Colonies from solid media

1. Place a drop of a sterile saline on slide. Distilled water may distort cellular morphology of fragile organisms and has to be avoided.

2. Transfer a small portion of colony with a sterile applicator stick, wire needle, or loop (preferably).

3. Gently mix to emulsify. The resulting smear should be slightly cloudy and homogenous (adjust saline drop size, area of smear, and/or inoculum to achieve optimum results). If swirl lines are evident as the smear dries, the inoculum was too heavy, the drop of saline was too small, and/or the smear was spread over too small an area.

Note: Never mix vigorously. Avoid creation of aerosols.

Smear fixation

Smears may be fixed with heat.

1. Air-dry smears on a flat surface, or place them on a 60°C electric slide warmer until dry (photo).

2. If smears are air-dried, pass them two or three times through a flame. To avoid distortions do not overheat. Allow slide to cool before staining.

7.3. Gram staining

This differential stain divides the majority of bacteria into two groups: gram-positive and gram-negative bacteria. Hucker's modification of the Gram stain is widely used for routine work.

In general, do not apply stains, water or decolorizer directly to specimen area. Drops may be applied to the frosted end of the slide, allowing reagent to flow over the remaining surface.

Technique

1. Flood the fixed smear in the crystal violet solution. Allow the stain to remain for 30 seconds.

2. Decant crystal violet, and rinse slide gently with running tap water. *Caution: excessive rinsing in this step could cause crystal violet to be washed from gram-positive cells.* The flow of water may be applied to the underside of the angled slide to ensure a gentle flow across the smeared side. Some recommend immersing the slide in a beaker of water under running water for 5 seconds.

3. Rinse off excess water with iodine solution, and then flood the slide with fresh iodine solution. Allow iodine to remain for 30 seconds.

4. Rinse gently with flowing tap water.

5. Decolorize by letting the reagent flow over the smear while the slide is held at an angle. Stop when the runoff becomes clear. Adjust decolorization time to thickness of smear and type of decolorizer used.

6. Remove excess decolorizer with gentle flow of tap water. Caution: Excessive rinsing in this step could cause dye-iodine complex to be washed from gram-positive cells. See recommendations in step 2 above.

7. Flood the slide with safranin, and allow counterstain to remain for 30 seconds. Some laboratories may prefer to use 0.1% to 0.2% basic fuchsine as a counterstain.

8. Remove excess counterstain with a gentle flow of tap water.

9. Drain slide, and air-dry it in an upright position, or use a commercial slide drier.

10. Examine the smear microscopically.

Variant with Carbo-fuchsine counterstain

Carbo–fuchsine is recommended for detecting faintly staining gram-negative organisms. The procedure is similar to that for Hucker's modification except that the recommended decolorizer is 95% ethanol and the counterstain is carbo fuchsine or 0.8% basic fuchsine.

- Crystal violet – 30 s
- Gram 's iodine – 30 s
- 95% ethanol – 30 s
- Carbo-fuchsine or 0.8% basic fuchsine > 1 m

Results interpretation:

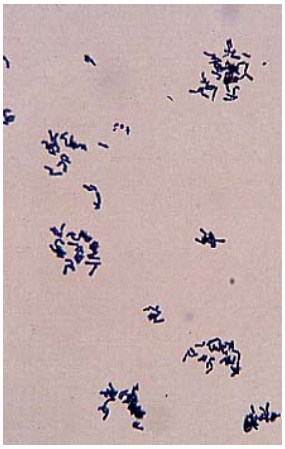
In the Gram stain procedure, cells are:

- Stained with crystal violet
- Treated with iodine to form a crystal violet / iodine complex within the cell
- Washed with an organic solvent (acetone–alcohol)
- Stained again with the red counterstain safranin.

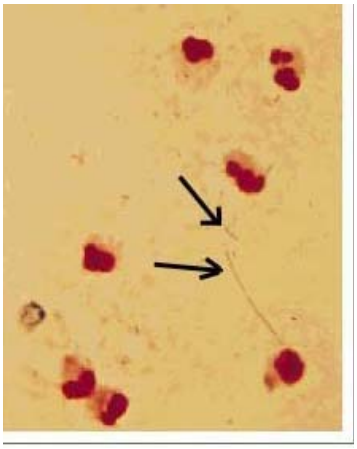
In gram-positive bacteria the purple crystal violet/iodine complex is retained within the cell after washing with acid–alcohol because thick peptidoglycan layer doesn't allow crystal violet iodine complex to be washed out of the cell.

In gram-negative bacteria, the crystal violet/iodine complex is leached from the cell (i.e., cell becomes colorless) due to disruption of the lipid-rich outer membrane by the acetone–alcohol organic solvent. These colorless cells must be counterstained to be seen under the light microscope; this counterstain is provided by safranin or carbo-fuchsine or 0.8% basic fuchsine

- Gram-positive bacteria appear blue–purple
- Gram-negative bacteria are stained red



Corynebacteria,
Gram positive



E. coli,
Gram negative

Gram stain reagents

Crystal violet, Gram's iodine, acetone–alcohol, Safranin

Gram stain for general bacteriology

- **Crystal violet**

1. Crystal violet stock solution

Crystal violet (90–95% dye content) – 40g
Ethanol, 95% – 400ml
Dissolve and mix in a glass bottle, label with a one-year expiration date, and store at room temperature. Caution: *Ethanol is highly flammable.*
2. Ammonium oxalate solution (1%)
Ammonium oxalate (reagent grade) – 16 g
Distilled water – 1, 600 ml
Dissolve and mix in a brown glass bottle, label with a one–year expiration date, and store at room temperature.
3. Crystal violet working solution
Crystal violet stock solution – 40ml
Ammonium oxalate solution (1 %) – 160ml
Filter crystal violet stock solution into a glass bottle. Allow to filter completely, and then filter ammonium oxalate solution. Label with the earliest expiration date of stock solution.

- **Gram's iodine**

1. Stock Lugol's iodine solution
Iodine crystals (reagent grade) – 25g
Potassium iodide (reagent grade) – 50
Distilled water – 500ml
Mix or let stand until dissolved in a brown glass bottle, label with a six-month expiration date, and store at room temperature.
2. Sodium bicarbonate, 5% (wt/vol.)
Sodium bicarbonate (NaHCO3) , reagent grade – 25g
Distilled water – 1, 000ml
Dissolve in a glass bottle, label with a one-year expiration date, and store at room temperature.
3. Gram's iodine
Stock Lugol's iodine solution – 60 ml
Distilled water – 220 ml
Sodium bicarbonate, 5% – 60ml
Mix in a brown glass bottle. Label with a six-month expiration date, and store at room temperature. Caution: Iodine and potassium iodide are corrosive. Avoid inhalation, ingestion, or skin contact.

- **Decolorizer**

Slowest: ethanol, 95%
Intermediate: acetone–alcohol
Ethanol, 95% – 100ml
Acetone (reagent grade) – 100ml
Combine in a brown glass bottle, label with a one-year expiration date, and store at room temperature.
Fastest: acetone (reagent grade). *Caution: Ethanol and acetone are flammable*.

- **Counterstain**

1. Safranin
Safranin stock solution
Safranin O (certified) – 5,0
Ethanol, 95% – 200 ml
Dissolve in a glass bottle, label with a one-year expiration date, and store at room temperature.
Caution: ethanol is flammable.
Safranin working solution
Safranin stock solution – 20 ml
Distilled water – 180 ml
Combine in a glass bottle, label with a one-year expiration date, and store at room temperature.
2. Alternatively: basic fuchsine, 0.1 or 0.2 % (wt/vol)
Basic fuchsine (reagent grade) – 0.1g or 0.2 g
Distilled water – 100ml
Add basic fuchsine in a brown glass bottle. Slowly add distilled water, thoroughly mix in to dissolve. Label with a one-year expiration date, and store at room temperature.

8. Analytical procedures – bacterial culture and sensitivity

8.1. General culture identification procedure

Inoculation of selective agar

- Fecal specimens should be plated as soon as possible after arrival in the laboratory.
- Selective media may be inoculated with a single drop of liquid stool or fecal suspension.
- Alternatively, a rectal swab or a fecal swab may be used. If a swab is used to inoculate selective media, an area approximately 2.5 cm (1 inch) in diameter is seeded on the agar plates, after which the plates are streaked for isolation.
- Media of high selectivity such as XLD require more overlapping when streaking than media of low selectivity.
- When inoculating specimens to a plate for isolation, it is important to use the entire plate to increase the chances of obtaining well-isolated colonies.
- Incubate the plates for 18 to 24 hours at 35° to 37°C.

Tube inoculation technique

- Carefully select at least one of each type of well-isolated colony on each plate.
- Using an inoculating needle, lightly touch only the very center of the colony.
- Do not take the whole colony or go through the colony and touch the surface of the plate. This is to avoid picking up the contaminants that may be on the surface of the agar. If there is doubt that a particular colony is sufficiently isolated from the surrounding colonies, purify the suspicious colony by streaking on another agar plate. Afterwards, inoculate KIA, LIA by stabbing the butt and streaking the surface of the slant.

8.2. Culture identification tube test

A simple method for bacteria identification consists of the preparation of a “tube test” gallery using a Kligler media.

Kligler Iron Agar (KIA)

Kligler Iron Agar may be used as an aid in the differentiation of enteric gram-negative bacilli on the basis of carbohydrate fermentation and H_2S production. The medium is similar to TSI Agar.

Preparation:

- Suspend 52 g of the medium in one liter of distilled water.
- Mix well and heat with frequent agitation.
- Dispense in volumes of 3 ml in tubes of 13 x 100 mm.
- Sterilize at 121°C for 15 minutes.
- The tubes should be cooled in a slanted position to obtain a butt of 1.5 - 2.0 cm depth.

Test Procedure

Inoculate medium by stabbing base of tube butt and streaking slant with a needle.

Loosely cap the tube to ensure aerobic conditions. Incubate at 35°C for 18 - 48 hours.

Examine at 24 and 48 hours for growth and color changes in tube butt and slant, and for blackening at the apex of slant.

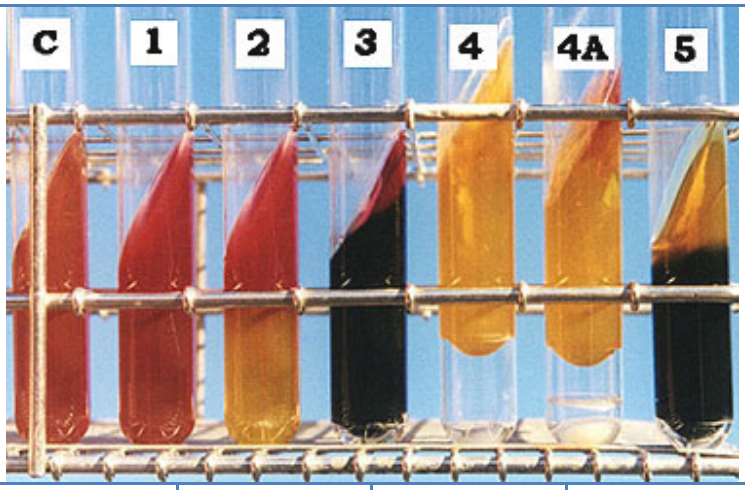
- Test samples of the finished product for performance using stable, typical control cultures.

Test Result:

- Lactose fermenting organisms totally acidify the medium, resulting in a yellow color.

- Lactose non-fermenting organisms acidify only in the bottom of the tube, with the slanted surface remaining the same original cherry red color.
- Formation of hydrogen sulfide blackens the medium. The results are interpreted the same as TSI Agar.

Visual example



Tube number	1	2	3	4*	5
amino acids deamination	+	+	+	+	+
glucose fermentation	–	+	+	+	+
lactose fermentation	–	–	–	+	+
H_2S production	–	–	+	–	+**
typical examples	*Pseudomonas* (a non-enteric)	*Morganella Providencia Shigella*	*Citrobacter Salmonella Proteus Edwardsiella*	*E. coli Enterobacter Klebsiella*	H_2S+ *E. coli* lactose+ *Salmonella*

* **Tube 4:** Much gas is often seen for this tube, evidenced by cracks in the medium. Also, lactose fermenters, which are **methyl red-negative,** may show a "reversion" toward an alkaline reaction as neutral products are formed from some of the acid. This appears as shown in Tube 4A, where a slight reddening of the slant occurs, as the alkaline deamination reaction becomes no longer over-neutralized by acid from fermentation.

** **Tube 5:** Enough acid can be produced to cause the black iron sulfide precipitation to break down and not be seen. In this case, the tube will look like No 4.

Table 22. Summary table

ORGANISMS	SLANT	DEPTH	GAS	H_2S
Enterobacter	yellow	pink	+ +	-
Hafnia	pink	yellow	+	-
Klebsiella	yellow	yellow	+ +	-
Escherichia coli	yellow (pink.)	yellow	+ (-)	-
Shigella	pink	yellow	-	-
Salmonella typhi	pink	yellow	-	+ (-)
S. paratyphi	pink	yellow	+	-

S. choleraesuis	pink	yellow	+	-
Other Salmonella	pink	yellow	+	+ + +
Citrobacter	pink (yellow.)	yellow	+	+ + + (-)
Edwarsiella	pink	yellow	+	+ + +
Serratia	pink (yellow)	yellow	-	-
Proteus				
P. vulgaris	yellow (pink.)	yellow	+	+ + +
P. mirabilis	pink (yellow)	yellow	+	+ + +
P. morganii	pink	yellow	-	-
P. rettgeri	pink	yellow	-	-
Providencia	pink	yellow	+ or -	-

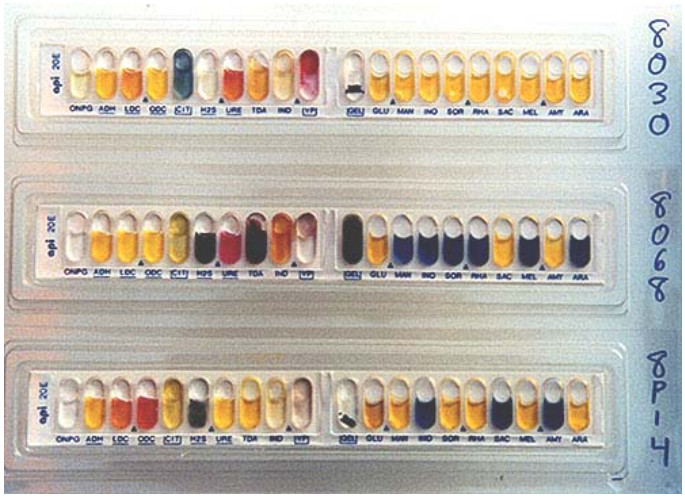
() = Occasional reactions;

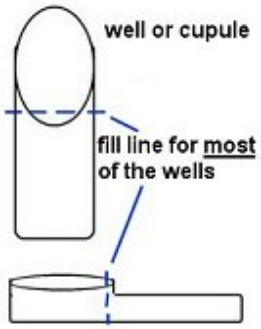
8.3. API identification systems

Introduction

API-20E test strip **(bioMérieux)** is used to identify the enteric gram-negative rods. 20 separate test compartments are included on a single strip, all dehydrated. A bacterial suspension is used to rehydrate each of the wells. Some of the wells will have color changes due to pH differences: others produce end products that have to be identified with reagents. A profile number is determined from the sequence of + and - test results, than looked up in a codebook having a correlation between numbers and bacterial species.

Example: API 20E





Materials needed:

- Agar plates of bacterial species
- 0.85% NaCl solutions (5ml)
- Sterile PSIpettes (5ml)
- McFarland standards
- Sterile mineral oil
- API 20E test strip (oxidase- gram- rods)
- API test strip incubation chamber
- API regents kit
- API 20E analytical Profile Index

Procedure:

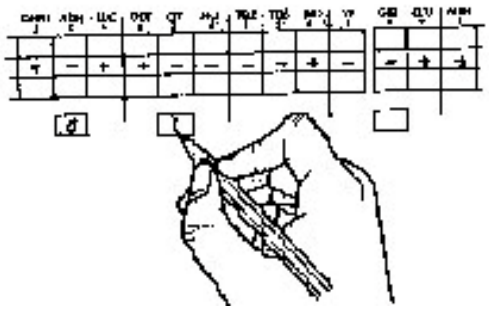
- Prepare a suspension of the bacteria in the saline tube.
- Inoculate a large colony (2-3mm diameter) of the bacterium (pure culture) into the 0.85% NaCl solution, making sure that the suspension is homogenous and without clumps of floating bacteria.
- Use a McFarland barium sulfate standard #3 to standardize the suspension.
- Inoculate the API strip as recommended.
- Holding the strip at a slight angle up from the table top, you will now inoculate the bacterial suspension into each well with the sterile pipette.
- Touch the end of the pipette to the side of the cupule, allowing capillary action to draw the fluid into the well as you slowly squeeze the bulb. This should eliminate any bubbles forming in the wells. Each well should be filled up to the top (see diagram).
- CIT, VP, and GEL have boxes around their names. These test wells will be filled all the way up to the top of the well.
- LDC, ODC, ADH, H2S, and URE are filled as described in step B, but they will then be filled up to the top with sterile mineral oil.
- Incubate the strip in its chamber as follows:
- The bottom of the incubation chamber has small indented wells in the bottom: fill it with water just enough to fill these indentations.
- Place the strip into this bottom. There should not be so much water that it slops onto the API strip.
- Place the top of the incubation chamber over the bottom, and label it.
- Place the strip at 37o C for 18-24 hours.

Interpretation:

Add the proper reagents to the compartments:

- 1 drop of Kovac's to the IND (read within a couple of minutes)
- 1 drop of Barritt's A and B to VP (a + reaction may take up to 10 minutes)
- 1 drop of FeCl3 to TDA

Read all other tests as described (chart below) without reagents.



Record results on the diagram handed out to you in lab (1, 2, or 4 points for + reaction, 0 points for – reaction, as shown on the picture). The oxidase test reaction should be negative, and is added as the last test result.

Three test reactions are added together at a time to give a 7-digit number, which can then be looked up in the API codebook.

Reading the API 20E

TESTS	*SUBSTRATE*	*REACTION TESTED*	*NEG RESULTS*	*POS RESULTS*
ONPG	*ONPG*	*beta-galactosidase*	*colorless*	*yellow*
ADH	*arginine*	*arginine dihydrolase*	*yellow*	*red/orange*
LDC	*lysine*	*lysine decarboxylase*	*yellow*	*red/orange*
ODC	*ornithine*	*ornithine decarboxylase*	*yellow*	*red/orange*
CIT	*citrate*	*citrate utilization*	*pale green/yellow*	*blue-green/blue*

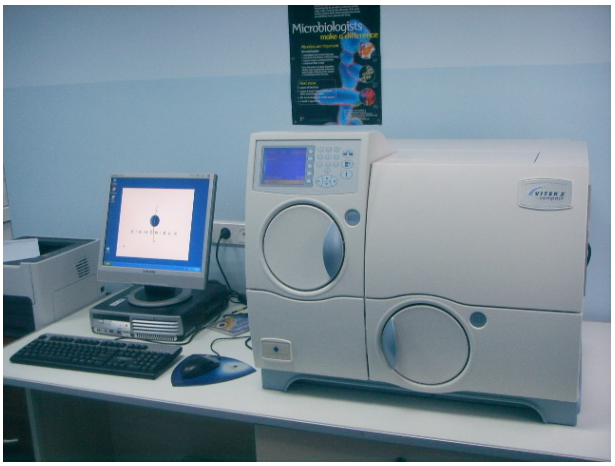
H2S	*Na thiosulfate*	*H2S production*	*colorless/gray*	*black deposit*
URE	*urea*	*urea hydrolysis*	*yellow*	*red/orange*
TDA	*tryptophane*	*deaminase*	*yellow*	*brown-red*
IND	*tryptophane*	*indole production*	*yellow*	*red (2 min.)*
VP	*Na pyruvate*	*acetoin production*	*colorless*	*pink/red (10 min.)*
GEL	*charcoal gelatin*	*gelatinase*	*no diffusion of black*	*black diffuse*
GLU	*glucose*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
MAN	*mannitol*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
INO	*inositol*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
SOR	*sorbitol*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
RHA	*rhamnose*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
SAC	*sucrose*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
MEL	*melibiose*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
AMY	*amygdalin*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
ARA	*arabinose*	*fermentation/oxidation*	*blue/blue-green*	*yellow*
OX	*oxidase*	*oxidase*	*colorless/yellow*	*violet*

8.4. Culture, identification and AST automated systems

VITEK 2 Compact is a new generation automated analyzer. Some other automated analyzers can be used for the same purpose.

VITEK 2 Compact

- Reduces time to identification and antibiotic susceptibility results.
- Offers an extensive analysis menu.



- Benefits from a miniaturized card-format consumable (10 cm x 6 cm x 0.5 cm) consisting of 64 wells for increased system analytical capacities.

Microbial Identification

Fluorescent technology provides broad profiles for the reliable identification of the most clinically relevant organisms. However, with the growing number of clinically significant pathogens and their rapidly emerging resistance to antimicrobials, identification must be fast and accurate.

Antimicrobial Susceptibility Testing

Sophisticated data analysis uses algorithms to look at a variety of parameters and test conditions to ensure accurate test results. Combined with the Advanced EXPERT System, the VITEK 2 provides state-of-the-art technology for AST and detection of resistance mechanisms.

Growing System

- After primary isolation, an organism suspension is prepared in a tube of saline and verified with the DensiChek densitometer.
- The inoculum tube is then placed into a rack, called a cassette.
- The sample identification number is entered into the Smart Carrier via barcode or keypad and electronically linked to the supplied barcode on each test card.
- ID and AST test cards can be mixed and matched in the cassette to meet your specific laboratory needs.

All information entered at the bench is then transported to the instrument in a memory chip attached to the cassette. This provides positive test tracking from the bench to the report.

Standardization and Enhanced Productivity

A new generation of test standardization and workflow productivity is now possible with the full automation of the VITEK 2. From start to finish, all processing steps are completely autonomous, standardized, controlled and checked:

- Test setup verification
- AST inoculum dilution
- Test inoculation
- Card sealing
- Incubator loading
- Optical reading and data transmission
- Card disposal

Fast Reports

The VITEK 2 knows when to check on each card. The optical system reads all 64 wells every 15 minutes. This kinetic monitoring provides an extended database for analysis and interpretation. This allows the VITEK 2 to provide rapid results without compromising biological performance. The simple point-and-click user interface is as easy to use as many similar windows-style applications.

Antimicrobial Resistance Detection with the Advanced EXPERT System

Antimicrobial Susceptibility Testing (AST) continues to become increasingly complex due to emerging and low-level microbial resistances. EXPERT systems provide various levels of security, validations and in-depth analysis of results to most accurately predict the clinical outcome of a therapy. This new approach allows us to use the most universal and quantitative value: the MIC. This is combined with the microbial identification and compared to a global knowledge base that has been constructed from published articles and bioMérieux research.

The Advanced EXPERT System has a three-step action:

1. Biological validation of the quality of the results from a technical, IN VITRO, point of view.

2. Result interpretation to facilitate correction for improved clinical outcomes.

3. Systematic addition of recommendations that your laboratory wishes to communicate to the physicians. These may be committee footnotes (CLSI, CASFM, DIN) or specific recommendations defined by your laboratory.

8.5. Disk diffusion AST method

Disk Diffusion Susceptibility Testing (Kirby-Bauer Method)

Introduction

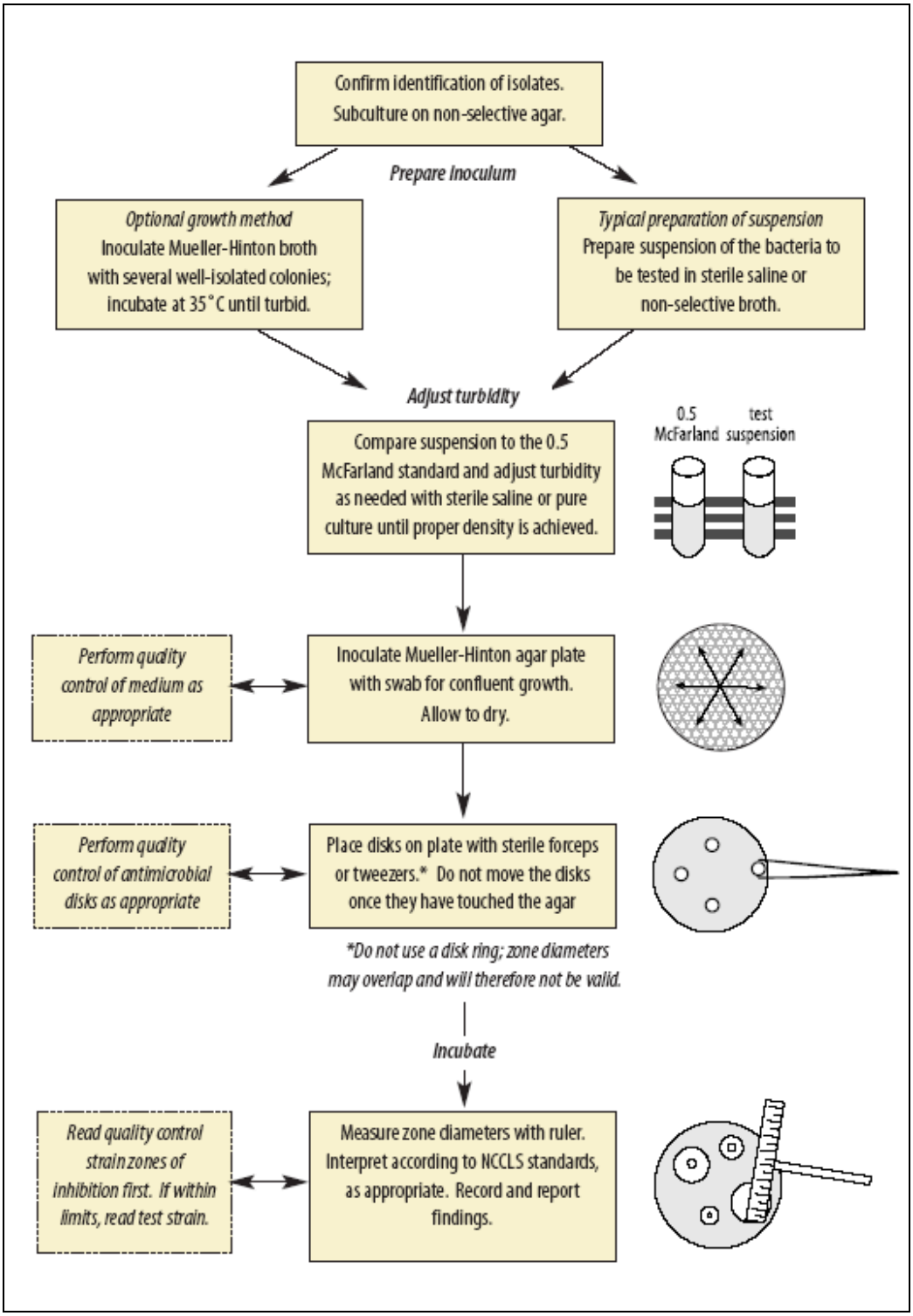
AST is becoming more and more important as more and more bacteria are developing new resistance patterns and mechanisms.

Different techniques have been used in the past, but the only manual method that is currently recommended by most of the worldwide scientific societies is the disk diffusion method.

Nevertheless, stringent quality assurance and standardization need to be applied at all steps of the process in order to get a reproducible result, comparable with previous results and with other laboratories. The following points represent all the standardization processes needed to be implemented:

- Standardization of the guideline to be used (CLSI is recommended)
- QC of the culture media
- QC of the disks
- Work on pure strains, well isolated on the agar
- Standardization of the inoculum and the media swabbing
- Standardization of the ATB to be used depending on the micro-organism concerned
- Standardization of the incubation conditions
- Standardization of the inhibition zones reading (using caliper ONLY)
- Standardization of the interpretation rules, skills of the senior microbiologist
- Overall technical skills of the technician
- Standardization of the result form provided to the clinicians
- Weekly use of at least 3 ATCC strains in order to validate the process

Summary diagram:



Warning:

When the nature of the infection is not clear and the specimen contains mixed growth or normal flora, in which the organisms probably bear little relationship to the infection being treated, susceptibility tests are often not necessary and the results can be grossly misleading.

Of the many media available, the CLSI recommends Mueller-Hinton agar due to: it results in good batch-to-batch reproducibility; it is low in sulfonamide, trimethoprim, and tetracycline inhibitors; it results in satisfactory growth of most bacterial pathogens; and a large amount of data has been collected concerning susceptibility tests performed with this medium.
The agar medium should have pH 7.2 to 7.4 at room temperature. The surface should be moist but without droplet of moisture. The antibiotic disks should be maintained at 8°C or lower or freeze at -14°C or below until needed, according to the manufacturer's recommendations. Allow the disks to warm to room temperature before use. Don't use expired disks.

The steps of the standard disk diffusion method:

- Select at least 4 to 5 well-isolated colonies of the same morphological type from an agar plate.
- Touch the top of each colony with a wire loop and transfer the growth to a tube containing 4 to 5 ml of saline.
- Adjust the turbidity to 0.5 McFarland either using a turbidimeter, or a visual scale (to be kept away from direct light).
- Dip a sterile non-toxic swab on an applicator into the adjusted suspension. Rotate the swab several times, pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.
- Inoculate the dried surface of a Muller-Hinton agar plate by streaking the swab over the entire sterile agar surface.
- Repeat this procedure two more times, and rotate the plate 60° each time to ensure an even distribution of inoculum. Replace the plate top and allow 3 to 5 minutes for any excess surface moisture to be absorbed before applying the antibiotic disks.
- Place the appropriate disks evenly (no closer than 24 mm from center to center) on the surface of the agar plate either by using a sterile forceps or the dispensing apparatus. No more than 12 disks should be placed on one 150 mm plate or more than 5 disks on a 90 mm plate. A disk is not to be moved once it has come in contact with the agar surface since some of the compound diffuses almost instantaneously.
- Invert the plate and place them in an incubator at 35°C, in aerobic conditions during 18-24 hours.
- Examine each plate and using a caliper, measure the diameters of the zones of complete inhibition, including the diameter of the disk. Measure the zones to the nearest millimeter using a ruler.
- Using your validation rules, try to interpret the possible resistance mechanisms of the bacteria.
- Modify the S/I/R results eventually, following the interpretation rules.
- Prepare the result form.

Mueller-Hinton agar plates key control elements

- **Sterility** (48-72 hours of incubation of a sample randomly chosen from the in-house lots)
- **Growth capacity** under normal conditions
- **Thickness:** 4 mm (very important)
- **pH** between 7.2 and 7.4 (aminoglycosides, macrolides, phenicols)
- **Humidity:** Dry the Petri dishes before use
- **Thymidine/thymine concentration**, if too high, diminish the diameter with sulphanilamide (test it with E. faecalis ATCC 29212)

- Concentration on **divalent cations** (Mg++ & Ca++ with aminoglycosides, Zn++ with penems)
- Special precautions for **MH with blood**

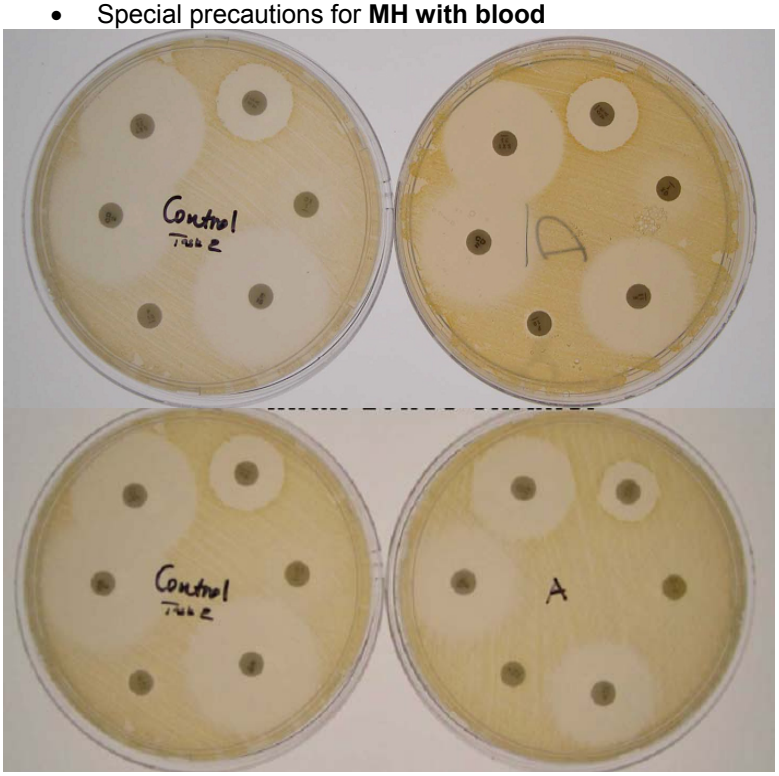


Illustration 3

On the right plate, the agar is too thick → all inhibition zones are smaller than on the control plate (left).

On the right plate, the inoculum is too strong → all inhibition zones are smaller than on the control plate (left).

Antimicrobial disks

The working supply of antimicrobial disks should be stored in a refrigerator (4°C).Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate; this reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before use. Do not forget to clean the dispenser between 2 Petri dishes (using hypochlorite) in order to avoid cross-contamination.

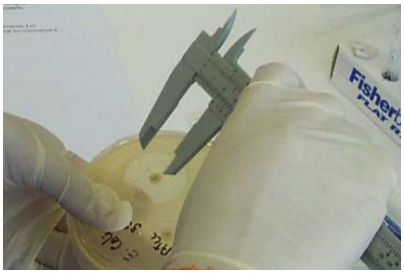
McFarland turbidity standards

If no specific McFarland densitometer is available, manual standards can be used. 5 different McFarland solutions are usually sold together in a small kit.

Such McFarland scales can be used during one year if kept away from the light (a drawer is usually a good place). The saline prepared with the bacteria is compared to the correct McFarland. Usually, a specific card containing alternated black and white stripes is used as a good background for better reading.
Commercially prepared 0.5 McFarland turbidity standards are available from various manufacturers, but it is also possible to prepare a home made set:

Table 23. Composition of McFarland turbidity standards

Turbidity standard number	Barium chloride dihydrate (1.175%)	Sulfuric acid (1%)	Corresponding approximate density of bacteria
0.5	0.5 ml	99.5 ml	1×10^8
1	0.1 ml	9.9 ml	3×10^8
2	0.2 ml	9.8 ml	6×10^8
3	0.3 ml	9.7 ml	9×10^8
4	0.4 ml	9.6 ml	12×10^8
5	0.5 ml	9.5 ml	15×10^8
6	0.6 ml	9.4 ml	18×10^8
7	0.7 ml	9.3 ml	21×10^8
8	0.8 ml	9.2 ml	24×10^8
9	0.9 ml	9.1 ml	27×10^8
10	1.0 ml	9.0 ml	30×10^8



Reading the zones

Measure the diameter from the back of the plate using a caliper (**Illustration 4).**

Use the correct standard to compare the diameter to the various thresholds.

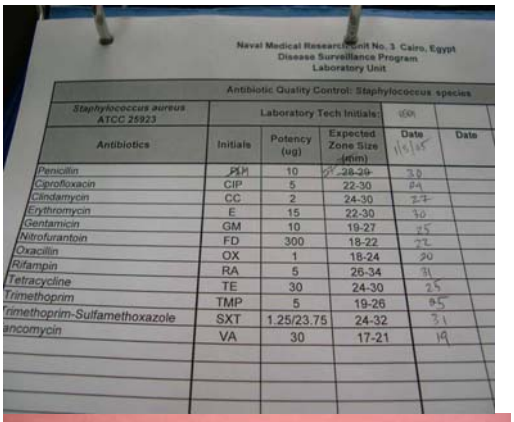
Testing reference strains

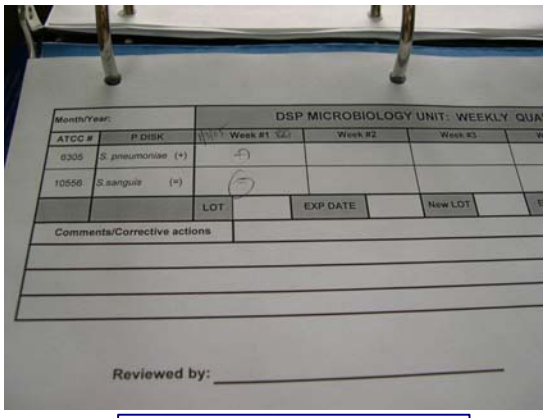
Ideally, the appropriate reference strain should be tested from a fresh overnight culture at the same time as the clinical isolate or at least once a week to ensure that all components of the test are in good working condition. Zone diameters obtained for ATCC 25922 should be compared with CLSI published limits; Table 24 includes the diameters of the zones of inhibition for ATCC 25922 (*E. coli*)

Table 24. Flowchart of the general procedure for antimicrobial susceptibility testing by disk diffusion

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml)			NCCLS QC strain
		Susceptible	Intermediate	Resistant	*E. coli* ATCC 25922
Ampicillin	10 µg	≥ 17 mm (≤ 8 µg/ml)	14 – 16 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	16 – 22 mm (2–8 µg/ml)
Chloramphenicol	30 µg	≥ 18 mm (≤ 8 µg/ml)	13 – 17 mm (16 µg/ml)	≤ 12 mm (≥ 32 µg/ml)	21 – 27 mm (2–8 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 16 mm (≤ 2/38 µg/ml)	11 – 15 mm (4/76 µg/ml)	≤ 10 mm (≥ 8/152 µg/ml)	23 – 29 mm (≤ 0.5/9.5 µg/ml)
Nalidixic acid	30 µg	≥ 19 mm (≤ 8 µg/ml)	14 – 18 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	22 – 28 mm (1–4 µg/ml)
Ciprofloxacin	5 µg	≥ 21 mm (≤ 1 µg/ml)	16 – 20 mm (2 µg/ml)	≤ 15 mm (≥ 4 mg/ml)	30 – 40 mm (0.004–0.016 µg/ml)

Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087 USA.





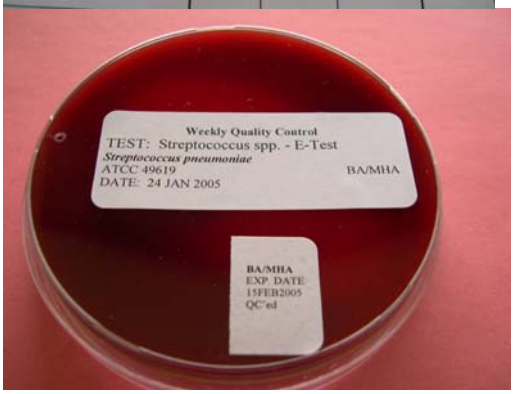


Table 25. Antibiotic disks recommended by CLSI for the INTESTINAL INFECTIOUS DISEASE related agents:

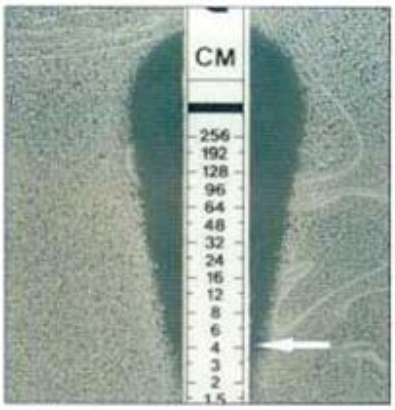
	Ampicilline	Amoxicilline	Chloramphenicol	Quinolones	Fluoroquinolones	Cotrimoxazole	Cephalosporine 2	Cephalosporine 3	Amoxicillinclav	Tetracycline	Gentamicin	Erythromycin	Doxycyline
Salmonella	X		X	X		X		X					
Shigella	X		X	X		X		X					
Escherichia coli	X	X			X	X	X	X	X		X		
Campylobacter	X			X	X	X				X	X	X	
Cholera			X			X				X	X	X	X

8.6. Minimum inhibitory concentration method using E-test

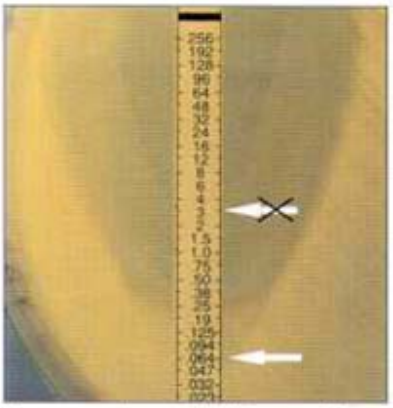
Introduction

E-test consists of a plastic strip calibrated with MIC values covering 15 two-fold dilutions. A predefined antibiotic gradient is immobilized on the surface opposite the MIC scale. When transferred to the agar, the continuous antibiotic gradient established under the strip remains stable over a period covering the critical times of most micro-organisms subjected to susceptibility testing.

E-tests should be kept at -20°C.



Look for microcolonies when reading enterococci. MIC 4 µg/ml.


Ignore swarming caused by *Proteus* species. MIC 0.064 µg/ml.

CLSI recommendations for E-test use should be followed.

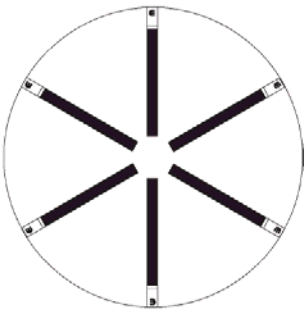
Method

Refer to the explanations provided on the next page.

- Remove package from the freezer and allow strips to reach room temperature for 30 min.
- Prepare a standardized inoculum (0,5-1 McFarland) from 1-2 days old colonies.
- Swab the plate as for AST determination, wait 15 minutes for the plate to dry.
- Apply the strips on the plate with forceps. Do not move it once on the plate.
- Up to 6 different strips can be used on the same plate (see Illustration 5).

- Incubate according to the micro-organism needs.
- Read the MIC where the ellipse intersects the scale.
- Always read the end point at complete inhibition of all growth including hazes and isolated colonies.

Illustration 5: Strips placement on the plate



Quality control

E-test can be very precise but is easily subject to mistakes if not done correctly, or if the strips are not preserved correctly.

CLSI procedures should be used for QC purpose. Different micro-organisms with no MIC for several antibiotics should be used on a regular basis.

Handling and Storage of Etest Strips

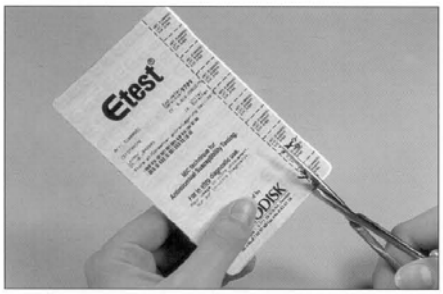


Figure 1.

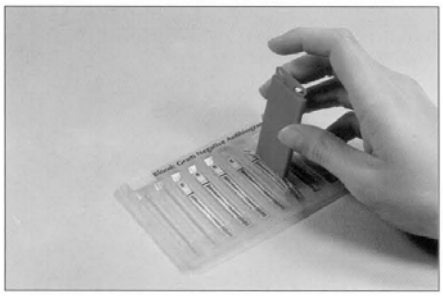


Figure 2.

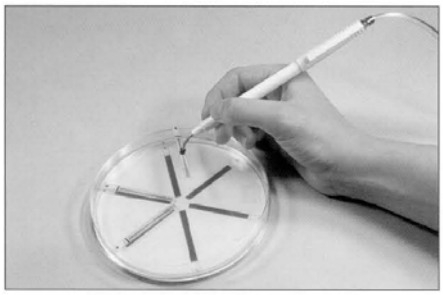


Figure 3.

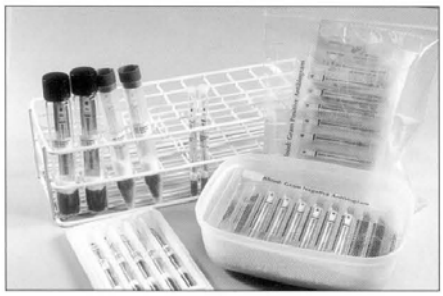


Figure 4.

Etest strips are shipped at ambient temperature.
On receipt store immediately at -20°C.

Opening an Etest package

1. Remove the package from the -20°C freezer.
2. Equilibrate to room temperature. This takes approx. 30 minutes if stored at -20°C and approx. 1 hour if stored at -70°C. **Ensure all moisture has evaporated before opening.**
3. Inspect the package for holes or cracks. Do not use if damaged.
4. Cut along the broken line at the top of a blister. **Do not cut in between blisters** (Fig. 1).
5. Tip the strips out of the opening slightly and take them out with forceps or your fingers.
6. If strips stick together, twist them apart with your fingers. Touch only the handle, i.e. the area labelled E.
7. Place the strips to be used into a dry clean petri dish or applicator tray (Fig. 2) supplied with the Etest applicator kit.

Application of strips

1. Apply Etest strips with your fingers, forceps or the Etest applicator. **Ensure the MIC scale is facing upwards**, i.e. towards the opening of the plate.
2. When using the Etest applicator, ensure the adhesive tape is still sticky. Replace the tape after approx. 50 applications.
3. Press the applicator onto a strip to pick it up (Fig. 2), then place it onto the agar surface and push the piston down to release the strip.
4. Be firm when applying the strip. **Once in contact with agar, do not move the strip.**
5. Use templates to position 4 to 6 strips onto a 150 mm plate or one to two strips on a 90 mm plate.
6. Place the handle of the strip closest to the rim of the plate.
7. The Etest Vacuum Pen Applicator is suitable for applying larger numbers of strips (Fig. 3).

Storage of unused strips

1. Always store unused strips in airtight containers e.g. storage tubes with silica gel (Fig. 4). **Ensure that the desiccant is blue during storage and before use.**
2. Reactivate the desiccant when it turns light blue or white. Remove the cap, replace the styrofoam with a plug of cotton-wool and heat the tube to 160°C for one hour. Cool the tube, replace the cap and store in a dry place.
3. Mark the storage tube with the label from the blister pack, showing the expiry date, batch number and antibiotic code.
4. Store the tube at -20°C and **always allow it to reach room temperature before opening.**
5. If stored at -70°C, leave the tubes in the styrofoam outer packaging during equilibration to room temperature to prevent the caps from popping out of the tubes.
6. **Strips in airtight storage tubes have the same expiry date as the original package, if properly stored, handled and always kept dry.**
7. Store only one type of antibiotic strip per storage tube.
8. Home-made storage tubes must use silica gel and be airtight.
9. Applicator trays with strips must be stored in airtight containers e.g. a Tupperware® box with a bed of silica gel (Fig. 4).

9. Analytical procedures – bacterial infections

9.1. Salmonellosis

Clinical manifestation

Salmonellosis is a bacterial disease commonly manifested by **acute enterocolitis**, with sudden onset of headache, **abdominal pain, diarrhea, nausea** and sometimes **vomiting**. Fever is almost always present. Anorexia and diarrhea often persist for several days.

S. typhi and *paratyphoid* serotypes (*S. Paratyphi* A, B and C) usually also cause septicemia and "enteric fever". Other serotypes of *Salmonella* can cause enterocolitis or local infection. Deaths are uncommon, except for the very young, very old, debilitated and immuno-depressed cases.

Incubation period: From 6 to 72 hours, usually about 12-36 hours.

Epidemiological characteristics

Salmonellosis is classified as a foodborne disease because contaminated food, mainly of animal origin is the predominant mode of transmission. Source of infection could be:

- Incriminated food
- Meat / meat products
- Poultry / poultry products
- Raw and undercooked eggs/egg products
- Raw milk and milk products
- Cereals
- Raw fruits / vegetables
- Contaminated water
- Cross-contamination of any food during
- Preparation
- Transport
- Storage
- Serving
-

Pathogen

Short description:

- *Salmonella* is a genus of rod-shaped Gram-negative enterobacteria.
- *Salmonella* species are motile (the non-motile exceptions are *S. gallinarum* and *S. pullorum*).

Most strains are prototrophs, not requiring any growth factors. However, auxotrophic strains do occur, especially in host-adapted serovars such as Typhi and Paratyphi A.

Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter.

Usually do not ferment lactose.

Most of them produce hydrogen sulfide which, in media containing Ferric Ammonium Citrate, reacts to form a black spot in the centre of the creamy colonies.

Phylogenic Tree of Salmonella species evolution within closely related families

The six subspecies of S. enterica are:

- *S. enterica subsp. enterica (I or 1)*
- *S. enterica subsp. salamae (II or 2)*
- *S. enterica subsp. arizonae (IIIa or 3a)*
- *S. enterica subsp. diarizonae (IIIb or 3b)*
- *S. enterica subsp. houtenae (IV or 4)*
- *S. enterica subsp. indica (VI or 6)*

Nomenclature and classification of these bacteria have changed a lot in recent years. Serovar names of *S. enterica* are not italicized and begin with a capital letter, e.g. the strain formerly known as S. typhimurium is now known as Salmonella enterica serovar Typhimurium. This can be shortened to Salmonella Typhimurium. Other subspecies of S. enterica (except for some

subspecies of salamae and houtenae) and those of S. bongori are not named, and are designated by their antigenic formula.

Cross References: Kauffmann-White classification scheme

Pre-analytic steps

Fecal specimens should be collected in the early stages of enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started. Refer to procedure 5.6.

Exception to this rule – persons with febrile illness – in case of typhoid fever, the etiologic agent *Salmonella* typhi may be presented with higher concentration in the stools during the second and third weeks of the disease.

Salmonella suspected samples should be transported in Cary Blair transport media, stored at 2-8°C. Refer to procedure 5.8.

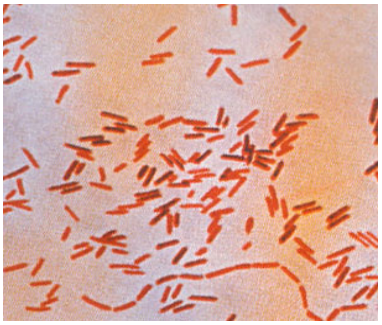
Equipment needed

- Biological safety cabinet (ideally, but not compulsory)
- Incubator (35° to 37°C)
- Refrigerator
- Microscope and
- Scale
- Micropipette
- Vortex
- Hot plate

Reagents and consumables needed

- Sterile Petri dishes
- Sterile Pasteur pipettes
- Sterile test tubes
- Sterile swabs
- Inoculating needle and inoculating loops
- Microscope slides
- Gram stain reagent, see procedure 7.3

Illustration 6. *Salmonella* on a microscopic preparation



Microscopy of the gram stain

On a microscopic preparation, *Salmonella* appear to be Gram-negative rods. Refer to 7.3 for Gram staining.

Analytical procedures

Initial media inoculation

Fecal specimens to be examined for *Salmonella* may be inoculated onto standard enteric plating media such as Hektoen enteric agar (HE), Salmonella-Shigella agar (SS), MacConckey agar (MAC).

The selective enrichment medium usually used for *Salmonella* typhi isolation from fecal specimens is Selenite broth. See section 6.2 for nutriment media.

- A rectal swab or stool swab/loop may be used to inoculate the plates.
- Inoculate the medium heavily with the specimen; plates must be streaked carefully to permit growth of discrete and separated colonies.

- In addition to the SS Agar plate, inoculate a tube of Selenite Broth Enrichment Medium, incubate it for 24 hours at 35-37 °C, and sub-culture onto another SS Agar plate.
- Plates/tubes must be freshly prepared and used within 36 hours.

Table 26. Appearance of Salmonella colonies

Type of media	Grams per liter of water*	Autoclave needed Y/N	Growth characteristics	IQC / ATCC strains
MacConkey agar (MAC)	51.5gr	121°C for 15 minutes	transparent/white/colorless colonies	*Enterococcus faecalis* 29212 *Escherichia coli* 25922 *Proteus mirabilis* 12453 *Salmonella typhimurium* 14028
Salmonella-Shigella agar (SS)	57gr	No	transparent/colorless, and larger with improved blackening centre.	*Enterococcus faecalis* 29212 *Escherichia coli* 25922 *Salmonella typhimurium* 14028 *Shigella flexneri* 12022
Hektoen enteric agar (HE)	76gr	No	blue-green with or without black centers	*Escherichia coli* 25922 *Enterococcus faecalis* 29212 *Shigella flexneri* 12022 *Salmonella typhimurium* 14028
Kligler iron agar (KIA)	52gr	121°C for 15 minutes	alkaline slant (red), acid butt (yellow), with or without production of gas and H_2S	*Escherichia coli* 25922 *Morganella morganii* 8019 *Pseudomonas aeruginosa* 27853 *Salmonella choleraesuis* serotype Typhi 19430 *Shigella flexneri* 12022
Lysine iron agar (LIA)	34gr	121°C for 15 minutes	slant and butt are alkaline (purple colour), H_2S positive	*Enterobacter aerogenes* 13048 *Proteus mirabilis* 29906 *Enterobacter cloacae* 23355
Selenite Broth (SE)	23gr	No	Cultural response is exhibited on MacConkey Agar after enrichment in Selenite Cystine Broth at 35°C for 24 - 48 hours good growth / colorless colonies	*Escherichia coli* 11775 *Escherichia coli* 25922 *Salmonella typhi* 19430 *Salmonella typhimurium* 14028 *Shigella sonnei* 25931

* Each manufacturer should provide its own reference strains. Above example is taken from bioMérieux.

Culture identification tube tests

Suspected isolated colonies from selective media plates should be inoculated to Kligler Ion Agar (KIA) or Triple Sugar Agar (TSI) and other screening media.

Identification details can be found in procedure 8.2.

Table 27. Characteristics shared by most Salmonella strains

Screening medium	Growth characteristics
Lactose	Negative
From glucose, mannitol, maltose, and sorbitol	Acid and gas positive
From adonitol, sucrose, salicin, lactose	Acid and gas negative
ONPG	Negative
Indole	Negative

Methyl red test	Positive
Voges-Proskauer	Negative
Citrate (growth on Simmon's citrate agar)	Positive
Lysine decarboxylase	Positive
Urease	Negative
Motility test	Positive
Ornithine decarboxylase	Positive
H_2S production from thiosulfate	Positive
Grow with KCN (potassium Cyanide inhibiting E. coli)	Negative
Phenylalanine and tryptophan deaminase	Negative
Gelatin hydrolysis	Negative

Table 28. Aspect of most *Salmonella* strains on the following culture media

Kligler iron agar (KIA)	alkaline slant (red), acid butt (yellow), with or without production of gas and H_2S
Lysine iron agar (LIA)	slant and butt are alkaline (purple color), H_2S positive
SS	transparent/colorless, and larger with improved blackening centre.
Hektoen	blue-green with or without black centers
MacConkey	transparent/white/colorless colonies

API identification systems
API20E is usually used for this semi automated identification process. It replaces the tube identification process.

Refer to procedure 8.3 for API20E manual test system application.

Serotyping of Salmonella strain using slide agglutination method

Purpose
The purpose of serotyping is to determine which of the >2300 Salmonella serovars a specific isolate belongs to. This is necessary for epidemiological purposes and for looking for evidence of links between different cases.

Serotypes of *Salmonella* are defined based on the antigenic structure of both somatic or cell wall (O) antigens and flagellar (H) antigens. These antigens are detected using slide agglutination with commercially produced antisera, the O antigens using a suspension of growth from an agar plate, while the H antigens using a suspension of broth culture. The serotype is deduced from the specific pattern of agglutination reactions using the Kauffmann-White classification scheme.

Specimen Requirements

A pure growth of the bacterial culture on a non-selective agar plate for O antigen detection and a broth growth from the bottom of an agar slope for H antigen detection.

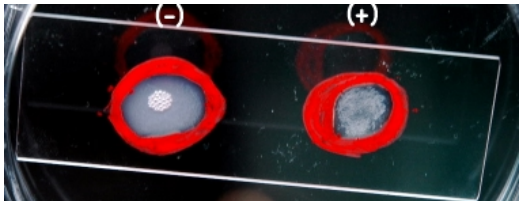


Illustration 7. Salmonella Serological reactions to various antigens

Illustration shows:
- Negative reaction to an anti-B serum
- Positive reaction to an anti-C1 serum

Note the grainy appearance of the positive reaction.

Principle of Examination

- On a clean glass slide, draw two circles (about 1 cm in diameter) heavily with a red wax marker.
- Place the slide in an empty Petri dish.
- Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, LIA, or other nonselective agar medium.
- **Important note:** Serologic testing cannot be done on growth from selective media (*e.g.*, MAC, Hektoen or SS agar) because selective media may yield false-negative serologic results.
- Emulsify the growth in three small drops of physiological saline and mix thoroughly.
- With a Pasteur pipette place a drop of the cell suspension within each circle. Make sure the drops appear as even, cloudy suspensions.
- Without touching the dropper to the cells, place a drop of the antiserum on one suspension and a drop of the physiological saline on the other. The third suspension is used as a control for autoagglutination. If autoagglutination occurs (agglutination with saline), the culture is named "rough" and cannot be serotyped. Strong agglutination reactions only are read as positive.
- After several minutes, hold up the Petri dish and observe the slide from the bottom. Gently tap the dish to effect some mixing of the cell suspension. Where there is a reaction between anti-bodies in the antiserum and their homologous antigens on the cell wall of the bacteria, the cells will agglutinate, and the drop will appear to contain many small particles. Where there is no agglutination, the cell suspension will maintain its original, evenly-cloudy appearance.
- Discard the slide appropriately (or clean it off for reuse, after disinfection) and discard the Petri plate into the proper container (do not place glass Petri plates into the pails with the disposable plastics!).
- Cultures that have a TSI/KIA reaction typical of *S. typhi* and that react serologically in either the Vi or the D antisera can be presumptively identified as *S. typhi*. The tube agglutination for the "d" flagellar antigen or further biochemical tests may be conducted by reference laboratories to confirm the identification as *S. typhi*.

AST procedures

Disk diffusion method

The disk-diffusion method (Kirby-Bauer) is suitable for routine testing in a clinical laboratory where a large number of isolates are tested for susceptibility to numerous antibiotics.

See section 8.5 for disk diffusion method to be used, along with the antibiotics to be tested for each pathogen.

Other methods for Antibiotic susceptibility test

- **Minimal Inhibitory Concentration (MIC) dilution method**

Can be used as an alternative method to identify the minimal inhibitory concentration of the antimicrobials. See chapter 8.6 for more details.

- **Manual antibiotic susceptibility test ATB strip**

Can be used as a rapid testing for antibiotic susceptibility test.

Reporting

- Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Salmonella spp*.
- Serologically negative isolates that are biochemically identified as *Salmonella spp.* may be sent to a reference laboratory.
- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using the special laboratory reporting form.
- Refer to chapter 12.4 for disease reporting **to the public health system**.

9.2. Shigellosis

Clinical manifestation

Shigella spp. cause a wide spectrum of clinical diseases, which vary **from asymptomatic infections to diarrhea without fever to severe dysentery**. Symptoms consist of **abdominal cramps, ineffectual and painful straining to pass stool, and frequent, small-volume, bloodstained inflammatory discharge**. Diarrhea usually appears after 48 hours, with dysentery supervening about 2 days later.
Infective dose: Shigella has very low infective dose from 80 - 100 cells.

Epidemiological characteristics

Shigellosis is a waterborne and foodborne disease. Foods most often incriminated in the transmission have been potato salad, shellfish, raw vegetables, etc. Shigella can be also transmitted by fingers, feces, and flies.

Four groups of *Shigella* organisms, with a total of 39 serotypes and subtypes, are recognized. Group A (*S. dysenteriae*), group B (*S. flexneri*), and group C (*S. boydii*) contain multiple serotypes; there is only one serotype for group D (*S. sonnei*). *S. dysenteriae, S. sonnei* and *S. flexneri* are the most commonly isolated species. Severe epidemic dysentery, with high death rates, is usually caused by unusually virulent pathogen - Shigella *dysenteriae* serotype 1 (Sd1). A more frequent complication is the hemolytic-uremic syndrome (HUS), which is characterized by the classic triad of hemolytic anemia. HUS may be mild with rapid recovery, or severe, leading to kidney failure and death.

Pathogen

Shigella species are Gram-negative, facultatively anaerobic, non-sporulating, non-motile rods in the family *Enterobacteriaceae*. They do not decarboxylate lysine or ferment lactose within 2 days. They utilize glucose and other carbohydrates, producing acid but not gas.

Pre-analytic steps

Fecal specimens should be collected in the early stages of enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started. Refer to procedure 5.6.

Shigella suspected samples should be transported in Cary Blair transport media, stored at 2-8°C. Rectal swabs can be used for isolation of Shigella. Refer to procedures 5.6.

Equipment needed

- Biological safety cabinet (ideally, but not compulsory)
- Incubator (35° to 37°C)
- Refrigerator
- Microscope and
- Scale

- Micropipette
- Vortex
- Hot plate

Reagents and consumables needed

- Sterile Petri dishes
- Sterile Pasteur pipettes
- Sterile test tubes
- Sterile swabs
- Inoculating needle and inoculating loops
- Microscope slides
- Gram stain reagent, see procedure 7.3

Analytical procedures

Isolation Methods

For optimal isolation of *Shigella*, two different selective media should be used: a general purpose plating medium such as MacConkey agar (MAC), and Hektoen enteric (HE). Xylose lysine desoxycholate (XLD) agar and Desoxycholate citrate agar (DCA) are suitable alternatives to Hektoen agar. SS agar can be used unless ***S. dysenteriae* serotype 1** is not suspected, because it frequently inhibits the growth of *S. Dysenteriae* serotype 1**.**

Note: There is no enrichment medium for *Shigella* that consistently provides a greater recovery rate than use of direct plating alone.

Culture examination

After plating and incubation, record the amount and type of growth (e.g., lactose-fermenting or lactose-non-fermenting) on each isolation medium for each patient specimen.

Table 29. Appearance of Shigella colonies on selective plating media

Selective agar medium	Color of colonies	Size of colonies
MAC	Colorless	2-3 mm
XLD	Red or colorless	1-2 mm
DCA	Colorless	2-3 mm
HE	Green	2-3 mm

- S. dysenteriae 1 colonies may be smaller.
- S. dysenteriae 1 colonies on XLD agar are frequently very tiny, unlike other Shigella species.

Select suspect colonies from the MAC and XLD plates and inoculate to appropriate screening media such as Kligler iron agar (KIA) or triple sugar iron agar (TSI) and other tests (refer to chapter XX about tube identification)

Biochemical Screening Tests

Identification of Shigella spp. involves both biochemical and serologic testing.
The use of biochemical screening media is usually advisable to avoid wasting antisera. Most laboratories will find KIA (or TSI) to be the single most helpful medium for screening suspected Shigella isolates. If an additional test is desired, motility medium can be used to screen isolates before doing serologic testing.

***Note*:** *Shigella* characteristically produces an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H_2S. A few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI.

Catalase Test for *S. dysenteriae* type 1
Screening based on catalase: since *S. dysenteriae* type 1 differs from most of the Enterobacteriaceae as it does not produce catalase. A test for catalase may provide a useful screening method for rapid identification of *S. dysenteriae* because of its high predictive value, simplicity and speed. It would be particularly useful during dysentery outbreaks, when other Shigella would be uncommon.

Serologic Identification of Shigella

Serologic testing is needed for the identification of *Shigella* isolates. The genus *Shigella* is divided into four serogroups, each group consisting of a species that contains distinctive type antigens. Serologic identification is performed typically by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype, which is the most frequent cause of severe epidemic dysentery. Once one colony from a plate has been identified as *Shigella*, no further colonies from the same specimen need to be tested.

Slide agglutination procedure
Emulsify the growth in two small drops of physiological saline and mix thoroughly.

Add a small drop of antiserum to one of the suspensions.

Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum.

To conserve antiserum, volumes as small as 10 µl can be used.

An inoculating loop may be used to dispense small amounts of antisera if micropipettes are not available.

Mix the suspension and antiserum well and then tilt the slide back and forth to observe for agglutination.

If the reaction is positive, clumping will appear within 30 seconds to 1 minute. Examine the saline suspension carefully to ensure that it is even and does not show clumping due to autoagglutination. If autoagglutination occurs, the culture is termed “rough” and cannot be serotyped.

Notes
- Because *S. dysenteriae* 1 (followed by *S. flexneri* and *S. sonnei*) is the most common agent of epidemic dysentery, the isolates that react typically in the biochemical screening should be screened first with a monovalent A1 antiserum, then with a polyvalent B antiserum, and finally with a polyvalent D antiserum.
- Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of KIA, TSI, heart-infusion agar (HIA), or other **non-selective** agar medium (serologic testing should not be done on growth from selective media such as MAC or XLD, because this may give false-negative results).

Reporting

- Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Shigella*.
- Serologically negative isolates that are biochemically identified as *Shigella* may be sent to a reference laboratory.
- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using the special laboratory reporting form.

- Refer to chapter 12.4 for disease reporting **to the public health system**.

9.3. Escherichia coli infection

Clinical manifestation

Several distinct classes of Escherichia coli are recognized as being associated with a diarrhoeal disease. These include enterotoxigenic, entero-invasive, Shiga toxin-producing, enteroaggregative, and enteropathogenic. At least six different classes of diarrhea-producing *Escherichia coli* have been identified:

- Enteropathogenic *E. coli* (EPEC)
- Enterotoxigenic *E. coli* (ETEC)
- Enterohaemorrhagic or verotoxin-producing *E. coli* (EHEC or VTEC)
- Enteroinvasive *E. coli* (EIEC)
- Enteroadhesive *E. coli* (EAEC)
- Enteroaggregative *E. coli* (EAggEC).

Table 30. Symptoms caused by pathogenic E. coli

Organism	Symptoms
EHEC	Bloody, watery diarrhea, slight fever, abdominal pain
ETEC	Profuse watery diarrhea
EIEC	Small amount of diarrhoeal stool with blood and mucus
EAEC	Diarrhea
EPEC	Fever, vomiting, and diarrhoeal stool with mucus; malaise

Escherichia coli Enterohaemorrhagic *E. coli* (EHEC) includes serotype O157:H7.
Ingestion of contaminated food or water containing EHEC producing Verotoxin blocks protein synthesis and induces a bloody diarrhea and in severe cases can lead to hemolytic uremic syndrome (HUS).

Epidemiological characteristics

Outbreaks of infantile gastroenteritis caused by enteropathogenic E. coli (EPEC) were common worldwide until the early 1970s. Serotypes isolated from infants included O114:H2, O119:H6, O127:H6, O128:H2, and O142:H6. There were usually high attack rates in these outbreaks, often with a high mortality. Since the 1970s, there has been a significant change in the epidemiology of EPEC. Nevertheless, few surveys have been made within past years in Georgia.

Outbreaks of infantile diarrhea have become rare because of improvements in hospital hygiene and baby formulas, although sporadic cases of EPEC infection in children are reported in small numbers that peak in the summer months.

Pathogen

E. coli is a gram negative, facultative anaerobic, non-sporulating, non-motile rod belonging to the family of *Enterobacteriaceae*.
E. coli O157:H7 is the most common serotype associated with hemorrhagic colitis. However, other serotypes can produce the verotoxin causing this syndrome, as O26:H11 and O111.

Pre-analytical procedures

Fecal specimens should be collected in the early stages of enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started. Refer to procedure 5.6.
Precautions when handling samples:

- Ideally, stool specimens should be examined as soon as they are received in the laboratory. If whole stool specimens are not processed immediately, they should be either refrigerated or frozen at -70 C as soon as possible after collection.
- Refrigerated specimens should be examined within 1-2 hours.
- If stools cannot be examined within this time, they should be placed in a Cary Blair transport medium (see procedure 5.8).
- If specimens in transport medium are being examined within 2-3 days, they should be refrigerated. If specimens cannot be examined within 3 days, they should be frozen immediately at -20°C, or preferably at -70 C.

Equipment needed

- Biological safety cabinet (ideally, but not compulsory)
- Incubator (35° to 37°C)
- Refrigerator
- Microscope and
- Scale
- Micropipette
- Vortex
- Hot plate

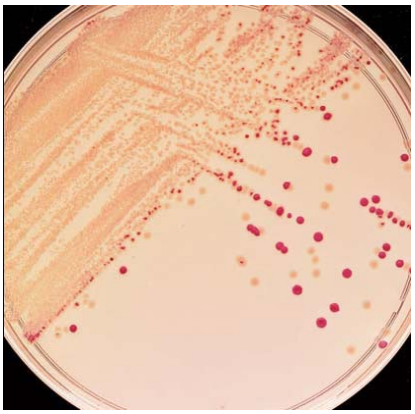
Reagents and consumables needed

- Sterile Petri dishes
- Sterile Pasteur pipettes
- Sterile test tubes
- Sterile swabs
- Inoculating needle and inoculating loops
- Microscope slides
- Gram stain reagent, see procedure 7.3

Specific culture media: Sorbitol Mac Conkey Agar

SMAC is a selective Agar for the direct isolation and differentiation of Enterohaemorrhagic (EHEC) E. coli 0157:H7 strains from foodstuffs and clinical specimen. Refer to chapter 6.2 for full details about SMAC media.

Illustration 8. *E. coli* colonies on SMAC



Analytical steps

Gram stain:
See procedure 7.3

E. coli is a gram negative rod.

E. coli identification
Refer to procedures 6.2 for regular identification of *E. coli.*

Inoculation on SMAC

- Inoculate stool specimens onto SMAC and incubate 18-24 hours at 35-37C.
- Sorbitol-negative colonies will appear colorless on SMAC.

E. coli O157:H7 rapidly ferments lactose and is indistinguishable from most other E. coli on traditional lactose-containing media. However, unlike approximately 80% of other E. coli, nearly all isolates of E. coli O157:H7 ferment D-sorbitol slowly, or not at all.

Sorbitol-MacConkey (SMAC) agar was developed to take advantage of this characteristic by substituting the carbohydrate sorbitol for lactose in MacConkey agar and is the medium of choice for isolation of E. coli O157:H7.

Serotyping
Note: There are no reliable and specific biochemical tests, which allow for differentiation of pathogenic E. coli from the non-pathogenic strains present in normal stool samples. The only possibility is to use specific antisera.
If your laboratory owns E. Coli O157 antiserum or latex reagents (O157 antibody-coated latex and control latex), test sorbitol negative colonies selected from SMAC according to the procedures recommended by the manufacturer.
Note: Colonies may be tested with antisera directly from the plate, or subcultured to another nonselective medium (blood agar, for example) and tested the next day.

If your laboratory doesn't own these antisera, place the colorless colonies into a Cary Blair transport media and send them rapidly to NCDC for further testing

Reporting

- Report: "Culture negative for (specific E. coli serotype requested)". For example: "Culture negative for E. coli O157:H7". If the laboratory is only utilizing the sorbitol screening method for detecting the hemorrhagic colitis syndrome, this report will tell the physician that it is the only pathogenic E. coli serotype for which the laboratory screened. The report may prompt the clinician to order further pathogenic E. coli studies if the patient's condition warrants it.
- Refer "Culture positive for E. coli O157 H7" to NCDC for further serological testing for O157 antiserum and H7 flagellar antigen.
- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using a special laboratory reporting form.
- Refer to chapter 12.4 for disease reporting **to the public health system**.

9.4. Campylobacteriosis

Clinical manifestation

Symptoms of *C. jejuni* or *C. coli* infection usually include fever, abdominal cramping, and diarrhea that last from several days to more than 1 week. *Campylobacter* spp. can cause mild to severe diarrhea, with loose, watery stools often followed by bloody diarrhea.

The intestinal disease varies from brief self-limiting enteritis to a fulminant enterocolitis with severe diarrhea, abdominal colic, fever, and muscle pain. Stools are at first mucoid and liquid and may progress to profuse watery diarrhea containing blood and pus. Gastroenteritis is usually self-limiting and does not require antibiotic therapy, although relapses may occur in 5 to 10% of untreated patients. Deaths attributed to *C. jejuni* infection are uncommon.

Epidemiological characteristics

Campylobacter jejuni and *C. coli* have emerged as major enteric pathogens that can be isolated as often as *Salmonella* and *Shigella* spp. in most parts of the world. *C. jejuni* and *C. coli* are the most

common *Campylobacter* species associated with gastrointestinal infection and are clinically indistinguishable.

Seasonal occurrence:
Campylobacter infections are usually sporadic and tend to occur in summer and early fall.

Source of infection:
Outbreaks are associated with ingestion of contaminated milk and water. Ingestion of improperly handled or undercooked food, primarily poultry products, raw milk, or contaminated water is a common source for human infections.

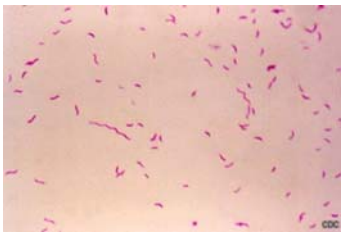
Infective dose:
These pathogens are highly infective. Poultry is a primary reservoir of *Campylobacter* spp. and may cause contamination of more than 80% of chicken carcasses.

It takes relatively few Campylobacter cells to cause illness and/or symptoms of gastroenteritis in humans. It is thought that the infective dose of *C. jejuni* ranges from 500 - 10,000 cells, depending on the strain, damage to cells from environmental stresses, and the susceptibility of the host.

Infants and young children are most susceptible. Travelers to developing countries are also at risk for Campylobacter infections.

Pathogen

Illustration.9 *Campylobacter spp.*



Campylobacter species are microaerophilic organisms that inhabit the gastrointestinal tracts of various animals, including poultry, dogs, cats, sheep, and cattle. They are small, slightly curved, gram – negative rods (see Illustration) that appear as simple curved or spiral-shaped rods (seagull wings or "S"-shape). They are oxidase-positive and motile with a darting, tumbling motility, *Campylobacter jejuni, Campylobacter coli* and *Campylobacter laridis* are main *Campylobacter* species associated with diarrhea.

Table 31. Diarrhea caused by *Campylobacter* species

Organism	Primary infection process	Association with diarrhea
C. jejuni	Diarrhea	Frequent
C. coli	Diarrhea	Occasional
C. laridis	Diarrhea	Occasional

Pre-analytical steps

- Fecal specimens are the preferred samples for isolating *Campylobacter* species from patients with gastrointestinal infections; however rectal swabs are acceptable for cultures.
- Cary-Blair or Campy-Thio medium transport media, should be used if there is a delay of more than 2 hours to the lab, and for transport of rectal swabs (see procedure 5.8).
- Specimens received in transport medium should be processed immediately or stored at 4°C until processed.
- Enrichment broths are used to enhance the recovery of *Campylobacter* from stool samples, such as Preston enrichment and Campy-Thio media.
- Enrichment cultures may be beneficial when low numbers of the organisms are expected. Use of enrichment cultures as part of routine stool culture setup is probably not necessary. Only reference laboratories surveying carriers and cured patients should use this technique.

Equipment needed

- Biological safety cabinet (ideally, but not compulsory)

- Incubator (42°C). See growth requirements below
- Refrigerator
- Microscope and
- Scale
- Micropipette
- Vortex
- Hot plate

Reagents and consumables needed

- Sterile Petri dishes
- Sterile Pasteur pipettes
- Sterile test tubes
- Sterile swabs
- Inoculating needle and inoculating loops
- Microscope slides
- Gram stain reagent, see procedure 7.3

Analytical procedures

Growth requirements for successful isolation of Campylobacter species
Microaerophilic conditions:

- Incubation at an elevated temperature (42°C).
- Proper incubation atmosphere (5 % oxygen, 10% CO_2, 85% nitrogen) is required.

Microaerophilic conditions can be generated by using the following:

- Commercially available gas–generating envelopes in anaerobe jars
- Evacuation replacement with a gas mixture of 10% CO2, 10% H2, and 80% N2 which results in the required microaerophilic atmosphere
- Bio–bags with microaerophilic gas generators

Commercial solutions:

- A hydrogen and CO2-generator envelope with a self-contained catalyst specifically for the isolation of *Campylobacter* spp. is commercially available. The envelope is placed in an anaerobic jar, and a new envelope must be used each time the jar is opened. No more than six plates should be stacked in the jar to obtain maximum isolation.
- A plastic bag incubation system is also commercially available. It consists of a plastic bag, which is collapsed two or three times by hand or vacuum, and refilled each time, with 5% O2, 10% CO2 and 85% N2.
- The evacuation–replacement system uses an anaerobic jar without a catalyst. The container is evacuated twice to 38cm (15mmHg) pressure and refilled each time with 10% H2 and 90% N2 mixture.

Selective media
Several selective media for successful isolation of Campylobacter species are commercially available as prepared plates or powder. However, a blood agar base with 5–10% sheep blood containing a combination of a cephalosporin (15mg/ml), vancomycin (10mg/ml), amphotericin B (2mg/ml) and 0.05% ferro-sulfate–sodium metabisulfite–sodium pyruvate (FBP) is acceptable. This media is called Campy CVA media (see chapter 6.2).

Direct Inoculation:

- **Swab:** Inoculate a Campy CVA Agar plate using the four quadrant streak technique for maximum isolation.

- **Diarrhoeal Stool:** Inoculate a Campy CVA Agar plate with three drops of stool and streak for isolation. At the same time, make a direct smear and look for small curved gram-negative bacilli and fecal leucocytes.
- **Solid Stool:** Prepare a 1:10 suspension of stool by placing pea-sized amount into 5ml of physiological saline (0.85%). Vortex the sample. Inoculate a Campy CVA Agar plate with three drops of this suspension, and streak for isolation.

Indirect Inoculation:

- **Swab:** Place the swab into an appropriate transport medium and refrigerate overnight.
- **Diarrhoeal Stool:** Place five drops of the specimen approximately one centimeter below the surface of a Campylobacter Thioglycollate Medium and refrigerate overnight. If no media is available, refrigerate overnight.
- **Solid Stool:** Prepare a 1:10 suspension of stool by placing pea-sized amount into 5ml of physiological saline (0.85%). Vortex the sample. Place five drops of the specimen approximately one centimeter below the surface of a Campylobacter Thioglycollate Medium and refrigerate overnight. If no media is available, refrigerate overnight.

Plating of Campy CVA Plates

- Place a one-inch Pasteur pipette below the surface of the broth medium.
- Place three drops onto a Campy CVA Agar plate and streak for isolation.
- Incubate Campy CVA Agar plates at 42°C for 48-72 hours in a microaerophilic atmosphere.

Culture examination

Examine the Campylobacter plates at 24 and 48 h for characteristic colonies (see photo provided in procedure 6.2), which can range from flat, spreading colonies that can cover the entire surface of the plate to very small, convex, translucent colonies. Colony color can range from gray to yellowish or pinkish.

Campylobacter jejuni colony morphology may appear as small, mucoid, grayish, flat colonies with irregular edges and no hemolytic patterns at 24-48 hours. They may also appear as round, convex, entire, glistening colonies 1-2mm in diameter. Certain strains of *C. jejuni* may appear lightly pink or tan in color.

Presumptive identification of Campylobacter from colonies

The appearance of gray, small, sometimes “spready“ colonies on Campylobacter selective agar, which exhibit characteristic “S” shape on Gram stain and are oxidase and catalase positive will be very much in favor of campylobacteriosis in patients with a diarrhoeal syndrome. Nevertheless, regular conformation is mandatory.

Presumptive identification from culture

Presumptive identification conventional method: Gram stain suspicious colony morphologies
Routine counterstain: Allow the safranin to remain on the slide up to 3 min, since Campylobacter spp. stain very faintly with routine Gram staining.
Alternative counterstain: An alternative counterstain that stains Campylobacter spp. more readily is 0.1 % basic fuchsine, this allows the fuchsine to remain on the slide for up to 3 min.
Culture Microscopy: *Campylobacter* are small, slightly curved, gram–negative rods that show an “S” or seagull shape.

Motility testing:

Suspend organisms in broth, such as nutrient broth or Mueller–Hinton broth, to observe motility by using phase microscopy or light microscopy. Saline and distilled water appear to inhibit motility. Campylobacter species reveal darting or tumbling motility.

Biochemistry characters

- **Oxidase test:** Campylobacter are oxidase positive.

- **Catalase test:** Campylobacter are catalase positive.
- **Hippurate test:** positive for *C. jejuni*

Supplemental identification method
Nalidixic acid and Cephalotin susceptibility

Table 32. Campylobacter biochemistry characters

Organism	Catalase	Hippurate	Susceptibility	
			Nalidixic acid	Cephalotin
C. jejuni	+	+	S	R
C. coli	+	-	S	R
C. laridis	+	-	R	R

Semi-automated identification gallery:
API Campy – Analytical profile index identifies Campylobacter species.

Automated Methods: VITEK 2 Compact GN cards – colorimetric automated method which identifies pathogen to species level.

Serotyping
Serotyping is performed using bacterial isolates previously cultured on 10% sheep blood agar for 24 to 48 h at 42°C in a microaerobic atmosphere. Heat-stable serotyping (O serotyping) is performed according to the Penner serotyping scheme with separate sets of sera for C. jejuni and C. coli. The C. jejuni strains are typed using all 47 *C,jejuni antisera* in the hemagglutination test. Commercial antisera for serotyping of Campylobacter jejuni and Campylobacter coli are available.

Antimicrobial Susceptibility Testing

- **Kirby Bauer Disk diffusion:** Kirby-Bauer agar diffusion method with disks on Mueller-Hinton agar supplemented with 5% sheep blood should be performed according to CLSI. Susceptibilities to nalidixic acid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), clindamycin (2 µg), tetracycline (30 µg), ampicillin (10 µg), imipenem (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), and trimethoprim-sulfamethoxazole (25 µg) should be checked.
- **VITEK 2 Compact ASTGN cards**

Reporting

- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using the special laboratory reporting form.
- Campilobacteriosis currently is reported under “Other bacterial intestinal infections (A04)” included in the list of reportable diseases to the public health system. As the list is regularly updated, you are recommended to follow up the updates with NCDC.

9.5. Cholera

Clinical manifestation

Cholera is an acute, bacterial enteric disease characterized in its severe form by sudden onset, profuse watery diarrhea, vomiting, and leg cramps early in the course of illness. In untreated cases, rapid dehydration, acidosis, circulatory collapse, hypoglycemia in children and renal failure can rapidly lead to death. In most cases, infection is asymptomatic or causes mild diarrhea, especially

with organisms of the El Tor biotype; in severe dehydrated cases (cholera gravis), death may occur within a few hours.

Incubation period: From a few hours to 5 days, usually 2-3 days.

Epidemiological characteristics

Epidemics and pandemics are strongly linked to the consumption of unsafe water, poor hygiene, poor sanitation and crowded living conditions.

Cholera is one of the 3 diseases requiring notification under the *International Health Regulations*. Only *Vibrio cholerae* serogroups O1 and O 139 are associated with the epidemiological characteristics and clinical picture of cholera. Serogroup O1 occurs as two biotypes - classical and El Top, each of which occurs as 3 serotypes (Inaba, Ogawa and rarely Hikojima).

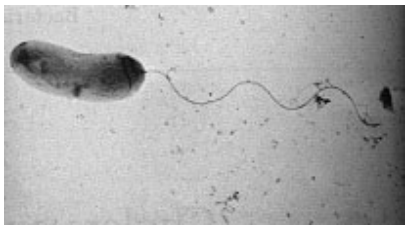
Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals *V. Cholerae* O1 and O139 can persist in water for long periods and multiply in moist leftover food.

Incriminated sources:

- Contaminated drinking water
- Cross-contamination of any food during
- Preparation
- Transport
- Storage
- Serving

Pathogen

Illustration 10. *V. cholerae*



The genus ***Vibrio*** consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. Vibrios are capable of both respiratory and fermentative metabolism. O_2 is a universal electron acceptor; they do not denitrify. Most species are oxidase-positive. They have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, since vibrios are typically marine organisms, most species require 2-3% NaCl or a seawater base for optimal growth. *V. cholerae* produces cholera toxin

Equipment / reagents consumables

Equipment

- Biological safety cabinet
- Incubator (35° to 37°C)
- Refrigerator
- Microscope and microscope slides
- Micropipette
- Vortex
- Hot plate

Consumables

- Sterile Petri dishes
- Sterile Pasteur pipettes
- Sterile test tubes
- Sterile swabs
- Inoculating needle and inoculating loops

Pre-analytical steps

Samples should be taken from the patients:

- Who have not received antibiotic treatment before collecting stool samples;
- Currently presenting acute diarrhea (either watery or bloody);
- Onset of illness less than four days before sampling
- Collect stools as specified in procedure 5.6 and place it into a Cary Blair transport media (procedure 5.8).

Cholera specimens should be sent refrigerated (at 4^0C to 8^0C). However, cholera specimens are less sensitive to temperature than dysentery specimens. If a cool box and ice are not available, cholera specimens can be sent at ambient temperature. Vibrio has an approx 90% chance of being alive if kept < 4 days at ambient temperature.

Cholera specimens in transport medium sent unrefrigerated will be useable in the laboratory for 7-14 days, depending on ambient conditions.

Analytical procedures

Gram staining
Gram stain will demonstrate typical small, curved gram-negative rods.7.3

Enrichment
Alkaline peptone water (APW) is recommended as an enrichment broth and can be inoculated with liquid stool, fecal suspension, or a rectal swab. The stool inoculum should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35° to 37°C for 6 to 8 hours maximum. Enrichment in alkaline peptone water (APW) can enhance the isolation of V. Cholerae, when few organisms are present.

Culture
Thiosulfate citrate bile salts sucrose agar (TCBS) is the selective agar medium of choice.

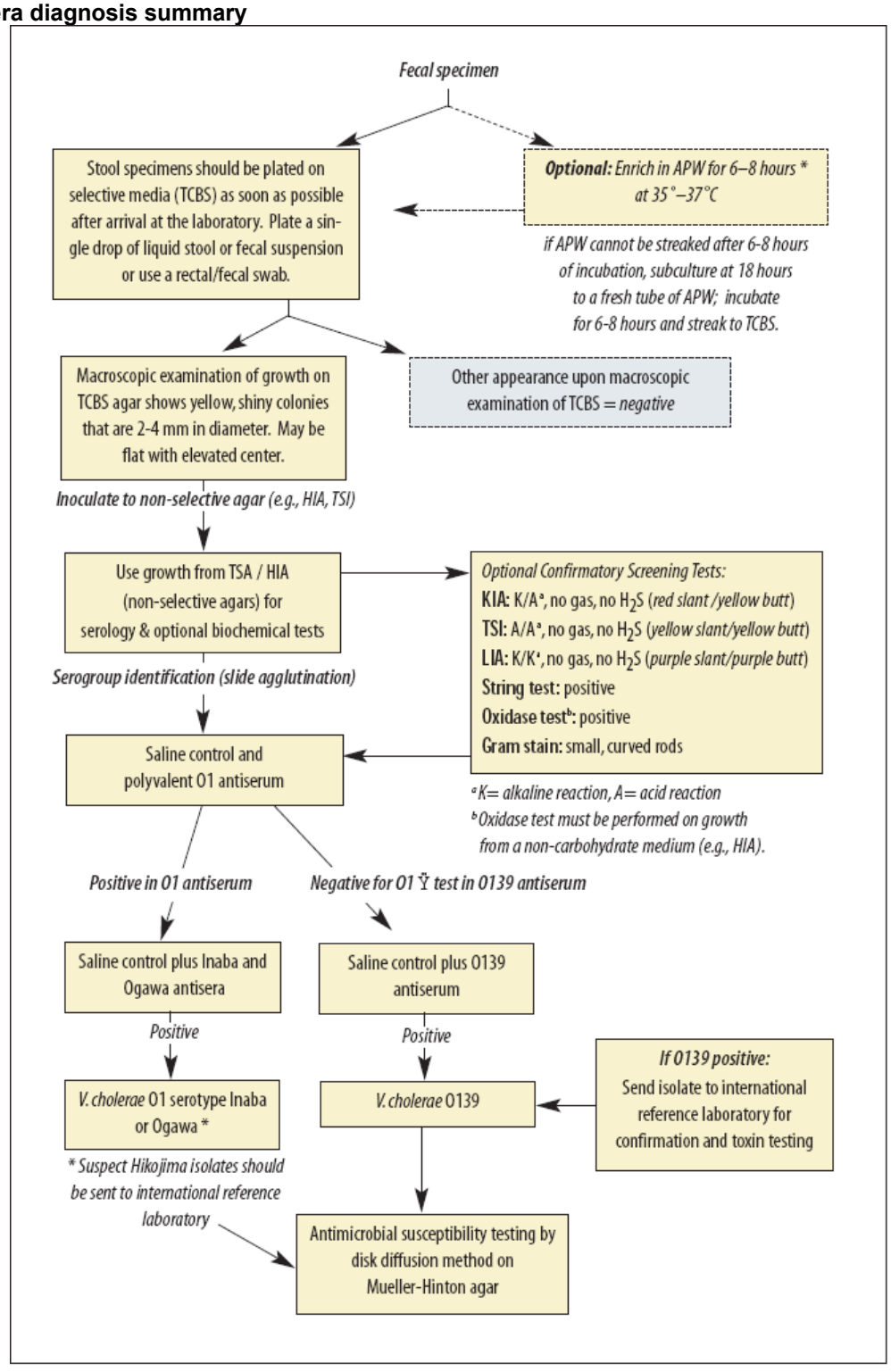
After incubation, subculture to TCBS should be made with one to two loops full of APW from the surface and topmost portion of the broth, since vibrios preferentially grow in this area. Do not shake or mix the tube before subculturing. If the broth cannot be plated after 6 to 8 hours of incubation, subculture a loop at 18 hours into a fresh tube of APW. Subculture this second tube to TCBS agar after 6 to 8 hours of incubation.

Illustration 11. Colonies suspicious for *V. cholerae* on TCBS agar

Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2 to 4 mm in diameter. The yellow color is caused by the fermentation of sucrose in the medium. Sucrose-non fermenting organisms, such as *V. parahaemolyticus* produce green to blue-green colonies.

TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective. Growth on this medium is not suitable for direct testing with *V. cholerae* antisera.

Cholera diagnosis summary



Isolation of suspected V. cholerae

Carefully select at least one of each type of sucrose-fermenting colonies from the TCBS plate to inoculate another non-selective medium. Do not use nutrient agar because it has no added salt and does not allow optimal growth of *V. cholerae.* Using an inoculating needle, lightly touch only the

very center of the colony. Do not take the whole colony or go through the colony and touch the surface of the plate. This is to avoid picking up contaminants that may be on the surface of the agar.

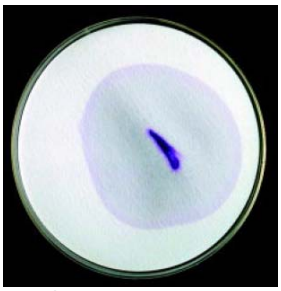
Culture identification with semi-automated systems
API 20E test strip can be used to identify *V. cholerae*. See section 8.3 for API 20E.

Biochemical tests

Table 33. Reactions of V. cholerae in screening tests

Screening test	*Vibrio cholerae* reactions
Oxidase test	Positive
String test	Positive
KIA	K/A, no gas produced (red slant/yellow butt)[a]
TSI	A/A, no gas produced (yellow slant/yellow butt)[a]
LIA	K/K, no gas produced (purple slant/purple butt)[a,b]
Gram stain	Small, gram-negative curved rods
Wet mount	Small, curved rods with darting motility

[a] K = alkaline; A = acid
[b] An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.


Oxidase positive

Serologic Identification of V. cholerae O1 and O139

Presumptive identification using O1 and O139 antisera
For slide agglutination testing with polyvalent O1 or O139 antisera, fresh growth of suspected *V. cholerae* from a nonselective agar medium should be used. Using growth from TCBS agar may result in false-negative reactions. Usually after 5 to 6 hours of incubation, growth on the surface of the slant is sufficient to perform slide serology with antisera; if not, incubate for a longer period. If the isolate does not agglutinate in O1 antiserum, test in O139 antiserum. If it is positive in the polyvalent O1 or in the O139 antiserum, it may be reported as presumptive *V. cholerae* O1 or O139.

Confirmation of V. cholerae O1 using Inaba and Ogawa antisera
The O1 serogroup of *V. cholerae* has been further divided into three serotypes: Inaba, Ogawa, and Hikojima (very rare). Serotype identification is based on agglutination in monovalent antisera to type-specific O antigens. A positive reaction in either Inaba or Ogawa antiserum is sufficient to confirm the identification of a *V. cholerae* O1 isolate. Isolates that agglutinate weakly or slowly with serogroup O1 antiserum but do not agglutinate with either Inaba or Ogawa antiserum are not considered to be serogroup O1.

Table 34. Serotypes of V. cholerae serogroup O1

	Agglutination in absorbed antiserum	
V. cholerae O1 serotype	Ogawa antiserum	Inaba antiserum
Ogawa	+	-
Inaba	-	+
Hikojima	+	+

Antibiotic Susceptibility testing

See section 8.5 for disk diffusion method and antibiotic to be tested for bacterial intestinal infections. We are also providing the antimicrobial agents suggested to be used for susceptibility testing of *Vibrio cholerae* O1 and O139:

- Trimethoprim-sulfamethoxazole (cotrimoxazole)
- Furazolidone
- Tetracycline[a]
- Nalidixic acid[b]

[a] *The results from the tetracycline disk are also used to predict susceptibility to doxycycline.*
[b] *If resistant to nalidixic acid, the isolate should be tested for susceptibility to ciprofloxacin, and will probably exhibit reduced susceptibility to ciprofloxacin.*

Reporting

- Cholera is included in the list of emergency infections which is regulated by the law; also it is included in IHR and accordingly it should be reported immediately.
- Refer to chapter 12.4 for reporting to the **public health system.**

When a peripheral laboratory is suspecting cholera, notification must be immediately (without any delay) sent to the local public health center. A clinician should also be immediately informed.

10. Analytical procedures – viral infections

10.1. Adenoviral infection – general facts

Clinical manifestation

There are 41 known adenovirus serotypes with diverse clinical manifestations ranging from:

- **Intestinal tract infections**

Acute pharyngo-conjunctival fever

Acute respiratory illness

Epidemic kerato-conjunctivitis

Epidemiological characteristics

Adenoviruses have a worldwide distribution, and they are ubiquitous in the environment, where contamination of human feces or sewage has occurred. Infections can occur throughout the year. Outbreaks have been more common in late winter, spring, and early summer. Adenovirus is easily transmissible to individuals without any specific serotype-specific antibodies (49 serotypes).

Pathogen

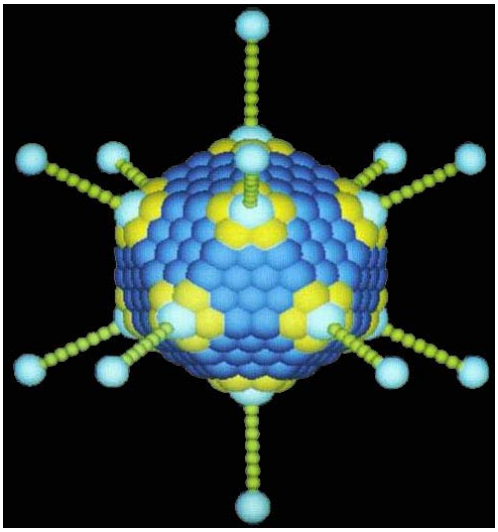


Illustration 12. Adenovirus

Adenoviruses are medium-sized (90-100nm), non-enveloped icosahedral viruses containing double-stranded DNA. There are more than 40 immunological distinct types (6 subgenera: A through F) that can cause human infections. Adenoviruses are unusually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival outside of the body.

10.2. Adenovirus – diagnosis using rapid tests

Pre-analytical procedures

Refer to procedure 5.6: Stool sampling.

Analytical procedures

The test used is immunochromatographic rapid assay for the Detection of Adenovirus Antigens in Human Stool Specimens.
Several commercial tests are available; we are providing the detailed description for one of them, performing adeno and rotaviruses diagnostic simultaneously.

Principle of the Assay:

- Rapid Adenovirus Test is an in vitro qualitative/sandwich solid phase immunochromatographic assay for the rapid detection of adenovirus antigens in human stool specimen.
- The test results are intended to aid in the diagnosis of adenovirus infection and to monitor the effectiveness of therapeutic treatment.

Assay procedure:

- Sample: To perform the test, an aliquot of diluted stool sample is added to the sample well of the test cassette.
- Procedure: The sample flows through a label pad containing adenovirus antibody coupled to red-colored colloidal gold. If the sample contains adenovirus antigens, the antigen will bind to the antibody coated on the colloidal gold particles to form antigen-antibody-gold complexes.
- These complexes move on the nitrocellulose membrane by capillary action towards the test line region on which adenovirus specific antibodies are immobilized.

Results and Validation:

- The complexes reach the test line; it will bind to the antibody on the membrane in the form of a line. A second red control line will always appear in the result window to indicate that the test has been correctly performed and the test device functions properly.
- If adenovirus antigen is not present or is lower than the detection limit of the test, only the control line will be visible. If the control line dose not developed, the test is invalid.

10.3. Adenovirus – diagnosis using ELISA

Pre-analytical procedures

Refer to procedure 5.4 for blood sampling

Equipment needed:

- Dispensing system and/or pipette (single or multichannel), disposable pipette tips
- Dry incubator or water bath, 37±0.5°C
- Microshaker for dissolving and mixing conjugate with samples
- Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm
- Microwell aspiration/wash system

Reagents and consumables:

- Freshly distilled or de-ionized water
- Disposable gloves and timer
- Appropriate waste containers for potentially contaminated materials
- Disposable V-shaped forceps
- Absorbent tissue or clean towel
- 96 well plate which contains anti-IgM antibodies
- Ready to use Standards
  - Standard A = Negative Control
  - Standard B = Cut-Off Control
  - Standard C = Weakly Positive Control
  - Standard D = Positive Control
- Horseradish peroxidase-conjugated adenovirus antigens
- Stock wash buffer solution
- Substrate solution
- Dilution buffers
- Stopping solutions
- RF Adsorbent

Analytical procedure

Principle of the Assay:
Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The wells are coated with antigen. Specific antibodies of the sample binding to the antigen-coated wells are detected by a secondary enzyme conjugated antibody (E-Ab) specific for human IgM. After the substrate reaction, the intensity of the color developed is proportional to the amount of IgM-specific antibodies detected. Sample results can be determined directly using the standard curve.

Assay Procedure
1- Reagents Preparation:
Allow all reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes before the initiation of the assay procedure.

Washing solutions should be checked for the presence of salt crystals. If crystals have formed in the solution, warming at 37°C is recommended. Wash buffers should be diluted 1 to 10 with distilled or de-ionized water.

All reagents are ready to use and should not be pretreated before adding to the wells.

2- Sample Preparation: Each sample is diluted 1:101 with the dilution buffer. The standards are ready for use and should not be diluted.

Note: In order to avoid interferences of specific IgG and rheumatoid factors, patient sera should be treated with RF absorbent. Pipette 20 µl of RF-Absorbent to 400 µl of 1:101 diluted sample. Mix

well. Incubate 1 min (< 15 min) at room temperature (18-25°C). Do not treat standards and controls with RF-Absorbent.

3- Adding Sample: 100µl of diluted and RF-Absorbent treated samples and non-treated standards should be added into their respective wells.

4- Incubating Sample: Microplate wells should be covered and incubated for 60 minutes at room temperature.

5- Washing: At the end of the incubation the content of the plate should be discarded. Each well should be washed 3 times with diluted washing buffer. After the final washing cycle, the plate should be taped on the clean towel or paper to remove any remaining liquids.

6- Adding Conjugate and Incubation: 100µl of HRP-Conjugate Reagent is added into each well except the blank one, and incubated for 30 min at room temperature.

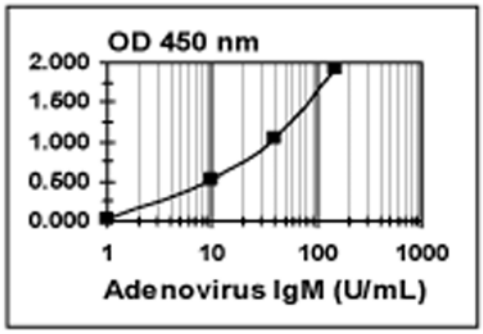
7- Washing: The same procedure as step 4

8- Coloring: 100µl of chromogen is added into each well including the blank and incubated for 20 minutes.

9- Stopping Reaction: 100µl stop solution is added, followed by the development of intensive yellow color in standards wells.

10- Measuring the Absorbance: The absorbance will be read at 450nm. If a dual filter instrument is used, the reference wavelength will be 630nm.

Illustration 13. cut of value



11- Validation of the assay: If the standards meet the criteria, then the cut of value is calculated. Illustration 13.

Note: If one of the standard values does not meet the control range specifications, the test is invalid and must be repeated.

12- Interpretation of Results: The results are calculated by relating each sample's optical density to the standard curve. The obtained ODs of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic graph paper or using an automated method.

After calculation of the sample U/ml, it should be interpreted according to interpretation table. The results are considered positive if the concentration is > 12 U/ml. Equivocal results should be re-assayed.

U/ML	Interpretation
<8	Negative
8-12	Equivocal
>12	Positive

Note: If one of the standard values does not meet the standard range specifications from the manufacturer's instructions, the test is invalid and must be repeated.

Reporting

- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using a special laboratory reporting form.
- There is no specific field for acute Adenovirus infection in the list of reportable diseases to the public health system. Nevertheless it should be reported under "Diarrhoea and gastroenteritis of presumed infectious origin (A09)" included in the list of reportable diseases. As the list is regularly updated, you are recommended to follow up the updates with NCDC.

10.4. Non polio enteroviral infection

Introduction

Human enteroviruses (family Picornaviridae) infect millions of people worldwide each year, resulting in a wide range of clinical outcomes ranging from unapparent infection to mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, **gastroenteritis**, and acute flaccid paralysis.

Note: The methodology described is a summary of the one recommended for the accredited WHO polio laboratories. Full version can be downloaded at the following address:
www.who.int/vaccines/en/poliolab/WHO-Polio-Manual-9.pdf

Pathogen

Group:	Group IV ((+)ssRNA))
Family:	*Picornaviridae*
Genus:	*Enterovirus*

On the basis of their pathogenesis the enteroviruses were originally classified into four groups:

1. Polioviruses
2. Coxsackie A viruses (CA)
3. Coxsackie B viruses (CB)
4. Echoviruses

Pre-analytical procedures

Sampling
Refer to procedure 5.6 - Stool sampling

Collection should preferably be carried out in the laboratory, otherwise at home. Bring the collection to the laboratory as soon as possible, preferably immediately after collection, but in any case not after two days.

If transport and/or analysis in the laboratory are not immediate, use VTM to avoid desiccation and expand the virus life (see procedure 5.9) and keep at 4°C.

Reception of the specimens in the recipient laboratory:
Upon arrival in the laboratory, shipping cartons or carriers must be immediately unpacked in a designated area equipped with a waste container, alcohol swabs and paper towels. If available, a Class II Biosafety Cabinet (BSC) should be used to limit exposure of laboratory staff to potential pathogens. If a BSC is not available a clean workbench should be used.

Biosafety:

Enteroviruses are not inactivated by alcohol per se, but a 70% solution is an effective antibacterial and antifungal disinfectant and will inactivate enteroviruses by desiccation, if the solution is allowed to completely dry.

Reagents & supplies needed

(Detailed list in the WHO polio laboratory manual)

Reagents

- Trypan blue
- Trypsin/EDTA
- FBS
- Nutritive media, buffers
- Cell lines are provided by NCDC Cell Culture laboratory

Equipment

- CO2 incubator, 5% CO2, 37ºC
- OR 28ºC, 5% CO2 for C6/36
- BSC
- Microscope, inverted
- Centrifuge
- Pipettor or pipet aid
- 20 and 200µl Pipetman
- Haemocytometer
-

Supplies

- 200µl pipet tips
- 1, 10 and 25 ml sterile pipets, individually wrapped
- T-75 Tissue culture flasks
- Tissue culture tubes
- Racks for tubes (64 slots)
- 1.5 ml Eppendorf tubes.
- Scrapper
- Clean, sterile, glass bottles

Buffers

- 70% Ethanol
- Maintenance media (listed in virus isolation protocols)
- Growth media
- PBS (sterile)

Preparation and control of the reagents

(Detailed procedures can be found in the WHO polio laboratory manual)

Analytical procedure

The diagnosis of enteroviruses is performed through manual viral culture method, split in two steps:

- **Virus isolation**- RD and Hep-2 Cell culture inoculation, which produce a characteristic Enterovirus Cytopathic Effect (CPE)
- **Virus serotyping**- Neutralization test for identification of enteroviruses

General issues for consideration when performing viral culture:
Limit access to the room in which you are working with cells.

Wipe all working surfaces and bottles to be placed in the hood with 70% ethanol.

Fill out the worksheet as indicated.

Cells are checked for growth on days 3 and 5. If they are growing too quickly (will be overgrown before day 7), they are placed in a 28°C incubator until day 5 or 6 and then transferred back to 37°C (to recover before being used).

Cells should be confluent (100%) on the 6th day after the passage.

Analytical steps summary
1-Trypsin/EDTA (trypsin) treatment
2- Cell counting

3- Preparing Tissue Culture (TC) tubes

4- Preparing TC plates

5- Preparation of faecal samples for virus isolation
6- Storage of faecal samples and suspensions
7- Isolation of viruses in continuous cell lines
8- Neutralization test for identification of enteroviruses

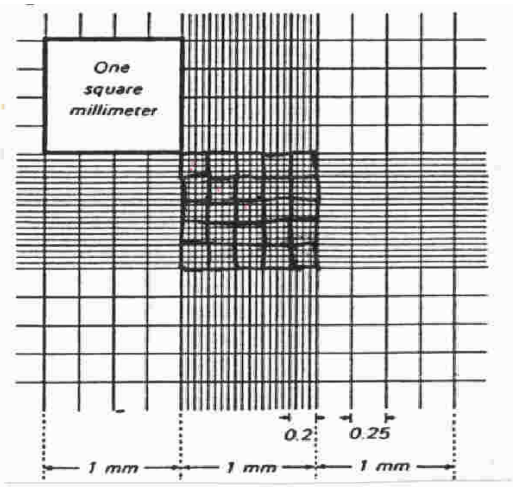
1-Trypsin/EDTA (trypsin) treatment:
Pre-warm the trypsin to room temperature. Mark the flask as Freeze/Thaw (F/T). Discard after the 3rd F/T.

Pour off the liquid media, add 5 ml of trypsin and rock the flask so that the trypsin completely covers the cells.

Remove the trypsin with a pipette, add 2.0 ml of trypsin, rock and let stand at room temperature until the cells start to come off. Most types of cells detach in 1-5 min. RD and Hep-2 cells are incubated at 37ºC; it may take more than10 min.

Strike the flask with your hand to completely dislodge the cells.
Add 8 ml of growth media. Use a 10 ml pipette to take up and expel the cells 4 times. For Hep2, pipette and expel 10 times. Expel the media with the tip of the pipette pressed against the bottom of the flask. Check a drop under the microscope to ensure that there are no clumps of cells and that no more than 10% of the cells are stuck.

2- Cell counting



At least once for each cell line, determine cell counts and viability of the above suspension using a haemocytometer as follows:

Add one volume of cell suspension to two volumes of trypan blue stain (non-vital stain i.e. only dead cells are stained). Dilution factor is 3.

Use a 20 μl pipette to add about 10 μl of cell suspension to each of the haemocytometer cells. Add the suspension slowly but continuously until the silver area is just covered. Read before the media evaporates – within 10 min.

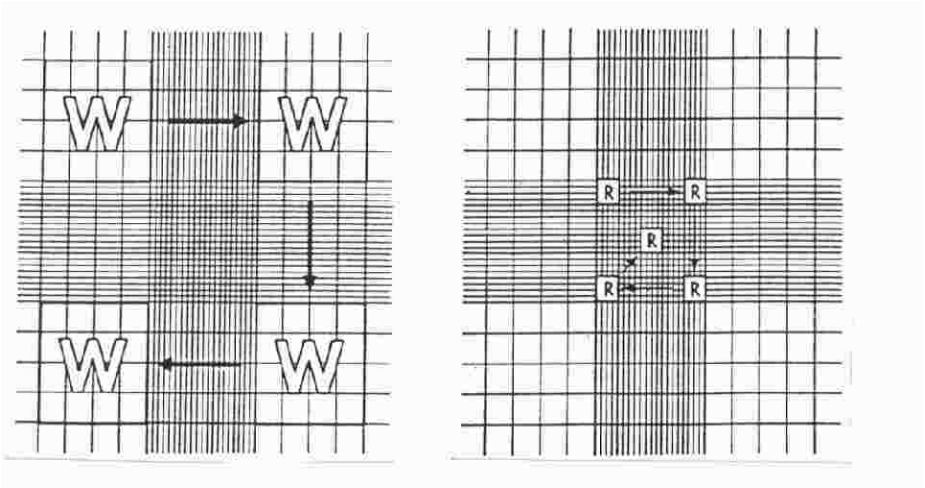
Count and average the four squares on opposite sides of the center square (smallest grid lines in the center of 9 squares) on both halves of the haemocytometer. Recount if the highest count is more than 150% of the lowest count.

Calculate cells per ml and cells per flask as follows:

Average of 8 cells x 3 (dilution factor) [cell suspension to trypan blue stain] x 10,000 = cells/ml.

Then multiply by 10 in order to get the number of cells per flask.

As example, if the 8 cell counts are 19, 25, 22, 23, 26, 18, 22, and 21. The average count is 22, and the total number of cells is 22 x 3 x 10,000 = 6.6 x 10^5cells/ml and 0.66 x 10^7 /flask.



3- Preparing TC tubes:

- Mark each tube with the type of cell and sample number to be inoculated. Mark the rack with the date and cell type.
- Add sufficient cells and growth media to make 35 ml. Vary the volume of cells to produce an 80% confluent monolayer on the day desired.
- Aliquot 1 ml of cell suspension into each tube. Within 2 minutes, place the tubes horizontally in the rack.
- Strike the rack (on one of its ends) to ensure that media completely covers the flat part of the tube.
- Remember to loosen the tube caps.
- Tubes are scored for confluence by observing the middle of the tube, exactly 1 cm from the end of the tube (the sides and bottom will contain more cells).

4- Passing cells:

Label each T-75 flask with:

- Date
- Type of cell
- Passage level
- Volume of inoculums

Add 25ml of growth media to the flasks. Place the flasks lying **down** so that the media covers the growth surface for at least 1 cm (FBS components bind to the flask surface and help cells attach) and pre-warm the flasks/media to 37°C (60-90 min).
Immediately after pipetting (to separate trypsin treated cells), add the volume of cells indicated (different depending on the cells used).

Since cells to be passed are in 10 ml of media per T-75 flasks, a 1:100 split means transferring 100µl of cells to a new T-75, etc. Incubate at 37°C.

Monitor cell growth:

On day +1, check the condition of the cells. In particular, note whether they have attached or not.

On day +3 and +5, check the cells and record the % confluence, as the percent of the flask surface covered by cells.

- Note that if cells are growing in clumps, cells at the center of clumps may “overgrow” before the flask becomes 100% confluent. Mark such flasks as O.G. (overgrown).
- If you are obtaining overgrown cells by day 7, transfer the flasks to 28°C for 2-3 days. Return the flasks to 37°C at least 24 hr before passing.

On day 6, check the cells. Slow growing cells (those passed less than 1:50) should be 90-95% confluent. Fast growing cells (passed greater than 1:200) should be 85-95% confluent. The idea is that the cells should be less than 1 day past just touching when passed. Results will always be better with 100% confluence.

5- Preparation of faecal samples for virus isolation

- Prepare first the following items:
- 15 ml or 50 ml polyethylene chloroform-resistant centrifuge tubes
- 1 or 5 ml glass pipette for aliquoting of chloroform
- 5 ml and 10 ml pipettes
- Wooden spatula
- Externally threaded, screw-capped storage vials (5 ml)
- Glass beads, approximately 3 mm diameter
- PBS with antibiotics
- Chloroform

Inside a BSC:

- Label centrifuge tubes with sample numbers.
- Add 10 ml PBS/ATB solution, 1 g of glass beads and 1 ml chloroform to each tube.
- Transfer approximately 2 g of each faecal sample to a labeled centrifuge tube.
- Retain the remaining original sample, preferably in its original container, for storage at -20°C.
- Close centrifuge tubes securely and shake vigorously for 20 minutes using a mechanical shaker.
- Spin for 20 minutes at 1500 g ideally in a refrigerated centrifuge (4-8°C).
- Transfer supernatant from each sample into two labeled externally threaded screw-capped storage vials (if supernatant is not clear, repeat chloroform treatment).
- Store one faecal suspension at -20°C as a backup and store the other at 4-8°C.

6- Storage of faecal samples and suspensions

Stool specimens should be processed, following the WHO protocol, within the first working day after arrival or as soon as possible after arrival in the laboratory, so that faecal suspensions will be available for inoculation onto cell cultures.

It is not always possible, however, to have cell monolayers ready for inoculation at all times, so faecal suspensions sometimes must be stored for a certain period of time before they can be used to inoculate cells.

If the expected delay before cell culture inoculation is 48 hours or less, the faecal suspension kept for inoculation should be refrigerated at 4-8°C. If the expected delay is greater than 48 hours, both aliquots of faecal suspension should be stored at -20°C.

To allow re-investigation of specimens giving anomalous or queried results, it is necessary to retain original stool specimens **for at least six months**, and **preferably 12 months** after reception. Ideally these specimens should be stored at -20°C in their original containers. Since many laboratories have restrictions on the volume of -20°C storage space available, it may be necessary to store original stool specimens in smaller 2 to 5 ml externally threaded screw-capped vials.

Great care must be taken to avoid cross-contamination of stool specimens during the transfer of material from the original containers to the storage vials.

Faecal suspensions should be stored at -20°C for **at least three months** after receipt of the specimen, or until complete results (including intratypic differentiation (ITD) and sequencing if necessary) are available. Faecal suspensions should not be stored for periods of greater than 12 months, as this is a waste of valuable -20°C storage space.

7- Isolation of viruses

Prepare the following items:

- Tube cultures of Hep2 and RD cells
- Maintenance medium
- 1 ml and 5 ml plastic disposable pipettes
- Specimen extracts

Do the following:

- Microscopically examine recently prepared monolayer cultures to be sure that the cells are healthy and at least 75% confluent (100% is ideal). A suitable monolayer would be the one formed within 2–3 days of seeding.
- Remove the growth medium and replace with 1 ml maintenance medium.
- Label two tubes of RD cells and two tubes of Hep2 for each specimen to be inoculated (write specimen number, date, and passage number).
- Label two tubes of each cell type as a negative control.

Note: Both cell lines must be inoculated at the same time.

Inoculate each tube with 0.2 ml of specimen extract and incubate in the stationary sloped (5°) position at 36°C. Do not rotate tubes.

Examine cultures daily, using a standard or inverted microscope, for the appearance of CPE.

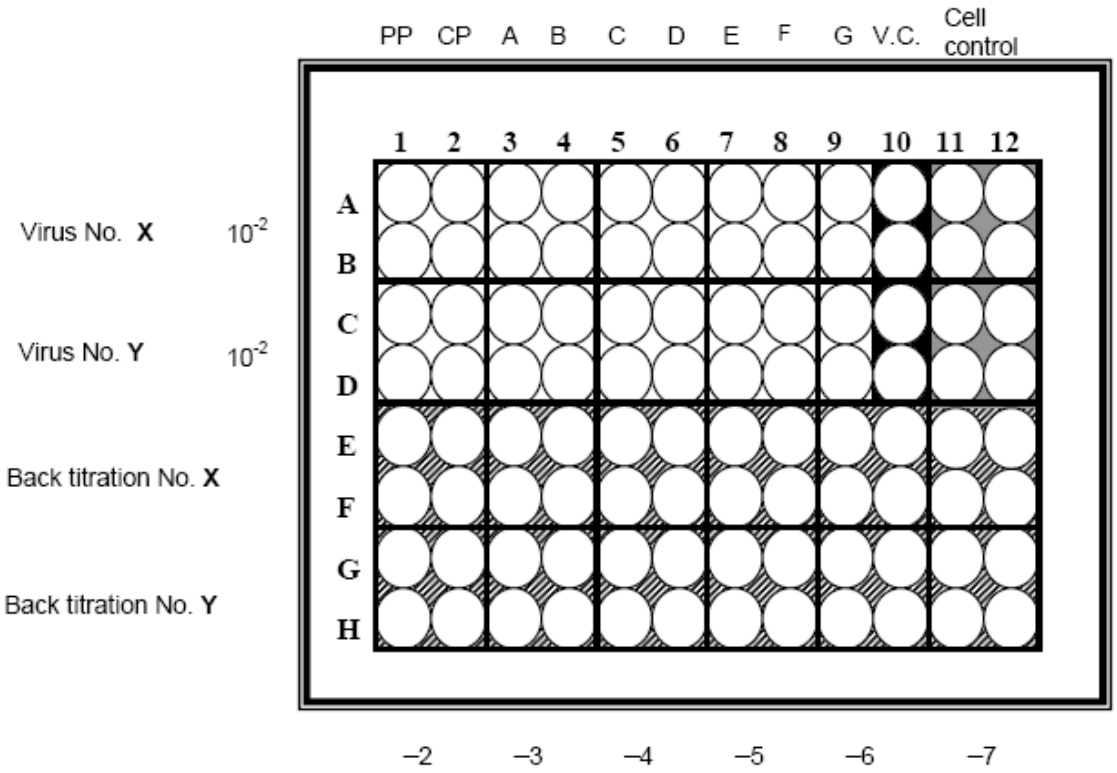
- Record all observations of inoculated and control cultures, recording CPE as 1+ to 4+ to indicate the percentage of cells affected (1+ represents up to 25% of cells; 2+ represents 25 to 50%; 3+ represents 50 to 75%; 4+ represents from 75 to 100%), toxicity, degeneration or contamination.
- If no CPE appears after at least 5 days of observation, perform a blind passage in the same cell line and continue examination for 5 more days (NB. Contents of duplicate negative cell cultures from an individual case should not be pooled for passage).

8- Neutralization test for identification of enteroviruses

Prepare the following items:

- Flat-bottomed cell culture microtitre plate with cover
- Non-toxic plate sealers (if non-CO2 incubator is used)
- 5 ml sterile tubes for dilution
- 1 ml and 2 ml pipettes
- Sterile 50 µl droppers or pipettors with aerosol resistant tips (ARTs)
- Flask of healthy RD cells
- Enterovirus serum pools, diluted for use
- Maintenance medium

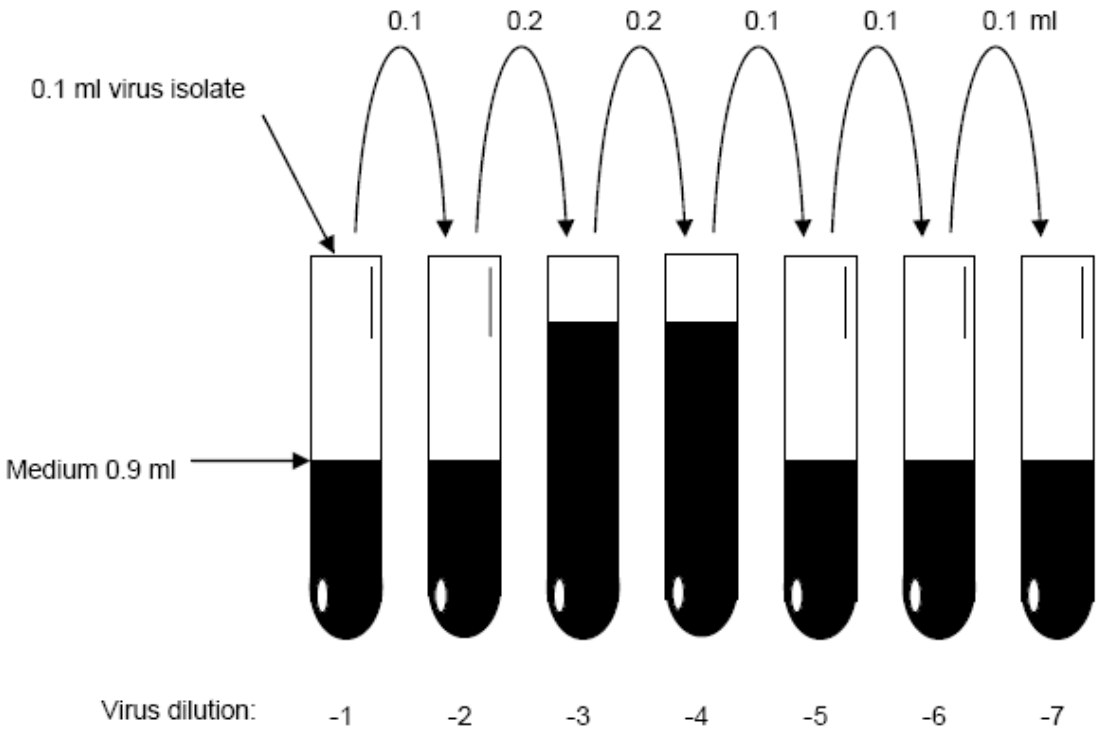
Each unknown virus is tested in duplicate against a trivalent pooled polio antiserum (PP), a coxsackievirus B1-6 pool (CP), and seven pools against coxsackievirus A9 and 20 echoviruses (A–G).



Freezing of the chloroform-treated isolates can result in re-aggregation of the virus; so chloroform treatment should be followed immediately (ideally) by typing, prior to freezing the isolate.

Do the following:

- Label the edge of the microtitre plate.
- Add 50 µl of antisera to the appropriate wells in columns 1–9.
- Add 50 µl medium to virus control wells in column 10 rows A to D.
- Add 100 µl medium to cell control wells in columns 11 and 12 rows A to D.
- Prepare 10^{-2} dilution of virus (it may be desirable to determine the virus titer and adjust the dilutions as needed).
- Dispense 0.9 ml medium to tubes 1–2 and 5–7, and 1.8 ml to tubes 3 and 4.
- Add 0.1 ml virus to the first tube (=10^{-1} dilution) using sterile pipette or pipettor with ART tip.
- Take another pipette/pipette tip, mix thoroughly but gently to avoid aerosols.
- Transfer 0.1 ml to the second tube and discard pipette/pipette tip.
- Add 50 µl virus to all wells in columns 1 to 10 of rows A to B for sample X and rows C and D for sample Y.
- Perform a back titration of virus X in rows E and F and of virus Y in rows G and H.



- Cover the plate with the lid and incubate for one hour at 36°C.

During this incubation period, trypsinize RD cells and prepare a suspension of approximately $1{,}5.10^{-5}$ cells per ml, calculating at least 10 ml per plate.

- Distribute 100 µl of cell suspension into all wells.
- Cover the plate with a non-toxic sealer and incubate at 36°C.
- Examine daily, using an inverted microscope, and record CPE.
- Continue recording until 24 hours after CPE in the virus control wells reaches 100%.
- The virus is identified by the pattern of inhibition of CPE by antiserum pools, as shown on the table accompanying the sera.
- If CPE is seen in all wells containing virus, the isolate should be reported.

Reporting

- **Reporting to the clinicians or a patient:** The positive and negative results are reported to the clinicians or a patient using the special reporting form.
- There is no specific field for non-polio Enterovirus infection in the list of reportable diseases to the public health system. Nevertheless, non-polio Enterovirus infection should be reported under “Diarrhoea and gastroenteritis of presumed infectious origin (A09)” included in the list of reportable diseases. As the list is regularly updated, you are recommended to follow up the updates with NCDC.
- In frames of the Polio virus lab surveillance, NCDC, which is a WHO Reference Laboratory in Georgia, reports on a weekly basis and sends specimens upon necessity to the WHO Sub-regional Office (Moscow).

10.5. Rotaviral infection – general facts

Clinical Manifestation

Rotavirus is a virus that infects the bowels. It is the most common cause of severe diarrhea among infants and children throughout the world and causes the death of about 600,000 children worldwide annually.

Pathogen

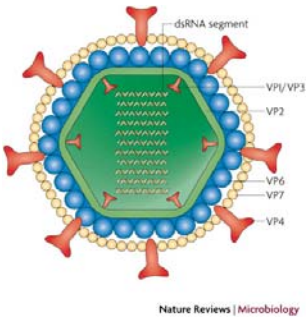


Illustration 14. Rotavirus

There are seven species of rotavirus, referred to as A, B, C, D, E, F and G. Humans are primarily infected by species A, B and C, but most commonly by species A. All seven species cause disease in animals.

Human serotypes of Group A Rotavirus are a major cause of gastroenteritis (diarrhea) in young children throughout the world. Rotavirus gastroenteritis may also occur in older children and elderly patients. The virus is commonly associated with nosocomial infections in pediatric wards and neonatal nurseries.
→ Commercially available kits only diagnose the A type of rotavirus.

Note: A rotavirus specific project is currently being implemented in Georgia.
Patient enrolment in the laboratories can begin before procurement of specific diagnostic kits and freezer. Until such equipment is available, stool samples from enrolled children will be delivered from the collection hospital to the NCDC lab, recorded in a laboratory logbook, divided into 3 aliquots at the NCDC laboratory, and frozen at -20°C in the freezer currently available at the NCDC laboratory. When the first ELISA kits become available, one of the three aliquots from each enrolled child will be obtained from freezer storage and tested for rotavirus. Results will be provided to Hospital and National Coordinator as per rotavirus-specific SOPs.

Pre-analytical phase

Refer to the following procedures:

- 5.6: Stool sampling (rapid tests)
- 5.4: Blood sampling (serology)

10.6. Rotavirus – diagnosis using rapid tests

See procedure number 10.2 (joined diagnostic kit adeno/rotaviruses)

Note: Several rapid tests exist, such as the one described in 10.2 Another kit is broadly used in Georgia, the **VIKIA Rota-Adeno** (20 tests kit).

10.7. Rotavirus – diagnosis using EIA

Several immuno-analysis kits and tests exist today. We are providing the example of the IDEIA Dako kit, but any other validated kit could be used. In this case, follow precisely the instruction provided along with the kit.

IDEIA Rotavirus Test uses a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotaviruses.

Specimen reception

Upon reception, divide the specimen into aliquots for performing different investigations as follows:

- Label 2 cryovials (externally threaded screw capped storage vials) with the appropriate storage label using the same unique patient ID.
- Using wooden spatula, transfer one-third of the stool specimen into each pre-labeled storage vial. These vials should be approximately half-filled (to avoid stool sample getting into threads or on cap). Record the specimen into the appropriate logbooks.

Specimen storage

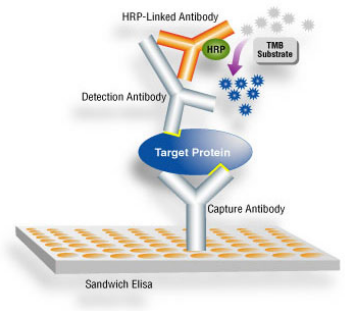
- Short term storage: store the aliquots for at 2-8^{o}C during maximum 1 month.
- Long term storage: store the two other cryovials at -20^{o}C. These samples will be used for genotyping. The vial will be stored until 1) transfer from the NCDC to the reference lab for genotyping; 2) protocol has been developed to determine which samples will be discarded and which ones will be stored. At the current time, no samples should be discarded and all should stay in long term storage at -20^{o}C.

Laboratory equipment needed

- Set of laboratory pipettes (Gilson, Eppendorf or Finnpipettes)
- Pipette tips (200ul, 1000ul)
- Absorbent tissue paper
- ELISA plate reader
- Automated plate washer

Reagents and supplies needed

- Wooden spatula
- Specimen containers for stool storage, such as 2 ml cryotubes with screw caps
- Absorbent tissue paper
- Laboratory disinfectants (e.g., 70% alcohol, sodium hypochlorite solution)
- Immuno-enzymatic kit



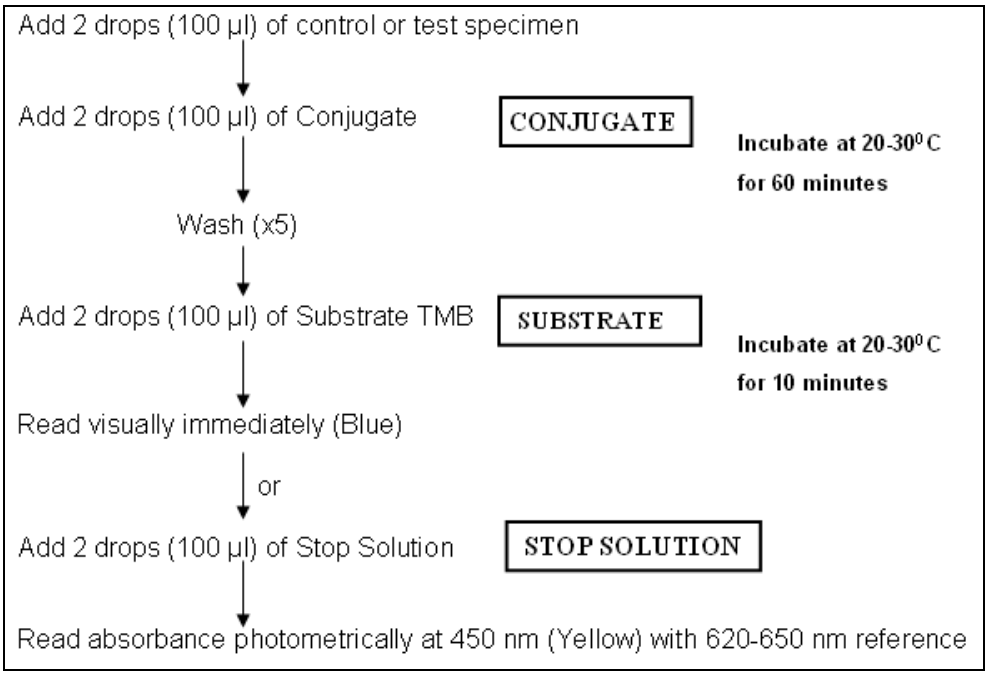
Analytical procedure

Reagents and specimens should be brought to ambient temperature (20-30^{o}C) before use.

Quality control of kits: Each DAKO IDEIA rotavirus EIA kit contains positive and negative control that has to be performed with each assay to ensure that kit reagents are functioning correctly and that proper assay procedures have been followed.

Summary of the IDEIA Rotavirus assay procedure

Ensure all reagents reach room temperature (15-30) before use.



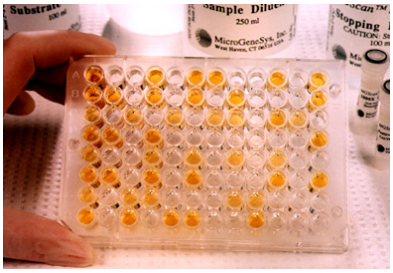


Illustration 15. IDEIAR Rotavirus reaction

Limitations of the procedure

A negative test result does not exclude the possibility of rotavirus infection. Failure to detect rotavirus in stool specimen may be a result of factors such as collection of specimen after the peak period of viral shedding (3-5 d), improper sampling and/or handling of specimen or another rotavirus group as the EIA test can not detect other rotavirus subgroups.

Results reporting

- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using the special laboratory reporting form.
- **Hospital-based Rotavirus Sentinel Surveillance** Projects are underway that require a specific reporting system, which is not included in this manual.
- In general, rotavirus infection is not currently included in the list of *reportable diseases* to the **public health system**. As the list is regularly updated, you are recommended to follow up the updates with NCDC.
- Report should first be made to a clinician and a patient.

10.8. Noroviral infection

Clinical manifestation

Norovirus infection is characterized by the sudden onset of **vomiting or diarrhea** or both symptoms**. Nausea, abdominal pain**, abdominal cramps, **anorexia**, malaise, and low-grade fever also occur. Bloody diarrhea is not seen with norovirus infection. The incubation period following exposure to norovirus is 1 to 2 days, and the illness duration observed has also been very short (1–3 days).

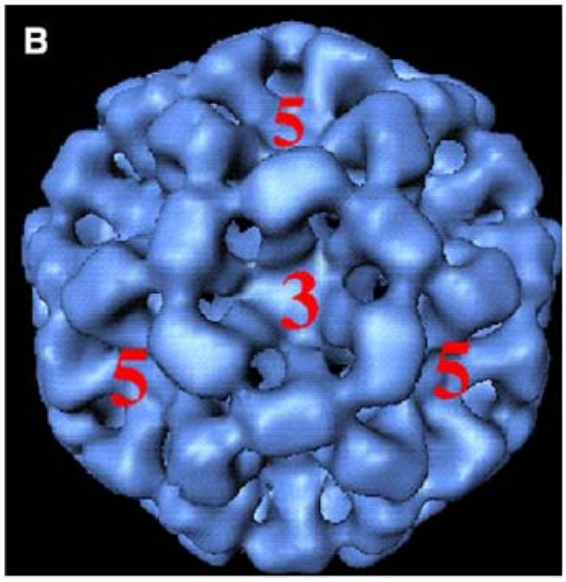
Epidemiological characteristics

The genetic heterogeneous groups of Noroviruses are the major cause of non-bacterial gastroenteritis documented in the USA as well as in western European countries. No prevalence data are available in the Caucasian area. Noroviruses cause infection throughout the year, although there is a peak incidence during the cold-weather months. The Center for Disease Control and Prevention (Atlanta) estimates that 23 billion cases of gastroenteritis/ per year may be attributed to human caliciviridae. Thus, 66 % of all food and water-borne infectious diseases are associated with Noroviruses in the USA.

Noroviruses are transmitted primarily through the fecal–oral route. Fecal contamination of food and water as well as direct person-to-person spread, accounts for most outbreaks.

Noroviruses are often called “Norwalk like” viruses, in relation to a large outbreak that happened in the town of Norwalk, USA in 1967.

Illustration 16. Norovirus



Pathogen

Human Noroviruses are small, non-enveloped viruses with an ssRNA (single stranded) genome.

Noroviruses belong to the family of Caliciviridae and are divided into genotype I and II. Five genetic groups of Noroviruses exist based on phylogenetic analysis of the complete capside (VP1) gene. Three of these genogroups (I,II, and IV) contain human strains, whereas genogroup III contains bovine strains and genogroup V contains mouse strains. Genogroups I, II, and III are divided further into genetic clusters, or genotypes, and contain 8, 17, and 2 clusters.

Immunity

Immunity to norovirus is group-specific, and during the infection immunity can be induced following challenge with a serologically distinct strain. Repeated exposure also seems to lead to resistance to re-infection. The mechanism of immunity is unclear, as according to the research studies, the antibody titers and the resistance to the norovirus infection do not correlate with each other.

Pre-analytical steps

Refer to the procedure 5.6: stool sampling

Analytical procedure

Real time RT-PCR assays are the most common approach for establishing a diagnosis of norovirus infection despite regular reverse transcriptase PCR could also be used. Virus-specific primers are used to amplify conserved regions of the genome (usually in the polymerase or VP1 genes). No single primer pair can detect all norovirus strains because of the high sequence diversity, but more than 90% of strains can be detected using two separate primer pairs for genogroup I and II Noroviruses.

The detection of the norovirus strains can be done using:

- Commercial kits, such as the AnDiaTec Norovirus test
- In house RT-PCR assays with the published primers and probes available in the market

We will use the AnDiaTec Norovirus test as an example:

The AnDiaTec Norovirus real time RT-PCR kit contains specific primers, TaqMan probes and additional material for the detection of the norovirus I and II in stool specimen.

Specific equipment and reagents for detection the norovirus by RT-PCR
The specific equipment detecting Norovirus with AnDiaTec Norovirus real time RT-PCR kit is listed below:

- LightCycler instrument
- LightCycler capillaries
- Capillary centrifuge
- Table centrifuge (10000 -12000 rpm)
- RNA-extraction kit

RT PCR assay procedure
The complete RT-PCR procedure is separated in three steps:

- RNA extraction: RNA-extraction from stool samples can be done using a commercially available RNA-Extraction kit according to the manufacturer's instructions.
- Reverse transcription of the RNA, amplification and cDNA detection is done in one step during the LightCycler PCR.
- Interpretation of the results using the LightCycler software is performed according to the manufacturer's instructions.

For more detailed information on the real time RT-PCR detection of the norovirus strains, please refer the manufacturer's instruction and assay manual.

Reporting

- There is no specific field for noroviruses infection in the list of reportable diseases to the public health system. Nevertheless, it should be reported under "Diarrhoea and gastroenteritis of presumed infectious origin (A09)" included in the list of reportable diseases. As the list is regularly updated, you are recommended to follow up the updates with NCDC.
- In the meantime, like any other diseases, patients and clinicians should be provided with the analysis reports.

10.9. Hepatitis A – general facts

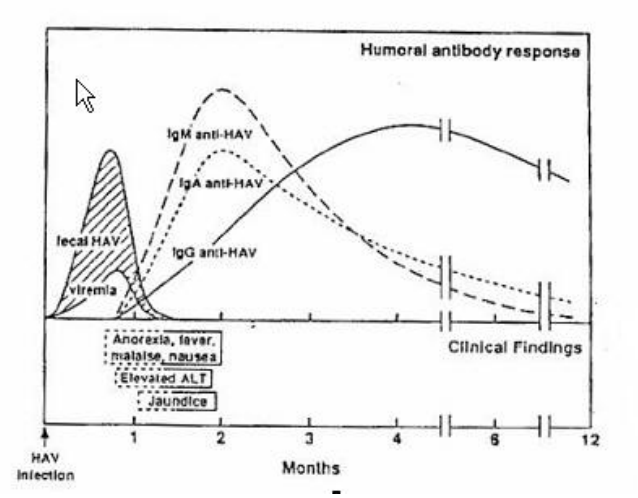
Clinical manifestation

Infection with Hepatitis A is a self-limited disease and chronic stage or other complications are rare. The clinical symptoms of the diseases are:

- Early influenza like symptoms (2 to 6 weeks)
- **Diarrhea**
- Fatigue
- Fever
- **Abdominal pain**
- **Nausea**
- **Appetite loss**
- Depression
- Jaundice
- Weight loss
- **Sharp pains in the right-upper quadrant of the abdomen**

Epidemiological characteristics

Infection with Hepatitis A virus is a self-limited disease, which is transmitted through the fecal-oral route. With improved sanitation and hygiene, infections are delayed and consequently the number of persons susceptible to the disease increases.



Pathogen

The cause of **hepatitis A is hepatitis A virus** (HAV)-non enveloped positive strand RNA virus with a linear single strand genome, encoding for only one known serotype. HAV has four major, structural polypeptides and it localizes exclusively in the cytoplasm of human hepatocytes.

Diagnosis strategy

Anti –HAV IgM are detectable for three weeks after exposure, their titer increases over 4-6 weeks and then declines to non-detectable levels generally within 6 months of infection.

Detection of HAV IgM is usually performed after the onset of the hepatitis clinical symptoms for confirmation of the diagnosis.
Anti HAV IgG are detectable later but persist lifelong after acute infection. Detection of the HAV IgG alone indicates a past infection; the test is usually requested by the physicians for different diagnosis purposes.

Pre-analytical procedures

Refer to procedure number 5.4: blood sampling

10.10. Hepatitis A – Diagnosis using ImmunoComb assay

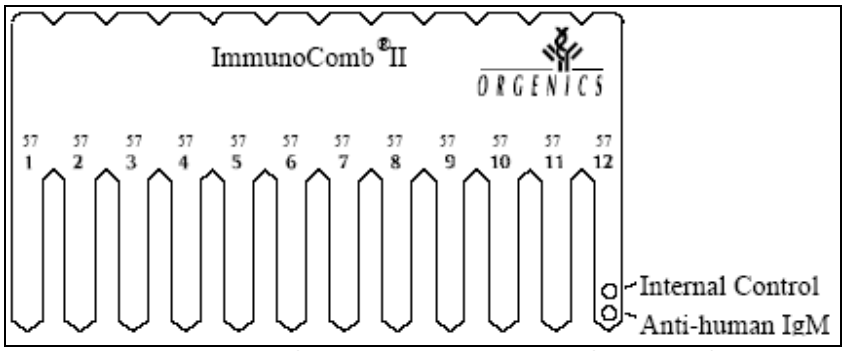
Identification of HAV IgM using ImmunoComb assay

Principle of the Assay:

ImmunoComb HAV IgM is a solid phase enzyme immunoassay (EIA) based on the sandwich principle. Solid phase is a card with 12 projections (teeth). Each tooth is sensitized at two positions:

- Upper spot – human IgM (Internal control)
- Lower spot – inactivated HAV antigen

The developing plate has 6 rows (A-F) of 12 wells, each row containing reagent solution ready to use at a different step in the assay. The test is performed stepwise, by moving the comb from row to row, with incubation at each step.



Note: We are providing a summary of the operating mode. Refer to the full documentation of the provider.

Precautions

- Bring all components, developing plates, combs, reagents and specimens to room temperature and perform the test at room temperature (22°–26°C).
- To ensure the proper functioning of the test, do not touch the teeth of the comb.
- Do not remove the foil cover of the developing plate. Break the foil cover by using the disposable tip of the pipette or the perforator only when instructed to do so by the test instructions.
- Unless especially mentioned, perform incubations at 37°C, when washings should be carried out at room temperature (22°–26°C).
- Use a timer and scrupulously respect timing recommended for each step.

Principle of the test:

- At the outset of the test, serum or plasma specimens are pretreated with anti-human IgG (stripping), in order to prevent interferences as a result of competition by anti IgG, and by rheumatoid factor.
- Pretreated specimens are further incubated with the solution in the wells of row A of the developing plate.
- The comb is than inserted in the wells of row A. Anti-IgM, if present in the specimens, will specifically bind to the antigens on the lower spot on the teeth of the comb.
- Unbound components are washed away in row B. In row C, anti-IgM captured on the lower spots of the teeth, and the human IgM on the upper spots (internal control), will react with alkaline phosphatase (AP)-labeled anti-human IgM antibodies.
- In the next two rows, unbound components are removed by washing. In row F, the bound (AP) will react with chromogenic components.
- The results are visible as gray-blue spots on the surface of the teeth of the comb.

Preparing the developing plate

Incubate the developing plate in an incubator at 37°C for 45 minutes. Cover the worktable with absorbent tissue to be discarded as biohazardous waste at the end of the test. Mix the reagents by shaking gently the developing plate.

Preparing the comb

Tear the aluminum pouch of the comb and developing plate or only a part. You may use the entire comb and developing plate or only a part.

→ To use part of a comb:

Determine how many teeth you need for testing the specimen and controls. You need one tooth for each test. Each tooth displays the code number ‘01’ of the kit, to enable identification of detached teeth. Bend and break the comb vertically or cut with scissors to detach the required number of

teeth (number of tests plus 2 controls). Return the unused portion of the comb to the aluminum pouch (with desiccant bag).

Close pouch tightly with a paper clip, to maintain dryness. Store the comb in the original kit box at 2°–8°C for later use.

Pretreatment of Specimens and Controls

- For each specimen and control, dispense 100 µl of stripping solution into a microtube or microtitre well.
- To each microtube or well, add 25 µl of a specimen, or of the Positive Control or Negative (untreated).
- Control supplied with the kit. Mix by repeatedly refilling and ejecting the solution.
- Set the timer and incubate for 10 minutes at room temperature (22°–26°C).

Adding Pretreated Specimens to Developing Plate

- Pipette 25 µl of a pretreated specimen. Perforate the foil cover of one well of row A of the developing plate with the pipette tip or perforator and dispense the specimen at the bottom of the well. Mix by repeatedly refilling and ejecting the well. Discard pipette tip.
- Repeat step 4 for the other pretreated specimens and the two pretreated controls. Use a new well in row A and change pipette tip for each specimen or control.
- Set the timer and incubate for 10 minutes at 37 C.

Analytical steps

1. Antigen – Antibody Reaction (Row A of the developing plate)
- Insert the comb (printed side facing you) into the wells of row A containing specimens and controls.
- Mix: Withdraw and insert the comb in the wells several times.
- Leave the comb in row A and incubate for 30 minutes at 37°C. Set the timer. Close to the end of 30 minutes, perforate the foil of row B using the perforator. Do not open more wells than needed.
- At the end of 30 minutes, take the comb out of row A.
- Absorb adhering liquid from the pointed tips of the teeth on clean absorbent paper. Do not touch the front surface of the teeth.

2. First Wash (Row B)
- Insert the comb into wells of row B.
- Agitate: Vigorously withdraw and insert the comb in the wells for at least 10 seconds. Repeat agitation several times during 2 minutes.
- Meanwhile, perforate the foil of row C. After 2 minutes, withdraw the comb and absorb adhering liquid as in step 7c.

3. Binding of Conjugate (Row C)
- Insert the comb into the wells of row C. Mix as previously. Incubate developing plate with comb for 20 minutes at 37°C. Perforate the foil of row D. After 20 minutes, withdraw the comb and absorb adhering liquid.

4. Second Wash (Row D)
- Insert the comb into the wells of row D; repeatedly agitate for 2 minutes.
- Meanwhile, perforate the foil of row E. After 2 minutes, withdraw the comb and absorb adhering liquid.

5. Third Wash (Row E)
- Insert the comb into the wells of row E. Repeatedly agitate for 2 minutes. Meanwhile, perforate the foil of row F. After 2 minutes, withdraw the comb and absorb adhering liquid.

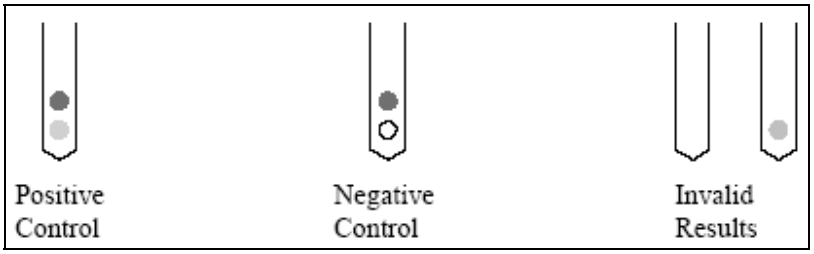
6. Color Reaction (Row F)
- Insert the comb again into row F. Mix. Incubate the developing plate with the comb for 10 minutes at 37°C. After 10 minutes, withdraw the comb.

7. Stop Reaction (Row E)

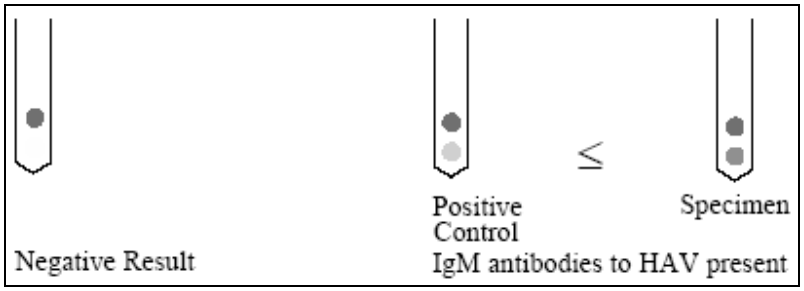
- Insert the comb again into row E. After 1 minute, withdraw the comb and allow it to dry in the air.

Validation of the results

In order to confirm that the test functions properly and to demonstrate that the results are valid, the following four conditions must be fulfilled.



- The Positive Control must produce two spots on the comb tooth.
- The signal of the lower spot of the Positive Control should be at least 35 when measured using program 91 of the Combscan refractometer (approximately equal to the second color frame starting from the left, if assessed using a Combiscale).
- The Negative Control must produce an upper spot (internal control). The lower spot will either not appear or appear faintly, without affecting the interpretation of the results.
- Each specimen tested must produce an upper spot (internal control). If any of the four conditions are not fulfilled, the results are invalid, and the specimens and controls should be retested.



Quantitative interpretation of results

Visual reading

- Compare the intensity of the lower spot of each specimen tooth with that of the lower spot of the Positive Control tooth.
- A spot presenting intensity higher than or equal to that of the Positive Control indicates the presence of IgM antibodies to virus. A spot with intensity slightly lower than that of the Positive Control should be considered an indeterminate result and the specimen should be retested. A faint spot or no spot should be considered a negative result.

Quantification

- Combscan refractometer (see the Combscan User Manual for detailed instructions) enables rapid and objective measurement of color intensity of the spots on the Immunocomb. Read test results as relative absorbance using program # 92 of the Combscan, designating reading of the lower spot by entering '1.' When the message 'spots: 1,2,3' appear on the screen.
- A specimen reading equal to or higher than 700 is considered positive for the presence of IgM antibodies to HAV IgM. A specimen reading between 600 and 700 should be considered an indeterminate result for the presence of IgM antibodies to Hepatitis A and should be retested. A specimen reading lower than 600 indicates a negative result.

10.11. Hepatitis A – Diagnosis using ELISA

Equipment needed

- Dispensing system and/or pipette (single or multichannel), disposable pipette tips
- Dry incubator or water bath, 37±0.5°C
- Microshaker for dissolving and mixing conjugate with samples
- Microwell plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm
- Microwell aspiration/wash system

Reagents and consumables needed

- Freshly distilled or deionized water
- Disposable gloves and timer
- Appropriate waste containers for potentially contaminated materials
- Disposable V-shaped forceps
- Absorbent tissue or clean towel
- Reagents: plate, controls, conjugate, buffer and chromogen (included in the ELISA kit)

Precautions

- All reagents and samples should **reach room temperature (18-30°C)** for at least 15-30 minutes before the initiation of the assay procedure.
- Washing solutions should be **checked for the presence of salt crystals**. If crystals have been formed in the solution, warming **at 37°C is recommended**. Wash buffers should be diluted 1 to 20 with distilled or de-ionized water.
- Mark three wells as Negative control (e.g. B1, C1, D1), two wells as Positive control (e.g. E1, F1) and one Blank well.

Analytical steps

ELISA reaction has already been largely described in procedure 4.10 about adenoviruses and we do not want to repeat it. We are only providing a summary table of the analytical steps. In any case, please refer to the full procedure provided along with the kit you own.

SUMMARY OF THE ASSAY PROCEDURE:	
Dilute the sample with normal saline	1:1000
Add sample	100ml
Incubate	20-60 minutes
Wash	5 times
Add HRP-Conjugate	100ml
Incubate	30-60 minutes
Wash	5 times
Coloring	50ml A + 50ml B
Incubate	15-20 minutes
Stop the reaction	50ml stop solution
Read the absorbance	450nm or 450/630 nm

Interpretation of Results

Note: Each commercial kit is provided with the algorithm for calculation of cut-off value. **If one of the control values does not meet the control range specifications, the test is invalid and must be repeated.**

If the positive and negative controls meet the criteria, then the cut-off value is calculated.

- The algorithm for calculation of cut-off value for the specific example used is :

  (C.O.) = Nc × 2.1

  Nc is the mean absorbance value of the three negative controls. If one of the negative controls does not meet the criteria, then it is disregarded, and the mean absorbance value is calculated using the remaining controls.

The results for each specimen are calculated by relating each sample (S) optical density (OD) value to the cut-off value (C.O.) of the plate.

- **Negative Results** (S/C.O. <1): samples giving absorbance less than the cut-off value are negative for this assay, which indicates that no IgM class antibodies to HAV have been detected. Therefore, there are no serological indications for recent infection, and the patient is probably not infected with hepatitis A virus.
- **Positive Results** (S/C.O.≥1): samples giving an absorbance greater than or equal to the cut-off value are initially positive, which indicates that IgM class antibodies to HAV have been detected, signing a recent infection.
- **Ambiguous results:** (S/CO =0.9-1.1) Samples with absorbance to cut-off ratio between 0.9 and 1.1 are considered equivocal. Retesting of these samples in duplicates is recommended. Repeatedly reactive samples can be considered positive for IgM antibodies to HAV. Another blood sampling should be performed 10 days after the first testing.

10.12. Hepatitis A – Results reporting

- **Reporting to the clinicians and/or a patient.** The positive, negative and equivocal results are reported to the clinicians and/or a patient using a laboratory reporting sheet.
- **Report to the local public health authorities and NCDC.** All laboratories must notify the local public health service whenever they diagnose, suspect, or even receive positive laboratory results for Hepatitis A virus. Acute infection with hepatitis A virus is included in the list of *urgently notifiable diseases*; therefore, the notification should be done to the local public health center on the same business day using the *urgent notification card (see below)*. The information is collected by the epidemiologist, analyzed and reported to the NCDC according to the established schedule.
- Consult the table for *urgently notifiable diseases* reporting and flow of notification (procedure 12.4).

11. Analytical procedures – parasitic infections

11.1. Amebiasis

Clinical manifestation

- Acute intestinal amebiasis: dysenteric syndrome without fever, liquid afecal stools, bloody phlegm with tenesms. Without treatment, can evaluate to a post amibia colitis and/or visceral spreading.
- Visceral amebiasis: usually located in the liver (more rarely in the lung or the brain). Clinical signs are variables, from classical hepatitis to liver abscess with high fever. Alteration of the general status, painful hepatomegalia, sometimes pleuropulmonary syndrome, on the right

Pathogen

Entamoeba histolytica, protozoa, is a pathogenic amibia of the intestine. Can be found in 3 different forms:

- Vegetative form “minuta”: non hematophages
- Vegetative form “histolytica”: hematophage
- Cystic forms

At least 6 different types of pathogenic ameba can be found in human. Only *E. histolytica* can induce visceral infection.

Epidemiology and context

- Contamination: water and food contaminated by cysts and/or ameba
- Cycle :
  - Non pathogenic (infestation or healthy carrier): intestinal presence of vegetative forms and cysts
  - Pathogenic (disease): penetration of the histolytica form in the intestinal mucus membrane, with possible visceral spreading
- Prophylaxis: water disinfection (drinking water, fruit and vegetables washing water), hand washing, isolation of the toilets

Equipment and reagents needed

Microscopy:

- Microscope
- Slides and coverslides
- Eventually, Lugol reagent

Stool formaldehyde concentration:

- Ether, glass conic tubes, glass agitator, Pasteur pipette, glass conic urine collector (250 ml)
- Ritchie reagent:
- 100 ml formaldehyde
- 9 g NaCl
- 900 ml distillated water
- Mix the three products and keep the reagent in a brown glass vial. Always put the lid back on the vial immediately (formaldehyde smokes are toxic)

Pre-analytical procedures

Refer to procedure about stool sampling (5.6). Handle the stools immediately.

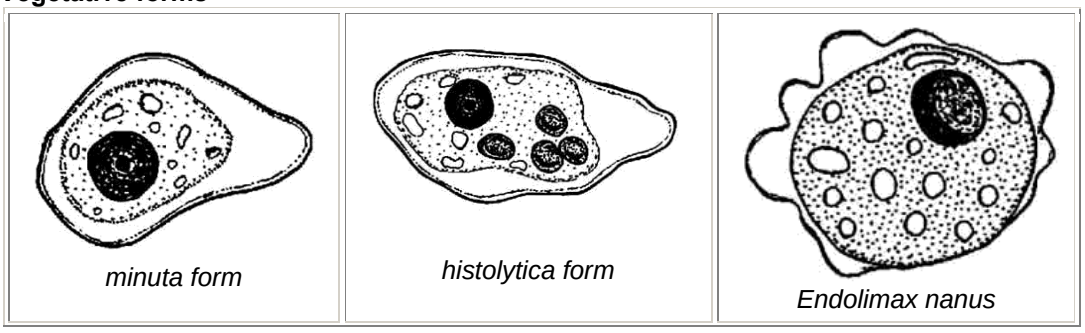
Routine diagnosis

Microscopy

Prepare a thin slide with the stool, including a cover slide. Pay attention to choose the parts of the stools either bloody or mucus parts. Observe using X100 lenses.

Below are several tables summarizing the different possible ameba that can be found in the stool:

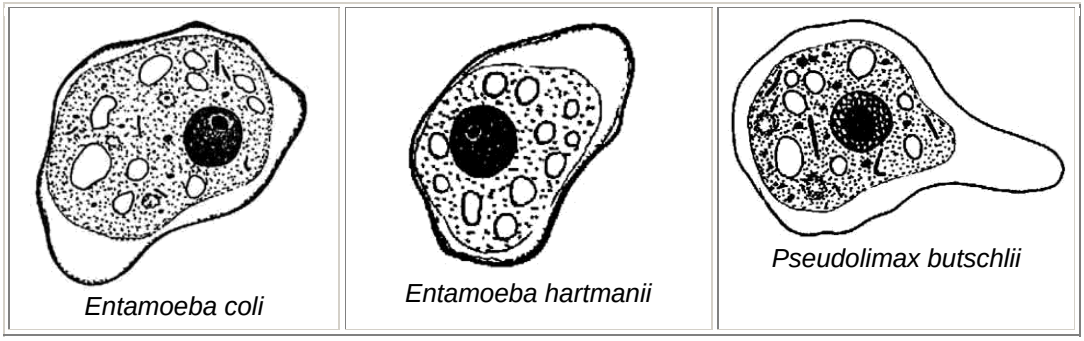
1- Vegetative forms



minuta form

histolytica form

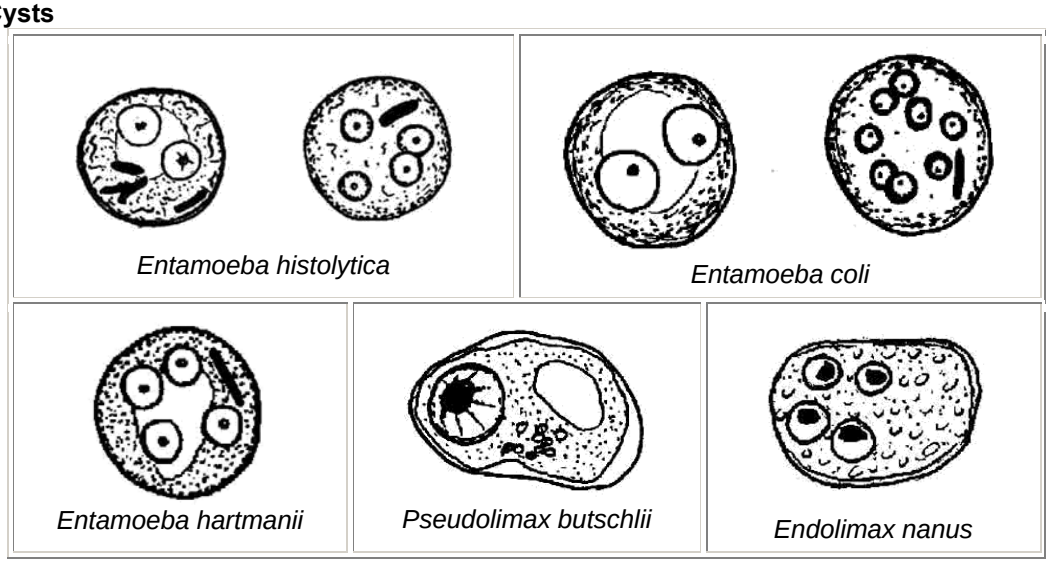
Endolimax nanus



Entamoeba coli | *Entamoeba hartmanii* | *Pseudolimax butschlii*

These vegetative forms are quite difficult to observe. It is possible to dilute some of the stool in 0,5ml of Lugol (see Gram stain procedure in paragraph 7.3) in order to make them more visible.

2- Cysts



Entamoeba histolytica | *Entamoeba coli*

Entamoeba hartmanii | *Pseudolimax butschlii* | *Endolimax nanus*

Illustration 17. Nucleus cyst colored by Lugol

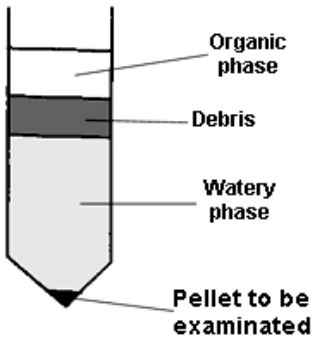
As for the vegetative forms, you can make these cysts more apparent by diluting the stools into couple of drops of Lugol. The photo shows a 4 nucleus cyst, colored by Lugol.

Cysts can be differentiated using 7 criteria, as shown on the table below:

Table 35. Criteria for differentiation of cysts

Name	*Entamoeba coli*	*Entamoeba histolytica*	*Entamoeba hartmanni*	*Pseudolimax butschlii*	*Endolimax nanus*
Size in µm	15-30	12-14	6-10	8-15	7-12
Number of nucleus	8 (16-32)	4	4	1	1-4

Refringency of the cysts	+++	+	+	+++	+/-
Crystalloids	needles	sausages	sausages	/	/
Vacuoles	/	/	/	large	/
Shapes	spherical - ovoid	spherical	spherical	variable	spherical - ovoid
Deformability	possible	rare	rare		



Nevertheless, direct microscopy sensitivity is usually quite low, and only the best laboratory technicians, with consequent stool parasites experience will be able to notify a large part of them.

This is the reason why a concentration method can be used, which will allow you a higher concentration of the cysts in the preparation.

Stool concentration technique: Ritchie concentration method (formaldehyde/ ether method)
CAUTION: Either handling is dangerous, pay attention to shut down your Bunsen burner and never smoke in the laboratory. Ideally, manipulate in a safe hood, or with laboratory windows wide opened.

Utilization:
This method is applicable for the cysts of ameba. The vegetative forms of ameba will be destroyed by the concentration and will not be seen anymore. Eggs of Ascaris and Ankylostome will also be destroyed by the method. The other egg parasites will be preserved and can be identified as well.

How to proceed:
In the 250 ml conic urine collector, dilute 1 volume of stool with 10 volumes of Ritchie reagent.

Let sediment stay during 30 seconds and pour into a glass conic tube that can fit into a centrifuge.

- Add ether in a proportion of 1/3 for 2/3 of liquid. Cap it with Parafilm or other ether-resistant cap.
- Close ether vial immediately.
- Vigorously agitate during 30 seconds. Let gently the gas to escape from time to time (be careful not creating stool aerosols).
- Centrifuge during 2 minutes at 2000g.
- Eliminate all 3 superior layers by rapid pouring, in order to keep the pellet only.
- Gather the pellet using a Pasteur pipette and prepare a new slide.

Observe it as for the direct microscopy.

Note: Some other concentration methods can be used for the same purpose, as the “Kato and Miura concentration method” (malachite green and glycerin).

Confirmation diagnosis

Confirmation of E. histolytica infection can be done through two different ways:

Rapid test:
Several rapid tests for the detection of E. histolytica can be used for confirmation.

As example, the rapid test "E. histolytica II" has already been used successfully in Georgia couple of years ago, when a large survey aiming at establishing E. histolytica prevalence was organized. This test is a rapid (3 hours) ELISA test detecting parasite adhesions (not the cysts) from stool specimens.

Nevertheless, its price is much more superior to the microscopy addressed above.

Serology:
Regular serological reaction on the blood can also be performed. It will only become positive after 8-10 days.

Reporting

As usual, results have to be provided to a clinician and a patient. Information to the public health system is transferred by a clinician on a monthly basis.

It is also recommended to perform a new examination at the end of the treatment, in order to confirm the total recovery and the absence of parasites in the stools.

Testing of other persons sharing the same food and life conditions with the patient can also be recommended.

12. Post – analytical procedures

12.1. Validation of results

Methodology

The analysis of the results will consist of checking whether the internal quality controls are correct, the quality management system functioned correctly and the results make sense.

Operating Mode

The microbiologist is in charge of validation. He will check:

If internal quality controls are correct. If this is not the case, the technician will discuss with the microbiologist, and if appropriate the analysis will be repeated.

The calibration of the equipment (if needed) was done correctly.

Make sure there has been no recent report of an eventual problem in the processing workflow.

The results concord with the clinical details given on the request form.

If the results are critical, discuss with the technician and proceed as indicated in SOP „Procedure of critical results report" 12.3 .

Abnormal laboratory results should always be verified by repeat testing and the microbiologist may request (at no cost for the patient) that the specimen be sent to a reference laboratory for confirmation.

Add a commentary according to the result of the analysis to facilitate their comprehension.

If the results need complementary analysis, the microbiologist will discuss with the clinician or the health provider. If they agree and if possible, extra analysis may be carried out. The microbiologist will then register the new analysis for the technicians to perform.

Print the reports.

Review his/her report and put his/her initials on the report; sign the report. By doing this, the microbiologist acknowledges he/she has reviewed the report and accepts responsibility for initiating any needed follow-up.

Validation and signature totally engages the microbiologist from medical, administrative and judicial points of view.

12.2. Printing - copying report of results

Methodology

The technician will check whether the internal quality controls are correct and that the analysis was carried out correctly before he/she prints or copies the results for the microbiologist to review for validation.

Operating Mode

Each laboratory will write out different steps according to its way of proceeding.

The technician will proceed as indicated in each specific SOP to verify the analysis was carried out correctly; he/she will check the internal quality controls.

If all is correct, he/she will copy or print out the results. Each result will be indicated with the identification number of the patient.

Once copied all the results written down must be checked for correct copying. Copying and recopying mistakes are the most common mistakes done in the laboratories.

12.3. Transmission of results (critical results reporting)

Each laboratory should have already prepared carefully:

- How to transmit critical results (to a clinician and to the public health authorities).
- How to transmit reports easily, safely and at low cost, being sure it will reach the correct recipient.

Example of critical results transmission:

The microbiologist should recheck the critical result if possible. All critical results should be transmitted to a licensed health care provider immediately (without delay).

Call the patient's appropriate location (unit or clinic). Identify yourself as clinical laboratory personnel and ask to speak to a licensed health care provider.

"Good morning (afternoon or evening. My name is _____, from the Clinical Laboratories. I have a critical result to report to you on patient _____, are you a licensed health care provider?"
At that time, inform the licensed health care provider that you are reporting a critical laboratory result (or results) on a patient (identify the patient by last and first names and Medical Record Number).

"I have a critical result on patient _____, medical record number _____." "Are you ready to record the critical result [test name and result]?" or "Here is the critical result."

Note: If a patient has more than one critical result, all critical results may be conveyed during the call.
Upon completion of the critical result notification, the licensed healthcare provider must verbally read back ALL of the reported critical result(s) and properly identify themselves (at minimum with the first initial of their name and their entire last name), including their professional title.

Request the healthcare provider to *"Please read back the critical result and patient name/medical record number that I just reported to you, and please provide me with your name and professional title."*

12.4. Transmission of results (reporting to public health authorities)

Which diseases should be reported and to whom?

The table below summarizes the notification process for the Intestinal Infectious Diseases diagnosed in Georgia:

Table 36: List of urgently notifiable Intestinal Infectious Diseases

#	Name	ICD-10 code	Notification should be sent to the rayon CPH		Notification should be sent from the rayon CPH to the regional CPH and NCDC
			by health care providers	by laboratories	
10	Acute viral hepatitis A	B15	X	X	
14	Cholera	A00	X	X	X
15	Typhoid fever	A01	X	X	
16	Paratyphoid fever A B C	A01.1-4	X	X	
17	Other Salmonella infections	A02	X	X	
18	Shigellosis	A03	X	X	
19	Other bacterial intestinal infections	A04	X	X	
20	Among them Escherichiosis	A04.4	X	X	
21	Yersiniosis	A04.6	X	X	
22	Food-borne bacterial intoxications	A05	X	X	
23	among them Botulism	A05.1	X	X	X
24	Unspecified Intestinal infectious Diseases	A09	X	X	

How to report them?

Laboratories detecting or confirming a case of a notifiable disease from the list in the table above must follow the same requirements: urgently (during the same business day, but under no circumstances more than 24 hours from identification) notify the local Public Health Center by any available means of communication.

A person responsible for the test result should send notifications using the standard Laboratory Confirmation of a Communicable Disease Result/Notification Form #58/2. If notification is made by phone, the same format should be used (to be recorded in journal 60/B at the receiving end). In case of a negative result of a test, there is no need to send a notification to the Public Health Center. In such a case, a response should be sent on the same form to the physician requesting the test. Submission of only one notification to the Public Health Center is required even if more than one specimen of a similar type may be taken from the patient during an episode of illness. Confidentiality of all laboratory notifications is regulated by the Law on Health Care.

Confidential	Laboratory Result/Notification of a Communicable Disease Form #58/2
Case: Last Name l First Name l Middle Name l Age l Sex l Address [Apt #; Street; City (village); Country] l Tel: l Date spec. was taken ----/----/----	
Referred by ○ Self-referral ○ Physician ○ Health facility/CPH ○ Laboratory ○ Other_____	Contact information of the referring physician or institution: Name_____ Address_____ Telephone_____ Fax_____ E-mail_____
Result (outcome) (indicate if pathogen is isolated) _____	

Type of specimen	Smear/lavash/scrape/swab/biopt	Type of test performed	
○ Blood /Serum ○ CSF ○ Stool ○ Urine ○ Sputum Other_____	○ Pharyngeal ○ Naso-pharyngeal ○ Vaginal ○ Oral ○ Skin ○ Eye	Culture ○ Bacteriology ○ Virology ○ Parasitology	○ Serology (specify) _____ _____ ○ Microscopy ○ Histology ○ Molecular identification

Date and time of result:	Date and time of CPH notification:	Name and address of laboratory	Name and signature of the person responsible for the result:
Notification sent by [name and signature] :			Notification recipient: [name and position]

12.5. Transmission of results (Classification of the Laboratory Interventions)

When reports are prepared on the laboratory interventions (e.g. financial reports for insurance companies) the Classification of the Laboratory Interventions should be used. This Classification involves the standardized list of laboratory tests organized under the coding system. The Classification is available on the web page of the Ministry of Labor, Health and Social Affairs.

12.6. Laboratory Information Management System

A **Laboratory Information Management System (LIMS)** is computer software that is used in the laboratory for the management of samples, laboratory users, instruments, standards and other laboratory functions such as invoicing, media production and work flow automation. LIMS and Laboratory Information Systems (LIS) perform similar functions.
Regulations often do not exist about LIMS (as example, it is not clearly described in the ISO standards for laboratory accreditation such as ISO 15189 and ISO17025). Some main characteristics can nevertheless be highlighted.

Security and Confidentiality

LIMS contain highly sensitive data, related to the health status of numerous persons. They cannot be accessed by any person outside the authorized laboratory staff. In addition, it is recommended to the LIMS:

- To be password protected (one different login and password for each person)
- To track each activity made by each person in order to be able to go back to any possible mistake or imprecision
- To get different authorization levels corresponding to the different activities to be carried out by the different laboratory staffs (reception, data entry, validation, printing, statistics, etc.)
- To request a specific master keyword for any deletion or cancellation

In addition, all staff members need to sign a confidentiality statement clearly specifying that no personal data can be discarded under any circumstances and to any third party.

Backup and archives

LIMS need to be backuped daily, ideally on two different supports. Any recovery should be immediate.

Archiving also needs to be scheduled and planned for.

Documentation

One of the criteria stated in the official laboratory accreditation norms and standards concerns the detailed documentation of the LIMS. When buying or preparing such software, always keep in mind that detailed documentation of the feature and architecture will be requested if any official recognition (accreditation) is wished or planned.

13. Quality Assurance

13.1. QAM Location

Once finalized, the quality assurance manual will be distributed to all laboratory technical staff. Staff in charge of cleaning, disinfecting and washing will be provided with biosafety and hygiene security procedures. It will be the same for the quality assurance manual updates.

A complete copy of the quality assurance manual will be kept in the laboratory director office or quality manager office. This copy will be confidential as all the details on the different staff are present.

Note: The current manual only addresses one part of the microbiology analysis that should be performed by a regular microbiology laboratory. Below is the generic table of contents of a full QAM:

1. Introduction
2. Organization and management
3. Quality policy
4. Document management
5. Personnel
6. Safety and hygiene
7. Premises and environment
8. Equipment, reagents, consumables management
    a. Equipment
    b. Reagents, media and consumables or supplies
9. Pre-analytical procedures
    a. Sampling
    b. Sample transportation
    c. Reception and conservation
10. Analytical procedures
    a. Reagent procedures
    b. Media procedures
    c. Culture Identification procedures
    d. AST procedures
    e. PCR procedures
    f. Serology procedures
    g. Staining procedures
    h. Subcontracting
11. Post-examination procedures
12. Quality Assurance
    a. External QC procedures
    b. General QA procedures

c. Internal quality control procedures

13. Laboratory information system
14. Occurrence management
15. Improvement and evaluation of the Quality Management System
    a. Corrective actions and Preventive actions
    b. Internal audits
    c. External audits
    d. Management review
    e. Continual improvement
16. Customer service
17. Communications, reports and other interactions

13.2. Procedure flow in the lab

General facts

The laboratory has a defined path of workflow, which represents the sequence of activities from the initiation of a request for a service through the provision of those services, and any necessary follow-up. The path of workflow begins with an order for a laboratory examination (test) and proceeds to provision of the report, and any necessary follow-up consultation.

The laboratory uses the quality system essentials to manage its respective path of workflow, thus developing one universal set of policies, processes, and procedures for the quality system essentials that apply to all in the entire organization through the Quality Assurance Manual.

The quality system essentials are developed in each chapter of the Quality Assurance Manual and details for each responsibility, reporting relationships of all the people involved and procedures to be carried out.

Implementation of new test

First, the specific pre-analytic, analytic, and post-analytic work processes and procedures for the new testing need to be identified.

The specific pre-analytic, analytic, and post-analytic work processes and procedures need to be developed, validated, and documented as detailed in SOP document management ref 2.1.

They will then be inserted in the Quality Assurance Manual and be distributed to all staff concerned for application.

13.3. General QA procedures

Quality Assurance is entirely under the responsibility of the QA Manager. Each laboratory in Georgia needs to designate such a QA Manager. The QA Manager is not part of the regular hierarchy (i.e. he can also control the QA promotion and maintenance of the laboratory director).

The QA Manager is helped by two other persons: the equipment manager and the cold chain manager. In his daily work he can rely on the head of units and senior technicians/technologists.

The QA Manager initiates the writing of a QA Manual; he is also in charge of updating this manual, as well as the relations with clients and patients and the links with eventual official recognition agencies.

In a small laboratory, these three persons can easily be replaced by a single one.

Profile of the QA Manager:

The QA Manager should ideally be a medical doctor or a high level scientist. Nevertheless, a motivated laboratory technician could easily fill the vacancy.

Ideally, this person should benefit from a week-long training in general QA issue for microbiology laboratories, as organized and provided by the WHO Office in Lyon[4] titled Quality Management in Public Health Laboratories.
This person should also look very carefully at various guidelines developed in the field of the QA in Georgia (VPD guidelines, Laboratory network proposal[5], etc.)

13.4. External QC procedures

The Laboratory Quality Assurance Unit is under establishment at the NCDC. It will manage a voluntary external QC program for the laboratories in various lab sciences. The program will assist the laboratories in monitoring the degrees of variability and bias in their assays. The information received from the program can then be used to a) eliminate bias or precision problems in the assay system and b) confirm the quality of analysis and increase the confidence level of the laboratory.

Services provided to participating laboratories will include: trainings, consultations, proficiency testing, reference materials.

This programme is still under development, but will be made available soon. Information will be provided to public laboratories upon its kick-off.

[4] More details as well as contact information can be found at:
http://www.who.int/csr/ihr/lyon/quality/en/index.html
[5] http:/www.curatiofoundation.org/

13.5. IQC procedures, logbooks, data management and representation

The IQC procedures imply the following:

- Quality control of staining methods
- Quality control of media
- Quality control of reagents

IQC of all above mentioned methods is performed using ATCC cultures and filling corresponding spread sheets (see Attachment 2) with every prepared lot or reagents, similarly is done with the Gram stain.

- Incubator use, control, and maintenance
- Refrigerator and freezer use, control, and maintenance
- Autoclave and oven use, control, and maintenance
- Incubators, refrigerators, autoclaves, oven are controlled by thermometers, temperatures are recorded by laboratory personal (usually technician) and corresponding forms are filled in.
- Sterility checking is performed by placing dishes or tubes from each lot into incubator for growth observation.
- All temperature control sheets are attached to corresponding equipment.

Media preparation procedures are part of the IQC (see Attachment 3).

13.6. Control material

The NCDC is working on the procedures and regulations for supply, distribution and management of the ATCC strains, in parallel to the future national EQC programme to be starting soon. Once both are finalized, public laboratories will be able to obtain the control materials for quality control purposes from NCDC.

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Attachment 1. Equipment Log Books

Temperature Chart

TEMP LOG FOR:						LAB NAME:							
JAN		FEB		APR		JUN		JUL		SEP		NOV	
1		23		15		6		29		20		10	
2		24		16		7		30		21		11	
3		25		17		8		31		22		12	
4		26		18		9		AUG		23		13	
5		27		19		10		1		24		14	
6		28		20		11		2		25		15	
7		29		21		12		3		26		16	
8		MAR		22		13		4		27		17	
9		1		23		14		5		28		18	
10		2		24		15		6		29		19	
11		3		25		16		7		30		20	
12		4		26		17		8		31		21	
13		5		27		18		9		OCT		22	
14		6		28		19		10		1		23	
15		7		29		20		11		2		24	
16		8		30		21		12		3		25	
17		9		MAY		22		13		4		26	
18		10		1		23		14		5		27	
19		11		2		24		15		6		28	
20		12		3		25		16		7		29	
21		13		4		26		17		8		30	
22		14		5		27		18		9		DEC	
23		15		6		28		19		10		1	
24		16		7		29		20		11		2	
25		17		8		30		21		12		3	
26		18		9		JUL		22		13		4	
27		19		10		1		23		14		5	
28		20		11		2		24		15		6	
29		21		12		3		25		16		7	
30		22		13		4		26		17		8	
31		23		14		5		27		18		9	
FEB		24		15		6		28		19		10	
1		25		16		7		29		20		11	
2		26		17		8		30		21		12	
3		27		18		9		SEP		22		13	
4		28		19		10		1		23		14	
5		29		20		11		2		24		15	
6		30		21		12		3		25		16	
7		31		22		13		4		26		17	
8		APR		23		14		5		27		18	
9		1		24		15		6		28		19	
10		2		25		16		7		29		20	
11		3		26		17		8		30		21	
12		4		27		18		9		31		22	
13		5		28		19		10		NOV		23	
14		6		29		20		11		1		24	
15		7		30		21		12		2		25	
16		8		31		22		13		3		26	
17		9		JUN		23		14		4		27	
18		10		1		24		15		5		28	
19		11		2		25		16		6		29	
20		12		3		26		17		7		30	
21		13		4		27		18		8		31	
22		14		5		28		19		9			

Pipette control form

Trade mark:

Model:

Identification N°

Date:

WEIGHT TEST					Electronic scale /precision 1mg /1µl										
Specification verification												Accuracy uncertainty		Repeatability	
Minimal volume Vo	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	V x µl	Vx-Vo µl	Vx-Vo %	SD	CV %
Maximal volume Vm															

Conclusion:

Signature:

Attachment 2. Tests IQC forms

Month/Year:	AFI Program: DAILY QUALITY CONTROL																																
ATCC #	**DAILY TESTS**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
	GRAM STAIN	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
25923	*Staph aureus* (GRAM +)																															
25922	*E.coli* (GRAM =)																															
	CATALASE	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
25923	*Staph.aureus* (+)																															
19615	*Strep pyogenes* (=)																															
	OXIDASE	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
27853	*P. aeruginosa* (+)																															
25922	*E.coli* (=)																															
The following QC tests are performed ONLY when patient samples are tested.																																
	DESOXYCHOLATE	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
6305	*Strep pneumoniae* (+)																															
10556	*Staph sanguis* (=)																															
	COAGULASE	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
25923	*Staph aureus* (+)																															
12228	*Staph epidermidis* (=)																															
	P-DISC	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
6305	*Strep pneumoniae* (+)																															
10556	*Strep sanguis* (=)																															
	INDOLE	**LOT**					**EXP DATE**							**New LOT**						**EXP DATE**							**CHANGED**					
25922	*E. coli* (+)																															
27853	*P. aeruginosa* (=)																															
* Please indicate a change in LOT NUM by placing an asterisk next to the corresponding test on the day it change.																																
COMMENTS / CORRECTIVE ACTION																																

REVIEWED BY : _____ **DATE** _____

Antibiotic Quality Control: *Staphylococcus* species								
Staphylococcus aureus **ATCC 25923**	**Laboratory Tech Initials:**							
Antibiotics	**Initials**	**Potency (ug)**	**Expected Zone Size (mm)**	**Date**	**Date**	**Date**	**Date**	**Date**
Penicillin	P	10	28-29					
Ciprofloxacin	CIP	5	22-30					
Clindamycin	CC	2	24-30					
Erythromycin	E	15	22-30					
Gentamicin	GM	10	19-27					
Nitrofurantoin	FD	300	18-22					
Oxacillin	OX	1	18-24					
Rifampin	RA	5	26-34					
Tetracycline	TE	30	24-30					
Trimethoprim	TMP	5	19-26					
Trimethoprim-Sulfamethoxazole	SXT	1.25/23.75	24-32					
Vancomycin	VA	30	17-21					
Comments/Corrective Actions								
Reviewed by:								

Antibiotic Quality Control: Gram Negative Rods Group 1								
Escherichia coli **ATCC 25922**	**Laboratory Tech Initials:**							
Antibiotics	**Initials**	**Potency (ug)**	**Expected Zone Size (mm)**	**Date**	**Date**	**Date**	**Date**	**Date**
Amikacin	AN	30	19-26					
Ampicillin	AM	10	16-22					
Ampicillin-Sulbactam	SAM	10/10	19-24					
Aztreonam	ATM	30	28-36					
Carbenicillin	CB	100	23-29					
Cefotaxime	CTX	30	29-35					
Ceftazidime-Clavulanic Acid	CAZ/CLA	30/10						
Ceftriaxone	CRO	30	29-35					
Cephalothin	CF	30	15-21					
Chloramphenicol	C	30	21-27					
Ciprofloxacin	CIP	5	30-40					
Gentamicin	GM	10	19-26					
Imipenem	IPM	10	26-32					
Nalidixic acid	NA	30	22-28					
Nitrofurantoin	FM	300	20-25					
Tetracycline	TE	30	18-25					
Ticarcillin-Clavulanic Acid	TIM	75/10	24-30					
Trimethoprim-Sulfamethoxazole	SXT	1.25/23.75	24-32					
Comments/Corrective Actions								
Reviewed by:								

Antibiotic Quality Control: Gram Negative Rods Group 2								
Pseudomonas aeruginosa **ATCC 27853**	**Laboratory Tech Initials:**							
Antibiotics	**Initials**	**Potency (ug)**	**Expected Zone Size (mm)**	**Date**	**Date**	**Date**	**Date**	**Date**
Ceftazidime	CAZ	30	22-29					
Ciprofloxacin	CIP	5	25-33					
Gentamicin	GM	10	16-21					
Piperacillin	PIP	100	25-33					
Comments/Corrective Actions								
Reviewed by:								

Attachment 3. Media preparation IQC forms

(From NAMRU-3, Cairo, Egypt)

Hospital Number	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Kliger's Iron Agar (KIA)

Date	Number	Sterility* Check	QC Organisms**			Initials	Remarks
			S. typhimurium (ATCC 14028)	E. coli (ATCC 25922)	P. aeruginosa (ATCC 27853)		
			K/A/H_2S/g	**A/A/g**	K/K		**Date/ #Discarded**

Expected QC Results: Alkaline/Acid H_2S Gas

Acid/Acid Gas Alkaline/Alkaline Supervisor Review:

_____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name: _____ _	Quality Control Program: Media preparation

Blood Agar (BA)

Date	Number	Sterility* Check	QC Organisms**				Initials	Remarks
			E. coli (ATCC25922)	*S. pneumoniae* (ATCC49619)	*S. aureus* (ATCC 25923)	*S. pyogenes* (ATCC 19615)		
			G	**G/α**	**G/β**	**G/β**		**Date/ #Discarded**

Expected QC Results: G =Growth G/α =Growth

G/ β =Growth G/ β =Growth Supervisor Review: _____

α =Alpha

β = Beta β = Beta

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name Bacteriology Section	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Cary-Blair (CB)

Date	Number	Sterility* Check	QC Organisms**		Initials	Remarks
			E. coli (ATCC25922)	*Sh. flexneri* (ATCC 12022)		
			G	**G**		**Date/ #Discarded**

Expected QC Results: G =Growth G =Growth

Supervisor Review: _____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name:_____	Quality Control Program: Media preparation

Chocolate Agar (CHOC)

Date	Number	Sterility* Check	QC Organisms**		Initials	Remarks
			H. influenzae (ATCC49247)	*N.* meningitidis (ATCC 13090)		
			G	**G**		**Date/ #Discarded**

Expected QC Results: G =Growth G =Growth

Supervisor Review: _____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Hektoen Enteric Agar (HE)

Date	Number	Sterility* Check	QC Organisms**			Initials	Remarks
			S. typhimurium (ATCC 14028)	*Sh. flexneri* (ATCC 12022)	*E. faecalis* (ATCC 29212)		
			G/GB	**G/G**	**NG**		**Date/ #Discarded**

Expected QC Results: G/GB =Growth G/G =Growth NG =No Growth Supervisor Review: _____

Grn/Blue w/Black center Greenish blue

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Lysine Iron Agar (LIA)

Date	Number	Sterility* Check	QC Organisms**			Initials	Remarks
			Sh. flexneri (ATCC 12022)	P. mirabilis (ATCC 25933)	*S. typhimurium* (ATCC 14028)		
			K/A	**R/A**	K/K H_2S		**Date/ #Discarded**

Expected QC Results: Alkaline/Acid Deep Red/Acid Alkaline/Alkaline H_2S Supervisor Review: _____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name: _____	Quality Control Program: Media preparation

MacConkey Agar (MAC)

Date	Number	Sterility* Check	QC Organisms**			Initials	Remarks
			E. coli (ATCC 25922)	P.vulgaris (ATCC 13315)	*S. aureus* (ATCC 25923)		
			LF	**NLF**	NG		**Date/ #Discarded**

Expected QC Results: LF =Lactose-Fermenting NLF =Non-Lactose- NG = No Growth

Supervisor Review: _____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name: _____ __	Quality Control Program: Media preparation

Mueller-Hinton Agar (MHA)

Date	Number	Sterility* Check	QC Organisms**				Initials	Remarks
			E. coli (ATCC25922)	*P. aeruginosa* (ATCC27853)	*S. aureus* (ATCC25923)	*E. faecalis* (ATCC29212)		
			G	**G**	**G**	**G**		**Date/ #Discarded**

Expected QC Results: G =Growth G =Growth G =Growth G =Growth

Supervisor Review: _____

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Salmonella-Shigella Agar (SS)

Date	Number	Sterility* Check	QC Organisms**			Initials	Remarks
			S. typhimurium (ATCC 14028)	*Sh. flexneri* (ATCC 12022)	*E. faecalis* (ATCC 29212)		
			Colorless/H_2S	**Colorless**	I		**Date/ #Discarded**

Expected QC Results: Colorless/H_2S
Colorless I =Inhibited Supervisor Review: _____

Procedures:
*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.
**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.

Hospital Name	Media Quality Control	
	Prepared by:	Date:
	Reviewed by:	Date:

Urea Broth (Urea)

Date	Number	Sterility* Check	QC Organisms**		Initials	Remarks
			E. coli (ATCC 25922)	*P. vulgaris* (ATCC 13315)		
			NEG	**POS**		**Date/ #Discarded**

Expected QC Results: NEG =No Color POS =Deep Red

Supervisor Review: _____ Change

Procedures:

*Place all media in the incubator at 37°C for 24 hrs. Look for bacterial growth and record the number of contaminated plates. Discard the entire batch if more than 5% of the plates are contaminated. If entire batch passes the sterility check, store all plates at 2-8°C.

**Inoculate media with the proper ATCC bacterial strains to determine proper biochemical reactions. Repeat quality control tests, using a fresh bacterial stock, if reactions are not within standard. If the media fails to react as expected, throw out the batch and inform the supervisor ASAP.