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manual

QUALITY ASSURANCE FOR MICROBIOLOGY IN FEED ANALYSIS LABORATORIES

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QUALITY ASSURANCE FOR MICROBIOLOGY IN FEED ANALYSIS LABORATORIES

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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Author
Richard A. Cowie
MIBMS FCQI CQP
Senior Quality Assurance Manager
SRUC
Ferguson Building
Craibstone Estate
Aberdeen
AB21 9YA
Scotland
E-mail: richard.cowie@sruc.ac.uk

Editor
Harinder P.S. Makkar
Animal Production Officer
Animal Production and Health Division
FAO, Rome, Italy

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Contents

Foreword

Animal feeding impacts on many areas of agriculture: productivity, environmental emissions, water pollution, land use, animal health, product safety, product quality and animal welfare.

Every sector of the livestock industry, the associated services and the wellbeing of both animals and humans are influenced by animal feeding. Proper animal feeding is the supply of a diet balanced in all nutrients and free from contaminants and undesirable substances, at a level that meets the production objective (considering the animal's physiological state) and generates animal products that are safe for human consumption.

One of the most significant contaminants of animal feed are microbiological agents. The detection and enumeration of harmful bacteria, yeasts, fungi and parasites is imperative for the health of the animals and of the humans consuming animal products. A robust Quality Management System, within microbiology laboratories engaged in testing animal feed, is vital to guarantee that only reliable data is produced which ensures confidence in the microbiological testing undertaken.

Reports received from international experts visiting animal nutrition laboratories which are engaged in analysing feeds and feed ingredients in developing countries, highlight the need to strengthen quality assurance systems in these laboratories. Without a robust Quality Management System in place, the microbiology laboratory personnel are unable to evaluate the quality of the results being generated. Various ring trials conducted in developed countries have shown an unacceptable variation for some matrices being routinely determined in feed analysis laboratories. Similarly evidence received from the feed industries in developing countries on the reliability of feed analysis data suggests this is inconsistent. Therefore an urgent need to produce a document covering quality assurance systems was realized.

A previous document (Quality Assurance for Animal Feed Analysis Laboratories) was developed and prepared by a panel of nine experts. The emphasis was on the basic analysis used for determining the nutritional value of feeds and feed ingredients. The document gave a comprehensive account of good laboratory practices, quality assurance procedures and examples of standard operating procedures as used in individual specialist laboratories.

At the time of preparation a need was identified for an additional document dealing specifically with microbiological procedures and quality assurance in microbiology laboratories. One of the original panel of experts, with suitable microbiology and quality assurance experience, was approached to write such a document. This document has been peer reviewed by a number of international experts. The adoption of the practices and procedures in the manual will assist microbiology laboratories in acquiring the recognition of competence required for certification or accreditation and will also enhance the quality of the microbiological data generated by feed analysis laboratories. In addition, ensuring good laboratory practices presented in the document will enhance the health and safety of the laboratory workers, protect the environment from laboratory-discharged pollutants and increase the efficiency of laboratories. The document will also provide a strong base for microbiology laboratories on which they can develop a system which will meet the requirements of international standards. It will be useful for Laboratory Practitioners, Laboratory

Analysts, Laboratory Managers, research students and teachers and it is hoped that it will enable workers in animal industry to appreciate the importance of proven reliable data and the associated quality assurance approaches. This document, through increasing skills and knowledge of laboratory personnel and researchers, will also result in quality assurance systems becoming an integral part of the functioning of a microbiology laboratory. It will assist countries to initiate the process of getting their feed analysis laboratories accredited to international standards.

An additional effect of implementing and adopting these quality control/assurance approaches will be strengthening of the research and education capabilities of students graduating from R&D institutions and promotion of a better trading environment between developing and developed economies. This will have long-term benefits and will promote investment in both feed industries and R&D institutions.

This document will also serve as a basis for developing a self-learning e-module and for organising training workshops aimed at Laboratory Managers and Technical Analysts on quality control/assurance approaches in microbiology laboratories.

Berhe G. Tekola
Animal Production and Health Division
Food and Agriculture Organization of the United Nations

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PART 1

The Quality Management System in a microbiology laboratory

Introduction

Availability of animal feed and efficient feeding are the foundations of successful livestock production. The feeding of a balanced ration and proper feed formulation increases animal productivity, animal product quality and animal welfare. Also to decrease livestock associated pollution of the environment feeding of a diet that matches the physiological status of the animal is essential.

For the best health protection of both the animal and human population and to facilitate trade between developing and developed countries, the harmonising of Quality Assurance approaches is imperative.

A wide range of microbiological organisms occur either naturally or as contaminants of cereal grains, forages and vegetable matter. Some of these microbes can have beneficial effects such as the fermentation of forages in the process of producing silage, or the probiotic properties of some bacteria and yeasts which may be added to animal feeds.

Animal feed may become contaminated with bacteria, yeasts or fungi which are harmful, such *as E. coli, Listeria* spp., *Salmonella* spp. or *Aspergillus* spp. as a result of faecal or slurry contamination or, in the case of *Aspergillus* spp., this may occur in the field or as a result of storage in damp conditions. Ingestion of contaminated animal feed can have adverse effects on animal health and production and may introduce infection to the human population.

A robust Quality Management System provides the mechanism to ensure confidence in the laboratory results issued to customers and provides a mechanism to constantly monitor microbiology laboratory results and identify any opportunities for improvement that may be noted.

A Quality Management System provides management, staff and customers with confidence that all technical, administrative and human factors that may influence the quality of the results being generated are constantly monitored with the aim to prevent any non-conformities and identify any opportunities for improvement. A robust quality assurance system ensures credibility of data produced by animal nutrition/feed analysis laboratories and satisfies the customer expectation that results will be reliable and trustworthy.

Mutual recognition and the harmonisation of laboratory standards facilitate international trade in animal products and will ultimately improve the health of both farm animals and the human population throughout the world.

This manual has been prepared to complement the previous publication 'Quality Assurance for Animal Feed Laboratories' (FAO, 2011) and describes additional procedures for detection and isolation of microbiological agents which may be found in animal feeds. Both documents may be used by animal nutrition/feed analysis laboratories and serve as a reference source which specific laboratory facilities can use to implement standard operating procedures (SOPs) appropriate to their specific situations. However the principles laid down are generalized and may not apply to every laboratory situation.

The Quality Management System described in this manual is based on ISO/IEC 17025:2005 principles and EA-04/10 'Accreditation for Microbiological Laboratories' and is intended to help laboratory personnel maintain the standards expected while providing a consistent, reliable, efficient and professional service with the level of quality required and expected by the laboratory's customers. This is achieved by the commitment of management and staff at all grades to apply laboratory practices that ensure the quality of testing services and results produced.

Since the work in individual laboratories varies greatly it is essential to have a flexible yet detailed Quality Management System. The laboratory personnel must have an understanding of the principles underlying quality assurance and must apply them in all areas of their work. Only in this way can they maintain credibility, which is the most important attribute of any laboratory. This manual provides a strong foundation for microbiology laboratories on which they can develop a Quality Management System which will meet requirements imposed by international standards.

This manual has been divided in two main sections. The first section presents general aspects of quality assurance procedures and good laboratory practices that must be put in place in a feed analysis laboratory performing microbiological testing. The second section contains some basic microbiology procedures for preparation and handling of microbiological samples and isolation and detection procedures for some common animal feed microbial contaminants. Most of the methods described have been taken from laboratories which hold ISO/IEC 17025:2005 accreditation, the workers in these laboratories have been using these methods for many years and the methods have proved reliable. However, other methods or variants of the method presented in this manual may also be used.

ISO/IEC 17025:2005 should be read in conjunction with EA-04/10 (European co-operation for Accreditation) Accreditation for Microbiology Laboratories. This document supplements ISO/IEC 17025:2005 and provides specific guidance for laboratories performing microbiological testing. EA-04/10 also provides guidance for microbiology laboratories working toward GLP (Good Laboratory Practice), GMP (Good Manufacturing Practice) and GCP (Good Clinical Practice).

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Glossary of terms

Accreditation: The confirmation by a third party accreditation body (usually governmental) that a laboratory meets the requirements of an accreditation standard e.g. ISO/IEC 17025:2005.

Accuracy: The difference between an observed or measured value and the accepted or 'true value'. Since accuracy is affected by both random and systematic errors, accuracy can also be defined as the sum of systematic plus random error.

Anomaly: An unexpected occurrence which has had (or had the potential to have) a negative effect on the work undertaken by the laboratory.

Biosecurity: A set of preventive measures designed and applied to reduce the risk of introducing a pathogenic agent into an enclosed laboratory.

Blank: A sample containing no added analyte or a sample treated in such a manner that the desired reaction does not take place, e.g., one of the reagents used to produce a reaction is omitted.

Certification: The confirmation by an independent, third party certification body that conformity is demonstrated with the specific requirements of a standard e.g. ISO 9001:2008 or ISO 14001:2004. Certification may also be referred to as 'Registration'.

Collection of Substances Hazardous to Health Regulations 2002 (COSHH): Documentation detailing specific hazards relating to exposure, health and incident planning associated with a substance in the workplace. (Statutory in the UK).

Complaint: An expression of dissatisfaction from a customer regarding the quality of work performed by the laboratory.

Containment Level (CL): The bio-containment precautions that are to be taken when handling potentially harmful biological agents in an enclosed laboratory. These range from CL 1 (minimum level) to CL 4 (the highest).

Corrective and Preventive Action (CAPA): Corrective actions are actions taken when a process deviates outside the specification of the Quality Management System. Corrective Actions removes the 'cause' and Preventive Action prevents recurrence. CAPA may be undertaken as result of an anomaly, non-conformance or customer complaint.

Document: A controlled written policy, procedure, or work instruction that defines what operators do and how to do it. Controlled means that the document states who wrote and/or authorized the policy or procedure, when it was issued and states a version number to avoid the use of a document that is no longer valid. Control of documentation will normally be the responsibility of the Quality Assurance Manager.

Gap Analysis: An audit with the purpose of establishing the current 'gap' between current activities and those which would meet the requirements of a standard or Management System.

High Efficiency Particulate Absorption (HEPA): A type of filter used in laboratories which can remove 99.97% of particles greater than 0.3 µm from the air that passes through it. Such filters are frequently used in biological safety cabinets in microbiology laboratories.

Impact assessment: The procedure of investigating and establishing the effect a non-conformance or anomaly may have had on work undertaken.

Integrated Management System (IMS): A combined management system which fulfils the requirements of the Quality Management System (ISO 17025:2005 or ISO 9001:2008) and an Environmental Management System (ISO 14001:2004) and/or Occupational Health and Safety Management System (BS OHSAS 18001:2007).

ISO The 'International Organization for Standardization': The international standard setting body which is based in Geneva, Switzerland. ISO comprises representatives from various national standards organisations from around the world and promotes and disseminates standards.

Internal Quality Assurance (IQA/ring trial): Samples sourced by the laboratory in order to demonstrate competence and may take the place of proficiency samples.

Internal Quality Control (IQC:) Samples of traceable known value which may be used to confirm that a procedure or process has worked as intended.

Limit of Detection (LOD): The lowest perceivable signal above the background for a particular procedure. The LOD is defined as the mean of the blank plus three standard deviations of the mean of the blank.

Material Safety Data Sheet (MSDS): Information supplied with a substance or chemical which provides workers and emergency personnel with information regarding safe working and handling. Information in MSDS can include physical data, toxicity, first aid, health effects, reactivity, storage, disposal, PPE requirements and information regarding spills.

Measurement Uncertainty (MU): An expression of confidence in the reliability of the results of laboratory tests.

Non-conformance: A finding that is noted during audit which contravenes the Quality Management System, Health and Safety Management System, Environmental Management System, an SOP or standard.

Personal Protective Equipment (PPE): Safety equipment supplied to laboratory workers and visitors to reduce the risk of injury or contamination. This includes laboratory coats, eye protectors, gloves etc.

Proficiency Sample (External Quality Assurance, EQA): Samples provided by an external source in order to compare laboratory results between similar laboratories. These may be used as an internal quality control sample. External Proficiency Providers should hold accreditation to ISO/IEC 17043:2010 – Conformity assessment – general requirements for proficiency testing.

Quality Assurance (QA): Planned and systematic activities implemented within the laboratory that provide confidence in the accuracy and reliability of results generated.

Quality Control (QC): Activities used to monitor a process or to check a result and provide assurance that all activities are performing within predetermined limits set by the laboratory.

Quality Management System (QMS): All documented and implemented processes within an organisation which describe work activity.

Records: Can be electronic or paper. Examples include chain of custody paperwork, sample results, worksheets, QA/QC data, audit results, calibration records, etc.

Standard Operating Procedure (SOP): Document describing specified steps taken in a method. This method can be a specific analytical procedure or a policy controlling a more generic aspect of the work performed (e.g. training records, handling complaints or using balances). SOPs may be paper or electronic but must be controlled and available at the point of use.

Traceability: The property of the result of a measurement whereby it can be related to stated references, usually international standards, through an unbroken chain of comparisons.

Trainee: A person receiving training in the workplace.

Trainer: A person who is trained and competent in a procedure and is training another.

Validation: The robust process of demonstrating and documenting that a procedure is fit for purpose and establishing the limits of testing that may apply.

Quality assurance purpose and guidelines

Laboratory quality programs are a critical part of improving the agriculture laboratories in developing countries. The Laboratory Quality Manual is the essential source for communicating to the laboratory staff the manner in which laboratory testing is to be conducted. Adherence to the Quality Manual by laboratory staff is essential to ensure both the quality and consistency of microbiology results generated. Recognizing that the Laboratory Quality Manual may not cover all situations and variables arising from the laboratory setting, any significant departures must have the concurrence of management and must be appropriately documented.

The management within the laboratory is responsible for the quality and integrity of all data generated in the laboratory. The management, collectively, assures this quality through adherence to the Laboratory Quality Manual, quality assurance plan, and through the development and adherence to standard operating procedures (SOPs).

Third party recognition (accreditation or certification) of a Quality Management System provides assurance to customers that the Quality Management System operated by a laboratory meets the requirements of internationally recognized standards. The international standard for the general requirements for the competence of testing and calibration laboratories is ISO/IEC 17025:2005.

Many organisations will hold certification (or registration) to ISO 9001:2008 (Quality Management System Requirements) which forms a basis for ISO/IEC 17025:2005. Testing and calibration laboratories that comply with ISO/IEC 17025:2005 will also operate in accordance with ISO 9001:2008.

Laboratories may also wish to consider certification to ISO 14001:2004 (Environmental Management System) and/or BS OHSAS 18001:2007 (Occupational Health and Safety Management Systems) which are compatible with ISO 9001:2008 and ISO/IEC 17025:2005.

If a laboratory holds accreditation/certification to more than one international standard it may develop an Integrated Management System (IMS) which has one Quality Manual and one set of SOPs which cover all requirements of the standards for which accreditation/certification is held.

Microbiology laboratory organization and responsibilities

Each member of the laboratory should have clearly identified and documented responsibilities (Job Description). An organisational chart which clearly demonstrates the line management and reporting structure in operation should be included in the laboratory quality documentation and made available in staff training records.

Laboratory Manager/Director Has ultimate responsibility for implementing the quality system. The Laboratory Manager/Director will sign the Quality Statement and Quality Manual to demonstrate commitment of senior management to the Quality Management System. Similarly the Laboratory Manager/Director will sign the Health and Safety Statement and Environmental Management Statement if they are in place.

Quality Assurance Manager Reports directly to the Laboratory Manager/Director and is responsible for maintaining and developing the quality procedures used in the laboratory. The Quality Assurance Manager will ensure that regular internal audits are conducted, facilitate external audits by certification and regulatory bodies, perform audits of suppliers and subcontractors (where necessary and appropriate) and manage anomalies, non-conformances and customer complaints. The Quality Assurance Manager may delegate these duties to competent laboratory staff as appropriate.

Laboratory Analysts Responsible for performing microbiology testing procedures, under the direction of the Laboratory Manager/Director, following all quality procedures and identifying any opportunities for improvement.

Laboratory Analysts may also be referred to as 'scientists', 'technicians', 'microbiologist' or another title used locally.

A **Health and Safety Manager** may also be identified (or this role may be undertaken by the Laboratory Manager/Director or Quality Assurance Manager). The Health and Safety Manager is responsible for maintaining and developing the Occupational Health and Safety Management System within the laboratory and ensuring legal and statutory compliance with Health and Safety law and statute. This is of particular importance in a microbiology laboratory which may be handling Containment Level 2 (CL 2) and Containment Level 3 (CL 3) organisms.

An **Environmental Manager** may also be identified (or this role may be undertaken by the Laboratory Manager/Director or Quality Assurance Manager). The Environmental Manager is responsible for maintaining and developing the Environmental Management System within the laboratory and ensuring legal and statutory compliance with environmental law and statute which is in place locally.

Microbiological testing must only be performed, or supervized, by an experienced person who is qualified in microbiology or has relevant knowledge and experience relating to

the microbiology work performed by the laboratory. Laboratory analysts must have relevant knowledge, skills and practical experience before being allowed to perform work unsupervised within the laboratory. All such training must be documented and made available.

All Laboratory Analysts must have received adequate training in the operation of all laboratory equipment and all such training must be documented.

Ongoing competence should also be monitored objectively and re-training provided when deemed necessary by supervising staff. If trained staff are to be absent for a prolonged period of time (e.g. maternity leave or long term sick leave) they must be signed off as competent by a supervisor upon returning to their duties.

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ISO 9001:2008. Quality management systems – requirements. Geneva, Switzerland.

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ISO 14001:2004. *Environmental management systems – requirements with guidance for use*. Geneva, Switzerland.

Personnel training, qualification and competence in the microbiology laboratory

Qualified and trained personnel are essential for producing analytical results of acceptable quality. The Laboratory Manager/Director must ensure that laboratory personnel have the knowledge, skills, and abilities to perform their duties. Competence is based on education, experience, demonstrated skills, and training. Staff training files contain the documentation of personal education, experience, skills, and training for the position held.

Consideration should also be given to ensuring confidentiality and independence of Laboratory Analysts when dealing with customer samples submitted to the laboratory.

Laboratory Analysts undergo a training program in accordance with the laboratory's training procedure. The analyst must demonstrate and document proficiency in a microbiological method before reporting any microbiology results to the laboratory's customers.

The first step for qualifying in a new analysis is for them to read the appropriate standard operating procedure (SOP). This document can be obtained from the Laboratory Manager/Director or Quality Assurance Manager and should be available at the point of use. The method should be reviewed with the trainee by someone familiar with the procedure. Subsequent to this, the trainee should observe samples being processed by the trainer. After observing the procedure the trainee may perform the procedure on known samples (previously processed samples, IQA or EQA submissions) under the supervision of the trainer. The trainee may also perform a parallel run with the trainer. The number of samples processed in the training exercise should be specified and justified by the trainer in the trainee's training file.

Only when the trainer and Laboratory Manager/Director are satisfied that the trainee has demonstrated competence in the procedure shall they be deemed competent. Competence shall be demonstrated by obtaining the desired results from known samples, or unknown samples processed in parallel where the same results are obtained by the trainer and trainee.

All aspects of the training should be documented in the individual's training file.

To assure the safety of everyone, the trainee must also read any Material Safety Data Sheet (MSDS) or COSHH forms that are applicable for information concerning any chemicals or reagents used in the analysis and be familiar with the Risk Assessment concerning handling Category 2 and Category 3 pathogens as appropriate. The toxicity levels and method of waste disposal both for any chemicals and biohazardous waste should be clearly understood before beginning any analysis. Similarly all aspects of this Health and Safety training should be documented in the individuals training file.

On-going competency should be demonstrated by participation in External Proficiency Schemes (often referred to as EQA), Internal Quality Assurance (IQA) or ring trials schemes at regular intervals and documented appropriately. If several operators are to participate in EQA or IQA a schedule can be created which gives each operator a regular opportunity to participate. EQA and IQA samples should be processed and reported exactly as routine samples and never as a joint effort by several operators. Consideration may be given to 'blind' IQA samples where only the Laboratory Manager/Director and Quality Assurance Manager are aware that the samples are part of an IQA distribution. This will ensure IQA samples are handled and processed exactly as routine samples.

Any anomalous results obtained from EQA or IQA should be investigated thoroughly by the Laboratory Manager/Director and Quality Assurance Manager to determine the cause and establish any corrective and preventive actions (CAPA) that may be required. Consideration should also be given to recalling of results that were issued prior to the anomalous EQA/IQA being noted and suspension of testing until the cause of the anomalous result is established.

All details of such an investigation should be documented along with any corrective and preventive actions and an impact assessment if necessary.

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ISO 14001:2004. *Environmental management systems – requirements with guidance for use*. Geneva, Switzerland.

Accommodation (facilities) and environment

There are some basic requirements for a microbiology testing facility which should be considered when setting up a microbiology laboratory or when considering accreditation of an existing laboratory.

The laboratory should be arranged so as to protect the integrity of all samples submitted, prevent cross-contamination between samples and prevent risk to staff. There should be restricted access by the public to all, except for sample reception areas and a clearly defined 'one way' route for samples from sample reception to sample holding, preparation, testing and then storage and disposal. Only authorized staff may be permitted access to laboratory areas and consideration may be given to secure access (e.g. key pad entry systems) especially for CL 3 facilities.

Laboratory areas must be suitable for purpose with smooth, non absorbent, wall and floor coverings which allow disinfection of spills. Benches must be non absorbent and resistant to disinfection. There should be no fabrics in the microbiology laboratory (i.e. no carpet, curtains or fabric covered chairs) chairs and stools should be metal or plastic and, if required, plastic or metal blinds should be used in windows. The use of bare wood should be avoided.

There should be a regular cleaning programme using a suitable microbiological disinfectant (e.g. 5% sodium hypochlorite) and a procedure for dealing with microbiological spills. 70% alcohol may be used as an alternative to 5% sodium hypochlorite where there may be possible contamination of tests sensitive to such disinfectant (e.g. ELISA tests). If there is a Containment Level 3 (CL 3) laboratory this should have its own cleaning programme.

Another option available for disinfecting of laboratory benches and rooms is the use of ultraviolet lights (black lights). This can be done by leaving ultraviolet lights on overnight, when laboratory activities have ended. Ultraviolet lights may be left on overnight, or a timer may be used to switch them off after a suitable period of time.

Ceiling should be smooth and lights fitted flush to the ceiling, if this is not possible regular cleaning and inspection should be performed and documented. Lighting should be adequate for staff to perform microbiological analysis.

Laboratory Analysts should wear suitable personal protective equipment (PPE) such as laboratory coats. These should be of a type that is fire resistant, fastened to the neck and have elasticated type cuffs. Laboratory coats should be worn fastened, at all times in the laboratory. There should be dedicated laboratory coats for any CL 3 facilities or molecular biology laboratories where DNA work may be conducted. Some laboratories may offer surgical type short sleeved tops and trousers in preference to laboratory coats.

Consideration should be given to temperature control as excessive heat can affect

microbiological testing and sample integrity. Opening of windows to cool a microbiology laboratory is inappropriate due to possible contamination from the environment and possible escape of pathogens to the outside (e.g. CL 2 and CL 3 pathogens). Fans should be avoided, due to the risk of contamination aerosols, but air conditioning (suitably filtered and maintained) is an appropriate solution. In tropical conditions the testing laboratories may be positioned facing north (in the northern hemisphere) or south (in the southern hemisphere) to reduce heat build up from direct sun and sun shades may be positioned on the outside of the building.

Consideration should be given to vector control (e.g. insects and rodents) to avoid contamination of the workplace and possible contamination of the environment from the microbiology laboratory (e.g. CL 2 and CL 3 pathogens).

It is advisable to have separate locations or clearly designated areas for different activities within the microbiology laboratory, i.e. sample receipt, storage, preparation, testing etc. It is also advised that testing is separated by space or time to avoid any cross-contamination. Equipment dedicated for 'clean' materials (e.g. media, reagents, test kits etc.) and 'unclean' materials (e.g. samples before and after testing) should be clearly identified to avoid contamination.

Work areas should be kept clean and tidy and Laboratory Analysts must have adequate space in which to work. Hand washing facilities must be provided and access to a shower should be considered, for decontamination of spills, by way of a risk assessment.

If the microbiology laboratory conducts CL 3 testing there must be a dedicated CL 3 laboratory. Only CL 3 work should be undertaken in this laboratory, which must contain dedicated equipment where practical. Windows in a CL 3 laboratory should be sealed and access must be restricted to trained authorized Laboratory Analysts only (this may be by way of a key pad entry system or similar). There must be an observation window allowing Laboratory Analysts to be viewed from outside whilst working and the CL 3 laboratory must have an air pressure negative to the atmosphere, this can be achieved by use of a microbiological safety cabinet. Wherever possible work should be performed in a microbiological safety cabinet within the CL 3 laboratory which is maintained on a regular basis and extracted air must be filtered using high efficiency particulate absorption (HEPA) or equivalent. Staff operating in the CL 3 laboratory must wear dedicated laboratory coats (or suitable protective clothing) and footwear protectors. Disposable gloves should be worn at all times. CL 3 laboratories must be sealed for fumigation when required (after a spillage or before routine maintenance).

An area must be made available for cleaning of contaminated laboratory equipment and disposal of biohazardous waste (including CL 3 if necessary). Only trained staff must perform these roles and operate any equipment (e.g. autoclaves).

There should be adequate first aid facilities, including first aid trained staff and fire extinguishers. Staff should also be familiar with the use of fire extinguishers.

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Microbiology testing – selection and verification of methods (including measurement uncertainty)

Appropriate test methods and procedures must be selected, documented, controlled and implemented in the laboratory for all tests within the scope of the Quality Management System. These methods and procedures are subject to appropriate validation and measurement where appropriate.

Wherever possible recognized standard methods such as methods published in international, regional or national standards shall preferably be used rather than laboratory developed methods or non-standard methods. The laboratory must ensure that it uses the latest valid edition of a standard method unless it is not appropriate or possible to do so. Methods published by reputable technical organisations or in recognized scientific texts or journals may also be used if they are appropriate and if they are validated

When deemed necessary and in agreement with the customer a standard method may be supplemented with additional information or details to ensure consistent application.

The Laboratory Manager/Director and the Quality Assurance Manager must select the most appropriate test that satisfies the customer's needs and expectations and also satisfies the requirements of the Quality Management System and ISO/IEC 17025:2005 (if appropriate). The Laboratory Manager/Director shall assign appropriately qualified and competent personnel to the development of test methods.

LABORATORY DEVELOPED AND NON STANDARD METHODS (INCLUDING MODIFIED STANDARD METHODS AND STANDARD METHODS USED OUT WITH THEIR INTENDED SCOPE)

Any non standard method used must be appropriately validated before use and any measurement uncertainty determined where relevant.

If a recognized standard method is to be used out with its intended scope or modified in any way it must be validated to confirm that it is still fit for use and this recorded as for a laboratory developed method.

All laboratory staff involved in the development of test methods must be appropriately qualified and experienced and shall document the results of each stage of development as it proceeds. It is the responsibility of the Laboratory Manager/Director to gain the agreement of the customer should it be necessary to use a non standard method and must ensure that the test method is capable of meeting the customer's requirements.

Before developing a new method the following information should be confirmed and agreed with the customer:

- scope of the test;
- sample types to be tested;
- parameters;
- quantities and ranges;
- apparatus and equipment, including technical performance requirements;
- reference standards and reference material required;
- environmental conditions required; and
- description of the procedure.

In describing the procedure the following should be considered:

- handling, transportation, storage, preparation and disposal of samples (including preservation of sample integrity);
- equipment maintenance and calibration;
- health and safety considerations and risk assessment;
- criteria for approval/rejection of test results and interpretation;
- reporting of results; and
- IQC, IQA, EQA and estimate of uncertainty.

TEST METHOD REQUIREMENTS

Validation is the confirmation by examination and provision of objective evidence that a method meets the requirements and is fit for its intended use. Validation is required for non standard methods, laboratory designed methods, standard methods used outside their intended scope and modifications of standard methods. On completion, the test validation will be approved by the Laboratory Manager/Director and a statement of approval included in the validation report. The extent of validation required will be determined by the Quality Assurance Manager and Laboratory Manager/Director. The validation process will be as extensive as is necessary to meet the requirements of application of the test method and may include procedures for sampling, handling, storage, transportation and preparation of test items.

The techniques used to validate test methods within the laboratory should include as many as possible of the following:

- testing using certified reference standards;
- comparison of results achieved with other validated methods;
- results of EQA, IQA and internal QC;
- inter-laboratory comparisons;
- systematic assessment of the factors influencing test results; and
- assessment of the uncertainty of the test results based on experience and scientific understanding of the principles of the method.

Should validated non-standard methods be modified, the influence of such changes will be determined and documented by the Laboratory Manager/Director, if appropriate ensuring the test method is revalidated.

The Quality Assurance Manager and Laboratory Manager/Director are responsible for ensuring that the range and accuracy of the values obtained from validated methods are relevant to the requirements of the customer.

ESTIMATION OF MEASUREMENT UNCERTAINTY (MU)

The laboratory must attempt to identify all the components of uncertainty that may have an effect on a result and make a reasonable estimation, based on knowledge of the performance of the method, of the reliability of the results. Where a component is identified as having the potential to affect results the laboratory must put in place procedures or controls to reduce that affect.

Unlike some other types of laboratory testing, qualitative microbiology tests do not require the rigorous metrological and statistically valid calculation of uncertainty of measurement. It is generally appropriate to base the uncertainty of measurement on repeatability and reproducibility of data, including proficiency testing results.

Each component which makes up uncertainty in microbiological testing should be identified (e.g. weighing, use of pipette, incubation etc.) and demonstrated to be under control within the Quality Management System. Some components such as sample preparation or sample condition cannot be controlled in such a manner but their importance to the variability of the final result should also be considered.

It is the responsibility of the Quality Assurance Manager and Laboratory Manager/Director to ensure that the procedures for the estimation of measurement uncertainty are applied where applicable and that appropriately qualified personnel are assigned to estimate the measurement uncertainty associated with test methods.

In assessing the rigour required in the estimation of measurement uncertainty the laboratory personnel assigned will consider:

- the requirements of the test method(s);
- the requirements of customers; and
- the existence of narrow limits on which decisions on conformance to specification are based.

CONTROL OF DATA

The Quality Assurance Manager and Laboratory Manager/Director will determine what checks should be applied to any calculations and data transfer. The requirement and procedure for such checks should be detailed in relevant SOPs.

When computers or automated equipment is used for the acquisition, processing, recording, reporting, storage or retrieval of test data, the laboratory will ensure that:

- any computer software developed in-house is documented in sufficient detail and is suitably validated as being adequate for use;
- procedures are established and implemented for protecting the data;
- hardware and software are kept secure with protected access; and
- computers and automated equipment are maintained to ensure proper functioning and are provided with the environmental and operating conditions necessary to maintain the integrity of the test results and internal calibration data.

Commercially produced software used within its designed application is regarded to be sufficiently validated (e.g. statistical packages, spreadsheets and word-processing packages). Should the laboratory introduce any modifications to commercially produced software packages the requirements for validation shall apply.

Test results shall be recorded in writing, as they are determined, unless direct data capture systems are implemented in the laboratories. All written test results will be accompanied by a signature or initials of the Laboratory Analyst performing the testing, date and sample identification to provide an audit trail. Batch numbers of media and reagents and details of critical equipment used should also be recorded to enable a procedure to be repeated, if necessary, recreating the exact conditions of the original testing. Recording of operator(s) and batch number(s) of media and reagents will also enable identifications of results which may be affected by any anomaly or failure concerning that operator, equipment or component as necessary.

The processing, storage and transmission of test results, including computer files, are only to be made by authorized laboratory personnel. Access to computer records and data is password protected for security and customer confidentiality.

REFERENCES

EURACHEM/CITAC. 2012. *Quantifying uncertainty in analytical measurement 3rd edition 2012*. Eurachem/CITAC guide CG4 2012. Uppsala, Sweden.

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 5 Technical requirements, 5.4 Test and calibration methods and method validation*. Geneva, Switzerland.

UKAS. 2000. *The expression of uncertainty in testing*. UKAS publication ref Lab 12 Edition 1, October 2000. Feltham, UK

Standard Operating Procedures (SOPs)

PRINCIPLE

The purpose of this procedure is to define the format and content of all Standard Operating Procedures and controlled documentation and to provide instructions for their review, approval and control.

Controlled documents may include SOPs, calibration tables, charts, text books, reference material, software etc. These may be hard copies or electronic documents. Controlled documents should be available at their point of use.

Controlled documents must not be copied (in full or in part) to avoid the possibility of an uncontrolled copy being in use which is no longer valid.

Standard Operating Procedures and controlled documentation may pertain to Quality Assurance, Health and Safety, Environmental Management or general administration and management within the microbiology laboratory.

SCOPE

This document applies to all controlled documents within the Quality Management System

RESPONSIBILITIES

- **Laboratory Manager/Director** – A technically competent person must authorize SOPs for use.
- **Quality Assurance Manager** – Must formally issue, highlight for review and withdraw controlled documents as appropriate. The Quality Assurance Manager will manage the Change Control procedure which records all changes made to controlled documentation.
- **Laboratory Analysts** – All staff may write or review SOPs.

PROCEDURE

Updating/issuing – A formal Change Control procedure must be followed when updating an existing controlled document or issuing a new controlled document. This should justify and authorize any changes to a controlled document.

An SOP should be written by an experienced operator who is proficient in the procedure and be passed to a technically competent person to approve for issue.

Format – SOPs should be written in a consistent format (font, layout etc.).

Each document will be formatted based on the specific details as follows:

- Header/Footer – The header will consist of the SOP number, title, author, approver, effective date, review date and version number. The footer will consist of pagination (i.e. page X of Y).

- Log of Updates – This will highlight recent changes/revisions.
- Principle – This section will record the objective of the procedure.
- Scope – This section details the area(s) to which the procedure pertains and includes any test limitations (sample criteria and testing limits).
- Responsibilities – Staff involved in the procedure and their roles.
- Equipment – Included only in test methods. List what equipment is required to carry out the procedure.
- Reagents – Included only in test methods. List reagents/kits used and detail storage and supplier details.
- Procedure – Detail in a clear concise manner, the actions necessary to perform each task and the person(s) responsible.
- Calculation – Any calculations or data manipulations that are required in the method.
- QC – Included only in test methods. Detail IQC, IQA and EQA measures in place.
- Remarks – Any additional useful information or comments.
- Interferences and troubleshooting – Included only in test methods. List any potential problems that may be encountered when performing the method detailed and how these may be dealt with.
- References – Record the reference number and title of any SOP or document that is relevant or is referred to in the procedure being written.

Definitions can also be included in addition to any specific COSHH forms, if applicable, and health and safety information including any risk assessments that may be applicable.

REVIEW, APPROVAL AND ISSUE

The author (or reviewer) should ensure that the document has been formatted correctly and reviewed by the relevant personnel, such as users of the procedure. When the draft document has been prepared the author or reviewer will gain the authorisation of an appropriate technically competent person. Once authorized, a new or revised document is no longer regarded as a draft and must be issued as soon as is practical by the Quality Assurance Manager. Previous versions should be withdrawn immediately to preclude the use of invalid or obsolete documents.

A locally agreed review date must be assigned to all controlled documents e.g. three years. When a document is due for review the Quality Assurance Manager must pass the document to the original author (or other suitably qualified person) and the above procedure followed to ensure the document is still valid and current.

If the document is still valid and current a new review date shall be assigned and the document reissued. If changes are required these should be made by the author and approved as before. A document may be changed whenever the need arises, each time following the above procedure to demonstrate control.

Each time a document is updated or reissued the version status should change to indicate this. A master list of current documents should be available to staff.

REFERENCES

ISO 9001:2008. *Quality management system - requirements*. Geneva, Switzerland.

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 4. Management requirements, 4.3 Document Control*. Geneva, Switzerland.

Equipment – maintenance and service

PRINCIPLE

The purpose of this procedure is to define the responsibilities and procedures for recording all equipment maintenance, calibration and identification and to ensure that critical equipment used for microbiological testing is capable of achieving the accuracy required.

SCOPE

This procedure applies to all critical equipment used in the provision of microbiological results.

RESPONSIBILITIES

- **Laboratory Analysts** – Must carry out routine maintenance and servicing that is required and that they are trained to perform.
- **Laboratory Manager/Director** – Must ensure that all necessary maintenance and servicing is carried out by suitably qualified and trained staff or subcontractors.
- **Quality Assurance Manager** – Must ensure all staff comply with the requirements of this procedure by way of internal audits.

Only trained personnel must use laboratory equipment and procedures must be in place which document the storage, use and planned maintenance of all laboratory equipment.

PROCEDURE

Equipment Records (Asset Register)

It is the responsibility of Laboratory Analysts to ensure that each item of critical equipment and any software used for testing and internal calibrations are uniquely identified and logged on the Asset Register.

The Laboratory Manager/Director is responsible for maintaining records of each item of equipment and its software significant to testing and internal calibration activities. The records maintained for each item of equipment include the following as a minimum:

- the unique identity of the equipment and any software;
- the manufacturer's name, equipment model, serial number (or equivalent);
- checks of the equipment's compliance with the specifications required;
- the current location (where appropriate) and contact;
- the manufacturer's instructions or user guide at point of use, or it's location is referenced;
- results and certificates of all calibrations, maintenance and safety checks and the due date of the next such check;
- any faults, malfunctions, modifications, damage or repairs to the equipment; and

- any defective equipment will be identified as such so as to prevent its use until it is repaired.

CALIBRATION STATUS OF EQUIPMENT

Equipment must be checked, and evidence documented, that it meets required specification before being put into service. The laboratory must have a documented programme of calibration for laboratory equipment.

Equipment requiring calibration will be calibrated and labelled by authorized and appropriately trained personnel, as evidenced in individual training records. Such equipment will be labelled with the unique identification assigned by the laboratory and will state the date of calibration and the date the next calibration is due.

Where it is not practical/feasible to label individual items of equipment with the calibration status the (e.g. glass pipettes) information regarding the date of calibration and the calibration due date will be readily available to operators.

EQUIPMENT CHECKS

The frequency and nature of any intermediate checks required to maintain confidence in the calibration status of equipment are detailed below.

CORRECTION FACTORS

Correction factors required as a result of equipment calibrations will be documented and applied to software used for calibration purposes or stated on individual items of equipment. Only corrected data should be recorded.

CALIBRATION AND MONITORING OF EQUIPMENT

The frequency of calibration and performance verification shall be determined by documented experience and be based on the need, type and previous performance of the equipment. The period between calibration and verification shall be shorter than the time the equipment has been found to drift outside accepted limits.

The following is a list of typical equipment found in a microbiology laboratory. It details recommended calibration, monitoring and certification that the laboratory should consider.

AUTOCLAVES

- **Calibration:** Annual.
- **Monitoring:** Chart Recorder and/or Browne's Tube. Regular (six monthly or three monthly service by approved service agent).
- **Certificate:** External Certificate of Calibration.
- Refer to specific Autoclave SOP for operating instructions, time and temperature tolerances (see 'Use of autoclaves' SOP).

AUTOMATIC PIPETTES

- **Calibration:** 3 monthly intervals (where less frequent calibration will suffice, the laboratory will provide documented evidence of 'fitness for purpose' of amended frequency).
- **Monitoring:** Monthly calibration check (spot check on single pipette).

- **Certificate:** Internal Calibration using recognized method.

(See 'Use of pipettors' SOP).

BALANCES/CHECK WEIGHTS

- **Calibration:** Annual.
- **Monitoring:** Check weights on a 'before use' basis.
- **Certificate:** External Certificate of Calibration.

Any correction factors to be applied to check weights are determined at the annual calibration of balances and are documented and clearly displayed where required. (See 'Use of balances' SOP).

DISPENSERS

- **Calibration:** 3 monthly intervals (where less frequent calibration will suffice, the laboratory will provide documented evidence of 'fitness for purpose' of amended frequency).
- **Monitoring:** as appropriate to use.
- **Certificate:** Internal Calibration.

(See 'Use of pipettors' SOP).

VOLUMETRIC GLASSWARE

- **Calibration:** Annual gravimetric to required tolerance.
- **Monitoring:** Visual checks.
- **Certificate:** Internal Calibration.
- **Verification:** Should not be necessary for glassware that has been certified to a specific tolerance.

(See 'Microbiology laboratory glassware' SOP).

pH METERS

- **Calibration:** Before each use with appropriate calibration buffers (i.e. pH 4, pH 7 and pH 10).
- Buffers should be stored in appropriate conditions with expiry date assigned.
- For details on specific pH meter operation, calibration and adjustment refer to manufacturers' instructions.

(See 'Use of pH meters' SOP).

THERMOMETERS/THERMOCOUPLES

- **Calibration:** Annual and traceable to national/international standards for temperature.
- **Monitoring:** Visual checks.
- **Certificate:** Internal Calibration using a certified reference thermometer.

Correction factors to be applied are detailed on each unit where the correction factor determined could invalidate test results. Reference thermometers/thermocouples require full traceable re-calibration at least every five years.

TIMERS

- **Calibration:** Annual with calibrated timer or national time signal.
- **Monitoring:** Visual checks/replace battery.
- **Certificate:** Internal Calibration.

CENTRIFUGES

- **Service:** Annual with external service agent (traceable calibration or check against independent tachometer, as appropriate).
- **Monitoring:** Visual checks/routine cleaning.
- **Certificate:** Annual service.

MICROSCOPES

- **Service:** Annual with external accredited service agent.
- **Monitoring:** Visual checks/routine cleaning.
- **Certificate:** Annual service.

TEMPERATURE CONTROLLED EQUIPMENT (Fridges, Freezers, Waterbaths, Ovens and Incubators)

- **Service:** Annual for low temperature (e.g. -80 °C) freezer and for any associated gas pressure systems for such freezers or CO_2 incubators.
- **Monitoring:** Daily temperature checks/routine cleaning. Electronic monitoring systems may be used which will alert staff when a temperature goes outside a pre set range. Alternatively a minimum/maximum thermometer can be used which can be reset at the start of every working day. The laboratory must ensure that the minimum and maximum temperatures recorded do not go out with the range stated for the item of equipment.
- **Certificate:** Annual service/gas pressure system check if appropriate.

When a new temperature controlled item of equipment is put into service or an existing item of equipment is moved from one location to another, or has major maintenance or repair work done, it must be validated (or profiled). This is done by monitoring that the desired temperature is attainable and consistent at various points within the item of equipment. If an item of equipment has an area within it where the desired temperature is not attainable, or can not be maintained, this area must be precluded from use. This validation work must be repeated at least every two years.

Fridges, freezers, waterbaths, ovens and incubators should be cleaned regularly at time periods determined by the laboratory. Use of a biocide is recommended for waterbaths. (See 'Use of incubators and temperature controlled equipment' SOP).

MICROBIOLOGICAL SAFETY CABINETS (including laminar flow cabinets)

- **Service:** Biannual with external accredited service agent.
- **Monitoring:** Visual checks/routine cleaning.
- **Certificate:** Biannual service.

DE-IONISERS, STILLS AND REVERSE OSMOSIS (RO) UNITS

- **Service:** Annual with external accredited service agent.
- **Monitoring:** Daily visual checks.
- **Certificate:** Annual service.

ANAEROBIC AND MICROAEROPHILIC JARS/BOXES

- **Service:** N/A.
- **Monitoring:** Visual check of seals before use and inclusion of control indicator.
- **Certificate:** N/A.

MOVEMENT OF EQUIPMENT

If equipment is moved from one location to another, this must be noted in the inventory/asset register. It is the responsibility of the Laboratory Manager/Director at the receiving site to ensure equipment is fit for the intended purpose. The Laboratory Manager/Director will ensure that any servicing, maintenance and calibration required is carried out prior to use. Equipment records will be held with equipment and maintained at the receiving site.

MAINTENANCE

The laboratory must have a documented programme of maintenance for critical laboratory equipment. This should include cleaning, servicing, inspection for damage and be performed by competent staff or, where necessary, an appropriately qualified and accredited external service agent.

DEFINITIONS

- **Calibration** – Set of operations, which establish, under specified conditions, the relationship between values indicated by a measuring instrument, measuring system, or values represented by a material measure and the corresponding values of a reference standard.
- **Calibration range** – Specified range across which the instrument will be calibrated.
- **Critical equipment** – Any equipment that has a direct influence on test results.

REFERENCES

ENAC. 2004. *ENAC NT-04 Rev. 2 Junio 2004*. Caracterización de medios isotermos. Madrid, Spain.

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 5.5 Equipment*. Geneva, Switzerland.

UKAS. 2006. *LAB 14 Calibration of weighing machines*. UKAS publication ref Lab 14 edition 4, November 2006. Feltham, UK.

UKAS. 2009. *LAB 15 Traceability: Volumetric apparatus*. UKAS publication ref Lab 15 edition 2, June 2009. Feltham, UK.

UKAS. 2012. *LAB 11 traceability of temperature measurement: platinum resistance thermometers, liquid-in-glass thermometers and radiation thermometers*. UKAS publication ref Lab 11 edition 4, November 2012. Feltham, UK.

Reporting microbiological results

PRINCIPLE AND SCOPE

The purpose of this procedure is to define the process, which ensures that the results of testing are reported accurately, clearly, unambiguously and objectively to the customer.

Results reported should be in accordance with any specific instructions detailed in the test methods (SOPs) to ensure that any necessary detail regarding testing activities are included and that results are reported consistently in a standard format.

RESPONSIBILITIES

- **Laboratory Manager/Director** – Must ensure that the results are reported accurately, clearly, unambiguously and objectively to the customer and in accordance with any specific instructions detailed in the test methods. The Laboratory Manager/Director may provide appropriate staff with the authority to report results on his/her behalf.
- **Laboratory Analysts** – It is the responsibility of all staff with the authority for reporting test results to ensure that the results are reported accurately, clearly, unambiguously and objectively to the customer and in accordance with any specific instructions detailed in the test methods.
- **Quality Assurance Manager** – It is the responsibility of the Quality Assurance Manager to ensure that the requirements of this procedure are met by way of regular internal audits.

PROCEDURE

Test reports must include all information requested by the customer and the necessary information required for the interpretation of the results.

Interpretation of test results

In addition and where necessary for the interpretation of test results, authorized laboratory personnel must ensure that the following information is listed in test reports:

- deviations from, additions to, or exclusions from the agreed test method;
- relevant information on specific sample conditions which may affect results. This should include, if applicable, a statement that results are not validated due to the submission of insufficient sample for analysis but where a pro-rata test has been performed;
- where relevant, a statement of compliance or non-compliance with requirements and/or specifications;
- where applicable, a statement on the estimated uncertainty of measurement;
- where appropriate and required, opinions and interpretations;
- additional information which may be required by specific methods, clients or groups of customers;

- details of sampling date, sample receipt date and date of analysis; and
- indication that the test results refer to the received sample, but not to the feed stock.

The interpretation of test results provided to customers must be clearly marked as such in test reports.

Opinions and interpretations included in a microbiology laboratory test reports may include the following:

- an opinion on the statement of compliance/noncompliance of the results with requirements;
- fulfilment of contractual requirements;
- recommendations on how to use the results; and
- guidance to be used for improvements.

Where it is appropriate to communicate additional opinions and interpretations not included in laboratory report, by direct dialogue with the customer the reporting officer must ensure that such dialogue is documented and retained in the relevant customer files.

Test reports from subcontractors

It is the responsibility of laboratory personnel receiving test results from subcontractors to ensure that the results are reported to the customer.

It is the responsibility of laboratory personnel authorized to report test results to ensure that results provided by subcontractors are clearly identified as such on test reports.

Electronic transmission of results

Test results may be provided to clients by post (mail), fax, telex, e-mail, telephone, and internet or by any other electronic means. Regardless of the method used to communicate the results the requirements of the Quality Management System must be fulfilled.

Format of test reports

It is the responsibility of laboratory personnel authorized to report test results to ensure that test data is presented in such a way as to minimize the possibility of misunderstanding by the customer and that the format of the report is such to prevent the possibility of addition or removal of pages or otherwise tampering with the information.

Where further results will be issued for a sample the test reports will be referred to as 'interim' prior to the 'final' report. Individual interim reports will be identifiable by report date.

Amendments to test reports

Should amendments be required to a test report after issue such amendments will only be made by the Laboratory Manager/Director or laboratory personnel authorized to report test results and in the form of a further document, or data transfer which includes the statement

'Supplement to test report, reference number…….', or an equivalent form of wording as appropriate.

Retest policy

Retesting or repeat testing of any sample(s) must always be undertaken where internal quality control results do not meet the expected criteria. In addition, on occasions where the IQC criteria are satisfactory but the results do not conform to expected results from the history provided retesting may be applicable.

The result of the retest should be compared with the original result, to ensure that it is similar, i.e. within the standard acceptance criteria for the test in question, before being reported to the customer.

In the unlikely event of the repeat test result being significantly different from the original, a further repeat test (or tests) should be performed until two consecutive similar results are obtained. This result must then be reported to the customer and an investigation conducted into the cause of the anomalous result(s).

Use of the accreditation mark

The appropriate accreditation mark must only be used on appropriate laboratory reports. It must not be used on reports where there are no tests covered by the scope of accreditation. If there is a mixture of accredited and non accredited results contained in a laboratory report the non accredited results must be identified clearly.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 5.10 Reporting the results*. Geneva, Switzerland.

UKAS. 2009. *LAB 1 Reference to accreditation for laboratories*. UKAS publication ref Lab 1 edition 5, August 2009. Feltham, UK.

Traceability of results

PRINCIPLE AND SCOPE

The purpose of this procedure is to ensure that all equipment, including equipment for subsidiary measurements (e.g. for measuring environmental conditions) having a significant effect on the accuracy or validity of test results, calibration or sampling is calibrated before use and that such calibrations are traceable to the International System of Units (SI) (Système international d'unités) where possible. This is to ensure that appropriate standards and reference materials are used in the calibration of equipment required for testing.

This procedure applies to any laboratory equipment used in the provision of testing services and requiring calibration.

RESPONSIBILITIES

- **Laboratory Analysts** – Must carry out routine calibration that is required and that they are trained to perform before using an item of equipment.
- **Laboratory Manager/Director** – Must ensure that all necessary calibration is carried out by suitably qualified and trained staff or subcontractors and is traceable to SI units.
- **Quality Assurance Manager** – Must ensure all calibrations are traceable to SI units by way of routine internal audit(s).

PROCEDURE

Calibrations performed in the laboratory are traceable, where the concept is applicable, through an unbroken chain of calibrations or comparisons to the International System of Units (SI). The link to SI units may be achieved by reference to national measurement standards. If calibrations are being carried out by an external service agent the Laboratory Manager/Director must ensure that the calibrations are traceable to SI units (e.g. accredited to ISO/IEC 17025:2005).

It is the responsibility of the Laboratory Manager/Director to ensure that internal calibrations are performed using appropriate reference standards and reference materials.

There are certain calibrations in the laboratory that cannot be strictly made in SI units. In such instances calibration provides confidence in measurements by establishing traceability to appropriate measurement standards such as:

- The use of certified reference materials (if available) provided by a competent supplier to give reliable characterisation of a material.
- The use of specified methods and/or consensus standards that are clearly described and agreed by all parties concerned.

It is the responsibility of the Laboratory Manager/Director to ensure participation in suitable programmes of inter-laboratory comparisons and proficiency schemes where possible.

Reference standards

Reference standards are calibrated by an external calibration laboratory capable of providing traceability to SI units of measurement.

It is the responsibility of the Laboratory Manager/Director to ensure that any reference standards used in the laboratory are traceable (where possible) to SI units of measurement or to certified reference materials. Laboratory Analysts must ensure that any internal reference materials are verified by comparison with certified reference materials.

Reference standards should be subject to intermediate checks to verify the calibration status of such standards.

Reference standards must be handled, stored and transported to the calibration laboratory to prevent damage, protect their integrity and to ensure confidence in their calibration status.

Reference materials

Reference materials should be traceable to SI units of measurement, where possible, or to certified reference materials. Any internal reference materials used should be checked for accuracy as far as is practicable.

Reference materials should be handled, transported and stored in accordance with suppliers' and manufacturers' instructions to prevent contamination or deterioration and to protect their integrity.

Liquid in glass reference thermometers are calibrated at least every 5 years and digital reference thermometers are calibrated at least every 2 years. Calibration certificates are provided by the calibration laboratory and should be maintained on file.

The laboratory ensures traceability to SI units for temperature measurement by maintaining and using calibrated liquid in glass and digital thermometers for internal calibration purposes only (i.e. using these calibrated thermometers to calibrate others within the laboratory). The reference thermometers (digital and liquid in glass) maintained in the laboratory are subject to annual ice-point calibrations (using crushed ice) to ensure confidence in their calibration status. If a thermometer does not have 0.0 °C on its scale an alternative to the ice point check is to check the thermometer at its normal operating temperature against a calibrated thermometer. Any uncertainty applicable to the reference thermometer must be taken into account when calibrating thermometers in the laboratory and included in the tolerances.

The laboratory should hold a set of traceable check weights of suitable weights (i.e. weights that are representative of the routine work that will be undertaken in the lab using balances). These check weights should be stored in a closed container when not in use and only handled using forceps or lint free gloves or tissue. At the annual service of laboratory balances the check weights can be weighed immediately after the service, their weights recorded and even if a slight drift is detected they may still be used and regarded as traceable to SI units. It should be ensured that if any drift has been detected that the revised weight is always assigned to that individual check weight.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 5.6 Measurement traceability.* Geneva, Switzerland.

UKAS. 2006. LAB 14 *Calibration of weighing machines*. UKAS publication Lab 14 edition 4, November 2006. Feltham, UK.

UKAS. 2012. LAB 11 *Traceability of temperature measurement: platinum resistance thermometers, liquid-in-glass thermometers and radiation thermometers*. UKAS publication Lab 11 edition 4, November 2012. Feltham, UK.

Proficiency testing

PURPOSE AND SCOPE

The purpose of this procedure is to ensure participation in relevant External Proficiency Schemes (External Quality Assurance (EQA)) or inter-laboratory QA Schemes (IQA) at appropriate frequency and to review all external QA results, ensuring that any unsatisfactory results are identified and appropriate action taken. This ensures the independent verification of the validity of the test results.

This procedure applies to all test procedures in the laboratory.

RESPONSIBILITIES

- **Laboratory Analysts** – Examines EQA or IQA samples regularly (as stipulated in any local schedule) ensuring that they are tested as routine samples and that their competency is kept up to date.
- **Laboratory Manager/Director** – Devises a schedule of participation in EQA and IQA schemes for all staff and ensures that all Laboratory Analysts involved in testing participate in EQA and/or IQA schemes to demonstrate compliance with the Quality Management System and maintain competency.
- **Quality Assurance Manager** – Records EQA/IQA data in such a way that any trends are detectable and results are reviewed regularly. Where EQA/IQA results are outside of pre-defined criteria an investigation should be initiated to establish corrective and preventive actions (CAPA). It may be necessary to perform an impact assessment and consider recalling results and suspend testing depending on the outcome of this investigation.

PROCEDURE

The laboratory should participate in EQA Schemes appropriate to the range of tests performed. All EQA samples should be tested as routine samples to ensure that any results produced mirror those which would be reported to customers. EQA scheme providers should hold suitable accreditation (e.g. ISO/IEC 17043:2010 Conformity assessment - general requirements for proficiency testing).

If a suitable EQA scheme is not available an appropriate IQA scheme (Ring Trial) may be set up at regular intervals. Suitable samples (mock samples, previously tested samples or QC samples) may be distributed to laboratory staff for analysis with results collated and analysed by the Quality Assurance Manager in the same way as for EQA samples. Results should be validated before issue as quantitative results may change during storage.

These Ring Trials may be performed 'blind' with the Laboratory Analysts unaware that the sample is part of an IQA exercise. This produces a more accurate representation of how a genuine sample will be handled and processed.

The EQA scheme provider will provide a schedule for the distribution of samples. If a Ring Trial scheme is set up a schedule should be prepared which meets the needs of testing, the regularity of the distributions may be increased or decreased at the discretion of the Laboratory Manager/Director and Quality Assurance Manager and recorded appropriately with justification.

EQA/IQA material may be used for training and competency assessment but results should not be compiled in collaboration between operators in a way that routine samples would not be handled. If more than one operator participates in processing of an EQA/IQA sample for training purposes, it must be agreed beforehand which operator's results will be submitted so as not to compromise the integrity of the EQA/IQA exercise.

EQA/IQA material should be challenging to the operators to best provide evidence of competence.

In the event that the results are unacceptable immediate investigative action is required by the Quality Assurance Manager and/or designated personnel. The investigation should clearly identify and document the cause of failure and the remedial action taken. Such action may include retesting the original QA sample (if available and the condition is acceptable) or sourcing additional QA material from the Scheme provider.

The supervising Laboratory Analyst must advise the Laboratory Manager/Director and Quality Assurance Manager who will determine what action should be taken and whether testing should be suspended or recalled.

The action taken and the outcome of the investigation is documented and retained on file. The Laboratory Manager/Director and Quality Assurance Manager should monitor subsequent EQA/IQA returns to ensure that any remedial actions taken remain effective. The Laboratory Manager/Director and Quality Assurance Manager should assess any possible impact on similar testing carried out prior to the EQA/IQA failure and if necessary recall such work for retest as soon as is practicable.

The Quality Assurance Manager should monitor trends in the EQA/IQA results for laboratory samples (where appropriate). Trend analysis of numerical data (where possible) should be used to identify areas where preventive action may be required.

In the event that EQA/IQA results have been within specification, although there is an evident bias the Laboratory Manager/Director must ensure that such information is considered in the interpretation of test results.

Preventive action may result in test methods, user guides or procedures being amended. If appropriate equipment should be re-calibrated or serviced, reagents or test kits rechecked and staff training or retraining provided.

REFERENCES

European co-operation for Accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 5.9 Assuring the quality of tests and calibration results*. Geneva, Switzerland.

ISO/IEC 17043:2010. *Conformity assessment - general requirements for proficiency testing*. Geneva, Switzerland.

Documentation and control of documents

PURPOSE AND SCOPE

The purpose of this procedure is to define the methods and responsibilities for the introduction, approval, review, amendment, and withdrawal of controlled documents.

Controlling documents prevents the possibility that different procedures for the same method are in use at any one time and that only the authorized method is used. The authorized method will be validated and reviewed to ensure it remains relevant. To further avoid the possibility of different procedures for the same method being in use at any one time personal laboratory notebooks and uncontrolled copies of documents should be prohibited.

Controlled documents may be any part of the Quality Management System including the Quality Manual, SOPs, User Guides, Logs or worksheets used in the laboratory.

This procedure applies to all controlled documentation within the Quality Management Systems. It may also apply to documentation within the Health and Safety Management System, Environmental Management System or general administration and management documentation.

RESPONSIBILITIES

- **Laboratory Analysts** – To ensure that controlled documentation at point of use are current, valid, relevant and is the correct version.
- **Laboratory Manager/Director** – To ensure all controlled documents are authorized for use and fit for purpose.
- **Quality Assurance Manager** – To facilitate the document control procedure, the correct issue, review and withdrawal of controlled documentation.

PROCEDURE

Introduction / Amendment of a controlled document

Controlled documents can be written by any member of staff who is trained and competent in the procedure to which the document refers.

The originator will assign a unique document reference number obtained from the Quality Assurance Manager (this number should be in compliance with any existing system in operation).

A Change Control procedure will be managed by the Quality Assurance Manager and is a formal way of tracking any changes made to a controlled document. Any changes requested must be detailed, justified and approved by a technically competent person before implementation. The change control must be documented and retained by the Quality Assurance Manager. The procedure may be a simple computer spreadsheet.

The name of both the author and person authorising the document must be recorded in the header of the document. An 'effective date' and review date must also be assigned. A version number should be included to easily track the correct version of controlled documents and preclude the possibility of using an invalid or obsolete document.

It is advisable to include a 'Log of updates' in controlled documents to track changes made. This makes it easier to establish quickly what changes have been made when a document is re-issued. This can be a simple box included below the header where changes are summarized.

The Laboratory Manager/Director must ensure all appropriate staff who may use the controlled document are made aware of the new/revised document. These staff must familiarise themselves with any relevant changes. The Laboratory Manager/Director must also consider if any training is required for a new procedure and if so ensure this is identified and delivered prior to a new procedure being adopted.

Test kit/reagent inserts (including microbiological media)

The Laboratory Manager/Director is responsible for ensuring that any changes in manufacturer's instructions are reviewed and implemented and that any amendments required to controlled documents are made using the Change Control procedure.

Evidence of review of test kit/reagent inserts will be signified by the initials and date of the relevant Laboratory Analysts on instructions/inserts and recording of lot number and date opened, which shall be retained on file at the relevant laboratory. Should a change be noted this will be communicated to the Quality Assurance Manager and Laboratory Manager/Director.

Calibration, Quality Control (QC) and Certified Reference Material (CRM)

The inserts/certificates provided with calibration, QC and CRM are reviewed between lots to ensure that the correct tolerance limits are achieved and implemented. Evidence of the review and action taken is signified by the date; calibrator or QC value; and initials of the relevant Laboratory Analyst on the inserts and retained on file in the relevant laboratory.

Equipment manuals

The Laboratory Manager/Director is responsible for ensuring that any updated equipment manuals are reviewed and implemented and that any amendments required to controlled documentation are made using the Change Control procedure.

Withdrawal of a controlled document

When a controlled document is revised or is deemed to be obsolete all controlled copies of the revised document must be withdrawn by the Quality Assurance Manager.

All master copies will be archived for a minimum of 7 years by the Quality Assurance Manager (or a time period agreed locally which meets legal and customer requirements).

Review of controlled documentation

Each controlled document will be allocated a review date (maximum five years). User Guides for equipment shall be valid for the lifetime of the equipment.

The Quality Assurance Manager will contact the author (or other suitably experienced or qualified person) when the review is due. The author will arrange for the controlled document to be reviewed.

If the document is found to be valid and current, this is noted as 'Reviewed on (date)' in the Log of Updates box. The document will be reissued with a new review date and version number.

If the document requires updating, the change control procedure must be followed.

When the review is complete, the reviewer(s) will notify the Quality Assurance Manager as appropriate and the revised document will be issued.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 4 Management requirements, 4.3 Document control*. Geneva, Switzerland.

UKAS. 2009. *LAB 31 Use of culture media procured ready-to-use or partially completed in microbiological testing*. UKAS publication Lab 31 edition 2, June 2009. Feltham, UK.

Health and safety (including risk assessment) in the microbiology laboratory

PRINCIPLE AND SCOPE

Laboratories can be one of the most hazardous places in which to work. Microbiological laboratories, due to the nature of work undertaken, pose a particular risk from the pathogens involved and potential zoonosis.

To minimise the risk of accidents and injury to staff, visitors and society at large as much as is practicable, the laboratory must have in place a Health and Safety Management System.

Injuries, accidents and ill health in the laboratory all have a financial component, in addition to damage to premises, property, equipment and the environment and lost productivity and potential liabilities. These can all be minimized with an effective Occupational Health and Safety Management System.

This may be separate to the Quality Management System or integrated with the Quality Management System (and Environment Management System if one exists) to form an Integrated Management System (IMS).

All Management Systems require policies, organisational and planning systems to be put in place and procedures to measure the performance and effectiveness (auditing) which identifies opportunities for improvement. This applies to a Health and Safety Management System as much as it does to a Quality Management System.

Certification to an appropriate Occupational Health and Safety Management System, such as BS OHSAS 18001:2007 may be considered.

A Health and Safety Manager may be identified or this role may be performed by the Quality Assurance Manager after suitable training.

RESPONSIBILITIES

- **Laboratory Analyst** – To comply with all requirements of the Health and Safety Management System and appropriate regulations and legislation.
- **Laboratory Manager/Director** – To ensure an effective management structure is in place and that all staff are committed, motivated and empowered to work safely.
- **Health and Safety Manager** – To manage the Health and Safety Management System, promote and communicate a positive occupational health and safety culture within the laboratory. Keep up to date with developments in Health and Safety management practice, legal and regulatory requirements relevant to the microbiology laboratory and ensure appropriate compliance. This responsibility may be that of the Quality Assurance Manager.

PROCEDURE

Health and Safety policies demonstrate commitment from senior management that the laboratory is engaged in developing and maintaining effective Health and Safety practices and in their continuous improvement. The policies demonstrate that the laboratory meets legal and regulatory requirements and instils confidence in employees, customers and society that the laboratory is committed to good Health and Safety practice.

The Laboratory Manager/Director, along with the Health and Safety Manager, must ensure that all staff are motivated to work in a safe manner and protect their long term health and that of their colleagues. Staff must feel empowered to be actively involved in the Health and Safety Management System by involving all staff in the development and continuous improvement of the Health and Safety Management System and encouraging engagement. An effective communication system and promotion of safe practices will greatly help in achieving this goal.

The Health and Safety Management System must encompass all aspects of the laboratory as hazards can exist wherever there is work activity. The Health and Safety Management System should cover:

- the premises, including offices, laboratories, entrances, exits, welfare facilities and all plant which are part of the fixed structure;
- plant and substances, including the receipt, handling, transport, storage and disposal of submitted samples, chemicals and reagents;
- procedures, including all routine and non routine processes performed in the laboratory; and
- people, including all employees, their competence and any health surveillance which may be required.

All laboratory work involves some risk and the aim of an effective Health and Safety Management System is to minimise these risks. The Risk Assessment procedure identifies hazards, evaluates possible risk and sets an objective of eliminating the hazard (if practicable) and reducing the risk to a minimum.

Monitoring

A system of self monitoring (audit) should be set up to measure the performance of the Health and Safety Management System. This should cover all aspects of the work conducted by the laboratory and include buildings, premises, equipment, reagents, procedures and people. This type of monitoring is referred to as 'active monitoring'.

Like auditing the Quality Management System (or Environmental Management System if available) when findings are noted corrective and preventive actions (CAPA) should be identified, agreed and put in place to correct the problem and prevent reoccurrence. These CAPA should then be monitored for effectiveness.

A system for investigating incidents when they occur (accidents, ill health or incidents which could cause injury) should also be in place. This type of monitoring is referred to as 'reactive monitoring' and like active monitoring should identify CAPA to minimise the possibility of reoccurrence.

Potential incidents (near misses) should also be investigated and CAPA identified to avoid future incidents. A near miss or minor incident may often appear trivial and it may

be tempting to ignore it but they often have the potential to become a major incident.

For instance if a laboratory analyst slips on a puddle from a leaking sample the result could vary from insignificant to catastrophic as follows:

- they are unhurt;
- they stain their clothing;
- they sprain their ankle;
- they break their ankle;
- they fracture their skull; or
- they die from a head injury.

Studies have shown that there will always be considerably more 'minor' incidents than 'major' ones but frequently minor incidents have the potential to have been major incidents. All incidents regardless of whether they are minor, major or a near miss demonstrate that controls have failed and merit investigation.

Control of health and safety

The Laboratory Manager/Director and the Health and Safety Manager shall work closely with all line managers to allocate responsibilities. Line managers must take responsibility for the working environment and develop Health and Safety policies pertaining to their own specialist area.

All staff allocated Health and Safety responsibilities should be suitably trained and competent.

These responsibilities may include:

- carrying out risk assessments;
- preparing Health and Safety policies and procedures;
- performing Health and Safety audits;
- accident investigation;
- providing first aid when required;
- supervising contractors; or
- fire safety.

DOCUMENTATION

A Health and Safety policy should be produced by the Laboratory Manager/Director and Health and Safety Manager which clearly demonstrates the commitment of the Laboratory to a robust Occupational Health and Safety Management System and shall provide a framework for any other Health and Safety documentation as necessary.

This policy should also set Health and Safety objectives and make a commitment to continuous improvement. The policy should identify the Health and Safety Manager (or person with that responsibility) and detail their responsibilities and those of other staff with regards Health and Safety. The policy should outline how Health and Safety matters will be effectively communicated, resourced and competencies achieved and maintained. The Health and Safety policy should be signed and dated by the Laboratory Manager/Director.

Other documentation which may be necessary may include:

- specific health and safety SOPs;
- risk assessments;

- Material Safety Data Sheets or COSHH data;
- emergency plans;
- accident report forms (including 'near miss' reporting);
- audit checklists and pro formas; and
- logs.

Planning and implementation of the health and safety management system

To best achieve commitment of staff at all levels planning and implementation of the Health and Safety Management System should be a collaborative effort involving all stakeholders.

Procedures and policies put in place should always be proportionate to the needs, risks and hazards of the laboratory.

A Gap Analysis is a useful tool to compare the current position, with regards Health and Safety Management, with the framework described in this manual and the appropriate Health and Safety legislation and regulations. After completing a Gap Analysis a plan for the implementation and ongoing maintenance of the Health and Safety Management System can be prepared with specific objectives and targets set.

Controlling health and safety risks

In addition to controlling occupational risks associated with safety in the workplace controls must be put in place to deal with occupational risks associated with the health of employees, visitors and society at large. The link between work activities and health issues is less apparent than the link between work activities and injuries at work. Ill health resulting from work activities can take a protracted time to become apparent but can be extremely serious or life threatening and to control such health risks requires a health strategy to be in place.

Risks to health in a microbiology laboratory include:

- zoonosis/infection by inhalation, ingestion or injection;
- skin contact with irritant substances;
- exposure to toxic chemicals;
- repetitive strain injury from extended periods of repetitive actions (e.g. pipetting);
- badly designed workstations; and
- burns.

Like all risks in the microbiology laboratory health risks need to be controlled to reduce the risk as much as is practicable. For both safety risks and health risks this is done by performing a risk assessment which will identify the health and safety hazard, assess the risk and decide on the most appropriate control measure.

RISK ASSESSMENTS

Risk Assessments should be conducted by competent individuals who are familiar with the processes involved. It may be advisable to engage a Health and Safety professional or an occupational health nurse or advisor to assist in conducting some Risk Assessments.

Relevant statutory requirements must be considered when conducting a Risk Assessment.

There are many ways of conducting a Risk Assessment but the simplest is to assign a score of 'likelihood' of an incident occurring multiplied by 'severity'. The score obtained is considered the 'risk rating'.

When considering the likelihood of an incident occurring, take into account any existing control measures, statistical data (e.g. accident records), personal knowledge and any other relevant data that is available.

When estimating severity, consider the worst foreseeable injury, damage or loss and the number of people who might be affected.

In the case of using a Bunsen burner the severity of a potential injury from an operator burning their hand in the flame whilst using the Bunsen burner could be regarded as 'minor', the burn may require first aid. The likelihood of this happening with an experienced Laboratory Analyst could be considered 'unlikely'. The risk rating would therefore be 2 x 2 = 4. It is probably not practicable to put any further controls in place.

In the case of handling *E. Coli O*157 samples on the open bench the possibility of infection from aerosol contamination when opening positive vials could be considered 'Possible'. The severity, if this was to happen and someone contracted an infection, could be considered 'Major'. The risk rating would therefore be 3 x 4 = 12. It would be advisable to put additional controls in place.

If the sample vials were processed in a safety cabinet in a dedicated Category 3 containment laboratory the likelihood would reduce to 'Very unlikely'. The risk rating would therefore be 1 x 4 = 4.

FOR EXAMPLE

	Very unlikely 1	**Unlikely 2**	**Possible 3**	**Likely 4**	**Very likely 5**
Insignificant 1					
Minor 2		X			
Moderate 3					
Major 4	X		X		
Catastrophic 5					

Risk rating	**Definition**
1–4 (low)	No further action is required in the short or medium term but the activity must be kept under review.
5–9 (medium)	Efforts must be made to reduce the risk within a specified time period but the cost associated with such prevention may be considered.
10–16 (high)	Urgent efforts are required to reduce the risk. If appropriate consider if the procedure should be suspended until control measures are in place.
17–25 (very high)	Activities must be suspended immediately and must not restart until the risk can be adequately controlled.

Once additional controls are in place the risk should be re-evaluated and the risk rating obtained should be acceptable. If it is not reduced the control measures can be reviewed to reduce the risk further.

When applying controls to reduce the risk rating a control hierarchy can be used which has increased management control required as the list progresses.

Eliminate the risk

- Substitute a less hazardous substance or procedure.
- Substitute a less hazardous piece of equipment or one that offers better protection.
- Avoid certain procedures or practices e.g. buy a product from a subcontractor rather than make it in the laboratory.

Combat the risk

- Separate the operator from risk of exposure by enclosing the process.
- Protect dangerous parts of equipment by guards or shields.
- Design activities to minimise or suppress airborne hazards e.g. segregate.

Minimise the risk

- Use of Personal Protective Equipment (PPE).
- Assign warning notices to equipment.

Provision of PPE and warning notices should be regarded as a last resort and should only be used if the hazard cannot be controlled in any other way.

General health and safety rules for a microbiology laboratory

- Eating (including chewing gum) is not permitted in the microbiology laboratory.
- Drinking is not permitted in the microbiology laboratory.
- Smoking is not permitted in the microbiology laboratory.
- Food and drink must not be stored in laboratory fridges.
- Mobile telephones and personal music players are not permitted in the microbiology laboratory.
- Laboratory doors must be kept closed to comply with fire regulations and increase air balance within the microbiology laboratory.
- Laboratory windows should be kept closed to prevent escape of pathogens or contamination from outside.
- Visitors must sign in at reception, be supplied with PPE and be accompanied by a member of laboratory staff at all times.
- White laboratory coats must be worn, fastened, at all times and removed when leaving the laboratory.
- Persons under working age are not permitted in the microbiology laboratory;
- Work areas must be kept clean and uncluttered.
- All Laboratory Analysts must be familiar with the hazards associated with any procedure they are involved in (i.e. COSHH, Risk Assessments etc.).
- Lone working is to be avoided at all times.

- Appropriate disinfectant (e.g. 5% sodium hypochlorite) must be available to deal with spills and bench areas cleaned at the end of a procedure (all spills of microbiology samples must be considered hazardous and treated as such).
- Hands must be washed thoroughly before leaving the laboratory.
- Long hair must be tied back.
- First aid kits and eye wash must be available in the laboratory and trained first aiders available on site.
- Adequate fire extinguishers should be available and staff familiar with their use.

Additional rules for Containment Level 2 (CL 2) Safety

- Access to the microbiological laboratory is limited to individuals who are working in the laboratory.
- Persons who are at increased risk of acquiring infection (immunocompromized), or for whom infection may have serious consequences (such as pregnant women), are not allowed in the microbiological laboratory or may have the work they perform restricted. The Laboratory Manager/Director and Health and Safety Manager have the final responsibility for assessing each circumstance and determining who may enter or work in the microbiological laboratory.
- Laboratory personnel will receive appropriate immunisations or test for the agents handled or potentially present in the microbiological laboratory.
- When appropriate, considering the agent(s) handled, a baseline serum sample for microbiological laboratory staff and other at-risk personnel will be collected and stored.
- The laboratory Manager/Director and Health and Safety Manager must ensure that laboratory personnel receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures. The training must be documented in the individuals training file.
- Personnel must receive additional training when procedural or policy changes occur.
- Personal health status may impact an individual's susceptibility to infection, ability to receive immunisations or prophylactic interventions. Therefore, all laboratory personnel and particularly women of child-bearing age should be provided with information regarding immune competence and conditions that may predispose them to infection. Individuals having these conditions should be encouraged to self-identify to the institution's healthcare provider for appropriate counselling and guidance.
- Individuals not listed on the authorized personnel list, located on the outside of the door, can only be admitted by the Laboratory Manager/Director and they must be accompanied by an authorized individual at all times.
- Avoid the creation of splashes or aerosols.
- Decontaminate all work surfaces on the completion of work or at the end of the day and after any spill or splash with an appropriate disinfectants (e.g. 5% sodium hypochlorite).
- A high degree of precaution must always be taken with any contaminated sharp item, including needles, syringes, slides, capillary tubes and scalpels.
- Plastic ware should be substituted for glassware whenever possible.

- Used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes or otherwise manipulated by hand before disposal; rather, they must be carefully placed in conveniently located puncture-resistant, autoclavable container used for sharps disposal.
- Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass are decontaminated before disposal, according to any local regulations.
- Cultures, tissues, specimens of body fluids, or potentially infectious wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.
- Contaminated equipment must be decontaminated according to any local regulations before it is sent for repair or maintenance or packaged for transportation in accordance with applicable local regulations, before removal from the facility. Decontamination should be documented.
- Immediately report all spills and accidents that result in overt exposures to infectious materials to the Laboratory Manager/Director and Health and Safety Manager.
- Provide medical evaluations, surveillance, and treatment as appropriate and maintain written records.
- An insect and rodent control program must be in place.
- A properly maintained biological safety cabinet must be used whenever conducting procedures with a potential for creating infectious aerosols or splashes.
- Cloth chairs are not permitted in the microbiological laboratory.
- Face protection (goggles, mask, face shield or other splatter guards) must be used for anticipated splashes or sprays of infectious or other hazardous material to the face when the microorganisms must be manipulated outside the biological safety cabinet.
- Gloves must always be worn whenever handling potentially infectious materials, contaminated surfaces or equipment.
- Dispose of gloves whenever they are overtly contaminated, when work with infectious material is completed, or when the integrity of the glove is compromized. Disposable gloves are not to be washed, reused, or used for touching 'clean' surfaces (keyboards, telephones, etc.).
- Dirty laboratory coats should be sterilized and washed as appropriate. If an operator is planning to wear a laboratory coat again, it must be hung up in the microbiological laboratory. Laboratory coats should never be worn outside the microbiological laboratory once they have been worn inside the microbiological laboratory.
- Laboratory coats will be put in the washer by laboratory personnel.
- Individuals must wash their hands after they handle viable materials, after removing gloves and before leaving the microbiological laboratory.
- Exit Procedure
  - Remove gloves.
  - Remove laboratory coat.
  - Hang up laboratory coat or send for sterilising and washing.
  - Wash hands.

- Materials to be decontaminated outside of the immediate microbiological laboratory are placed in a durable, leak proof, autoclavable biological waste bag and closed for transport from the laboratory.
- All containers and vials will be wiped down with 5% sodium hypochlorite or 70% alcohol before exiting the CL 2 lab for storage.

Note: avoid using alcohol on vials to prevent the ID markings from being removed.

Additional rules for Containment Level 3 (CL 3) safety

- Access to the Containment Level 3 laboratory is limited to individuals who are working in the lab and are identified as approved to work in the facility. A notice listing these individuals should be posted outside the Containment Level 3 Laboratory and controlled by the Laboratory Manager/Director.
- Dedicated laboratory coats or surgical suits must be worn at all times in the Containment Level 3 facility and removed before leaving the facility.
- Dedicated equipment should be available in the Containment Level 3 facility and not shared with other parts of the laboratory.
- A window should allow viewing of the individual(s) working in the Containment Level 3 facility.
- Windows should be sealed shut.
- Containment Level 3 waste will be autoclaved before disposal and treated separately to Containment Level 2 waste.

Additional rules for Containment Level 4 (CL 4) safety

There are specific rules and guidelines for operating a Containment Level 4 (CL 4) laboratory which should be sought from appropriate experts should there be a need to operate such a facility. It is extremely unlikely that an animal feed laboratory would be required to operate such a facility.

In addition, the microbiology laboratory should have an effective maintenance programme for all equipment, regular checks of any gas pressure systems and monitoring of fire safety procedures (e.g. fire drills etc.) and equipment (e.g. fire extinguishers, fire doors, alarms and smoke detectors).

The microbiology laboratory should prepare emergency (contingency) plans to deal with events such as:

- fire;
- gas leak;
- biosecurity;
- bomb scare; and
- chemical spill.

REFERENCES

BS OHSAS 18001:2007. *Occupational health and safety management systems – requirements*. BSI, London, UK.

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G*: 2002. Paris, France.

Health and Safety Executive (HSE). 2001. *HSE Management, design and operation of microbiological containment laboratories*. HSE Books, Sudbury, UK.

Audits, corrective actions and management review in the microbiology laboratory

PRINCIPLE AND SCOPE

The purpose of this procedure is to describe the methods and responsibilities for performing periodic Internal Audits of all activities to demonstrate continual improvement and to verify compliance with Quality Management Systems requirements and all relevant international standards.

External audits (third party audits) may be conducted by accreditation, certification or regulatory bodies and will follow a similar process to that described in this procedure. Any third party audits will be managed by the Quality Assurance Manager who will liaise directly with the third party accreditation or certification body regarding audit plans, logistics and any corrective and preventive actions (CAPA) that may be identified.

The same procedure may be used for audits within the Health and Safety Management System (and Environmental Management System should this exist) that are in operation in the microbiology laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To comply with all requirements of the Quality Management System and appropriate regulations and legislation and to demonstrate this through accommodating periodic internal or external audits of procedures and systems.
- **Laboratory Manager/Director** – To ensure any internal or external audits are accommodated and that CAPA identified are addressed within timescales agreed.
- **Quality Assurance Manager** – To manage the internal audit system and ensure an effective audit schedule exists that covers all aspects of the Quality Management System and testing within the laboratory. The Quality Assurance Manager may train other staff to conduct internal audits on his/her behalf. The Quality Assurance Manager will co-ordinate any external (third party audits) that may be conducted in the laboratory.
- **Health and Safety Manager** (or person identified with this responsibility) – To manage the internal audit system and third party audits for the Health and Safety Management System if applicable. Alternatively this may be managed as part of an Integrated Management System.
- **Environment Manager** (or person identified with this responsibility) – To manage the internal audit system and third party audits for the Environmental Management System if applicable. Alternatively this may be managed as part of an Integrated Management System.

PROCEDURES

Audit schedule

The Quality Assurance Manager shall prepare an internal audit schedule which covers all aspects of the Quality Management System and testing performed in the laboratory. Each test performed in the laboratory should be audited on a regular basis which is agreed between the Laboratory Manager/Director and Quality Assurance Manager. This should be based on the perceived 'risk' associated with the procedure with the frequency being reduced as fewer findings are noted. Normally a test should be audited once per year. A trained auditor will be identified for each audit who is independent of the activity to be audited. Where resources do not permit a trained auditor may audit in his/her area but not audit their own work.

Additional audits may be added to any schedule as and when required, such as following corrective actions, complaints etc.

Audit preparation

The Auditor will agree the audit date with the relevant Laboratory. The Laboratory Manager/Director will ensure that all information and a suitably qualified member of staff to accompany the auditor are available on the pre arranged date.

The Auditor will review any previous audit findings in order to identify systematic or protracted problems at the site to be audited or previous CAPA that need to be checked for effectiveness since they were implemented.

The Auditor may prepare an Internal Audit Checklist to act as an 'aide memoir' when conducting the audit. A copy of the relevant procedure will assist the auditor in identifying non conformances.

Performing the audit

The Auditor will arrange an opening meeting with the auditee(s), where the scope of the audit will be agreed. The Auditor will confirm the status of any previous audit findings.

Any findings noted during the audit will be discussed with the auditee and recorded. When a finding is identified the auditor should consider, by checking previous findings and in discussion with the auditee, whether the finding is systematic at the site being audited.

If an audit finding casts doubt on the integrity of a test performed by the laboratory, the laboratory must take immediate corrective action and notify customers in writing if investigations demonstrate that laboratory results may have been affected.

The auditor may also note 'Observations' or 'Opportunities for Improvement' which are not non-conformances against a standard or procedure but may be considered by the auditee(s) and Laboratory Management as appropriate.

When the audit is complete, the Auditor will review any findings with the auditee(s) and CAPA will be agreed with a timescale. All notes taken and documentation supplied, including any copies of laboratory worksheets, will be filed by the Quality Assurance Manager, after completion of the audit report.

Audit report

The Auditor will compile a brief audit report using a standard agreed format (which will be provided by the Quality Assurance Manager).

The report will contain the following:

- audit reference number;
- audit title;
- scope of audit;
- names of auditor(s) and auditee(s);
- audit date;
- introduction, briefly describing the overall impression of the site being audited;
- any systematic or protracted problems noted;
- list of findings which are non conformances against a standard or management system;
- list of any observations or opportunities for improvement;
- date of issue; and
- circulation list.

The completed report will be copied to the relevant Laboratory Manager/Director, Quality Assurance Manager and the supervisor of the area being audited. Department managers may distribute the report to any staff that they deem appropriate.

Audit follow up / close out

The auditee and auditor will ensure that each agreed corrective action is completed before the due date (maximum of one month from the date of the audit or other suitable, agreed local timescale). Progress of findings should be monitored by the auditee(s) and auditor at local meetings. For certain serious findings immediate close out may be required

When satisfied that evidence demonstrates completion of CAPA, the auditor will close each finding.

The Quality Assurance Manager will monitor all CAPAs and report any trends in findings to the Laboratory Manager/Director.

Follow up audits can verify and record the effectiveness of any previous CAPA that have been implemented.

Microbiology laboratory internal audit checklist

It may prove useful to use a checklist when conducting an audit of a testing procedure. A simple checklist ensures all relevant questions are asked and provides a useful opportunity to compare different testing activities (to identify systematic problems) and to ensure consistency in audit approach, which may be useful when different auditors may be used.

DEFINITION

- **Auditee** – Person being audited.
- **Auditor** – Person conducting the audit.

Internal Audit Checklist (Example)

Audit Reference Number: _____

Audit Scope: _____

Auditor: _____ **Audit Date** _____

Question	Comments
Organisation and management:	
- Is there an organisational chart available?	_____
- Is it current?	_____
Quality Management System:	
- Are SOPs current?	_____
- Are SOPs available at point of use?	_____
- Is there an index of SOPs available?	_____
- Are documents controlled effectively?	_____
- Is the test method being witnessed validated?	_____
Complaints:	
- Have there been any recent complaints?	_____
- Have there been any recent anomalies?	_____
Non conformances:	
- Are there any open non conformances?	_____
- Are there any systematic failures?	_____
EQA/IQA:	
- What EQA/IQA is available?	_____
- How is EQA/IQA reviewed?	_____
- Have there been any recent failures?	_____
- If so how were these addressed?	_____
Test records:	
- Availability?	_____
- Is there a clear audit trail?	_____
- Traceability?	_____
- Are they held securely?	_____
- What is the retention policy?	_____
Training files:	
- Is/are the operator(s) trained in the procedure?	_____
- Do all the operators have a job description?	_____
- Do all the operators have a CV?	_____
- How is competency maintained?	_____
- Do all the operators participate in EQA/IQA?	_____
- Are there any trainee staff?	_____

Question	Comments
Accommodation and environment:	
- Are the facilities fit for purpose?	
- Are the facilities clean?	
- Is there evidence of good housekeeping?	
- Are there cleaning records?	
- Is there controlled access?	
- Are customer records secure?	
- Are maintenance/service records available?	
- Is calibration/servicing up to date?	
- Is equipment uniquely identified?	
- What PPE is available?	
Balances:	
- Is the balance serviced regularly?	
- Are check weights traceable to SI units?	
- Are daily checks performed?	
Temperature sensitive equipment:	
- Are temperature ranges stated?	
- Are temperature logs up to date?	
- Is the temperature range being maintained?	
- Have fridge/freezer/incubator(s) been profiled?	
- Are thermometers calibrated?	
- Are correction factors applied?	
- Are annual ice point check (or other suitable temperature checks) performed?	
Pipettes:	
- Are pipettes calibrated?	
- Are calibration records up to date?	
Media/reagents:	
- Batch numbers/expiry dates of media/ reagents	
- QC records	
- Is storage of media/reagents appropriate?	
Test procedure:	
- Correct SOP used?	
- Version number of SOP?	
- Is SOP authorized?	
Sample integrity:	
- How is sample integrity preserved?	
- How are samples stored before/after testing?	
- How are samples disposed of?	

Question	Comments
Test report:	
- Do report details match correct sample?	
- Has the correct testing been performed?	
- Is an appropriate audit trail visible?	
- Do the results match the lab records?	
- Was the test reported within timescale?	
- Has the report been authorized?	
- How was report issued?	
Health and Safety:	
- Are there any Health and Safety concerns?	
Environmental:	
- Are there any environmental concerns?	
Additional comments:	

Management review

Periodically (at least once per year) the laboratory management should conduct a review of the Quality Management System, laboratory testing activities and the results of internal audits, external audits and any anomalies or complaints that have been raised. The purpose of this review is to ensure the continuing suitability and effectiveness of the Quality Management System and seek opportunities for improvement.

Attendees at the Management Review should include, at least, the Laboratory Manager/Director, Quality Assurance Manager and supervisory staff from the laboratories.

Similarly a management review should be conducted of the Health and Safety Management System and Environmental Management System, if appropriate, and be attended by the Health and Safety Manager and Environmental Manager or persons with that responsibility.

The Management Review should be recorded and action points assigned as appropriate. The agenda should include the following:

- the suitability of current policies and SOPs;
- report from Laboratory Manager/Director;
- report from Quality Assurance Manager (and Health and Safety Manager and Environmental Manager if appropriate);
- report(s) from supervisory Laboratory Analysts;
- findings from recent internal audits;
- findings from recent external (third party) audits;
- corrective and preventive actions;
- results of EQA and IQA (and relevant IQA issues);
- anomalies;
- customer complaints;
- changes in volume or type of work undertaken and resources;
- customer feedback; and
- opportunities for improvement.

REFERENCES

BS OHSAS 18001:2007. *Occupational health and safety management systems – requirements*. 4.5 Checking. BSI, London, UK.

ISO 9001:2008. *Quality management systems – requirements. 8.2 Monitoring and measurement*. Geneva, Switzerland.

ISO 14001:2004. *Environmental management systems – requirements with guidance for use. 4.5 Checking*. Geneva, Switzerland.

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 4. Management requirements, 4.14 Internal audits*. *4.15 Management review*. Geneva, Switzerland.

ISO 19011:2011. *Guidelines for auditing management systems*. Geneva, Switzerland.

UKAS. 2009. *LAB 3 The conduct of ukas laboratory assessments*. UKAS publication ref Lab 3 edition 4, August 2009. Feltham, UK.

Corrective and Preventive Actions (CAPA)

PURPOSE AND SCOPE

The purpose of this procedure is to describe the methods for investigating failures of control within the Quality Management System.

Although having a robust Quality Management System in place will greatly improve the confidence in the work performed within the microbiology laboratory no system will ever prevent all anomalies or complaints from customers.

A failure may result from within the microbiology laboratory (anomaly) and can be defined as an unpredicted event which has had, or had the potential to have, an affect on the integrity of reported results from the microbiology laboratory or the Quality Management System. Like Health and Safety 'near miss' reporting, potential anomalies can be useful in preventing a true (and potentially more serious) anomaly occurring.

A failure can be received directly from a customer who is dissatisfied with the product (microbiology laboratory report) that they have received (complaint).

Anomalies and complaints are handled in the same way, by an investigation which establishes the root cause of the failure, corrects the failure and puts in place systems to prevent a recurrence of the same failure. This demonstrates continuous improvement of the Quality Management System.

Without a thorough investigation anomalies and complaints will generally be dealt with by way of a 'quick fix' which does not address the route cause or prevent a repeat occurrence of the same issue.

The same procedure may be used for the Health and Safety Management System (and Environmental Management System should this exist) that are in operation in the microbiology laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To comply with all requirements of the Quality Management System and initiate the internal anomaly or complaints investigation system whenever this is required.
- **Laboratory Manager/Director** – To ensure any internal anomalies or customer complaints are addressed appropriately within timescales agreed. The Laboratory Manager/Director must also respond directly to customers when a complaint is received.
- **Quality Assurance Manager** – To manage the internal anomaly and customer complaints system and ensure effective investigations are conducted which establish a root cause and corrective and preventive actions (CAPA) whenever failures of control within the Quality Management System are identified.

- **Health and Safety Manager** (or person identified with this responsibility) – To manage the internal accident reporting (including 'near miss' reporting) and ensure effective investigations are conducted which establish a root cause and corrective and preventive actions (CAPA) whenever failures of control within the Health and Safety Management System are identified.
- **Environment Manager** (or person identified with this responsibility) – To manage the internal environmental anomaly and incident reporting system and ensure effective investigations are conducted which establish a root cause and corrective and preventive actions (CAPA) whenever failures of control within the Environmental Management System are identified.

PROCEDURE

Anomalies

Any member of staff can raise an anomaly with the Quality Assurance Manager. When the member of staff has decided to do this they must inform the Quality Assurance Manager immediately. The Quality Assurance Manager will assign a unique reference number to the incident and initiate the investigation with the operator.

An anomaly can be identified within the Quality Management System, Health and Safety Management System or Environmental Management System.

A brief description of the anomaly must be recorded and how this has, or may have, affected the integrity of results reported from the microbiology laboratory or the Quality Management System.

The Quality Assurance Manager should liaise with the Laboratory Manager/Director and establish if similar such anomalies have occurred previously and if so cross reference previous events.

Together the investigation team should identify a root cause for the anomaly and evaluate any potential impact on laboratory results (impact assessment) issued prior to the identification of the anomaly. It may be necessary to recall results and/or notify customers of the anomaly. Consideration should also be given to suspending testing to avoid any further anomalies until such time as CAPA are implemented.

Once the root cause has been established corrective actions should be identified to eliminate the problem and prevent any recurrence. Preventive actions, once identified, should be implemented to reduce the likelihood of the occurrence identified ever happening again.

All details of the root cause analysis, impact assessment, investigation and any CAPA identified should be recorded for future reference. Once any corrective and/or preventive actions are implemented they should be monitored for effectiveness.

The Quality Assurance Manager should use review of root cause analyses to establish systematic problems, identify training needs and provide trends for the Laboratory Manager/Director.

An example of an anomaly form which could be used is below:

Anomaly reference number _____

Date raised _____

Raised by _____

Details of anomaly:

Include a description of the anomaly, sample ID, test/procedure being performed, equipment details, customer etc.

Root cause analysis:

Impact assessment on results reported prior to anomaly being raised:

Include possible impact on any tests, or opinions and interpretations, issued prior to the identification of this anomaly. *Cross reference any similar anomaly if possible.

Corrective actions:

Preventive actions:

Review of effectiveness of CAPA:

Date anomaly closed _____

Authorized by _____

Complaints

Complaints received from customers should be handled in a similar way to anomalies.

Any member of staff may receive a complaint from a customer and should notify the Quality Assurance Manager immediately. Complaints received by telephone or in person should immediately be passed to either the Quality Assurance Manager or Laboratory Manager/Director. The Quality Assurance Manager will assign a unique reference number for the complaint.

Once all the details of the complaint have been established the customer should be notified in writing that the matter is being investigated. It may be necessary to clarify some details or seek further information directly from the customer. This should be done by either the Quality Assurance Manager or Laboratory Manager/Director.

The Quality Assurance Manager will establish if similar complaints have been received and will conduct an investigation into the complaint with the operators concerned. Together the investigation team should identify a root cause for the complaint and evaluate any potential impact on laboratory results (impact assessment) issued prior to receiving the complaint. It may be necessary to recall results and/or notify other customers of the investigation. Consideration should also be given to suspending testing to avoid any further complaints until such time as CAPA are implemented.

Once the root cause has been established corrective and preventive actions (CAPA) should be identified to eliminate the problem and prevent any recurrence of a complaint.

All details of the root cause analysis, impact assessment, investigation and any CAPA identified should be recorded for future reference. Once any corrective and/or preventive actions are implemented they should be monitored for effectiveness.

The Quality Assurance Manager should use review of root cause analyses to establish systematic problems, identify training needs and provide trends for the Laboratory Manager/Director.

The customer who made the complaint should be provided with the outcome of the investigation in writing by the Quality Assurance Manager or Laboratory Manager/Director as soon as it is concluded.

It may be that after investigating a complaint there is no corrective and preventive action required. This may be as result of confusion or mistake on the part of the customer who has complained. This should still be documented in the complaints procedure for future reference.

An example of a complaint form which may be used is below:

Complaint reference number _____

Date raised _____

Received by _____

Customer details:

Complaint details:

Include details of the following where relevant: unique identification, areas of the business involved, the test or procedure being deviated from, equipment details etc.

Impact assessment on results reported prior to anomaly being raised:

Include possible impact on any tests, or opinions and interpretations, issued prior to the identification of this anomaly. *Cross reference any similar anomaly if possible.

Investigation:

Root cause analysis:

Impact assessment on results issued prior to complaint being received:

Include possible impact on any tests, or opinions and interpretations, issued prior to the identification of this complaint. *Cross reference any similar complaints if possible.

Corrective and preventive actions identified:

Review of effectiveness of CAPA:

Date customer notified of outcome _____

Date complaint closed _____

Authorized by _____

REFERENCES

ISO/IEC 17025:2005. *General requirements for the competence of testing and calibration laboratories. 4.11 Corrective action. 4.12 preventive action*. Geneva, Switzerland.

ISO 19011:2011. *Guidelines for auditing management systems*. Geneva, Switzerland.

PART 2

Quality assurance and general laboratory procedures

Microbiological media, reagents and chemicals

PRINCIPLE AND SCOPE

The purpose of this procedure is to ensure that all bacteriological media, reagents and chemicals are appropriate for the tests concerned and are prepared, quality controlled and stored appropriately in order to maintain the standards and performance of all bacteriological techniques.

RESPONSIBILITIES

- **Laboratory Analysts** – To comply with all local procedures regarding the purchasing, receipt, storage, preparation and use of all chemicals, reagents (including commercial kits) and microbiological media used in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure the correct chemicals, reagents (including commercial kits) and microbiological media are available for use in the microbiology laboratory and that facilities exist for their correct storage.
- **Quality Assurance Manager** – To ensure that chemicals, reagents (including commercial kits) and microbiological media are validated before use and that appropriate quality control procedures are used to maintain confidence.
- **Health and Safety Manager** (or person identified with this responsibility) – To ensure appropriate controls are in place (COSHH, Risk Assessment etc.) for all chemicals and reagents in use in the microbiology laboratory.
- **Environment Manager** (or person identified with this responsibility) – To ensure appropriate environmental controls are in place to deal with potential spills or escapes and to dispose of chemicals and reagents in the microbiology laboratory appropriately.

EQUIPMENT

- Balance and weighing boats.
- Autoclave.
- Steamer and/or microwave oven.
- Browne's tubes as required.
- pH meter.
- Microbiological loop.
- Spatula.

REAGENTS

- Dehydrated culture media and appropriate supplements.
- Distilled or deionised water.

Ensure that all dehydrated media, supplements and other ingredients are stored according to the manufacturer's instructions and used before the expiry date stated. Label all containers with the date of receipt and date first opened. After use, ensure that lids remain tightly sealed to prevent moisture ingress. Discard any media that has deteriorated in condition.

PROCEDURE

Microbiological media

To grow and isolate bacteria, yeast and fungi artificial growth media are used which provides the microbiological agent with optimum growth conditions. To minimise contaminants some media may contain inhibitory substances that suppress bacteria other than the target organism(s). Others may only allow growth of a particular type of organism or have indicators which change colour when certain organisms are present.

The specific media for the procedure will be stated in the appropriate SOP and must always be used.

To avoid the cost and time required in producing microbiological media many microbiology laboratories purchase pre-prepared microbiological media from a variety of production companies. Ideally this should be purchased from a reputable supplier who holds accreditation to ISO/IEC 17025:2005 for production and quality control of microbiological media and thus avoid the need for time consuming validation and quality control.

Batches of pre prepared media purchased should be identifiable and be accompanied by evidence that they meet the specified quality requirements. The user should ensure arrangements are in place to be notified by the manufacturers of any changes to specification.

Should the laboratory wish to manufacture its own microbiological media then the freeze dried media and constituents should be purchased from a reputable company and the media must be prepared and sterilised as follows:

- Prepare each microbiological media according to manufacturer's instructions or published formula.
- Use a clean spatula to weigh out the amount required (± 0.1 g for dehydrated media up to 100 g) into a clean weighing boat on a calibrated balance.
- Suspend the weighed powder in the required volume (± 2%) of distilled or deionised water.
- Check the pH of the media where specified and adjust if necessary.
- Distribute into final containers if appropriate.
- Sterilise according to specific instructions. (See 'Use of autoclaves' SOP).
- Ensure that media are not overheated during sterilisation.
- If necessary, allow media to cool to required temperature for addition of supplements and other additives. Agar media solidify at temperatures below 50 °C.
- Distribute aseptically into Petri dishes or bottles as required (see Asceptic technique procedure).
- Label with medium identifier, batch number and expiry date as appropriate. The base of the Petri dish (containing the media) should be labelled and not the lid, to avoid lids being accidentally swapped.

- Dry poured plates before use by placing the lid upside down in an incubator, a plate drying cabinet, or laminar flow cabinet. Angle the media-containing dish (media downward) either within or on the edge of the lid for up to 20 min or until there is no moisture visible on the agar.

Storage of microbiological media

- Store all prepared plates at 2–8 °C in the dark and in sealed containers or bags to avoid dehydration.
- Ensure that media are used in rotation, using oldest first, and discard any media outwith the expiry date.
- Dehydration during storage will cause concentration of ingredients, which may affect the performance and shelf life of the medium. The extent of dehydration may be determined by check-weighing during storage. The weight loss should not exceed 5%.
- Specific stock bottled liquid media and agar bases may be stored at 15–30 °C in line with manufacturer's or supplier's instructions.
- Check for signs of deterioration or contamination before use.

Microbiological media quality control

Internal quality control (IQC) is carried out on all media produced in-house. Quality control must only be performed using verified control organism (e.g. NCTC or ATCC) which are preserved and sub cultured in accordance with supplier guidelines. A control organism must not be sub cultured repeatedly or it may become 'lab adapted' loosing some of its characteristics. A stock of control culture should be held (i.e. cryopreserved) and cultured directly on a weekly basis, or as required.

The following parameters are checked on randomly selected units from each batch and recorded on the appropriate log sheets:

- pH is measured with a calibrated pH meter using a flat-head probe. If outside the acceptable range, the medium is discarded and the reason investigated;
- sterility is checked by incubation at 37 ± 1 °C for 2 days and 2–8 °C for 10 days respectively. Evidence of contamination should be investigated and the batch rejected;
- growth support is checked by a quantitative comparison of a representative target organism on the test batch with a non-selective control medium and/or previous batch;
- selectivity is checked using a representative organism to check the efficacy of selective agents;
- where a medium contains an identification indicator (e.g. MacConkey, lactose fermentation), representative organisms are used to check positive and negative reactions; and
- shelf life is checked by retaining sufficient aliquots of the requisite media to repeat the above checks at expiry date.

QC growth comparison method

This method is used to compare batches of non-selective media and for performance testing of selective media for target recovery.

- Prepare a suspension of the appropriate test organism in saline equivalent to McFarland turbidity standard 0.5.
- Prepare a 1:100 dilution by transferring a 10 µl loopful to 1 mL saline (Dilution A).
- Prepare three serial tenfold dilutions from Dilution A and label B, C and D.
- Divide the test plate and a nutrient or blood agar plate (control) into quadrants using a permanent marker on the underside.
- Transfer 25 µl of each dilution (A, B, C and D) to each plate quadrant.
- Spread each drop over the plate segment using a sterile loop.
- Incubate as appropriate to the test organism.
- After incubation, compare the growth on each plate. Select the quadrant which gives isolated colonies and count for each plate. Record the counts obtained in a Media QC Log.
- All in-house prepared media are deemed to be satisfactory if there is a minimum 50% recovery on the test medium when compared to the control medium.
- For selective broths, prepare dilutions A–D as above and inoculate 25 µl into each of four broths from the test batch and the control (previous) batch.
- Incubate as appropriate and then subculture to the required agar medium.
- Each batch should yield growth from the same dilution.
- Selective Media - efficacy of selective (inhibitory) agents
- Prepare a suspension of the appropriate test organisms in sterile saline equivalent to McFarland turbidity standard 0.5.
- Streak a charged loop from each suspension as appropriate onto the surface of the selective plate or inoculate a loopful into a selective broth and also onto a control nutrient or blood agar to check viability.
- Incubate as appropriate to the medium usage.
- Examine after incubation for growth of the test organisms.
- Growth should normally be totally inhibited. Any growth indicates failure of the selective agents. The medium should be rejected and the cause of the failure investigated.

Note: E. coli on BGA may only be partially inhibited and show trace growth, record as such in the Media QC Log. This is acceptable provided that the growth is significantly inhibited when compared with the viability control plate.

Chemicals and reagents

Chemicals and reagents used in the preparation of microbiological media must be stored as stated in the manufacturer's instructions. MSDS and COSHH data must be retained at point of use and passed to the Health and Safety Manager (or nominated person) for reference. Chemicals and reagents must not be used after their expiry date and disposed of in accordance with local Health and Safety and Environmental legislation.

Reagents that are produced in house must have appropriate quality control checks performed before use to ensure they are fit for purpose and meet the requirements. Any quality control checks should be performed on verified reference cultures as for microbiological media. Results of quality control checks should be recorded in an appropriate reagent log.

Commercial kits

Commercial kits should only be purchased from reputable manufacturers and stored as stated in the manufacturer's instructions. Commercial kits should only be used as intended and any modification to procedure or scope should be regarded as a modification to a standard method and validation performed (see Microbiology testing – selection and verification).

When opening a kit for the first time the expiry date should be checked to ensure it is still valid and the date the kit was opened written on the box or container. Constituent parts of kits should not be swapped from kit to kit.

Commercial kits are usually supplied with controls which should be used on each occasion. Periodic checks can also be performed using verified reference cultures, routine cultures should never be used to verify performance of a commercial kit.

Manufacturers instruction inserts should be retained with the kit and each time a new kit is started the new instruction insert should be compared with the previous one to ensure there have been no changes in methodology. If there have been changes this should be highlighted to the appropriate staff and if necessary the appropriate SOP changed.

If there are no changes the Laboratory Analyst should indicate this on the instruction insert and retain the insert in the kit. Previous insets should be disposed of to ensure the correct insert is available at all times.

QUALITY CONTROL

Commercially available McFarland standards may be purchased with which to compare turbidity.

HEALTH AND SAFETY

Minimise the production of dusts when handling dehydrated media or chemicals. Use a disposable half-mask respirator approved to EN149:2001 + A1:2009 standard and CE marked when identified in MSDS, COSHH or Risk Assessment documentation. Some formulations may contain specific hazards. Pay particular attention to all hazard labels and refer to product safety data sheets, relevant COSHH forms and risk assessments.

Quality control testing of media involves the handling of Category 2 (CL 2) organisms. Where media are specifically targeted as Category 3 (CL 3) organisms, non-toxigenic controls should be used where possible.

Special care is needed when handling hot media after autoclaving or during boiling.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for Microbiology Laboratories EA-04/10 G: 2002*. Paris, France.

UKAS. 2009. *Lab 31 Use of culture media procured ready-to-use or partially completed in microbiological testing*. UKAS publication Lab 31 edition 2, June 2009. Feltham, UK.

Receiving microbiological samples

PURPOSE AND SCOPE

The purpose of this procedure is to document the procedure which should be followed by staff receiving microbiological samples for analysis. This procedure applies to all samples received and will preserve the integrity of submitted samples and ensure the safety of those handling them.

RESPONSIBILITIES

- **Laboratory Analyst** – Receives laboratory samples, ensures they are suitable for testing requested and preserves their integrity.
- **Laboratory Manager/Director** – Ensures sample reception staff are proficient in receipt, handling and documentation of samples. Ensures appropriate Health and Safety procedures are available and are followed (this may be delegated to the Health and safety Manager or other nominated individual).
- **Quality Assurance Manager** – Ensures sample integrity is preserved at all stages of handling and storage of samples. To periodically perform internal audits of the sample receipt procedure to ensure compliance.

EQUIPMENT

- PPE (gloves and eye protection as appropriate).
- Safety cabinet (as appropriate).
- Palate knife.
- Fridge.

REAGENTS

Appropriate disinfectant (e.g. 5% sodium hypochlorite).

PROCEDURE

Samples submitted for testing may be received in many different ways; in the post (mail), by courier or submitted directly by the customer or their agent. Upon receipt it must be ensured that full details of the customer are recorded and the testing required. This may be by way of a submission form that accompanies the sample and will form a contract between the laboratory and customer. Any missing details that are required must be completed by the customer before they leave the sample or, if the sample is received by post or via a courier, by contacting the customer and noting the missing details on the submission documentation. If missing details are collected in this way it must be annotated on the submission documentation with the date and time that the information was noted.

Appropriate PPE (e.g. gloves and eye protection) must be available for staff who receive laboratory samples.

Each sample must arrive in a suitable condition for testing, in an appropriately labelled container with appropriate documentation. Samples submitted for microbiological testing may contain pathogens hazardous to health and it is the responsibility of the customer to ensure that there is no risk to sample reception staff, couriers or post office workers who may handle the sample. Local regulations may dictate the sample containers and labelling which is required for biohazardous material.

If appropriate the laboratory must check and record the temperature of samples on receipt, as this may have had an effect on the integrity of the sample in transit. Also consider if the sample was frozen, defrosted or suffered high temperatures during transport as this may influence the results of future analysis.

Upon receipt, if a sample is leaking it must be immediately transferred to a safety cabinet along with any contaminated documentation and packaging. A suitably trained person may now open the package and using an appropriate disinfectant (e.g. 5% sodium hypochlorite) the container should be cleaned and if necessary the contents transferred into another suitable container.

No attempt should be made to clean documentation or packaging. Packaging must be disposed of as biohazard waste. The documentation, if contaminated, should be placed inside a clean 'poly pocket' or clear plastic bag and photocopied. The original can then be disposed of as biohazard waste and photocopy used in its place.

In the event that a sample has been submitted in a glass container and has broken in transit no attempt should be made to clean or recover the sample. It should be disposed of appropriately and the customer notified.

All details of leaking samples should be recorded on the submission documentation and communicated to the customer as a comment on the laboratory report. All incidents of leaking microbiological samples should be communicated to the Health and Safety Manager.

In the event that a sample is too heavily contaminated or compromised and unsuitable for testing, it must be disposed of as biohazard waste and the customer notified.

Samples may also be rejected if there is physical deterioration, potential contamination due to leakage or mingling of samples submitted together, insufficient sample for testing or an inappropriate sample.

If an inappropriate or insufficient sample is received it may still be processed but this must first be agreed with the customer by the Laboratory Manager/Director and all reports generated with such samples will include a statement indicating their status as insufficient or inappropriate and tested outwith the agreed scope of testing.

Samples must be assigned a unique laboratory identification number immediately upon receipt. This number will be assigned both sample and documentation and will be used throughout testing and for all storage containers, laboratory reports, worksheets etc. The date and where necessary the time of receipt and sample condition may also be recorded.

Upon receipt the sample must be transferred to the microbiology laboratory immediately. In the microbiology laboratory the Laboratory Analysts can review the testing requested and either begin sample processing, or store the sample pending testing.

The sample integrity must be preserved at all times and the sample must be stored in a way to preserve it in the most suitable manner to minimise any change in microbial load. For microbiological samples this is usually at 2–8 °C in the dark. Samples stored at 2–8 °C must be brought to room temperature (18–21 °C) before processing begins (See Handling and preparation of microbiological samples). Samples should not be frozen on receipt.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

ISO 6498:2012. *Animal feeding stuffs – Guidelines for sample preparation*. Geneva, Switzerland.

Handling and preparation of microbiological samples

PURPOSE AND SCOPE

This procedure describes the handling and any preparatory work that is required of animal feed samples submitted to the laboratory for microbiological analyses. Specific handling requirements are dictated by the nature of each sample. Correct handling and preparation of samples preserves the integrity of the sample, minimises any change in microbial load and ensures the safety of those handling them.

This procedure applies to all laboratory feed and feed ingredient samples submitted to the laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To handle and prepare samples submitted to the laboratory for microbiological testing in the correct manner.
- **Laboratory Manager/Director** – To ensure laboratory staff are trained and competent to handle and process samples submitted for microbiological testing.
- **Quality Assurance Manager** – To ensure sample integrity is preserved at all stages of handling and preparation and performs regular audits to ensure compliance.
- **Health and Safety Manager** (or person identified with this responsibility) – To ensure all staff who handle and prepare microbiology samples are suitable trained with regards appropriate hazards and have appropriate PPE available.

EQUIPMENT

- PPE (gloves and eye protection as appropriate).
- Safety cabinet (as appropriate).
- Sterile palate knife.
- Sterile knife or scalpel.
- Sterile forceps.
- Sterile scissors (to trim long material if necessary).
- Fridge at 2–8 °C.
- Vortex.
- Mechanical shaker.
- Grinders or mills.
- Rifflers.
- Homogenisers.
- Sieve.
- Balance.

- Dividing or quartering apparatus.
- Tools and hammer (for hard solid samples) which may be sterilised.

REAGENTS

Appropriate disinfectant (e.g. 5% sodium hypochlorite).

SAMPLING PROCEDURE

Note: There are specific guidelines for sampling of material submitted for detection of Processed Animal Protein (PAPs). See Section 12 Detection of Processed Animal Protein (PAPs) in animal feed samples.

The volume of sample used to test for a microbiological agent will be stipulated in the relevant SOP. When selecting a subsample for microbiological examination care should be taken not to contaminate the sample with organisms from the environment, which may overgrow the organisms in the original sample or produce false results.

Unless the sterility of sampling equipment (grinders, mills, rifflers etc.) can be guaranteed they should not be used when processing microbiology samples.

The sample used must be representative of the whole sample submitted. Ideally customers should be instructed on the required volume and sampling procedure that is required for testing to avoid additional sub sampling and storage/disposal of large quantities of feed for analysis by the laboratory.

If large samples of feed are submitted the following procedures can be used to achieve a suitable representative or selective sample:

Sampling can be one of two types; representative sampling or selective sampling.

Representative sampling

Representative sampling obtains a small fraction from a larger volume in such a way that a determination of a required characteristic will represent the mean value of the characteristic of the entire sample.

Selective sampling

If a noticeable difference is observed in a portion of the sample to be analysed this portion shall be separated from the entire sample and treated as a separate lot. If this is not possible the entire sample shall be processed and the proportion of sample which had a noticeable difference recorded.

In either case details shall be recorded in the final report to the client.

Statistical consideration

Acceptance sampling is the usual method of sampling for animal nutrition laboratories. For sampling by attributes, there is a theoretical sampling plan based on binomial distribution. This plan has been simplified to a square root relationship between lot size and the number of increments.

With bulk product sample variances are expected to be uniform if, for lots of up to 2–5 tonnes at least seven increments are taken and for between 2–5 and 8 tonnes the number

of increments taken is at least equal to √20 m (where m = mass in tonnes of the sample). If the lot exceeds 80 tonnes, the square root relationship is still applicable but will be less accurate.

Sampling equipment

Grinders or Mills. These should be capable of grinding feedstuffs without causing any noticeable changes in moisture in the sample and should not generate excessive heat (which may have a detrimental effect on the sample). If the feedstuff to be analysed is likely to lose or gain moisture a correction factor should be applied to the results. This is determined by comparing the moisture content of the prepared (ground or milled) sample against a portion of the original sample before processing. Once ground or milled the sample should be suitable to pass through an appropriate sieve. Grinders or mills should be thoroughly cleaned after use to avoid cross contamination.

(Examples of grinders or mills include: Retsch SR-3, ZM 200 & SR-300 Rotor Beater Mill, Romer Model R.A.S. Mill, Retsch ZM-1 Mill, Robot Coupe).

- Rifflers. Examples include: Carpco SS-16-25, Carpco SS-32-12X, Retsch PK1000.
- Homogeniser (or mechanical stirrer), to homogenise moist feedstuffs.
- A mincer with a 4 mm plate may also be useful for frozen feedstuffs.
- Sieves, of aperture size 1.0 mm, 2.8 mm and 4.0 mm made from woven metal wire cloth or similar.
- Mechanical shaker, used to shake viscous liquid molasses sample.
- Dividing or quartering apparatus. Equipment such as a conical divider or a multiple slot divider with a sorting system will ensure uniform dividing of laboratory samples.
- Tools including hammer (used to hit screwdriver/chisel) screwdriver/chisel (used to break molasses block into smaller pieces) and a spatula may also be used.
- Transfer pipette, used in the delivery of wet feed and liquid feed.
- Sample containers, these should be suitable for preserving the sample integrity avoiding any changes or effects from moisture, temperature or light. The sample container should be sterile, of suitable size to allow storage of sufficient sample to complete all determinations required (not less than 100 g) and there should be some air space left once filled (to allow effective mixing prior to sampling for all tests required). The container should have a securely fitting lid and should be uniquely identified with a sample identifier on the container and not the lid.

If a sample is to be examined microbiologically it should be handled under sterile conditions to preserve the microbiological load.

To avoid exposure to the atmosphere grinding or milling should be as rapid as possible. It may be necessary to break or crush the sample prior to using the grinder or mill.

Fine samples

If a sample can pass through a 1.0 mm sieve it should be mixed thoroughly and divided successively using dividing or quartering apparatus. Lumps should be removed, crushed in a disinfected mortar, returned to the sample and mixed.

Coarse samples

If the sample does not pass through a 1.0 mm sieve but passes through the 2.8 mm sieve it should be ground until it does pass through the 1.0 mm sieve and divided as for fine samples. Likewise if a sample passes through the 4.0 mm sieve but not the 2.8 mm sieve. Lumps should be removed, crushed in a disinfected mortar, returned to the sample and mixed.

Difficult to grind samples

If a sample is difficult to grind using the grinder or mill the moisture content of a subsample from the initial sample should be determined and the remaining sample crushed with a mortar and pestle until it passes through the 1.0 mm sieve. The moisture content of the crushed sample should then be determined to allow a correction factor to be assigned.

Grass or cereal silage

The entire sample should be milled and mixed. Some samples may require chopping finely beforehand. If required the moisture content of the sample may be determined beforehand by drying a portion in an oven overnight at 60–70 °C.

Liquid samples

The sample should be mixed using the homogeniser or stirrer to ensure complete dispersal of separated material and the sample transferred using a sterile wide bore pipette.

Sample handling

Dry feed sample preparation

- Riffle the sample. Generally, riffle the original sample until a satisfactory sized sub-sample is obtained. Place the riffled, unground sample in a sterile labelled Ziplock (or similar) bag and the other portion(s) of unground sample back in the sample bag to be retained. Label each container (bag, bottle, etc.) with a matching identification number (e.g. a barcode).
- Grind one of the portions (bag portion) through the appropriate mill.
- Grinding or milling of some samples may lead to a loss or gain of moisture and volatile matter, allowances should be made for this. Grinding should be as rapid as possible to avoid exposure to the atmosphere.
- Place ground sample on sterile paper or a sterile flat bottomed dish.
- Thoroughly mix the sample before taking the laboratory portion.
- Fill a sterile laboratory sample bottle with sufficient ground sample by passing through the mixed sample.
- Return to the sample storage area immediately after analysis.

Procedure for obtaining laboratory sample

- Allow sample to warm to room temperature if stored in a freezer or refrigerator.
- Mix the sample by rolling the bottle and tilting the bottle from the left to right (twirl) for several seconds. Do not shake.
- Weigh sample by means appropriate for the desired method.
- Return the sample to the appropriate storage area.

Liquid molasses samples

- Samples should be stored refrigerated.
- Allow sample to warm to room temperature.
- Shake sample vigorously for 1 minute to provide thorough mixing. For those samples that are too viscous to be shaken by hand, a mechanical shaker needs to be used. Shake the sample for no less than 15 min or as described in the appropriate SOP to provide thorough mixing.
- Weigh sample by means appropriate for the desired method. (Clean all spillage from the outside of the bottle).
- Return the sample to the appropriate refrigerator.

Molasses/lick block samples

- Samples should be stored refrigerated. Do not freeze.
- Allow sample to warm to room temperature.
- For soft blocks using a spatula cut off small portions of the sample from different locations around the block until the desired weight is achieved. Mixing is not practical.
- For hard blocks chisel off small portions of the sample from different locations around the block until the desired weight is achieved. (Use a hammer and screwdriver/chisel, ensure appropriate personal protective equipment is used).
- Return the sample to the refrigerator.

SAMPLE HANDLING PROCEDURE

Testing shall begin on the day of receipt of samples or on the first working day afterwards which allows the method to be completed in accordance with the appropriate SOP. If testing is not to begin on the day of receipt the samples must be stored in accordance with the appropriate SOP (usually 2–8 °C) in the dark. If samples have been refrigerated before testing they should be removed to room temperature (18–21 °C) for a minimum of one hour prior to the start of the procedure. Samples should be stored in such a way that no cross contamination can occur with other diagnostic samples.

Micro-organism may be viable in dry feed with low pH (as low as 5.5) and in high pH (above 10.0) for long periods due to their inactive state. In prepared solutions for microbiological analysis micro-organisms may become active and an unfavourable pH may affect their viability. In dry feed samples the pH should be adjusted as specified in the appropriate method using sodium hydroxide solution or hydrochloric acid as necessary.

Trace elements (especially copper) in compound, mineral feed, premixtures and supplementary feedstuffs when present in their ionic form, are toxic in high concentrations for micro-organisms. The toxic threshold lactic acid bacteria and yeasts is approximately 20 mg Cu/L in suspension. A selective, water soluble chelating agent (e.g. iminodiacetic acid) may be used to chelate the copper as specified in the appropriate method.

For fine samples, coarse samples, grass or cereal silage samples and semi solid samples the sample must be well mixed using a sterile palate knife, spoon or stirring rod before sub sampling and the required portion removed using a sterile spatula or spoon and placed in a sterile container or sterile foil which is suitably identified. Long material, such as hay, may be cut using sterile scissors to a more appropriate length if required. The sterile container, or foil, can be placed on a balance to obtain the exact amount required for testing.

Liquid, or semi liquid, samples should be thoroughly mixed by inverting (not shaking) or placing on a vortex mixer to ensure complete dispersal of any separated material and the required volume transferred using a wide bore sterile pipette. For samples that are too viscous to be mixed by hand or on a vortex (e.g. molasses) the sample may be placed on a mechanical shaker for 15 min or as described in the appropriate SOP.

Solid samples should have small pieces cut from them using a sterile knife or scalpel. Ideally the sample should be cut to expose an uncontaminated side and portions taken from this 'clean' area to avoid contamination which may have been introduced when the sample was initially taken. The sub sample should be placed in a sterile pot suitably identified.

The sample should be manipulated using sterile forceps or, if by hand, using sterile laboratory gloves. Non sterile laboratory gloves may be washed in alcohol after putting them on to sterilise.

After the required portion of sample has been taken this should be passed immediately to the Laboratory Analysts who will commence testing. Any remaining sample should be returned to storage for the agreed time period before being destroyed. The retention period will be stated in the appropriate SOP and should be such a time period that allows any subsequent retesting to be performed after the laboratory report has been issued to the customer. Samples submitted to the microbiology laboratory should never be returned to the customer after testing.

Samples should be disposed of in a way that they pose no biological harm (i.e. autoclaving or incineration before disposal through normal locally agreed waste channels).

HEALTH AND SAFETY

Minimise the production of dusts when handling fine powders. Use a disposable half-mask respirator approved to EN149 standard and CE marked when identified in Risk Assessment documentation.

When using a knife or scalpel care must be taken. Consider using cut resistant gloves (e.g. Kevlar™ or similar) under normal laboratory gloves. If using tools to chip portions of solid material appropriate eye protection should be worn.

REFERENCES

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ISO 6498:2012. *Animal feeding stuffs – Guidelines for sample preparation*. Geneva, Switzerland.

ISO 21528-2:2004. *Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae Part 2: Colony-count method*. Geneva, Switzerland.

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Microbiological identification using traditional and commercial methods

PRINCIPLE AND SCOPE

The purpose of this procedure is to provide a framework for the identification of bacterial isolates which may be of veterinary or zoonotic significance.

This procedure applies to all bacterial isolates in the animal feed microbiology laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the controlled procedures available in the microbiology laboratory and their experience to identify organism isolated in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the experience and competence to identify organism which are isolated using the resources that are made available.
- **Quality Assurance Manager** – To ensure controlled procedures are available for all processes involved in the identification of organisms in the microbiology laboratory and that staff have the required competence. Also to perform regular audits to ensure compliance.

EQUIPMENT

- Slides.
- Microscope.
- Bunsen burner.
- Microbiological loops.
- Forceps.

REAGENTS

Appropriate commercial kits and routine microbiological identification reagents as required.

PROCEDURE

Taking account of information supplied by the customer (the type of sample and test procedure used) the Laboratory Analyst, using their knowledge of the colonial appearance of bacteria, augmented if necessary by microbiology texts, records the colonial morphology and/or suspected identity of relevant growths on isolation media.

Where indicator media or highly selective media are used it can be assumed that growth observed on a plate is that of the target organism. In this case the Laboratory Analyst must confirm the identification using techniques stated in the relevant SOP.

Where growth is judged to be a mixed growth resembling normal flora or of contaminants unlikely to be of clinical significance, then the growth may be so recorded without further attempts at colonial description or identification.

The microbiologist, using knowledge and skills augmented by relevant texts, will decide which organisms are relevant and should be pursued further, using a range of primary characterisation tests which may include: Gram stain, catalase, oxidase, indole, motility and atmospheric growth requirements (see appropriate procedures).

Primary identification tests are assessed to determine if the information is sufficient to make a presumptive identification and whether further tests should be carried out.

Where minimum criteria are insufficient for a presumptive identification, further testing will be carried out using appropriate microbiological tests as identified in appropriate SOPs.

If the results give a positive identification, they are recorded.

If the results are not satisfactory, further testing should be carried out until a satisfactory identification is made. When a satisfactory identification of an organism deemed to be significant cannot be made, the organism should be sent for referral to a specialist laboratory.

ENTEROBACTERIACEAE

Enterobacteriaceae are facultative, Gram negative rods which are catalase positive, oxidase negative and ferment carbohydrates.

Members of the Enterobacteriaceae are widely found throughout the environment and some are normal inhabitants of the intestinal tract of man and animals. The genera contain organisms that can be pathogenic to animals and some are zoonotic.

Isolates with the typical colonial appearance and Gram reaction of Enterobacteriaceae for which further identification is deemed appropriate should be tested for their oxidase reaction.

Oxidase negative isolates may be tested using a commercial biochemical index system (e.g. API 20E™) following the manufacturer's instructions.

When the test is completed, a numerical profile is obtained which is entered into a database to obtain the probable identity of the isolate along with a % probability.

The group Enterobacteriaceae contains *E. coli*. For further identification of *E. coli* O157 refer to the SOP for isolation and identification of *E. coli* O157.

For further identification of *Salmonella* refer to the SOP for isolation of *Salmonella* spp.

GRAM NEGATIVE RODS THAT GROW AEROBICALLY OTHER THAN ENTEROBACTERIACEAE AND PASTEURELLACEAE

Gram negative rods for the purposes of this SOP are a broad group of bacteria, a few of which may be significant veterinary pathogens. They include both strictly aerobic and facultative genera. The majority of isolates will be oxidase positive but a few may be oxidase negative.

Isolates for which further identification is deemed appropriate should be tested for their oxidase reaction and may be further tested using a commercial biochemical index system (e.g. API 20NE™) following the manufacturer's instructions.

When the test is completed, a numerical profile is obtained which is entered into a database to obtain the probable identity of the isolate along with a % probability.

See 'Enterobacteriaceae detection and enumeration from feed samples' SOP.

NON-SPOREFORMING GRAM POSITIVE BACILLI

The Gram positive non-sporeforming bacilli represent a large and diverse group of organisms, which contain many organisms of significance for animals. Cellular morphology permits separation into two groups comprising regular and irregular bacilli.

Regular Gram positive Bacilli

There are two genera of major importance, *Listeria* and *Erysipelothrix*, which can be separated by catalase test.

Listeria

Regular bacilli that are catalase positive are suspect *Listeria* spp. The genus contains pathogenic and non-pathogenic members, the major pathogens being *Listeria monocytogenes*, which infects a range of hosts, and *Listeria ivanovii,* which is normally recovered from sheep only.

These organisms can be identified using a commercial biochemical index system (e.g. API™, MICRO-ID™ or similar) following the manufacturer's instructions with the addition of the tests listed in Table 1.

Listeria monocytogenes hydrolyse aesculin.

Listeria spp. (particularly *L. monocytogenes*) demonstrate characteristic 'tumbling motility' when a 2–4 hour broth culture (incubated at 25 °C is examined using the hanging drop method). See 'Gram stain and primary characterisation tests' SOP.

See '*Listeria* testing of feed samples' SOP.

QUALITY CONTROL

Primary characterisation tests

- Catalase – according to manufacturer's instructions.
- Oxidase – checked with appropriate positive and negative controls and the results recorded in the appropriate laboratory log.
- Indole – checked with appropriate positive and negative controls and the results recorded in the appropriate laboratory log.

Commercial biochemical index system

- Strips and reagents are deemed fit for purpose upon receipt until the manufacturer's expiry date, provided that they are returned immediately to the correct storage conditions immediately after use.

TABLE 1

Organism	**ß-Haemolysis**	**CAMP Test** *S. aureus*	**CAMP Test** *R. equi*
L. monocytogenes	Narrow zone often not extending beyond the colony edge.	+	-
L. ivanovii	Wider more pronounced zone.	-	+

Other commercial identification tests

- Reagent tablets, antisera, commercial kits and sensitivity discs are tested with positive and negative controls. Results are recorded on the appropriate laboratory log.

Reporting the results

Organisms will be reported to the genus or species dependant upon the requirements of the case and as stipulated in the appropriate SOP.

REFERENCES

Carter, G.R. & Cole, J.R. 1990. *Diagnostic procedures in veterinary bacteriology and mycology*, 5th edn. Academic Press. ISBN 0-12-161775-0. Virginia, USA.

Quinn, P.J., Carter, M.E., Markey, B. & Carter, G.R. 1994. *In*: Clinical veterinary microbiology, Wolfe Publishing. ISBN 0 7234 1711 3. Dublin, Ireland.

Gram stain and primary characterisation tests

PRINCIPLE AND SCOPE

The purpose of this procedure is to describe primary characterisation tests which may be used for the identification of bacterial isolates which may be of veterinary or zoonotic significance.

This procedure applies to all bacterial isolates in the animal feed microbiology laboratory.

This procedure includes the following methods:

- Gram reaction;
- oxidase reaction;
- catalase reaction;
- indole test;
- glucose fermentation test;
- sugar fermentation test (i.e. L-rhamnose, mannitol, D-xylose etc.);
- aesculin hydrolysis test (also referred to as Esculin or Æsculin);
- motility test; and
- nitrate reduction test.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the controlled procedures available in the microbiology laboratory and their experience to identify organism isolated in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the experience and competence to identify organism which are isolated using the resources that are made available.
- **Quality Assurance Manager** – To ensure controlled procedures are available for all processes involved in the identification of organisms in the microbiology laboratory and that staff have the required competence. Also to perform regular audits to ensure compliance.

EQUIPMENT

- Slides and coverslips (including slides with well for hanging drop motility test).
- Microscope with oil immersion objective (X100) and dry objective (X400).
- Bunsen burner.
- 3 mm, 10 µl Microbiological loops (and straight wire) made from nickel/chromium or platinum/ iridium (plastic loops should be used for Oxidase and Catalase tests).
- Forceps.

REAGENTS

- Appropriate microbiological identification reagents as required.
- Crystal violet or methyl violet.
- Lugol's or Gram's iodine.
- Acetone.
- Carbol fuchsin.
- N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD).
- Hydrogen peroxide (H_2O_2).
- Kovac's reagent (Isoamyl alcohol, para-Dimethylaminobenzaldehyde).
- Aesculin agar slope.
- Nitrate reduction broth.
- Sulfanilic acid.
- N,N-dimethyl-1-naphthylamine.
- Zinc powder.
- Tryptophan or peptone broth.
- Nitrate Motility Medium (NMM).
- Saline.
- Distilled or deionised water.

PROCEDURES

Gram reaction

Bacterial cell walls confer rigidity to the cell and protect it against osmotic damage. Bacterial cell walls differ in structure, some have a simple structure made of peptodoglycan and teichoic or teichuronic acid, and others also have an outer membrane with surface lipopolysaccharide.

These differences produce different reactions when stained with biological stains, the less robust cell walls absorbing the first stain (crystal violet or methyl violet) and staining purple. The more robust cell walls with lipopolysaccharide outer membrane do not absorb the first stain and can be decolourised with acetone, these bacteria can then be counterstained red with carbol fuchsin. Bacteria which stain purple are referred to as 'Gram positive', those that stain red are referred to as 'Gram negative'.

Gram negative bacteria will often require higher concentrations of disinfectant to destroy them due to their more robust cell walls.

Reagents

- Crystal violet or methyl violet (0.5%).
- Lugol's iodine (or Gram's iodine):
  - Iodine 1 g
  - Potassium iodide 2 g
  - Distilled water 100 mL
- Acetone.
- Carbol fuchsin (10%).
- Commercial Gram reagents may be purchased, ready to use, from a suitable scientific reagent supply company.

- Prepare a film of the organism to be identified by emulsifying a small amount of a colony in a loopfull of distilled or deionised water on a microscope slide. Allow to dry and heat fix by passing through a Bunsen flame.
- Flood the slide with 0.5% crystal violet (or methyl violet) for 30 to 60 seconds.
- Wash off with Gram's iodine (Lugol's iodine) and flood the slide. Leave for 30 to 60 seconds.
- Rinse in tap water. Where the cleanliness of local tap water cannot be guaranteed suitable laboratory water should be used instead (see 'Laboratory Water' SOP).
- Decolourise with acetone (until no more violet colour emanates from the smear). Decolourisation is very rapid and is usually complete in two to three seconds. After this period of contact with acetone the smear must immediately be washed thoroughly with water under a running tap. Longer exposure to acetone may cause decolourisation of some Gram positive organisms.
- Counterstain with dilute carbol fuchsin (10%) for 10–30 seconds.
- Rinse in tap water and blot dry using clean blotting paper then allow to air dry completely before examining microscopically by oil immersion (X100).
- Gram positive organisms will be stained purple.
- Gram negative organisms will be stained red.

Quality Control

New batches of stain are checked for suitability before use by staining reference organisms (e.g. NCTC or ATCC).

Oxidase reaction

The Oxidase test is used to determine if bacteria produce certain cytochrome c oxidases. Filter paper impregnated with N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) has a plastic loop full of suspect bacterial colony smeared onto it. The paper will either turn purple when oxidised (Oxidase test positive) or will remain colourless if there is no oxidation (Oxidase test negative).

Procedure

- Use dried filter paper impregnated with N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD).
- Filter paper may be placed in a Petri dish and flooded with freshly prepared TMPD. Once impregnated the paper may be transferred to a fresh Petri dish and dried in a 37 °C incubator. Such paper must be stored away from direct light and checked with an appropriate reference culture (e.g. NCTC or ATCC) before use.
- Use a plastic loop to aseptically transfer a loopfull of suspect pure colony onto the filter paper and smear onto the surface.
- Observe the disk for up to three min.
- If a dark blue to purple reaction is observed the results is Oxidase positive.
- If no colour change is observed within three min the result is Oxidase negative.
- Normally a colour change will be noticed within a few seconds.

Commercial Oxidase paper or Oxidase sticks (compacted paper sticks which are dabbed onto suspect colonies) can be purchased from a suitable scientific reagent supply company and used as indicated in manufacturer's instructions.

Quality Control

New batches of reagent/paper are checked for suitability before use by staining appropriate reference organisms (e.g. NCTC or ATCC).

Catalase reaction

Catalase is an enzyme found in nearly all living organisms which are exposed to oxygen. It can catalyse hydrogen peroxide (H_2O_2) into water and oxygen. The ability of bacteria to catalyse H_2O_2 is a useful identification tool. Those bacteria that catalyse H_2O_2 are Catalase positive, those that do not are Catalase negative.

Procedure

Media containing whole red blood cells will contain catalase and could therefore give a false positive result. Only bacterial colonies from media which contain no red blood cells should be used for the Catalase test (Chocolate agar contains only lysed red blood cells and may still be used).

The enzyme is present in viable cultures only and the test should not be performed on cultures older than 24 h as older cultures may give false-negative reactions.

A pseudocatalase reaction may be produced by some strains of *Aerococcus* species and *Enterococcus* species.

Metal inoculating loops can react with H_2O_2 and produce a false positive reaction. Therefore only plastic loops or glass rods, or similar, should be used.

- Place approximately 0.2 mL of H_2O_2 solution in a test tube or bijoux bottle.
- Carefully pick a colony to be tested with a plastic disposable loop or glass Pasteur pipette.
- Rub the colony on the inside wall of the bottle above the surface of the H_2O_2 solution.
- Cap the tube or bottle and tilt it to allow the H_2O_2 solution to cover the colony and observe for vigorous bubbling of Oxygen occurring within 10 seconds.
- Vigorous bubbling within 10 seconds is regarded as Catalase positive.
- No bubbling observed within 10 seconds is regarded as Catalase negative.
- H_2O_2 can be purchased from a suitable scientific reagent supply company.
- H_2O_2 is unstable and should be stored in a spark proof fridge away from direct light.

Quality control

Due to the unstable nature of H_2O_2 a positive and negative reference strain control (e.g. NCTC or ATCC) should be tested daily or before each test.

Indole test

The Indole test determines the ability of an organism to ferment indole from the amino acid tryptophan.

Procedure

A pure bacterial growth is incubated at 37 ± 1 °C in sterile tryptophan or peptone broth for 24–48 h. After incubation five drops of Kovac's reagent can be added to the broth.

A positive reaction is indicated by a red colour (Indole positive).

A negative reaction is indicated by a yellow colour (Indole negative).

Commercially available indole fermentation test reagents are available which may be used as indicated in manufacturer's instructions.

Quality control

Positive and negative reference strain organisms (e.g. NCTCC or ATCC) should be tested with every batch of tryptophan or peptone broth. Kovac's reagent should be tested regularly with reference strain organisms to ensure stability.

Kovac's reagent

Isoamyl alcohol, para-dimethylaminobenzaldehyde, concentrated hydrochloric acid.

Kovac's reagent is flammable, harmful by inhalation, irritating to the eyes, skin and respiratory system. Appropriate Health and Safety precautions should be taken and reference made to appropriate COSHH and MSDS.

Glucose fermentation test

The glucose fermentation test is used to test for the ability of an organism to ferment glucose.

Procedure

- Glucose agar is prepared in a capped test tube.
- Using a sterile wire (nickel/chromium or platinum/iridium) stab the glucose agar.
- Incubate at 37 ± 1 °C for 24 ± 2 h.
- A yellow colour around the stab indicates a positive reaction.
- Commercially available glucose fermentation test reagents are available (e.g. DIATABS™) which may be used as indicated in manufacturer's instructions.

Quality control

Appropriate reference organisms (e.g. NCTCC or ATCC) should be inoculated as positive and negative controls.

Sugar fermentation tests (i.e. L-rhamnose, mannitol, D-xylose etc.)

Bacteria can be identified using their ability to ferment various carbohydrates. Liquid sugar broths containing a pH indicator can be prepared in the laboratory or commercial sugars can be purchased for this purpose and used as directed by the manufacturer. A positive reaction is indicated by a change in pH resulting in a colour change after incubation.

Procedure

- Inoculate the sugar broth from a nutrient agar plate using a microbiological loop.
- Incubate at 37 ±1 °C for 24 ± 2 h.

- A colour change (depending on the individual sugar broth used) indicates a positive reaction.

Quality control

Appropriate reference organisms (e.g. NCTCC or ATCC) should be inoculated as positive and negative controls.

Aesculin hydrolysis test

Aesculin (sometimes written Esculin or Æsculin) is a glycoside that can be used to aid identification of some bacteria e.g. *Listeria monocytogenes* when incorporated into agar with ferric citrate. Hydrolysis of aesculin produces aesculetin and glucose, the aesculetin reacts with the ferric citrate to produce black or brown iron complexes.

Procedure

Using a microbiological loop inoculate an aesculin agar slope and incubate for up to 24 h at 37 ± 1 °C. Observe for black precipitate throughout the agar slope.

Commercial test kits are also available which may replace the above method.

Quality control

Appropriate reference organisms (e.g. NCTCC or ATCC) should be inoculated as positive and negative controls.

Motility test

Some bacteria are motile by way of flagella which are helical filaments protruding from the cell wall. These filaments produce motility by rotation. Some bacteria have a single flagella, a group of flagella at one end or may have numerous flagella distributed around the cell wall.

The motility test divides bacteria into two groups and can be performed by observation in a microscope preparation. Care must be taken to distinguish 'true' bacterial movement from Brownian motion which occurs naturally.

Procedure

- Hanging Drop Method (Using a slide with a central depression and a cover slip).
- Prepare a liquid culture in nutrient broth or prepare a suspension in saline. If preparing a saline suspension the saline should be warmed to the optimum culture temperature before inoculation.
- Place a small drop of liquid bacterial culture in the centre of a coverslip.
- Place a small drop of sterile water at each corner of the coverslip.
- Invert a slide with a central depression over the coverslip.
- The coverslip will stick to the slide and when the slide is inverted the drop of bacterial culture will be suspended in the well.
- Examine microscopically (x400) for motile organisms.
- True motility is visible as organised movement. This may be darting, zig-zagging or tumbling but must be differentiated from natural Brownian motion.

- If well slides are not available, a ring of Vaseline (applied with a syringe) or Plasticine may instead be made on an ordinary microscope slide and inverted over the 'loaded' coverslip.
- Motility for *Clostridia* spp. may be tested using Nitrate Motility Medium (NMM). This should be inoculated using a straight wire with half a colony of suspected *Clostridia* spp. and incubated anaerobically at 37 ± 1 °C for 24 ± 4 h.
- If growth is observed away from the stab line and throughout the NMM (producing a cloudy appearance throughout) the culture may be considered motile. If growth is restricted to the length of the stab line the culture may be considered non-motile.
- MMN may also be used for testing nitrate production.

Quality control

A suitable motile reference organism (e.g. NCTCC or ATCC) should be used to control the procedure.

Nitrate reduction test

The Nitrate reduction test detect the ability of bacteria to reduce nitrate (NO_3) to nitrite (NO_2) or another nitrogen compound such as ammonia (NH_3) or nitrogen gas (N_2).

Procedure

- Nitrate reduction can be checked by inoculating nitrate reduction broth with the suspect culture and incubating for 48 h at the appropriate temperature for the organism.
- Add 10–15 drops of sulfanilic acid and N,N-dimethyl-1-naphthylamine and observe for a red colour change within 5 min. A red color change at this step indicated a positive nitrate reaction.
- If there is no colour change it is possible that nitrate has been further reduced to ammonia or nitrogen gas so a further test is performed to detect unreduced nitrate.
- Zinc can reduce nitrate to nitrite and therefore detect unreduced nitrate.
- Add zinc powder to the broth and observe for a colour change to red (as sulfanilic acid and N,N-dimethyl-1-naphthylamine are already present). If the broth turns red after the addition of zinc the result is negative, as the nitrate was unreduced before the addition of the zinc powder.
- If the broth does not change colour there is no nitrate present, as it has been reduced to nitrite and then further ammonia or nitrogen gas and is recorded as a positive reaction.

Quality control

A suitable motile reference organism (e.g. NCTCC or ATCC) should be used to control the procedure.

REFERENCES

ISO 21528-2:2004. *Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae Part 2: Colony-count method.* Geneva, Switzerland.

Use of autoclaves

PRINCIPLE AND SCOPE

The purpose of this procedure is to describe the use of autoclaves for sterilisation of microbiological media and reagents and the sterilisation of microbiological waste before disposal.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures and guidelines available in the microbiology laboratory when using autoclaves in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the experience and competence to operate the autoclaves efficiently and safely.
- **Quality Assurance Manager** – To ensure controlled procedures are available for all processes involving the use of the autoclave and that staff have the required competence. Also to perform regular audits to ensure compliance.
- **Health and Safety Manager** (or person identified with this responsibility) – To ensure appropriate controls are in place (Maintenance plans, Risk Assessment, PPE etc.) for operation of the autoclave in the microbiology laboratory.

PROCEDURE

Autoclaves are an essential piece of laboratory equipment in any microbiology laboratory and may be used to sterilise microbiological media and reagents before use, to sterilise laboratory equipment (e.g. glass pipettes, other reusable glassware etc) and to sterilise, and therefore render harmless, microbiological waste before disposal through normal waste channels.

An autoclave at 121 °C or 134 °C will destroy all bacteria, fungi, viruses and bacterial spores. It will not necessarily destroy all prions. To destroy prions a holding temperature of 121–132 °C for 60 min or 134 °C for 18 min is recommended.

An autoclave uses a sealed pressure vessel to raise the temperature at which water boils to above 100 °C. At a pressure of 15 lb per in^2 (100 kPa) the temperature of steam produced is 121 °C.

Bacterial spore death rate is increased 8–10 times for each 10 degree rise in temperature over the range 100 °C to 135 °C. A holding time of 15 min is considered sufficient to sterilise a load at 121 °C. This decreases to a holding time of 3 min for a temperature of 134 °C.

A high vacuum autoclave consists of a double walled chamber where steam circulates between the double walls. Steam can be supplied to the inner chamber under pressure to sterilise goods placed within it, once air within the chamber is removed.

If required, drying is accomplished at the end of a sterilisation cycle by evacuating the steam from the chamber but continuing to allow it to circulate between the double walls,

which keep the chamber hot. Moisture evaporates from the load and filtered air is sucked through the chamber.

It is essential that space is left around items in the chamber to allow steam to penetrate and that the chamber is never over loaded. Microbiological waste should be in an appropriate autoclave bag which is loosely tied and held on a robust autoclavable tray, which is leak proof and has solid sides and base, to retain any spillage that may occur.

- Care should be taken when removing items from an autoclave as they will remain hot for a considerable period of time afterwards. Before unloading an autoclave, or releasing the door mechanism, ensure that the chamber is vented to atmosphere, and the load temperature has reduced.
- Never attempt to override door interlocking safety devices.
- Always stand clear when opening the door, as hot liquid or vapour may escape from the chamber.

Modern autoclaves will automate the various stages into a pre programmed cycle but training in the use, routine maintenance and health and safety requirements is essential.

Routine maintenance, cleaning and checks should only be carried out by suitably trained Laboratory Analysts with all regular servicing and maintenance performed by a suitably trained autoclave engineer.

It is considered good laboratory practice to sterilise 'clean' (e.g. laboratory media or glassware) separately from 'dirty' (e.g. microbiological waste).

A domestic pressure cooker is a crude autoclave but does not dry the load or expel all the air from the chamber. The use of a domestic pressure cooker for sterilisation should be avoided.

QUALITY CONTROL

It is essential that accurate records are kept of all loads. These should record the date and time of the load, what the load consisted of. The time, temperature and pressure should all be recorded along with any observations or faults noted. Many modern autoclaves provide a print out of the temperature cycle achieved.

To ensure an autoclave has reached the intended temperature indicators such as autoclave tape (self adhesive tape which indicates 121 °C has been attained with a colour change) or a chemical indicator tube which indicates the desired temperature has been attained with a colour change may be used. Other indicators use spores of *Geobacillus stearothermophilus* which are incubated after autoclaving with a colour change evident if the bacteria have not been destroyed.

All autoclave indicators may be purchased from a suitable laboratory supplier.

Validation

- A competent trained person must carry out the validation, using thermometric test equipment calibrated and traceable to national standards.
- The laboratory should carry out validation at least annually, and at any other times when the previous test might no longer be valid (for example, as part of recommissioning after maintenance work).

- The person responsible for the autoclave must check the validation certificates and print-outs to satisfy themselves that the autoclave meets the specified criteria.
- Validation records must be kept for seven years.

Carrying out validation

An autoclave cycle has three phases: warm-up, holding and cooling down. Validation should demonstrate that, during the holding phase, the necessary temperature has been attained throughout the load and is held for the requisite minimum time.

If the autoclave is used for waste cycles (also called 'make safe' or 'destruct' cycles) for loads that contain pathogens or harmful genetically modified micro-organisms, then validation must also show that the autoclave process achieves a 100% kill.

To carry out a waste cycle validation:

- place a 'worst case' load of simulated waste (that is, worst case volumes, materials and equipment, but not contaminated with pathogens) into the autoclave chamber;
- insert temperature probes at various points in the waste and connect them to recording equipment;
- start the process. The holding part of the cycle begins when all the sensors show that the prescribed sterilising temperature has been reached; and
- all sensors must then maintain at least that temperature for the prescribed holding time:
  - At least 121 °C (maximum 125 °C) for 15 min; or
  - At least 126 °C (maximum 130 °C) for 10 min; or
  - At least 134 °C (maximum 136 °C) for 3 min.

However loads with a variety of items and containers do not heat uniformly. There can be large temperature variations during short holding times (3 min), so avoid these if possible.

After validation ascertains the autoclave settings that enable the desired condition to be achieved. These settings vary considerably, depending on many factors, including the load characteristics, for example, one autoclave may need to be set at 121 °C for 50 min to achieve the first condition above, but another at 123 °C for 75 min to achieve the same.

Where an operator can alter the autoclave settings, the temperature and time requirements for a waste cycle must be clearly displayed on the autoclave.

HEALTH AND SAFETY

This document includes the use of autoclaves for sterilising material and equipment which may be contaminated with organisms from Containment Level 1, 2 and 3, (CL 1, CL 2 and CL 3) but not organisms in Containment Level 4 (CL 4), for which complete containment of condensate is considered to be essential.

To ensure the safety of all who operate the autoclave full training in the use of autoclaves must be provided to all operators and only those with appropriate training and competency should operate the equipment. Personal Protective Equipment (PPE) must be used as appropriate:

- an impervious apron;
- heat-resistant gauntlet gloves;

- suitable heavy-duty footwear or overshoes; and
- a full-face visor.

A maintenance log should be set up in accordance with manufacturer's guidelines where daily, weekly, quarterly and annual maintenance can be recorded. This schedule of maintenance can be created in discussion with an appropriate autoclave maintenance engineer and make reference to appropriate Health and Safety guidelines on the use of autoclaves (e.g. BS 2464-4 Autoclaves for sterilisation in laboratories, 1993).

The following describes the minimum maintenance requirements.

Operators must report, and record in the maintenance log, any defect noticed while using, maintaining or repairing an autoclave.

Daily maintenance

The operator must carry out the following checks and activities daily:

- check that steam pressure from the supply is correct;
- clean the chamber internally, as recommended by the manufacturer. Include all the internal fittings to the chamber, such as brackets, shelves and so on;
- clean the drain filter, if fitted;
- clean the door seal with a damp cloth, and examine it to ensure that it is in good condition with no cuts or abrasions;
- inspect the chart recordings for unusual traces, and report any abnormalities to the responsible person; and
- check visually for steam and water leaks.

Weekly maintenance

The responsible person must carry out the following checks weekly, and record the results in the maintenance log:

- check that the indicator lamps work properly;
- during an operating cycle, check that temperature and pressure gauges correlate; and
- inspect the cycle chart recordings for abnormalities.

Quarterly Maintenance

The maintenance/service engineer must carry out the following checks every three months, report any abnormalities and resulting corrective actions, and record these in the maintenance log:

- check all manual valve spindles, open and close all valves, then clean, lubricate and repack, as required;
- inspect all piping joints;
- visually inspect the chamber for any signs of corrosion or wear;
- clean the water and steam line main strainers, if fitted;
- inspect, and tighten if necessary, all electrical heater terminal points;
- clean out the pipework from the chamber drain;
- clean out the steam traps, then replace the elements and steam trap seat, as required;
- check that the main drain to waste is clear and operating;
- clean and replace parts, as required, on the steam pressure reducing valve, if fitted;

- check that the safety valves and ancillary pipework are not blocked;
- check the control instrumentation, including recorders. The instruments might require recalibration or replacement; and
- check that the door interlocks work correctly.

Annual maintenance and inspection

The maintenance/service engineer must carry out the following checks annually, then prepare a comprehensive report of the annual inspection, and refer to it in the maintenance log:

- check the service history for recurring faults, and, if necessary, ensure that corrective action has been taken;
- remove any scale from the chamber by a method approved by the manufacturer;
- if appropriate remove any scale from the steam generator by a method approved by the manufacturer;
- if appropriate inspect and remove any scale from the water level control and indicator systems;
- check the condition and operation of the pressure gauges;
- check the condition and operation of the temperature indicators;
- test the operation of the safety devices, including the safety valves and door interlocking system, under operating conditions;
- during a cycle, with the chamber empty, check all the control functions, including correlating the pressure and temperature gauges against known references;
- test all the functions of the autoclave under working conditions, to the satisfaction of the responsible person; and
- carry out thermometric tests of typical laboratory loads, as performed during the original commissioning and validation.

REFERENCES

BS 2646-3:1993. *Autoclaves for sterilisation in laboratories*. Guide to safe use and operation. BSI, London, UK.

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

Manufacturer's handbook as appropriate.

Use of incubators and temperature controlled equipment

PRINCIPLE AND SCOPE

The purpose of this procedure is to describe the use, profiling and monitoring of incubators and temperature controlled devices used for incubation of microbiological samples in the correct environment and for storage of microbiological samples, kits and reagents in the microbiology laboratory at the required storage temperature.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures and guidelines available when using incubators and temperature controlled devices in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the competence to operate and maintain incubators and temperature controlled devices efficiently and safely.
- **Quality Assurance Manager** – To ensure controlled procedures are available for all processes involving the use of incubators and temperature controlled devices. Also to perform regular audits to ensure compliance.

Temperature controlled devices include the following:

- incubators (including CO_2 Incubators);
- fridges (including cold stores);
- freezers (including low temperature freezer units);
- waterbaths;
- ovens;
- warm rooms; and
- analysers and equipment which have integral refrigeration or incubation.

PROCEDURE

All temperature controlled devices which may have an effect on a procedure or test result in the microbiology laboratory must be monitored to ensure that the temperature is maintained within a pre determined range. This pre determined range should be established from SOPs and displayed on equipment.

Temperatures should be monitored at least daily using a dedicated calibrated thermometer or temperature probe. Laboratory thermometers used to monitor the temperature of incubators and temperature controlled devices may be calibrated annually against a traceable calibrated thermometer. Any correction factor required must be clearly stated on the

thermometer and only the corrected temperatures must be recorded for an incubator or temperature controlled device. Electronic monitoring systems are available which can alert the laboratory if a temperature goes outside a pre set range.

Temperature controlled devices will fluctuate in their temperature during the working day and it is best practice to record the temperature at the start of the working day.

The temperature of a piece of equipment may be recorded on a daily recording chart attached to the equipment and initialled by the operator recording the temperature. The operator must ensure that the temperature is within the range specified on the equipment and that the temperature recorded has been corrected if necessary.

If a temperature is outwith the specified range the cause must be investigated and any temperature sensitive items moved immediately to another temperature controlled device. If the temperature is outside the specified range consideration should be given to repeating tests which may be affected or discarding temperature sensitive items which may have been stored (e.g. diagnostic kits, reagents or media).

Carbon dioxide (CO_2) is required by many bacteria and is usually available as a product of metabolism. Some bacteria, which are slow growing or fastidious, may not generate sufficient CO_2 and this will need to be supplied in the laboratory. In addition the requirement for additional CO_2 is increased by some bacteria when transferred to artificial growth medium. Many pathogenic bacteria require 5–10% CO_2 to grow in an incubator (capnophilic bacteria). 5–10% CO_2 can be supplied to the incubator by mechanical means from a cylinder through a reducing valve and flow meter.

Where a CO_2 incubator is not available commercial CO_2 gas generating kits are available and can be used in a sealed anaerobe jar.

A lighted candle in a sealed jar generates approximately 5% CO_2 and can be used if no other means of generating CO_2 is available.

Like temperature the level of CO_2 should be monitored and recorded. Most CO_2 incubators will have a CO_2 gauge which may be used for this purpose and calibrated by a suitably qualified engineer at regular intervals.

Incubators and temperature controlled devices must be subject to a schedule of cleaning and/or disinfection. Fridges, cold stores and incubators must be cleaned and/or disinfected at a frequency appropriate to their application. Fridges and freezers should also be defrosted as required. Records of cleaning and/or disinfection must be recorded on the relevant temperature log/equipment log. A suitable maintenance schedule for incubators and low temperature (e.g. -80°C) freezers should be considered with an appropriately qualified engineer.

Profiling

When an incubator or temperature controlled device is first used it must be profiled to verify that the temperature is constant throughout the unit. If part of the unit is identified that is outwith the required range (e.g. a portion of a shelf nearest the fan or nearest the heating elements in an incubator) this should be identified within the unit as 'out of range' to prevent use of that area.

Should an incubator or temperature controlled device undergo a major repair, service or be transferred from one location to another it should be reprofiled. All equipment should be reprofiled at least every two years.

Switch the unit on and adjust the thermostat to give the required temperature using a calibrated thermometer placed in the centre of the unit.

An additional calibrated thermometer is used to read the temperature at various points within the unit to ensure that there are no significant variations in temperature readings and that the span is within specification for the application.

The second thermometer is placed at five points, in sequence, on each of the top, middle and lower shelves, the points being towards each corner and at the centre. Avoid placing a thermometer too close to fridge/freezer cooling plates or incubator surfaces that may have heating elements. In a chest freezer consider the unit to be one 'shelf' and record the five points.

The first thermometer remains at its original central location for the duration of the exercise and is used as a reference to check for fundamental drift or shift in the set temperature.

The temperature of each thermometer is recorded on a suitable log sheet after a period sufficient to allow the unit to stabilise at its set point.

Having recorded both temperatures the second thermometer is moved to the next sequential point. The process is repeated to complete five readings per shelf. Alternatively several additional thermometers can be used in conjunction to complete the process in less time.

Any shift in the readings of the first thermometer out with the set tolerance for the unit invalidates the exercise. The thermostat must be readjusted and the readings repeated when the temperature has stabilised.

Ensure application of all appropriate correction factors during recording of result.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

Manufacturer's handbook as appropriate.

AN EXAMPLE OF A TWO MONTH TEMPERATURE RECORDING LOG.

Two Monthly Temperature Recording Log

Unit Reference			
Location			
Target Temperature	**°C**	**±** **°C**	
Thermometer No.		**Correction:** **°C**	**@ °C**

Month:

Year: 20

Date	Temp (°C) Corrected	Action	Initial
1			
2			
3			
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Month:

Year: 20

Date	Temp (°C) Corrected	Action	Initial
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Laboratory Manager must sign and date records sheet before archiving

Signature _____ Date _____

Basic microbiological techniques

PRINCIPLE AND SCOPE

The purpose of this procedure is to describe the basic laboratory techniques that are essential to allow Laboratory Analysts to isolate and identify microbiological organisms in the microbiology laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures available when processing samples in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the competence to process samples and identify microbiological organisms in the microbiology laboratory.
- **Quality Assurance Manager** – To ensure controlled procedures are available for all processes involving the identification of microbiological organisms and that all Laboratory Analysts can demonstrate appropriate competence through regular internal audit.
- **Health and Safety Manager** (or person identified with this responsibility) – To ensure all Laboratory Analysts are familiar with all Risk Assessments, COSHH data etc. and have received suitable training in handling of microbiological samples including Containment Level 2 (CL 2) and Containment Level 3 (CL 3) as appropriate.

EQUIPMENT

- Sterile forceps, scalpels and scissors.
- Microbiological loops (nichrome or platinum wire or sterile disposable plastic).
- Sterile Pasteur pipettes (glass or disposable plastic).
- Pipettor (with sterile tips).
- Bunsen burner.
- Balance (including weighing boats and spatula).
- Microscope slides.
- Incubator (including CO_2).
- Light microscope with X100 oil immersion objective.
- Appropriate PPE (laboratory coat, gloves etc.).

REAGENTS

- Approved microbiological disinfectant (e.g. 5% sodium hypochlorite).
- Microbiological culture media (agar plates, bottles or tubes of agar slopes or liquid media).
- Reference cultures for QC (e.g. NCTC or ATCC).

PROCEDURE

The aim of basic microbiological techniques is:

- the transfer to artificial culture media of viable organisms from the sample;
- avoiding the introduction of contaminants from the environment;
- avoiding aerosol production during the process; and
- obtaining pure, single colonies on solid media.

Bacteria can be grown in the laboratory easily if provided with the correct environment and nutrients with which to multiply. Most artificial growth media is prepared in Agar which is a carbohydrate derived from seaweed. Agar melts at 90 °C but then does not solidify until cooled to 40 °C meaning that heat sensitive ingredients can be added just before it solidifies. Agar is usually added at a concentration of 1.5% to produce media with the consistency of firm jelly.

Other constituents may include:

- water;
- sodium chloride (and other electrolytes);
- peptone (a protein digest prepared from animal or vegetable protein by enzyme action);
- meat or yeast extract (used to enrich media);
- blood or serum (usually horse or sheep blood);
- pH indicators (e.g. MacConkey agar);
- pH regulating substances;
- selective inhibitors or promoters; and
- chromophore indicators.

Most media are supplied as dehydrated powder which is reconstituted and sterilised before being poured into sterile plastic Petri dishes. Many commercial suppliers can also supply pre poured media ready to use. This has a distinct advantage in that, as long as the supplier holds appropriate accreditation for the production and quality control of pre poured media, the lab will not have to perform extensive quality control procedures before using the media.

The use of solid media allows separate, discrete colonies to be formed and used for identification by colonial morphology and for sub culture to produce pure growths for identification tests. It also allows for quantification of bacterial load.

Some media contain selective ingredients that will inhibit the growth of unwanted contaminants/non target organisms and allow certain pathogens to grow. Media may also have indicators which demonstrate the presence of particular bacteria by colour differentiation of colonies.

When making microbiological media follow manufacturer's instructions or published formula. Always use clean spatulas and weigh boats to measure constituents and only use sterile distilled or deionised water to avoid any impurities which may be present in domestic water supplies. The instructions may require that the pH of media is checked and adjusted as necessary when preparing.

When prepared and ready to distribute into sterile plastic Petri dishes, bottles or tubes this must be done using sterile technique (see below) and avoiding the formation of bubbles. Bubbles on the surface of Petri dishes may be removed before the media has set by briefly passing the flame of a Bunsen over the surface to pop them.

Once poured into sterile plastic Petri dishes media must be allowed to dry and solidify at room temperature (18–22 °C) for 20–30 min before being inverted and stored in the dark at 2–8 °C. Assign an expiry date to plates and identify the type of media on the base of the Petri dish. The expiry date can be established by performing validation of media to establish the 'shelf life'. Do not use media after its expiry date and always visually check media before use to ensure there are no contaminants growing on the surface or that the media has dried out.

QUALITY CONTROL OF MICROBIOLOGICAL MEDIA

All QC checks on media should be performed using traceable reference standards (e.g. NCTC or ATCC). These reference cultures must not be allowed to become 'laboratory adapted' and must be inoculated fresh from cryopreserved stocks on a regular basis. No more than two subcultures should be permitted from a cryopreserved culture.

pH is measured with a calibrated pH meter using a flat-head probe on a sample plate from a prepared batch. If outside the acceptable range the medium is discarded and the reason investigated.

Sterility should be checked by incubation at 37 ± 1 °C for 2 days and 2–8 °C for 10 days. Evidence of contamination should be investigated and the batch rejected.

For selective media compare appropriate target organisms on the test batch with a non-selective control medium for both enhancement and inhibition as appropriate.

Check any indicators (e.g., MacConkey, lactose fermentation) with representative organisms to check positive and negative reactions.

To determine shelf life retain sufficient aliquots of the media to repeat the above checks at the assigned expiry date.

Parallel growth comparison method for QC of microbiological media

- Prepare a suspension of the test organism in saline equivalent to McFarland turbidity standard 0.5. This suspension will contain 1.5 x 10^8 CFU per mL.
- Prepare a 1:100 dilution by transferring 10 µl, using a pipettor or 10 µl microbiological loop, to 1 mL sterile saline (Dilution A).
- Prepare three serial tenfold dilutions from Dilution A and label B, C and D.
- Divide the test plate and a nutrient or blood agar (control) plate into quadrants using a permanent marker on the underside.
- Transfer 25 µl of each dilution (A, B, C and D) to each quadrant.
- Spread each drop over the plate segment using a sterile loop.
- Incubate as appropriate to the test organism.
- After incubation, compare the growth on each plate. Select the quadrant which gives isolated colonies and count for each plate. Record the counts obtained in a Media QC log.
- Microbiological media is deemed to be satisfactory if there is a minimum 50% recovery on the test medium when compared to the control medium.
- For selective broths, prepare dilutions A–D as above and inoculate 25 µl into each of four broths from the test batch and the control (previous) batch.
- Incubate as appropriate and then subculture to the required agar medium.
- Each batch should yield growth from the same dilution.

Selective media – Efficacy of selective agents

- Prepare a suspension of the appropriate test organisms in sterile saline equivalent to McFarland turbidity standard 0.5.
- Streak a charged loop from each suspension as appropriate onto the surface of the selective plate or inoculate a loop-full into a selective broth and also onto a control nutrient or blood agar to check viability.
- Incubate as appropriate to the medium usage.
- Examine after incubation for growth of the test organisms.
- Growth should normally be totally inhibited. Any growth indicates failure of the selective agents. The medium should be rejected and the cause of the failure investigated.

Note: *E. coli* on BGA may only be partially inhibited and show trace growth, record as such in the Media QC Log. This is acceptable provided that the growth is significantly inhibited when compared with the viability control plate.

Asceptic (sterile) technique

It is important to ensure contaminants from the environment are not introduced when dealing with bacterial samples and that sterile technique is used at all times. All manipulations of bacterial colonies should be done with a standard 10 µl (3 mm) microbiological wire loop made of nickel/chromium or platinum/irridium wire. Microbiological wire loops are sterilised by holding vertically in a blue Bunsen flame until the wire glows orange, the loop should then be allowed to cool for a few minutes, by resting on a loop rest, before using. It is often useful to use two loops with one cooling whilst the other is being used. Alternatively sterile plastic disposable loops can be used.

To avoid contamination dust must be kept to a minimum in the microbiology laboratory and surfaces must be cleaned with a suitable laboratory disinfectant (e.g. 5% sodium hypochlorite) after any spillage and at the end of every procedure. To avoid contamination from outside (and biohazardous contamination from the laboratory to the outside) windows should be kept closed and the use of fans (which may spread dust and contaminants around the laboratory) should be avoided. To keep a microbiology laboratory at a comfortable temperature suitably filtered air conditioning should be considered.

Long hair must be tied back and a laboratory coat worn and fastened at all times. Laboratory coats with elastic wrists are preferred in microbiology laboratories as this prevents contamination from loose cuffs touching media etc.

When manipulating bottles and tubes in the microbiology laboratory (e.g. when transferring cultures to broths or reagents or distributing media) the neck of the bottle or tube can be passed briefly through the flame of a Bunsen to sterilise. Do not hold it in the Bunsen for more than one second.

To obtain discrete, single colonies from a mixed culture or to obtain pure colonies from a media that contains indicator or inhibitory substances it is desirable to obtain single colonies on a purity plate. Identification tests should only be performed from basic 'nutrient' type media such as nutrient agar or blood agar. Media plates should always be identified on the base (containing the media) using permanent marker.

Streak plate (to obtain discrete, single colonies).

The sterile loop is used to make a 'well' of inoculum on the agar plate by removing a single colony from the original growth on solid media, or a loop-full from broth culture, and streaking the loop back and forth over approximately one quarter of the plate to be inoculated. When inoculating the plate hold the loop horizontal to the media surface.

The loop is sterilised (or replaced if using a plastic loop) and, after rotating the plate a quarter turn, three or four parallel streaks are made from the inoculum. The loop should be held vertical to the media surface.

The loop should be sterilised (or replaced if using a plastic loop) or may be turned 180° to use the fresh side. The plate is again rotated a quarter turn and the process repeated.

This procedure is repeated a third time and finished with a zig-zag line to the centre of the plate. Each portion will dilute the inoculum and should produce discrete colonies after incubation.

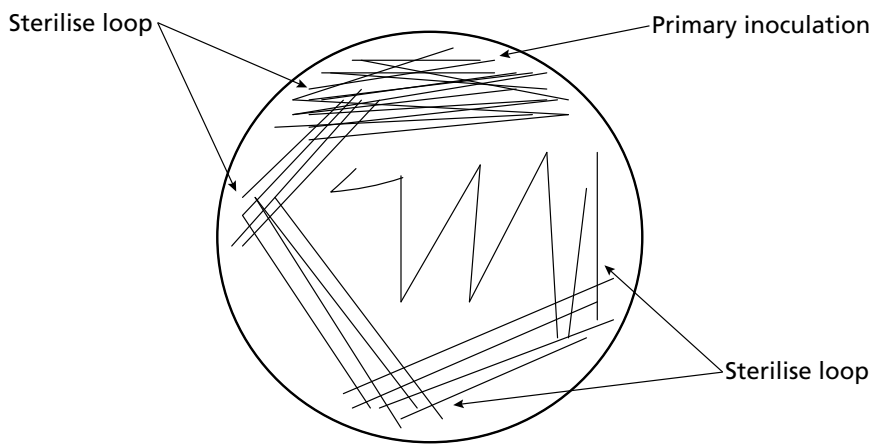
The optimal temperature of most bacteria that will be isolated in the animal nutrition/feed analysis laboratory is 37 ± 1 °C**.** Incubation should be done in an appropriate laboratory incubator away from direct light and the plate inoculated inverted to avoid drips of condensation falling onto the media. Overnight inoculation (20 ± 3 h) is usually sufficient to produce visible colonies.

After incubation overnight growth can be estimated by the number of colony forming units (cfu).

(Standard 90 mm plastic Petri dish)

- **Confluent** +++ > 1000 cfu
- **Heavy** ++ 100 – 1000 cfu
- **Moderate** + 10 – 100 cfu
- **Scanty** +/- < 10 cfu

Diagram indicating inoculation of solid media for single colonies



Other colonial characteristics can be noted which will aid identification:

- **Size** colony diameter in millimetres.
- **Shape** circular, irregular, crenated, spreading.
- **Edge** entire, crenated.
- **Elevation** flat, convex, domed, pitting.
- **Surface** smooth or rough, glossy or matt.
- **Colour** colourless, grey-white and translucent, pigmented and opaque.
- **Consistency** creamy, viscid, tough, gelatinous, dry, friable.
- **Haemolysis** α (alpha) haemolysis is a greenish zone on blood agar, ß (beta) haemolysis is a clear zone on blood agar. γ (gamma) haemolysis is also described in some texts (no haemolysis) but is rarely used.

HEALTH AND SAFETY

To minimise the production of dusts when handling dehydrated microbiological media or chemicals use a disposable half-mask respirator approved to EN149 standard and CE marked when identified in appropriate COSHH and Risk Assessment documentation. Some microbiological media may contain specific hazards and all hazard labels and product safety data sheets which accompany microbiological media and chemicals should be noted.

Special care is needed when handling hot microbiological media after autoclaving or during boiling.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

UKAS. 2009. *LAB 31 Use of culture media procured ready-to-use or partially completed in microbiological testing*. UKAS publication Lab 31 edition 2, June 2009. Feltham, UK.

Use of balances

PRINCIPLE AND SCOPE

The purpose of this procedure is to ensure the correct calibration, service, performance and use of balances for critical weighing in the microbiology laboratory.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures and guidelines available when using balances in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the competence to operate and maintain balances efficiently.
- **Quality Assurance Manager** – To ensure controlled procedures are available for processes involving the use of balances. Also to perform regular audits to ensure compliance.

EQUIPMENT

Check weight set, stainless steel, or similar and certified yearly.

PROCEDURE

Service and calibration

Balances in the microbiology laboratory should be serviced and calibrated annually (on site) by an appropriate service contractor accredited to ISO/IEC 17025:2005 for balance calibration. A certificate of calibration, traceable to National Standards, should be retained for each balance.

Any defects detected during servicing must be repaired prior to calibration and certification.

There is no need to have check weights used in the microbiology laboratory independently certified. These check weights may be weighed on the balance on the day of its annual service and calibration and the mass of each check weight recorded, they may then be considered traceable to SI units. Any correction factor to be assigned should be noted and used thereafter.

Monitoring performance

Balances must be positioned in a well-lit, draught-free room, on a level surface not subject to vibration. If excessive vibrations are likely the balance may be positioned on a heavy (granite, marble or concrete slab) to minimise interference.

Balances should not be moved and, when set to zero, the display should be steady.

The performance of the balance should be checked daily using check weights appropriate to the balance range and use. Check weights must be stored in the protective container

in which they are supplied. The weights should never be handled without the use of a lint-free wipe or tweezers. For the larger weights, powder-free gloves may be used when necessary. Fingerprints will affect the results of a weighing and will decrease the accuracy and the precision.

The selected weight is placed centrally on the balance pan and the weight recorded in the appropriate balance log. The operator must sign the balance log to confirm that the results fall within the tolerance stated. If the tolerance is not achieved the balance should not be used and the cause investigated. It may be necessary to have the balance serviced and calibrated before it is returned to service.

Cleaning

It is inevitable for the balances and weights to become dirty with everyday use. Therefore, it is important to follow the proper methods carefully to obtain the most accurate results. The follow methods for cleaning the balances and weights were developed to ensure quality results.

Unplug the balance before cleaning and do not use any harsh or abrasive cleaning agents. Allow nothing to enter the balance's internal mechanism. Also handle the balance pan with care, do not touch the balance pan feet on which it rests or the place where the pan contacts the balance. When cleaning the weighing pan with a liquid solution, remove the pan and clean it outside of the balance to prevent any liquids from running into the internal electronics and causing damage. If some material enters the balance housing, notify a supervisor immediately so proper care can be taken to limit the damage to the balance.

The balance should be cleaned as necessary. For simple cleaning, such as removing sample from the balance pan, a soft camel hair brush should be used. Make certain no small particles remain on the balance pan since even the smallest amount can affect a reading or corrode the pan.

If the balance pan cannot be cleaned simply by using a brush, then water may be used to wipe clean the weighing pan. A lint-free wipe should be utilized to wipe away any dirt and water on the pan. The water should be put onto the wipe, not the balance.

If necessary, a 1% soap solution may be used to help clean the weighing pan, but this must be used with care. The soap solution must be thoroughly washed off. Soap tends to leave a residue on the balance, therefore affecting the results of a weighing. A lint-free wipe should be used to wipe the soap solution from the balance pan.

For the analytical balances, the windows may be cleaned with a glass cleaner and a lint-free wipe. The glass cleaner should be sprayed onto the wipe and then applied to the windows. This will prevent any build up from the spray.

To clean the weights use ethyl ether and a lint-free wipe. The ether should only be used in a solvent hood. Wipe the weight until it is visually clean and no ether remains. The weights should be cleaned as needed.

Weighing substances

Appropriate PPE must be worn when handling hazardous (including biohazardous) material and any spillages must be cleaned immediately.

Ensure that the substance to be weighed is at room temperature to avoid condensation forming on the sample.

The material to be weighed must not be placed directly on the balance pan. Select the most appropriate vessel for the substance being weighed. When weighing small volumes, use the final container as the weighing vessel, if possible, to avoid the loss of mass in transfer. Weigh the vessel empty and tare out the weight before weighing the substance into the container.

Material being weighed in an encased balance must be at ambient temperature as warm objects may give rise to convection current within the case, resulting in weighing errors.

On completion, all substances must be returned to the appropriate storage facility, the balance and surrounding areas cleaned and logs completed.

Do not drop weights onto the balance pan as this may damage the internal structure. Do not leave samples or weights sitting on the balance pan for extended periods of time.

REFERENCES

European co-operation for accreditation. 2002. E*A-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

UKAS. 2006. *Lab 14 Calibration of weighing machines*. UKAS publication ref Lab 14 edition 4, November 2006. Feltham, UK.

Manufacturer's handbook as appropriate.

Use of pipettors

PRINCIPLE AND SCOPE

The purpose of this procedure is to ensure the correct calibration, service, performance and use of pipettors for critical measuring of liquids in the microbiology laboratory.

In addition to hand held pipettors (automatic pipettes) this procedure also applies to dispensers attached to bottles and those attached to dispensing units. Dispensers may not need to be calibrated but should be tested to ensure they are fit for purpose.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures and guidelines available when using pipettors in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the competence to operate and maintain pipettors efficiently.
- **Quality Assurance Manager** – To ensure controlled procedures are available for maintenance, calibration and processes involving the use of pipettors. Also to perform regular audits to ensure compliance.

EQUIPMENT

- Appropriate pipettors and tips.
- Balance (four-places) with valid calibration certificate from a suitably accredited service agent.
- Small volumetric flask.

REAGENTS

- Distilled or deionised water between 16 °C and 24 °C.

PROCEDURE

Service and Calibration

All pipettors must be uniquely identified in the microbiology laboratory and it is advisable to indicate on each when they were last calibrated and when the next calibration is due.

Laboratories may opt to send pipettors to a third party calibration service company for calibration and service or have a third party perform service and calibration 'on site'. Only reputable service agents should be used who hold appropriate accreditation for the service they provide.

Alternatively commercial computer packages are available which attach to a balance and provide an asset register of pipettors, provide prompts when calibrations are due and guide the Laboratory Analyst through the calibration process.

Before calibration, a dispenser should be rinsed with sterile water, or other weighing medium, to remove all of the previous solution. Optimum conditions for the calibration

measurements involve the type of weighing vessel, the environment, the testing medium, and the Laboratory Analyst's technique. The calibration weighing vessel should be cylindrical in order to keep the surface of the liquid sample constant. To minimize evaporation, the container should be covered. To minimize fingerprints, use tweezers, gloves or lint-free wipes when handling the weighing vessel. The room that the measurements take place in must be draft free and have no direct sunlight on the balance. The room must be normal ambient temperature (18–22 °C) or allow the pipette to equilibrate to room temperature before calibrating. The Laboratory Analyst's technique is important, follow the pipette's instruction manual and keep the timing between weights consistent.

For most pipettes, the testing medium is distilled or deionised water. It is necessary that a portion of the medium is placed into the sampling container to equilibrate for one hour prior to calibration. It is essential that the water temperature is measured to the nearest 0.1 °C for future calculations (see Table 1).

A calibration schedule should be prepared which identifies the frequency of calibration and the volume that should be checked for each pipettor. This should be prepared based on the accuracy required for the pipettor.

At a minimum, pipettes that are used on a regular basis for critical work must be calibrated three monthly with three sets of 5 weighings. The frequency may be reduced if a low risk is identified. For pipettes that are used less frequently, calibrate the pipette with each use. This determines the accuracy and the precision. It is also necessary to calibrate with three sets of 5 weighings after a major service is completed or when a new instrument has arrived. These weighings are to be done at three different pipette volumes. The weighing must span the range of use for the pipette in the microbiology laboratory. For example, a 1 000 µl pipette would be tested at 200, 500 and 1 000 µl volumes. The pipette may also be calibrated at a reading commonly used in the lab. For example, if a 300 µl measurement is needed every day, the pipette can be calibrated at 200, 300 and 1000 µl. The analyst needs to determine the three measurements to be taken based on the needs of their laboratory. If a pipette is set to only dispense a specific volume, then the calibration only needs to be done at that volume. If a pipette is used only to fill to volume or to dispense a non-critical volume, it need not be calibrated. These special pipettes must be labelled to inform other analysts of the pipette's status.

Specifications are determined from the measurements taken during the calibration. If the pipette does not meet the required specifications it should be examined in-house or be sent to the manufacturer or service agent for recalibration or be replaced. When the pipette does not meet specifications it must also be labelled with an out of service sticker.

TROUBLESHOOTING

Inaccurate volumes can occur if the proper pipetting procedure is not followed such as, the tips do not properly fit the pipettor being used, or they are not placed tightly onto the pipettor. These problems can be determined by observing the placement of the ejector arm on the pipettor, this should not interfere with pipette operation.

With all pipettes and dispensers, a loosened or cracked shaft will affect the volumes delivered.

TABLE 1
Z factors for converting sample weight to sample volume

Water Temperature (° C)	Z-Factor (mL/g)
15	1.002
15.5	1.002
16	1.0021
16.5	1.0022
17	1.0023
17.5	1.0024
18	1.0025
18.5	1.0026
19	1.0027
19.5	1.0028
20	1.0029
20.5	1.003
21	1.0031
21.5	1.0032
22	1.0033
22.5	1.0034
23	1.0035
23.5	1.0036
24	1.0037
24.5	1.0038
25	1.0039
25.5	1.004
26	1.0041
26.5	1.0042
27	1.0043
27.5	1.0044
28	1.0045
28.5	1.0046
29	1.0047

Any problems with the internal mechanisms of the pipette should be referred to an appropriate pipettor service and calibration agent.

Sample volume can also become inaccurate if the sample is splashed into the pipettor.

Care of pipettors

Pipettors must be stored upright, do not leave units lying on bench.

If using corrosive or large volumes, use appropriate filters.

REFERENCES

BS 1132:1987. *Specification for automatic pipettes*. BSI, London, UK.

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

ISO 8655-1:2002. *Piston operated volumetric apparatus. Terminology, general requirements and user reccomendations*. Geneva, Switzerland.

ISO 8655-2:2002. *Piston operated volumetric apparatus. Piston pipettes*. Geneva, Switzerland.

ISO 8655-5:2002. *Piston operated volumetric apparatus*. Dispensers. Geneva, Switzerland.

ISO 8655-6:2002. *Piston operated volumetric apparatus*. Gravimetric methods for the determination of measurement error. Geneva, Switzerland.

UKAS. 2009. *LAB 15 Traceability: Volumetric apparatus. UKAS publication ref Lab 15 edition 2, June 2009*. Feltham, UK.

Manufacturer's handbook as appropriate.

Use of pH meters

PRINCIPLE AND SCOPE

The purpose of this procedure is to ensure the correct calibration, service, performance and use of a pH meter in the microbiology laboratory. The pH meter in microbiology is essential when preparing microbiological media.

RESPONSIBILITIES

- **Laboratory Analyst** – To follow the appropriate procedures and guidelines available when using the pH meter in the microbiology laboratory.
- **Laboratory Manager/Director** – To ensure Laboratory Analysts in the microbiology laboratory have the competence to operate and maintain the pH meter efficiently.
- **Quality Assurance Manager** – To ensure controlled procedures are available for maintenance, calibration and processes involving the use of the pH meter. Also to perform regular audits to ensure compliance.

EQUIPMENT

- pH meter.
- Electrode (if solid media is to be tested an electrode for this purpose is available).
- Beaker.
- Small volumetric flask.

REAGENTS

- Distilled or deionised water.
- pH buffer 4.00 +/- 0.2
- pH buffer 7.00 +/- 0.2
- pH buffer 10.00 +/- 0.2

PROCEDURE

- All pH meters should be uniquely identified and it is advisable to indicate on each when they were last calibrated and when the next calibration is due.
- The electrode is stored in fluid as recommended by manufacturer or in pH 7 ± 0.2 buffer. Remove the electrode and rinse in distilled or deionised water before use.
- Place the electrode in pH 7 buffer. Take a reading and record.
- Repeat for pH 4 buffer and pH 10 buffer (as appropriate or unless otherwise specified for a specific model), rinsing in deionised water between. Before placing the electrode into buffer swirl by hand.
- If buffers do not give an acceptable reading, refer to the specific handbook for the particular model and adjust accordingly to ensure the accurate reading of all buffers (some models incorporate slope adjustment to allow for aging electrode).

- Record performance results in an appropriate QC log.
- Record the pH of the test fluid or media, rinsing the electrode before and after in distilled or deionised water.
- Rinse the electrode and return to storage fluid.
- If the pH of solid microbiological media is to be tested a specific probe for the pH meter should be purchased for this purpose. Microbiological media should be checked for pH at the temperature at which it will be used as pH can be affected by temperature.

QUALITY CONTROL

External proficiency schemes often include a pH distribution. Participation in such an EQA scheme should be considered.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

Manufacturer's handbook as appropriate.

Microbiology laboratory water

PRINCIPLE AND SCOPE

Purified water is essential in the microbiology laboratory and is obtained by distillation, ion-exchange treatment, reverse osmosis (RO), or other suitable processes.

This procedure applies to all water used by the microbiology laboratory to prepare microbiological media and reagents, wash glassware, or that comes in contact with samples.

RESPONSIBILITIES

- **Laboratory Analysts** – To ensure only laboratory water is used for the preparation of microbiological media and reagents.
- **Laboratory Manager/Director** – To ensure equipment is available to supply the microbiology laboratory with an adequate supply of sterile water or that suitable sterile water is purchased ready to use.
- **Quality Assurance Manager** – To specify procedures used are appropriate. Also to perform regular audits to ensure compliance.

EQUIPMENT

There are various processes available to provide laboratory water in the microbiology laboratory and the equipment required will be dependent on the process used. Most methods of producing laboratory water for use in the microbiology laboratory will use a dedicated self contained unit which required little maintenance from laboratory staff.

Purified laboratory water is water that has been physically processed to remove impurities and is then used in the microbiology laboratory. There are a large number of methods that are commonly used for laboratory water purification. Distilled water and deionised water are the most common forms of purified water.

Distillation involves boiling the water and then condensing the steam into a clean container, leaving most of the solid contaminants behind. This method produces very pure water, but leaves a mineral scale on the distillation apparatus. This requires the frequent cleaning of the apparatus. Deionisation is the process of passing tap water across ion exchange resins in order to remove mineral impurities. The ions in the water exchange with ions in the resin, neutralising the positive and negative ions with the use of an acid and a base. Deionisation is quick and straightforward but requires specific anion resins to remove organic molecules, such as bacteria, from the water.

Laboratory water can also be produced by other laboratory water purification processes. These include reverse osmosis (RO), carbon filtration, microporous filtration, ultrafiltration, ultraviolet oxidation or electrodialysis.

PROCEDURE

Commercially available apparatus can supply distilled, deionised, reverse osmosis (RO) water etc. and should be professionally installed. The apparatus should be used by suitably trained and competent staff as stipulated in the manufacturer's instructions. The apparatus will often have a display or indication of its status and this should be monitored to ensure that the apparatus is operating correctly. Any such apparatus should be serviced and maintained as stated by the manufacturer.

Some commercial suppliers can also supply purified water ready to use.

QUALITY CONTROL

Deionisers and reverse osmosis (RO) units should have conductivity checked weekly, or as appropriate and microbial contamination checked monthly.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

Manufacturer's handbook as appropriate.

Microbiology laboratory glassware

PRINCIPLE AND SCOPE

This procedure describes the methods to use and to clean glassware in the microbiology laboratory. Broken, chipped, cracked or etched glassware is set aside for repair by a glass blower or discarded in the waste container designated for broken glass. The attention to cleaning procedures is determined by the sensitivity and accuracy of the results required. Glassware used in the preparation of microbiological media must have all bacteriostatic or bactericidal material removed. Sterile disposable ware such as petri dishes and pipettes must have a certificate of sterility from the manufacturer prior to being used in the laboratory.

This standard operational procedure applies to all laboratory glassware used during the production of microbiology laboratory media, reagents and/or the preparation of samples. Some plasticware may be cleaned and reused. The same procedure applies.

RESPONSIBILITIES

- **Laboratory Analysts** – To clean and use laboratory glassware according to SOP.
- **Laboratory Manager/Director** – To ensure appropriate procedures are available.
- **Quality Assurance Manager** – To specify procedures used are appropriate.

REAGENTS

- Laboratory Detergent (DeSCAL, Contrad NF, Dri-CONTRAD or similar).
- Distilled or deionised water.

EQUIPMENT

- Laboratory glassware dishwasher.
- Deionised water purification system (or similar).
- Drying oven at 75 °C and 110 °C.

PROCEDURE

Classification of volumetric glassware

Volumetric glassware may either deliver or contain a stated volume. Each type may be further divided according to whether it is specified for single volumes or multiple volumes (one-mark or multi-mark).

Most items of volumetric glassware (with the exception of graduated cylinders) are available commercially in two classes, Class A and Class B. The distinction between the two Classes is based principally on the tolerance limits of the nominal volume of the glassware as specified in the relevant British Standards. Normally, for a given volume, the tolerance

for Class B is twice that for Class A. Glassware used in a microbiology laboratory does not normally need to be Class A. However the laboratory must ensure that it has glassware of the correct class appropriate to the measurements being performed and that if Class A volumetric glassware is appropriate that a certificate of calibration is available.

Commercially available volumetric glassware may be manufactured either from sodalime glass or borosilicate glass. Borosilicate glass is often distinguished by appropriate marking, either as the manufacturer's trade mark or a marking such as 'B', 'boro' or 'borosilicate'.

Each item shall be marked in accordance with the appropriate national/international standard to where it is purchased. This may include:

- tolerance: Class A or B (not applicable to automatic pipettes);
- capacity: either 'mL', 'mL' or 'cm^3' is permitted as the indicated unit of volume;
- reference temperature, i.e. calibration temperature: normally 20 ˚C (27 ˚C for tropical countries);
- identification number: all Class A glassware should bear a permanent identification number.

Cleaning of laboratory glassware

- Glassware that has contained biohazardous material.
- This should be autoclaved to destroy any infectious material before being cleaned and sterilised for reuse.
- Use acetone to remove any writing on the glassware.
- Remove any solid material.
- Rinse thoroughly with tap water, ensuring there are not any particles left on the glassware that may clog the washer. An appropriate laboratory detergent may be required if glassware has debris remaining (a suitable bottle brush may be required).
- Load in washer so that each piece will be washed and rinsed properly.
- Allow the washer to flush for approximately 3 min and then let it fill up with water and add 350 mL of detergent (DRI-CONTRAD or similar) to the wash. Start the wash cycle.
- After the wash cycle is complete, allow the glassware to cool and place them in the appropriate drying oven.
- Glassware should be placed in the 110 °C oven and plastic ware in the 75 °C oven. Glassware that contains rubber seals (i.e. bottles) should be dried in the 75 °C oven.
- Objects that are too large to be washed effectively in the washer, should first be soaked in a 10% detergent (Contrad NF or similar) bath.

Cleaning glass pipettes

- The pipette baths are made up of a 10% detergent (DeSCAL or similar) solution. To make a fresh pipette bath, add 13.5 L of water to the bath followed by 1.5 L of detergent.
- Make sure the glassware is completely submerged in the bath and allow it to soak for at least two hours.
- Carefully remove the glassware from the bath, making sure not to splash the solution onto oneself.

- Use the pipette washer to rinse the pipettes at least three times with distilled or deionised water.
- Place the pipettes in the 110 °C oven to dry.

Volumetric flasks

Be careful not to use excessive heat as this may invalidate the calibrated volume, checks with weighed water should be undertaken if excessive heat has been used.

REFERENCES

European co-operation for accreditation. 2002. *EA-04/10 Accreditation for microbiology laboratories EA-04/10 G: 2002*. Paris, France.

UKAS. 2009. *LAB 15 Traceability: Volumetric apparatus*. UKAS publication Lab 15 edition 2, June 2009. Feltham, UK.

PART 3

Microbiology procedures

Introduction

Evaluation of animal feed stuffs for the presence of bacteria, yeasts, moulds and *Dematiaceae* allows a quality assessment of the feed to be performed to establish its suitability for use.

Animal feed which contains harmful micro-organisms, or is spoiled due to their presence, is to be avoided. The reproduction of micro-organism in animal feed can result in a loss of important ingredients and production of by-products which reduce the effectiveness of the feed. The presence of micro-organisms in animal feed may also results in feed becoming unpalatable, leading to reduced intake of essential nutrients by livestock.

The presence of harmful micro-organisms in animal feed can harm the health of animals feeding on it or of those consuming meat, milk or eggs from infected animals. There may also be a risk of infection to those exposed to infected animals.

Animal feed should be in 'sound condition'. This means that the animal feed should be free from micro-organisms which could potentially harm animals feeding on it or consumers of animal products. It should be noted however that many animal feeds will contain micro-organism naturally (field flora) or as a results of storage (e.g. silage).

Some micro-organisms are added by animal feed processers for their beneficial effects, these are known as probiotics and can increase the health of animals consuming the feed. Animal feed laboratories may be required to detect and enumerate such probiotic micro-organisms.

Animal feed, due to its composition, provides a favourable environment for the growth of micro-organisms. These micro-organisms can be saprophytic, pathogenic, conditionally pathogenic or toxic and their growth is dependent on many factors affecting the feed. These factors include moisture, pH, temperature, type of feed, aerobic or anaerobic conditions, the chemical properties of the feed and storage conditions and environment.

Contamination by micro-organisms can occur during processing, storage, transport or from the environment in which they are grown or harvested. Contamination can frequently result in spread of infection and surveillance programmes for microbiological safety of animal feed are recommended.

Animal feed laboratories may be requested to determine 'germ numbers' of micro-organisms which are regarded as indicator organisms that animal feed is spoilt. They may also be requested to determine the presence of a specific bacteria, yeast, fungi, parasite or other agents which are undesirable in animal feed, or products used to manufacture animal feed.

Sound, validated standard operating procedures (SOPs) are essential to produce reliable laboratory results from such analysis and should be combined with robust internal quality control procedures and external proficiency schemes to provide confidence to customers in results reported.

The following procedures have been produced from validated procedures used by animal feed laboratories and reference facilities and have proved reliable. However, other methods or variants of the methods presented in this manual may also be used.

Isolation and enumeration of *Enterobacteriaceae* from animal feed samples

PRINCIPLE AND SCOPE

A number of processes are employed to eliminate bacteria from products to enable their use as animal feedstuffs. Submitted samples are tested using appropriate selective media, allowing the detection and enumeration of low numbers of Enterobacteriaceae in such samples. *Escherichia coli* is deemed an appropriate marker for such organisms and as such all Enterobacteriaceae will be enumerated, identified as *E. coli* or other genus of Enterobacteriaceae.

This procedure applies to all samples submitted to the microbiology laboratory for detection and enumeration of Enterbacteriaceae. Enterobacteriaceae are microorganisms that form characteristic colonies on violet red bile glucose agar, they ferment glucose and are Oxidase negative.

Separate specific procedures exist for the detection of *Salmonella* spp. and *E. coli* O157 in animal feeds.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA and Health and Safety requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Calibrated balance.
- Incubator at 37 ± 1 ˚C.
- Fridge at 2–8 ˚C (for sample storage).
- Water bath at 47 ± 1 ˚C.
- Pipettors (200–1000 µl and 50–200 µl with filtered sterile tips or plugged sterile).
- Sterile scoop or spatula.
- Sterile 90 mm Petri dishes.

REAGENTS

All microbiological media used is prepared according to the manufactures instructions or purchased pre poured.

- 90 mL Buffered Peptone Water (BPW).
- 15 mL aliquots of Violet Red Bile Glucose Agar (VRBGA).
- 10 mL aliquots of Violet Red Bile Glucose Agar as above.
- Peptone saline diluent for serial decimal dilutions.
- Reference culture *E. coli* (e.g. NCTC or ATCC).

Materials for confirmation of identification.

- Nutrient Agar plates.
- Glucose fermentation test (Glucose agar or commercial test kit).
- Oxidase test reagent (or commercial test kit).
- Commercial bacterial identification test (e.g. API™ or similar).

PROCEDURE

Sample management

Testing shall begin on the day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

Day 1

Samples must be well mixed using a sterile palate knife, spoon or stirring rod before sub sampling. One mL of liquid sample may be added directly to a sterile 90 mm Petri dish (in duplicate) without the need for BPW.

Ten g portions of each sample submitted for testing are weighed, aseptically.

Each 10 g portion is added to the 90 mL Buffered Peptone Water (BPW) and mixed thoroughly until evenly suspended.

Prepare a single decimal dilution series from the test sample, if liquid, or initial suspension in Peptone Saline Diluent (PSD) for all other sample types.

One mL is aseptically removed from each sample and transferred to a sterile 90 mm Petri dish (in duplicate). One mL is taken from each decimal dilution of the sample and transferred to a sterile 90 mm Petri dish (in duplicate). Petri dishes must be suitable identified with the sample identifier on the base prior to adding the sample.

Fifteen mL of Violet Red Bile Glucose Agar (VRBGA) at a temperature of 44–47 ˚C is added to each Petri dish and immediately mixed with horizontal movements. Plates are left undisturbed on a cool flat surface until the agar has set.

When the agar has set, each plate is overlaid with a further 10 mL of VRBGA at a temperature of 44–47 ˚C, to prevent spreading growth and achieve semi-anaerobic conditions.

When the overlay has set, the plates are inverted and incubated aerobically at 37 ± 1 ˚C for 24 ± 2 h.

Day 2

Negative control plates are checked for sterility. If at any stage the negative control plate demonstrates growth the procedure is invalidated.

Each set of duplicate plates is examined for colonies characteristic of Enterobacteriaceae (red/purple colonies with, or without a precipitation halo, 1–2 mm in diameter). Select a pair of plates with between 15 and 150 colonies and count. All characteristic colonies on each plate are counted and the mean of the duplicate plates calculated.

If characteristic colonies are observed five typical colonies should be subcultured onto Nutrient Agar plates for further identification. The sub cultured plates are incubated at 37 ± 1 °C for 24 ± 2 h.

If more than half the plate surface is contaminated the procedure is invalidated. If less than half the surface is contaminated the colonies in the clear portion may be counted and the count extrapolated to correspond to the total surface area of the media.

Some species of Enterobacteriaceae may cause decolourisation of their colonies or the medium. In the event that there are no characteristic colonies present, five whitish colonies should be selected for confirmation tests.

Day 3

The Nutrient Agar subcultures are tested for Oxidase and glucose fermentation. Isolates which are Oxidase negative and glucose fermentation positive are considered to be Enterobacteriaceae.

If requested all morphologically distinct types may be sub cultured and identified using suitable laboratory tests or a commercially available identification system, to specifically identify *E. coli* as a marker organism.

In the event of all of the colonies not being Enterobacteriaceae, the total count at Day 2 shall be proportionally reduced (prior to establishing whether or not the sample should fail).

QUALITY CONTROL

- Positive and negative controls are run on each day that testing is initiated.
- A 0.5 McFarland suspension of reference *E. coli* (e.g. NCTC or ATCC) in sterile distilled water is diluted to the forth decimal dilution (10^{-4}) by serial dilution in BPW. One mL of the suspension is added to 90 mL of BPW and processed as a sample to act as a positive control.
- This suspension (100 µl) is inoculated and spread evenly onto a Nutrient Agar plate. This is incubated and counted to act as a control count.
- Ten grams of autoclaved sample, therefore known to be free of Enterobacteriaceae, is added to 90 mL of Buffered Peptone Water and processed as a sample to act as a negative control.
- One mL of positive control suspension can be added to 90 mL Buffered Peptone Water and used as the positive control and target recovery verification. When it has been demonstrated that the process successfully detects appropriate numbers of *E. coli*, only media controls are required on future testing.

ENUMERATION AND REPORTING RESULTS

- Select the pair of plates which has <150 characteristic colonies and count these colonies.
- Each pair of plates is counted and the mean calculated.
- The number of colony forming units (cfu) per mL or gram of sample are expressed using the following calculation:

$$\frac{\text{Number of colonies confirmed}}{\text{Number of colonies tested}} \times \frac{\text{Number of colonies counted}}{\text{Volume tested x dilution}}$$

- Results are reported as the number of Enterobacteriaceae per millilitre or per gram of sample.
- If no colonies are counted the result should be expressed as <10 cfu/g or <10 cfu/mL.
- If the count is between 10 and 99 cfu report as x cfu/g or x cfu/mL where x is the count.
- If the count is greater than 100 cfu report to the appropriate power of ten and round the count up if the last figure is 5 or above and down if the last figure is below 5.

i.e. 1966 cfu/g is reported as 1.9×10^3 cfu/g
723000 cfu/g is reported as 7.2×10^5 cfu/g

REFERENCES

Edel, W. & Kampelmacher, E.H. 1973. *Bulletin of World Health Organisation*, 41: 297–306, World Health Organisation Distribution and Sales, Ch-1211, Geneva 27, Switzerland (ISSN 0042-9686).

ISO 21528-2:2004. *Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae Part 2: Colony-count method*. Geneva, Switzerland.

ISO 7218:2007. *Microbiology of food and animal feeding stuffs – general requirements and guidance for microbiological examination*. Geneva, Switzerland.

Isolation and identification of *Escherichia coli* O157 from animal feed samples

PRINCIPLE AND SCOPE

Escherichia coli O157 is a verotoxigenic strain of *E. coli* which poses a significant threat to human health from animal products contaminated with faeces. The presence of *E. coli* O157 in animal feeds may introduce the microorganism to previously non infective animals although the animals infected will demonstrate little or no affects.

On Sorbitol MacConkey agar (SMAC) or Sorbitol MacConkey agar supplemented with Cefixime and tellurite (CT-SMAC) VTEC *E. coli* O157 produces colourless, 2–3 mm diameter colonies which can be confirmed with commercial test kits.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *E. coli* O157. The procedure is designed to detect *E. coli* O157 in 1 g samples using immunomagnetic separation (IMS) and use of volumes less than 1 g will reduce the sensitivity.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

HEALTH AND SAFETY

Verocytotoxigenic *E. coli* (VTEC) are Containment Level 3 (CL 3) organisms. All isolation and identification work shall be carried out in an appropriate Containment Level 3 facility by suitably-trained workers. VTEC, including *E. coli* O157, are highly infective, are pathogenic for man and can cause severe laboratory-acquired infection, the infective dose is low.

EQUIPMENT

- Calibrated Balance.
- Incubator at 37 ± 1 °C.

- Fridge at 2–8 ˚C (for sample storage).
- Pipettor and tips (20 µl sterile).
- Sterile scoop (10 µl and 1 µl).
- Sterile 90 mm Petri dishes.
- Rotating mixer.
- Magnetic particle concentrator.
- Screw-capped 1.5 mL conical tubes (Eppendorf, or similar) appropriate for the magnetic particle concentrator used.
- Plastic pastettes (1 mL and 1 mL fine tip, or equivalent).

REAGENTS

- All microbiological media used is prepared according to the manufactures instructions or purchased pre poured.
- Buffered peptone water (BPW) 20 mL and 225 mL.
- Paramagnetic beads coated with antibody to *E. coli* O157 antigen (Dynal 710.04 or equivalent).
- Sterile phosphate buffered saline (10 mM PBS pH 7.4), with Tween 20–0.05% v/v (PBS-T).
- Sorbitol MacConkey Agar supplemented with Cefixime (0.05 mg/L), tellurite (2.5 mg/L) and Chromocult agar (Merck 1.10426 or equivalent) or MacConkey agar.
- *E. coli* O157 latex (Oxoid DRO620 or equivalent).
- Non-toxigenic positive control *E. coli* O157 (NCTC 10418 or similar).

PROCEDURE

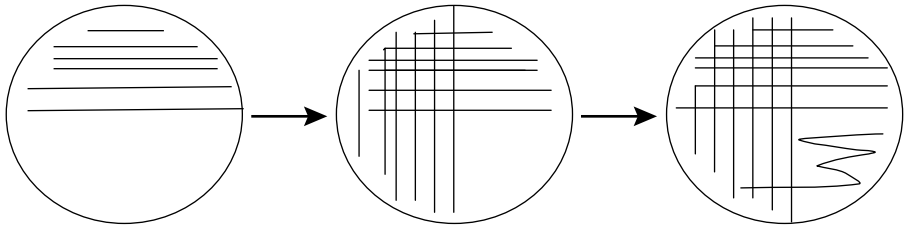
Sample management

Testing shall begin on the day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

- Add 25 g of sample to be tested to 225 mL Buffered Peptone Water (BPW), mix and incubate at 37 ± 1 °C for six hours. Incubated BPW may be stored overnight at 2–8 oC before proceeding but must be brought to room temperature before commencing the procedure.
- For each enriched sample or control, label a 1.5 mL conical tube and add 20 µl *E. coli* O157 paramagnetic beads.
- Taking care not to disturb the sediment, transfer 1 mL enrichment broth to its appropriately labelled tube.
- Ensure each tube is firmly capped and mix the beads and broth by inversion until the beads are suspended.
- Firmly secure each tube on a rotating mixer and mix for 30 min at room temperature.
- Place each tube in a magnetic separator and leave for 3–5 min at room temperature.

- Gently rotate the rack three times to concentrate the beads onto the side of the tube at the magnet.
- Using a sterile Pastette, remove all the liquid in the tube leaving the beads behind. Any liquid in the cap should also be removed.
- When this has been done for all samples, remove the magnet and add 1 mL PBS-T to each tube individually using a different pastette for each tube. Gently invert the rack four or five times to resuspend the beads.
- Replace the magnet and leave for three min.
- Repeat washing steps twice more.
- Gently rotate the rack three times to concentrate the beads onto the side of the tube at the magnet.
- Using a sterile pastette, remove all the liquid in the tube leaving the beads behind. Any liquid in the cap should also be removed.
- Resuspend the beads in approximately 50 µl PBS-T.
- The bead suspensions are inoculated onto CT-SMAC agar as shown below and incubated at 37 ± 1 oC for 18 h to a maximum of 24 h.



Plates are examined for non-sorbitol fermenting colonies typical of *E. coli* O157. Representative colony types are sub cultured to a labelled section of Chromocult or MacConkey agar and incubated at 37 ± 1 ˚C overnight. *E. coli* O157 generally lack ß-D-glucuronidase and are seen as red/pink colonies on Chromocult or pink lactose fermenting colonies on MacConkey agar. Other *E. coli* are seen as dark blue/violet colonies on Chromocult.

Confirmation of suspect colonies

Suspect colonies are confirmed as *E. coli* O157 using a latex agglutination test according to the manufacturer's instructions. Half a suspect colony is emulsified into a drop of *E. coli* O157 latex reagent and the slide rocked back and forth and examined for agglutination. Colonies which agglutinate within one minute are regarded as *E. coli* O157; and the remaining half colony is spread onto a fresh MacConkey agar plate using a sterile microbiological loop and incubated at 37 ± 1 ˚C for 18 to 24 h.

Typing of *E. coli* O157 isolates may be sought from an appropriate *E. coli* O157 reference laboratory.

Automated IMS recovery systems are also available (e.g. Kingfisher ML automated bead retrieval system) and if used the appropriate manufacturer's instructions should be followed.

QUALITY CONTROL

QC of media

Pre-poured media may be supplied from an accredited supplier with QC data. If media is to be made on site appropriate QC should be in place. One plate from each batch should be inoculated with appropriate reference organisms (e.g. NCTC or ATCC) to check enhancement, indicator and suppression as required. Media should not be used until internal QC checks are completed satisfactorily.

QC of IMS

Positive and negative controls should be included in each batch of tests. A negative result in the positive control invalidates the batch tested and requires investigation and re-testing of the failed batch. If all other controls are satisfactory, it suggests that the para-magnetic beads are not capturing the organism. This can be confirmed by culturing the BPW containing the positive control directly onto CT-SMac.

QC of latex agglutination

Latex agglutinations are carried out using the *E. coli* O157 positive control and a reference *E. coli* (e.g. NCTC or ATCC) as a negative control. The kit is supplied with negative and positive latex.

QC of new batches of immunomagnetic beads

Duplicate tests are carried out comparing new batches of IMS beads against the in-use batch.

EQA

Regular participation in an appropriate accredited external proficiency scheme is advisable.

REPORTING RESULTS

Results should be reported as '*E. coli O157* detected' or '*E. coli* O157 not detected'.

REFERENCES

HPA. 2011. *UK standards for microbiology investigations: Identification of* Escherichia coli *O157*. HPA Bacteriology identification ID 22 Issue No 3.1, October 2011. London, UK.

Isolation of *Salmonella* spp. from animal feed samples

PRINCIPLE AND SCOPE

Salmonella is a single species which is found predominantly in animal gut. It is both an animal and human pathogen and foodstuffs contaminated with animal faeces are an important vehicle in transmission of the infection. Animal feeds are thought to be an important source of *Salmonella* infection due to faecal contamination (particularly in animal feeds containing blood, bone, fish meal or protein rich feed materials of plant origin such as oil seed meals). In some parts of the world poultry manure may be used as a feed for ruminants. The bacteria may also be spread by way of manure used on pasture or as a fertilizer. The presence of *Salmonella* in animal feeds may introduce the microorganism to previously non infective animals.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *Salmonella.*

Salmonella species are classified and identified into serotypes according to the White-Kauffmann-Le Minor scheme, which currently contains in excess of 2600 serotypes. Primary subdivision is into 'O' serogroups (those which share a common somatic antigen), and these are then subdivided on the basis of 'H' (flagella) antigens. A few species, notably *Salmonella typhi*, may produce a capsular 'Vi' antigen which causes the bacteria to agglutinate with 'Vi' antiserum and may mask 'O' antiserum.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

HEALTH AND SAFETY

Salmonella are zoonotic and may cause severe and sometimes fatal disease. Most *Salmonella* species belong to Containment Level 2 (CL 2) with some exceptions (*Salmonella typhi* and *Salmonella paratyphi* A, B and C). All isolation and identification work shall be carried out in an appropriate Containment Level 2 (CL 2) or Containment Level 3 (CL 3) facility, avoiding aerosol formation and ingestion, by suitably-trained workers.

EQUIPMENT

- Calibrated Balance.
- Incubator at 37 ± 1 ˚C.
- Incubator at 41.5 ± 1 ˚C.
- Fridge at 2–8 ˚C (for sample storage).
- Sterile loop (10 µl and 1µl).
- Sterile 90 mm Petri dishes.
- Glass slides.

REAGENTS

All microbiological media used are prepared according to the manufactures instructions or purchased pre poured.

- Buffered peptone water (BPW) 90 mL and 225 mL volumes (including double BPW if necessary).
- Rappaport-Vassiliadis Medium (RVS broth).
- Muller-Kauffman Tetrathionate-Novobiocin broth (MKTTn).
- Xylose Lysine Deoxycholate media (XLD).
- Another suitable solid selective media for *Salmonella* complimentary to XLD*.
- Nutrient agar.
- Salmonella monovalent and polyvalent agglutinating antiserum (as required).
- Commercial microbiological identification system (e.g. API ™ or similar).
- Suitable Salmonella reference culture (e.g. NCTC or ATCC) for positive control.
- Saline.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

Day 1

- Aseptically weigh five 25 ± 0.2 g (or 25 ± 0.2 mL for liquid samples) portions of each feed sample and add to 225 mL Buffered Peptone Water (BPW). BPW may be used as a negative control and a suitable reference culture (e.g. NCTC, ATCC) should be used as a positive control. The final ratio of sample to BPW should be 1:10 and may be adjusted if there is insufficient sample. If this is the case it should be noted in the final report to the customer.

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* It is recommended that a selective media with biochemical properties different from XLD agar (H_2S production, black colonies), e. g. BPLS (Oxoid CM 329), Rambach agar (Merck 1.07500), chromID Salmonella (bioMérieux 43291), etc. is used.

- When using light weight animal feed (e.g. hay) or feed stuffs which swell (e.g. line seed products) the amount of sample may be reduced (e.g. to 10 g) as needed, so that the total sample weight can be taken up in 225 mL of BPW.
- For acidic or acidifying feed stuffs (e.g. silages) double buffered peptone water may be used to ensure that the pH does not fall below pH 4.5.
- Incubate at 37 ± 1 °C for 18 ± 2 h.

Day 2

- Aseptically remove 100 µl from each of the aliquots of BPW and inoculate into 10 mL Rappaport-Vassiliadis Medium (RVS broth).
- It is important not to disturb the BPW and the 100 µl should be taken from an area at or near the surface, away from any floating debris.
- Incubate the RVS broth at 41.5 ± 1 ˚C for 24 ± 3 h.
- Aseptically remove 1000 µl from each of the aliquots of BPW and inoculate into 10 mL Muller-Kauffman Tetrathionate-Novobiocin broth (MKTTn).
- It is important not to disturb the BPW and the 1000 µl should be taken from an area at or near the surface, away from any floating debris.
- Incubate the MKTTn broth at 37 ± 1 ˚C for 24 ± 3 h.

Day 3

- Using a 10 µl microbiological loop inoculate the RVS broth and MKTTn broth onto four selective plates each. Two plate should be Xylose Lysine Deoxycholate (XLD) and two an appropriate selective solid media which is complementary to XLD. Each pair of plates should be considered as one and the loop should not be recharged between each plate.
- Incubate the four pairs of plates at 37 ± 1 ˚C for 24 ± 3 h.
- If after 24 ± 3 h at 37 ± 1 ˚C the plates are negative, or only have very small colonies, incubate at 37 ± 1 ˚C for a further 24 ± 3 h.

Day 4 (or Day 5 if plates incubated for a further 24 ± 3 h at Day 3)

- Examine the plates. Subculture a minimum of three colonies from each plate showing suspicion of *Salmonella* growth to a Nutrient Agar and incubate at 37 ± 1 °C overnight. *Salmonella* typically produce colonies with a black centre (due to H_2S production) and a slightly transparent zone of reddish colour, although Lactose positive strains produce yellow colonies, either with or without blackening. Some serovars e.g. *Salmonella paratyphi* A (a human serotype) or *Salmonella senftenberg* are H_2S negative and produce no blackening.

Note: Colonies from selective plates may contain a minority population of viable but inhibited contaminant organisms that will grow on subculture and cause erroneous biochemical reactions.

Day 5 (or Day 6 if plates incubated for a further 24 ± 3 h at Day 3)

- If after overnight incubation purity plates show pure growth, the suspect isolates are tested using a commercial microbiological identification system (e.g. API™ or similar)

to confirm the biochemical characterisation of *Salmonella* spp.

- Perform agglutination test with antisera, as directed by manufacturer's instructions. Antisera reactions should be observed by emulsifying a single, well isolated colony grown on Nutrient Agar into a drop of antisera on a glass slide. 'O' antisera produce a granular agglutination reaction on a glass slide. 'H' antisera produce a floccular agglutination on a glass slide. 'Vi' antigen may be present and mask the 'O' antigen and cause the bacteria not to agglutinate 'O' antisera.
- Perform a check for auto agglutination by emulsifying a single, well isolated colony grown on Nutrient Agar into a drop of saline (0.85% NaCl) and observe for granulation. A positive auto agglutination indicates that a strain cannot be investigated for serotyping.
- Serological identification is complex and may be performed using polyvalent antisera which contain 'pools' (e.g. OMA and OMB). If a strain indicates a positive result it may then be tested with the components of that 'pool'.
- In many cases it will be sufficient to identify a *Salmonella* as *Salmonella* Group 'B', 'C', 'D', 'E' or 'G' using a limited range of 'O' antisera with further serotyping performed by a reference laboratory.
- If purity plates show mixed organisms, repeat biochemical tests and slide agglutinations from a *Salmonella* colony on the purity plate.
- The second set of inoculated BGA plates is examined and the same procedure followed.
- Where both sets of BGA plates have no (suspicious) growth the results can be reported and a final report issued.

Day 6

- The microbiological identification system (e.g. API™ or similar) is read and where biochemical results confirm *Salmonella* spp., this is reported.
- Local arrangement may exist for *Salmonella* isolates to be sent for confirmation and further identification to a reference laboratory or reported for disease surveillance purposes.

REPORTING THE RESULTS

The results should be reported in the following manner:

- '*Salmonella* spp. not detected (25 g of sample used)' if no *Salmonella* is isolated from the sample; or
- '*Salmonella* spp. detected (25 g of sample used)' if *Salmonella* is isolated from the sample. Serotyping, if known, should also be reported.

Any departure from the method used (e.g. use of a smaller sample weight) should also be reported to the customer.

QUALITY CONTROL

Suitable reference cultures should be grown on Nutrient Agar and used to ensure the viability of *Salmonella* agglutination sera on a regular basis.

All media should be controlled with suitable reference cultures (e.g. NCTC or ATCC).

To control MSRV media an appropriate reference strain of *Salmonella* should be inoculated at 10^4 CFU/0.1 mL as a positive control and an appropriate strain of non target organism (e.g. *E. coli* or *E. faecalis*) inoculated at $10^5 – 10^6$ CFU/0.1 mL

Aberrant *Salmonellae* spp.

There are several serotypes of *Salmonella* which differ phenotypically from other members of the genus. Many of these are host-adapted to a particular animal species and may not grow in selective broth and agar media routinely used for *Salmonella*, visible growth only appearing on less selective isolation media such as MacConkey agar. In addition, profiles for these serotypes are frequently excluded from the databases provided by manufacturers of commercial kits. It is important that these atypical characters are borne in mind if successful culture and identification of these serotypes from samples is to be achieved.

Some of those atypical *Salmonella* serotypes of veterinary significance are listed below:

- *S. choleraesuis* (natural host swine);
- *S. pullorum* (natural host poultry);
- *S. gallinarum* (natural host poultry);
- *S. abortusovis* (recovered from ovine and caprine abortion material);
- *S. arizonae* (recovered from cold blooded animals).

Procedure for serotyping an unknown isolate of *Salmonella* to group level using a limited range of 'O' antisera.

If a polyvalent antisera of another antibody composition to OMA and OMB is used the group specific steps at step 2 and 3 will have to be altered appropriately.

Steps 'O'antigen

Step 1 'O' antigen	Use of polyvalent antisera	1.1 OMA If positive: 2.1.1 If negative: 1.2	1.2 OMB If positive: 2.2.1 If negative *
Step 2 'O' antigen	Use of group specific antisera and allocation to a serogroup.	2.1.1 O:4,5 or group B If positive: 3.1.1 If negative: 2.1.2 2.1.2 O:9 or group D If positive: 3.1.2 If negative: 2.1.3 2.1.3 O:3,10,15 or group E If positive: 3.1.3	2.2.1 O:6,7,8 or group C If positive: 3.2.1 If negative: 2.2.2 2.2.2 O:13,22,23 or O:13 If positive: 3.2.2 If negative: 2.2.3 2.2.3 O:11
Step 3 'O' antigen	Use of monospecific antisera. Identification of serovar specific O antigens	3.1.1 Isolate of group B: O:5, O:27 3.1.2 Isolate of group d: O:46 for group D_2 O:27 for group D_3 3.1.3 Isolate of group E: O:10, O:15, O:34 for group E_1 O:19 for group E_4	3.2.1 Isolate of group C: O:7 for group C_1 O:8, O:6, O:20 for group $C_{2\text{-}3}$ O:14, O:24 for Group H 3.2.2 Isolate of group G: O:22, O:23

* In the case of a negative reaction with both polyvalent 'O' antisera test the isolate with further polyvalent or group specific antisera, if available, or send to a reference laboratory.

REFERENCES

ISO 6579:2002. *Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella species*. Geneva, Switzerland.

HPA. 2011. UK *standards for microbiology investigations: Identification of Salmonella species*. HPA Bacteriology identification ID 24 Issue No 2.2, October 2011. London, UK.

VDLUFA. 2012. *Methods book III 8th Supplement 2012, No 28.3.1. Detection of Salmonella*. Speyer, Germany.

Isolation of *Listeria* spp. from animal feed samples

PRINCIPLE AND SCOPE

Listeria spp. are non sporing, short (0.5–2.0 µm in length), Gram positive rods which are widely distributed in the environment and can be isolated from animal products, soil, vegetables, food, silage and other animal feeds. They are both animal and human pathogens and foodstuffs contaminated with animal faeces are an important vehicle in transmission of the infection. The presence of *Listeria* spp. in animal feeds may introduce the microorganism to previously non infective animals. Silage is commonly implicated in outbreaks of Listeriosis in farm animals.

Listeria spp. can be divided into ten species:

- *L. murrayi* and *L. grayi* are non-haemolytic and are rarely isolated. Both are considered to be non pathogenic.
- *L. monocytogenes* and *L. ivanovii* are haemolytic and pathogenic for man and animals.
- *L. seeligeri* is also haemolytic but is considered to be non pathogenic.
- *L. innocua* and *L. welshimeri* are non haemolytic and are considered to be non pathogenic
- *L. fleischmannii, L. marthii* and *L. rocourtiae* are newly described species of unknown pathogenicity.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *Listeria* spp.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

HEALTH AND SAFETY

Listeria spp. are zoonotic and may cause severe and sometimes fatal diseases. *Listeria* species belong to Containment Level 2 (CL 2). All isolation and identification work shall be carried out in an appropriate Containment Level 2 facility by suitably-trained workers.

Listeria spp. can produce severe infection and abortion in individuals exposed to infection, therefore pregnant laboratory workers and immunocompromised individuals should be prohibited from working with known or suspect cultures of *Listeria* spp.

EQUIPMENT

- Sealable sterile bags.
- Calibrated balance.
- Incubator at 30 ± 2 ˚C (and 37 ± 1 ˚C for CAPM test).
- Stomacher or similar.
- Fridge at 2–8 °C (for sample storage).
- Sterile loop (10 µl).
- Appropriate reference cultures (e.g. NCTC or ATCC).

REAGENTS

All microbiological media used is prepared according to the manufacturer's instructions or purchased pre poured.

- Listeria selective agar (containing aesculin).
- Blood agar (5%).
- Listeria Enrichment Broth (UVM formulation).
- Commercial microbiological identification system (e.g. API or similar).
- Listeria polyvalent O antiserum.
- D-Xylose fermentation broth.
- L-Rhamnose fermentation broth.
- Nitrate reduction broth (also sulfanilic acid, N,N-dimethyl-1-naphthylamine and zinc powder).
- Saline.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

Listeria spp. grow on Listeria Selective Agar (containing aesculin) as black colonies with a surrounding dark brown/black zone produced by hydrolysis of the aesculin. To isolate *Listeria* from animal feed samples the sample must be homogenated and incubated in enrichment broth before a secondary enrichment and growth on selective media.

- Aseptically place 25 ± 1.0 g of representative sample into a sterile stomacher, or similar, type bag. Ensure the bag is sealed and if necessary double bag inside an appropriate bag.

- Add 225 ± 5 mL (or 225 ± 5 g) Listeria Enrichment Broth (UVM formulation) blend or stomach for approximately 2 min and aseptically transfer the homogenate to a sterile container. Incubate at 30 ± 2 ˚C for 48 ± 4 h.
- A standard quantity of a *Listeria* reference culture (e.g. NCTC or ATCC) should be inoculated into 225 mL Listeria Enrichment Broth as a positive control. An uninoculated Listeria Enrichment Broth should be used as a negative control.
- Streak a loop full of the two day enrichment broth across a Listeria selective agar (containing aesculin) plate in a zigzag pattern. The space between the loop streaks should be 0.5 to 1cm. Incubate the Listeria selective agar plate at 30 ± 2 ˚C for 24–48 h.
- Examine plates for purity and colony morphology. Sub culture suspect colonies onto 5% blood agar and incubate at 37 ± 1 ˚C for 24–48 h.
- Confirmation of the genus *Listeria.*
- From the blood agar plate, perform a catalase test (most strains positive) on a single colony and a Gram stain (Gram positive rod) on a single colony. Test for agglutination with Listeria polyvalent O antiserum.

Appropriate reference cultures (e.g. NCTC or ATCC) should be used as positive and negative controls for Catalase test and agglutination with Listeria polyvalent antisera.

Perform a check for auto agglutination by emulsifying a single, well isolated colony grown on blood agar into a drop of saline (0.85% NaCl) and observe for granulation. A positive auto agglutination indicates that a strain cannot be investigated for serotyping.

Further identification (if required)

Commercially available identification systems (e.g. API™ or MICRO-ID™ or similar) may be used to identify isolates or appropriate laboratory biochemical tests may be used.

If required to further identify *Listeria* spp. the following tests can be performed:

Species	Production of acid			CAMP test	
	Mannitol	L-Rhamnose	D-Xylose	*Staph aureus*	*R. equi*
L. monocytogenes	-	+	-	+	-
L. innocua	-	v	-	-	-
L. ivanovii	-	-	+	-	+
L. seeligeri	-	-	+	w+	-
L. welshimeri	-	v	+	-	-
L. grayi	+	v	-	-	-
L. murrayi	+	-	-	-	-

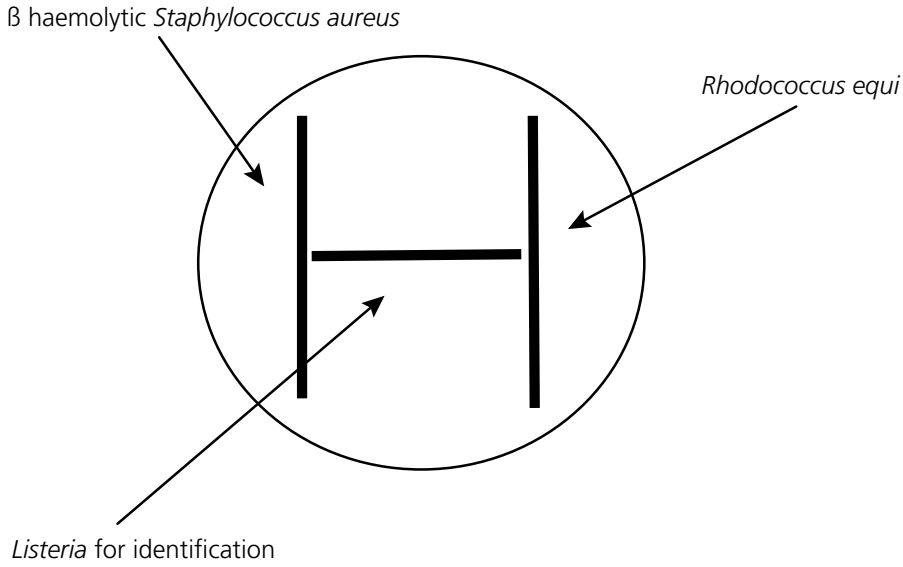
V = variable reaction w+ = weak positive reaction
Additionally only *L. murrayi* reduces Nitrate.

CAMP test

- Onto a 5% blood agar plate streak an appropriate reference strain (e.g. NCTC or ATCC) of ß haemolytic *Staphylococcus aureus* in a single line at one side. On the same plate streak an appropriate reference strain (e.g. NCTC or ATCC) of *Rhodococcus equi* in a single line parallel to the ß haemolytic *Staphylococcus aureus.* Between

these parallel streaks inoculate the suspect *Listeria* at right angles, without touching the ß haemolytic *Staphylococcus aureus* or *Rhodococcus equi*.

- Incubate at 37 ± 1 ˚C for 24–48 h and examine for enhanced haemolysis at the intersection of the *Listeria* and the ß haemolytic *Staphylococcus aureus* and *Rhodococcus equi*.
- Appropriate reference cultures (e.g. NCTC or ATCC) should be inoculated as positive and negative controls.



Sugar fermentation

Innoculate 5 mL amounts of mannitol, L-rhamnose and D-xylose fermentation broths from the 5% blood agar plate. Incubate at 37 ± 1 ˚C for 24 ± 2 h and examine for acid production. Mannitol, L-rhamnose and D-xylose fermentation broths may be purchased from a suitable laboratory supplier.

Appropriate reference cultures (e.g. NCTC or ATCC) should be inoculated as positive and negative controls.

If required *Listeria* isolates may be sent for further typing to a Reference Laboratory.

Nitrate reduction can be checked by inoculating nitrate reduction broth with the culture and incubating for 48 h.

Add 10–15 drops of sulfanilic acid and N,N-dimethyl-1-naphthylamine and observe for a red colour within five min. A red color change at this step indicated a positive reaction. If there is no colour change add zinc powder to the broth and observe for a colour change to red. If the broth turns red after the addition of zinc the result is negative. See 'Gram stain and primary characterization tests' SOP.

Calculation of results

Results should be expressed as *Listeria* spp. present in 25 g or 25 mL of original sample.

REFERENCES

ISO 11290-2:1998. *Microbiology of food and animal feeding stuff. Horizontal method for the detection and enumeration of Listeria monocytogenes*. Geneva, Switzerland.

HPA. 2011. *UK standards for microbiology investigations: Identification of Listeria species, and other non-sporing Gram positive rods* (except *Corynebacterium*). HPA Bacteriology identification ID 3 Issue No 2.1, October 2011. London, UK.

Isolation and enumeration of yeasts (excluding probiotic yeast), moulds, *Dematiaceae* and aerobic/mesophilic bacteria from animal feed samples

PRINCIPLE AND SCOPE

Colonies of yeasts, moulds, *Dematiaceae* and aerobic/mesophilic bacteria detected in animal feeds may be diagnostically differentiated as indicator micro-organisms and data used regarding their growth may be used to describe the feed quality as regards its condition as microbiologically sound and unspoilt.

Yeast can be described as round to oval unicellular fungi which reproduce by budding. Some are able to produce pseudohyphae (chains of elongated budding cells) but only a few are able to produce true hyphae. Yeasts are characterised, classified and identified by their morphology and biochemical laboratory tests.

Yeasts are widely distributed in the environment and are part of the normal flora of man and animals. Yeasts may contaminate a wide variety of animal feeds (including dry feeds) and may cause spoilage of silage.

A number of microbial products may be added to animal feeds to provide beneficial effects on health and production but are not attributed with a nutritional role (probiotics). Yeast cultures may be used as probiotics with *Candida pintolopesii*, *Candida saitoana* and *Saccharomyces cerevisiae* commonly used. The presence of such probiotics should be established with the animal feed supplier/manufacturer if detection of yeasts is requested.

C. albicans is commonly associated with animal disease and grows as a budding yeast cell on many different agar cultures at a wide variety of temperatures and differing pH. They produce distinctive convex colonies with a shiny waxy appearance.

Mouldy animal feed does not necessarily contain dangerous mould poisons or mycotoxins but the presence of considerable mould in animal feed will adversely affect production and health of animals feeding on it. Digestibility may be affected for ruminants and energy values will be reduced.

Dematiaceae are saprophytic fungi which are widely distributed in soil, water and decaying vegetable material.

Mesophilic bacteria are bacteria which have an optimum temperature of 20–45 °C and typically are food contaminants (including animal feeds).

This procedure applies to all samples submitted to the microbiology laboratory for detection of Yeasts (excluding probiotic yeasts), moulds, *Dematiaceae* and aerobic/mesophilic bacteria in animal feeds.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA and Health and Safety requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Pipettor capable of measuring 100–1000 µl.
- Incubator at 25 ± 1 °C (and 20 ± 1 °C for aerobic/mesophilic bacteria).
- Microscope.
- Microbiological spreader.
- Horizontal shaker capable of maintaining 120–180 rpm.
- Stomacher and stomacher bags.
- Blender or homogeniser capable of up to 10 000 U/min.

REAGENTS

- All microbiological media used is prepared according to the manufacturer's instructions or purchased pre poured.
- Peptone water broth (0.1% mass concentration) at pH 7 ± 0.2 with Tween™ 80.
- Diluent buffer.
- Dichloran Rose Bengal Chloramphenicol (DRBC) agar with tergitol.
- Dichloran 18% glycerol agar (DG18).
- Tryptose agar with 2,3,5 – triphenyltetrazolium chloride (TTC).
- Iminodiacetic acid (98%).
- Sodium hydroxide solution (5 mol/L).
- Silicone antifoaming agent (e.g. Wacker Silicon AS-EM.SE 2).

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure. Samples for yeast testing should not be frozen.

Copper may have a toxic effect on some micro-organisms. If samples submitted contain more than 400 mg Cu/kg add 350 mg of iminodiacetic acid and 0.53 mL sodium hydroxide solution to the suspension solution to chelate the copper.

Add the required sample quantity (± 0.1 g or 0.1 mL) to the required volume of peptone water and shake for 20 min on a horizontal shaker at 120–180 rpm. If foaming is noted a few drops of suitable silicone antifoaming agent may be added to the solution.

Alternatively the sample and diluents may be placed in a stomacher bag and after being left for 10–15 min, it may be treated in the stomacher for 3–5 min. Liquid feeds may be treated for a minimum of three min without the holding time of 10–15 min.

If appropriate the sample may be treated with a blender in a sterile beaker. First at 5 000 U/min for one minute followed by 8 000–10 000 U/min for five min.

Recommended sample weight and diluents volumes for suspension of animal feeds

Type of sample	Sample	Suspension diluent	Dilution factor
Feed additives	4 g	196 mL	1:50
Premixtures, mineral feeds	20 g	380 mL	1:20
Single and compound feeds	20 g	180 mL	1:10
High swelling feeds	20 g	380 mL	1:20
Hay, straw and silage	20 g	380 mL	1:20
Single and compound feeds	20 g	180 mL	1:10
Liquid feeds	20 mL	180 mL	1:10
Pasty or oily products	5 g	90 mL + 5 g Tween	1:20

Dilutions of 1:100 are required from the initial dilutions to enable preparation of serial dilutions. Serial dilutions should be made in a diluent buffer.

Use the following preparation to achieve 1:100 (10^{-2}) first dilution.

Dilution factor (from Table above)	Volume of initial suspension	+ volume of diluent
1:50	5 mL	5 mL
1:20	5 mL	20 mL
1:10	5 mL	45 mL

Produce further decimal dilutions based on the expected micro-organism load and sample type. Generally three dilution count plates are produced.

Recommended dilution steps for count plates

Feed type					
Pelleted and extruded feed, extraction meals and milk replacers	10^{-1}	10^{-2}	10^{-3}		
Ground feed and grain		10^{-2}	10^{-3}	10^{-4}	
Silage		10^{-2}	10^{-3}	10^{-4}	10^{-5}
Hay, straw, brewery and bakery by-products		10^{-2}	10^{-3}	10^{-4}	10^{-5}
Liquid feed		10^{-3}	10^{-4}	10^{-5}	10^{-6}

Inoculate 100 µl of the dilution onto the appropriate media (DRBC or DG18) in duplicate with a pipettor and spread over the surface with a sterile microbiological spreader. If serial dilutions have been made 100 µl of each dilution should be spread onto two suitable identified plates. Incubate at 25 ± 1 ˚C for three days with the lids uppermost. Yeast may benefit from standing in diffuse daylight for an additional 1–2 days at room temperature (18–22 ˚C).

If low populations of yeast are suspected up to 300 µl may be inoculated and spread onto suitable plates depending on the feed sample.

Alternatively grains or kernels may be applied directly to the surface of the media with sterile forceps. To quantify the growth (as a percentage) 100 grains or kernels should be placed onto the media.

Examine after incubation for characteristic yeast growth (or growth of other significant fungi) using suitable mycological atlas.

Yeasts grow on suitable agar as distinct matt or shiny domed cream to white coloured colonies with a waxy surface. Confirmation may be done by microscopic examination using either a Gram stain, where yeasts stain Gram positive as large round to oval cells (3–5 x 5–10 µm), or by preparing a drop of saline into which a portion of growth is mixed with a microbiological loop and examined under a coverslip for distinct round to oval cells (3–5 x 5–10 µm).

Select the plates with the highest number of colonies that can be counted and count these. To distinguish between yeasts and bacterial or other fungal contaminants it may be necessary to use a stereo microscope.

If added by the manufacturer probiotic bacteria and yeasts will grow from feeds (> 10^5 cfu/g) and should be differentiated from contaminant organisms. On DRBC and DG18 media *Saccharomyces cerevisiae* will grow.

REPORTING THE RESULTS

Yeasts, moulds and *Dematiaceae* may be reported as the number of colony forming units (cfu) per gram or millilitre of feed by multiplying the number of colonies by the respective dilution factor or by stating the number of grains or kernels showing viable growth.

Yeasts, mould and *Dematiaceae* are usually reported as 10^3 cfu/g or cfu/mL.

Commercial testing systems are available which can reduce the incubation time to 48–72 h by using membrane filters, or dried culture media enclosed in plastic or fabric films. If these systems are used the manufacturer's instructions should be used.

ENUMERATION OF AEROBIC/MESOPHILIC BACTERIA

The procedure noted above may also be used to enumerate aerobic/mesophilic bacteria in feed samples by substituting DRBC and DG18 with tryptose agar which includes 2,3,5-triphenyltetrazolium chloride (TTC). The incubation temperature should be changed to 30 ± 1 °C for two days.

TTC is reduced to red formazan by bacteria considered as indicator micro-organisms of spoilage in animal feed. After three days only colonies with a reduction of TTC should be counted.

Bacteria which are considered indicators of spoilage in animal feed include:

- Yellow coloured bacteria;
- *Pseudomanas* spp.*;*
- Enterobacteriaceae*;*
- *Bacillus* spp.*;*
- *Staphylococci;*
- *Micrococci;*

- *Streptomycetes.*
- Aerobic/mesophilic bacteria may be reported as the number of colony forming units (cfu) per gram or millilitre of feed by multiplying the number of colonies by the respective dilution factor.
- Bacteria are usually reported as 10^6 cfu/g or cfu/mL.

QUALITY CONTROL

Suitable reference cultures (e.g. NCTC, ATCC) should set up in parallel to act as positive control.

External proficiency schemes are available (e.g. European Feed Microbiology Organisation).

REFERENCES

ISO 7218:2007. *Microbiology of food and animal feeding stuffs – general requirements and guidance for microbiological examinations*. Geneva, Switzerland.

ISO 21527-1:2008. *Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95*. Geneva, Switzerland.

ISO 21527-2:2008. *Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 2: Colony count technique in products with water activity less than or equal to 0.95*. Geneva, Switzerland.

VDLUFA. 2012. *Methods book III 8th Supplement 2012, No 28.1.2.* Standard operating procedure to enumerate bacteria, yeasts, moulds and *Dematiaceae*. Speyer, Germany

Isolation and enumeration of *Aspergillus* spp. from animal feed samples

PRINCIPLE AND SCOPE

Fungal contamination of animal feed can occur in the field, during processing or storage of harvested products, or feed, if environmental conditions are suitable for fungal growth. This can lead to loss of nutrient value and have adverse effects on animal health and production if fed to animals or if spores are inhaled. The human population may also be affected by consumption of meat, eggs or milk from affected animals or from inhalation of spores from contaminated feed (Farmer's Lung).

One of the most frequent fungal contaminants of animal feed are *Aspergillus* spp. which may produce mycotoxins, secondary metabolites that can have an adverse effect on animal health. When consumed or inhaled mycotoxins can affect feed intake, reproduction, growth, immunological function or may be carcinogenic, teratogenic or mutagenic. Aspergilli are aerobic and widespread in almost all oxygen rich environments and are common contaminants of food and plants.

Important fungal species which produce mycotoxins include, but are not limited to, the following:

- **Aflatoxin** — *Aspergillus flavus* and *Aspergillus parasiticus*
- **Trichothecenes** — *Fusarium graminearum* and *Fusarium sporotrichioides,* among others
- **Zearalenone** — *Fusarium graminearum, Fusarium culmorum*
- **Ochratoxin** — *Aspergillus ochraceus* and *Penicillium verrucosum,* among others
- **Ergot alkaloid** — *Claviceps purpurea* and *Claviceps paspali*
- **Fumonisin** — *Fusarium verticillioïdes* (formerly *Fusarium moniliform*), among others

Aspergillus spp. may be enumerated using the previous procedure for yeasts, moulds, *Dematiaceae* and aerobic/mesophilic bacteria.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *Aspergillus* spp. The same procedure may be used for isolation of other fungal contaminants from animal feed, including *Fusarium, Penicillium* and *Cladosporium*.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA and Health and Safety requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Balance capable of weighing 1 g.
- Pipettor capable of measuring 1 mL.
- Incubator at 25 ± 1 ˚C.
- Microscope.
- Microbiological loop or spreader.
- Sterile forceps.
- Glass slides.
- Clear self adhesive tape.

REAGENTS

All microbiological media used is prepared according to the manufacturer's instructions or purchased pre poured.

- Sterile distilled or deionised water.
- Sabouraud dextrose agar (SAB).
- Czapek-dox agar (CZ).
- Potato dextrose agar (PDA).
- 2.5% sodium hypochlorite (for sample surface sterilisation).
- Lactophenol cotton blue.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure. Samples for *Aspergillus* detection should not be frozen.

Add 10 g of sample to 90 mL sterile distilled water and lightly shake. Serial dilutions may be made in further 10 mL containers of sterile distilled water up to 10^{-4} if heavy growth is suspected.

Inoculate 100 µl of the suspension to the media with a pipette and spread over the surface with a sterile microbiological spreader. If serial dilutions have been made each dilution should be spread onto a suitable identified plate. Incubate at 25 ± 1 ˚C for up to fourteen days.

Alternatively grains or kernels may be applied directly to the surface of the medium. To avoid overgrowth of bacterial contaminants grains and kernels may be surface sterilised in 2.5% sodium hypochlorite, by immersing in 2.5% sodium hypochlorite and then rinsing in sterile water, before placing on the agar surface with sterile forceps. To quantify the growth 100 grains or kernels should be inoculated.

Sabouraud dextrose agar (SAB), Czapek-dox agar (CZ) and Potato dextrose agar (PDA) are routinely used for isolation of *Aspergillus* and may be used singly or in combination. Suitable control cultures (e.g. NCTC or ATCC) should be set up to ensure the suitability of media and as a comparison for identification purposes.

When bacterial overgrowth is anticipated antibiotics may be added to media before it is poured. 50 mg/L chloramphenicol and 50 mg/L chlortetracycline are routinely used. Chloramphenicol should be prepared in 100 mL amounts and filtered for sterility prior to use. Chlortetracycline hydrochloride should be prepared immediately before use and filtered for sterility as it is unstable in solution.

Examine daily for characteristic *Aspergillus* growth (or growth of other significant fungi) using suitable mycological atlas.

Aspergillus spp. grow on suitable mycological agar with distinct velvety, powdery surface and have characteristic colours (*A. fumigatus* is dark green, *A. niger* is black, *A. flavus* is yellow-green). The fungal growth must be examined microscopically using Lactophenol cotton blue as a stain to demonstrate septate hyphae and typical structures e.g. the conidiophores (stalks on which conidia are borne).

The spores of *Aspergillus* spp. disperse in the air and Petri dishes should be handled with care to avoid development of satellite colonies, which could give an overestimation of population in the sample, or displacement of spores to the atmosphere which may be harmful to laboratory workers. To avoid cross contamination between Petri dishes self adhesive tape may be used to seal each Petri dish before incubation.

Staining using lactophenol cotton blue

- Place a drop of Lactophenol cotton blue on a slide using a pipette or microbiological loop. Using a small portion of clear adhesive tape lightly place the sticky side on a typical colony, gently touching it onto the colony surface. Place the adhesive tape onto the glass slide over the drop of Lactophenol cotton blue and allow it to attach (acting as a coverslip). Examine for distinctive branching, fern-like structures using a suitable mycological atlas.

The presumptive species identification of *Aspergillus* spp. is made on the basis of colonial pigmentation and the microscopic appearance of the distinctive fruiting heads. Cultures should be referred to a mycology reference laboratory for confirmation if required.

REPORTING THE RESULTS

Aspergillus may be reported as colony forming units per gram (CFU/g) by multiplying the number of colonies by the respective dilution factor or by stating the number of grains or kernels showing viable growth.

QUALITY CONTROL

Suitable reference cultures (e.g. NCTC, ATCC) should set up in parallel to act as positive control.

REFERENCES

ISO 21527-1:2008. *Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95*. Geneva, Switzerland.

ISO 21527-2:2008. *Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 2: Colony count technique in products with water activity less than or equal to 0.95*. Geneva, Switzerland.

VDLUFA. 2012. *Methods book III 8th Supplement 2012, No 28.1.2*. Standard operating procedure to enumerate bacteria, yeasts, moulds and *Dematiaceae*. Speyer, Germany.

Isolation and enumeration of probiotic bacteria and yeasts from animal feed samples

PRINCIPLE AND SCOPE

Bacteria and yeasts with probiotic properties may be added to animal feeds to provide beneficial properties. The feed analysis laboratory may be requested to enumerate these bacteria and yeasts to indicate the levels of the added micro-organisms in the feed.

The common probiotic bacteria and yeasts that are added to animal feeds include:

- *Bacillus cereus;*
- *Bacillus licheniformis;*
- *Bacillus subtilis;*
- *Pediococcus acidilactici;*
- *Enterococcus faecium;*
- *Lactobacillus rhamnosus;* and
- *Saccharomyces cerevisiae.*

This procedure applies to all samples of feed additives, premixtures and compound feeds submitted to the microbiology laboratory for detection of probiotic bacteria and yeasts.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA and Health and Safety requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Homogeniser or blender (blender capable of attaining 800–12 000 rpm).
- Pipette capable of measuring 5 mL, 20 mL and 45 mL.
- Pipettor capable of measuring 100–250 µl.
- Microbiological spreader.
- Incubator capable of maintaining 37 ± 1 ˚C (30 ± 1 ˚C for *S. cerevisiae*).
- pH meter.
- Light-optical microscope.

- Blender capable of speeds between 5 000–10 000 rpm.
- Stomacher and stomacher bags.

REAGENTS

- Sodium hydroxide solution (5 mol/L) pH ≥ 8.5.
- Buffer solution containing Tween® 80 (50 g Tween, 18.17 g Tris, 5 g peptone, 1 L water). Antifoaming agent (e.g. Wacker Silicon AS-EM.SE 2).
- Tryptic Soy agar (with Chloramphenicol and Polymyxin B when isolating *B. cereus*).
- Bacillus cereus selective medium (PEMBA) with Polymyxin B and egg yolk emulsion.
- Enterococci selective agar.
- ROGOSA agar (Lactobacillus selective agar).
- Malt extract agar.
- Hydrochloric acid (5 mol/L).
- Sodium phosphate buffer with peptone and Tween® 80
- Tris-Tween-Peptone solution.
- Potassium phosphate buffer (0.08 mol/L).
- Sodium phosphate buffer (pH 7.3 prior to autoclaving).
- MRS-agar.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

An initial suspension is prepared from the sample using a sodium hydroxide solution (for *B. cereus, B. licheniformis* and *B. subtilis*) or a buffer solution containing Tween 80 and peptone (for *Pedicoccus acidilactici, Enterococcus faecium, Lactobacillus rhamnosus* or *Saccharomyces cerevisiae*) which should be adjusted to pH 8.1 with hydrochloric acid before autoclaving.

Documentation accompanying samples submitted will normally state which micro-organism is to be enumerated and occasionally samples will require more than micro-organism to be enumerated from the same sample (e.g. *B. licheniformis* and *B. subtilis*)

Bacillus spp.

The following method is normally used for the enumeration of *B. cereus*, *B. licheniformis* and *B. subtilis* but it may also be used for other *Bacillus* spp. as necessary.

The initial suspension should have a pH of ≥ 8.5. This should be adjusted as necessary with sodium hydroxide solution (5 mol/L).

Use the following quantity of sample and sodium hydroxide solution for enumeration of *Bacillus* spp.

Sample type	Sample (g or mL)	Suspension diluent	Dilution factor
Feed additive	2	198	1:100
Premixture	2	198	1:100
Mineral feed	10	190	1:20
Compound feed	20	380	1:20

Treat the initial suspension for 4–5 min in a homogeniser or blender. An antifoaming agent (e.g. Wacker Silicon AS-EM.SE 2) may be added if foaming is observed during treatment.

Prepare the first dilution (1:1000 for additives and premixtures or 1:100 for mineral feeds and compound feeds) by removing 5 mL of the initial suspension with a pipette, whilst keeping it homogeneous. Add the 5 mL to 45 mL of suspension diluents to attain a 1:1000 dilution or to 20 mL to attain a 1:100 dilution as required.

Prepare further decimal dilutions in suspension diluent based on the expected number of colony forming units (cfu) of *Bacillus* spp.

Each decimal dilution (100–250 µl) should be placed onto three plates of detection medium, Tryptic Soy agar (with the addition of Chloramphenicol and Polymyxin B when isolating *B. cereus*) and spread using a sterile microbiological spreader. To confirm the identity of the Bacillus spp. it is recommended to also inoculate 100 µl onto the confirmation media (Bacillus cereus selective medium) and spread using a sterile microbiological spreader.

The plates should be incubated at 37 ± 1 °C for 24 ± 2 h. *B. cereus* may be slow growing and if required plates may be incubated for up to three days if required.

Select the plates producing 20–200 cfu and count these. Results should be reported as cfu/kg or cfu/L for compound feeds and mineral feeds and as cfu/g or cfu/mL for premixtures and additives. *B. licheniformus* and *B. subtilis* may be present together and should be reported as an approximate ratio to one another.

The characteristics of the colonies on both the detection media and confirmation media should be examined to confirm the identity of the Bacillus spp.

- *B. licheniformis* produce mucoid, gleaming colonies which resemble lichen on the detection media. On the confirmation media they produce typical colonies which are coloured turquoise to blue with precipitation of egg yolk.
- *B. subtilis* produce round colonies with either a matt, fine grained or uneven folder surface. *B subtilis* does not grow on the confirmation media.
- *B. cereus* produces grey-white round colonies. The edges are occasionally irregular and notched. On the confirmation media *B. cereus* produce typical colonies which are coloured turquoise to blue with precipitation of egg yolk.

QUALITY CONTROL

Suitable reference cultures (e.g. NCTC or ATCC) should be used to confirm the method. Participation in a suitable proficiency scheme (EQA) should be considered.

ENTEROCOCCUS FAECIUM AND *LACTOBACILLUS RHAMNOSUS*

The sample should be adjusted to pH 7.3 ± 0.1 using sodium hydroxide or hydrochloric acid.

Copper ions have a toxic effect on *E. faecium* and they should be chelated without delay. Copper concentrations of ≥ 20 mg/L in the initial suspension are considered critical and the declaration of copper content, which accompanies the sample, should be examined. If the copper content is not known it should be checked. If the copper content is considered critical 0.35 g iminodiacetic acid and 530 µl sodium hydroxide solution can be added to a 20 g sample to chelate the copper. If the copper content exceeds 8 300 mg/kg a higher quantity will be required.

Use the following volumes of sample and diluents to prepare an initial suspension in sodium phosphate buffer with peptone and Tween® 80 (or Tris-Tween-Peptone solution for samples encapsulated with fats). If required add silicone antifoaming agent.

Sample	Critical Cu content mg/kg	Weight of sample g or mL	Diluent mL	Initial suspension dilution factor
Additive	N/A	5	495	1:100
Premixture	>400	20	380	1:20
Compound feed and mineral feed	>400	20	380	1:20
Milk replacer (powder/granules)	N/A	20	380	1:20
Milk replacer (micro encapsulated)	N/A	100	900	1:10
Compound feed (pellets)	>200	100	900	1:10
Pastes/oily products	>400	5	95	1:20

Weight the required volume of sample into an appropriate container and add the required volume of sodium phosphate buffer with peptone or Tris-Tween-peptone solution as appropriate. Blend the mixture at approximately 5 000 rpm for one minute and then increase the speed to 8 000–10 000 rpm for approximately four min.

For pasty or oily products treat the prepared sample in a stomacher for five min rather than using a blender.

Prepare the first dilution (1:100) by removing 5 mL of the initial suspension with a pipette, whilst keeping it homogeneous. For initial suspensions of 1:20 add the 5 mL to 20 mL of sodium phosphate buffer diluent to attain a 1:100 dilution. For those with an initial suspension of 1:10 add the 5 mL to 45 mL of sodium phosphate buffer diluents to attain a 1:100 dilution.

Prepare further decimal dilutions in suspension diluent based on the expected number of colony forming units (cfu) of *E. faecium* or *L. rhamnosus*.

Pipette 100–1000 µl of each dilution (with the aim of achieving 20–200 cfu per plate) into three Petri dishes and add 10 mL of detection media at approximately 50 °C. Mix and allow to solidify before overlaying with approximately 8 mL of the same detection media at approximately 50 °C.

- For *E. faecium* use Enterococci selective agar as a detection medium.
- For *L. rhamnosus* use ROGOSA (Lactobacillus selective agar) as a detection medium.

Incubate the plates at 37 ± 1 °C for at least two days (Enterococci selective agar) or at least four days (ROGOSA agar).

- On Enterococci selective agar *E. faecium* grow as small (0.5–1.5 mm) dark red colonies.
- On ROGOSA agar *L. rhamnosus* grow as larger (2.5–3.5 mm) colonies.

Select the plates producing 20–200 cfu and count these. Results should be reported as cfu/kg or cfu/L for compound feeds and mineral feeds and as cfu/g or cfu/mL for premixtures and additives. If both *E. faecium* and *L. rhamnosus* enumeration are requested they may also be reported as an approximate ratio to each other.

Confirmation of the micro-organism can be made by examining the cell form under a light-optical microscope:

- *Enterococci* produce cocci in pairs and short chains; and
- *Lactobacilli* produce rod-shaped bacilli in chains.

QUALITY CONTROL

Suitable reference cultures (e.g. NCTC or ATCC) should be used to confirm the method. Participation in a suitable proficiency scheme (EQA) should be considered.

It should be noted that Enterococcus selective agar will grow all Lancefield group-D Streptococci (including *E. faecalis*) although if present these should be in very low numbers (10^6–10^7 cfu/kg) compared to added *E. faecium* (> 0.2 x 10^9 cfu/kg).

PEDICOCCUS ACIDILACTICI

For detection of *Pedicoccus acidilactici* in animal feeds the same procedure as for *E. facium* and *L. rhamnosus* may be followed but with the replacement of potassium phosphate buffer (0.08 mol/L) as a diluent for serial dilutions. Also the initial sample should have the pH adjusted to 8.1 ± 0.1 before preparation.

Use the following volumes of sample and diluents to prepare an initial suspension in Tris-Tween-Peptone solution. If required add silicone antifoaming agent.

Sample	Critical Cu content mg/kg	Weight of sample g or mL	Diluent mL	Initial suspension dilution factor
Additive	N/A	5	495	1:100
Premixture	>2000	5	495	1:100
Compound feed	>200	40	360	1:10
Milk replacer	N/A	40	360	1:10
Mineral feeds	>400	40	760	1:20

ROGOSA agar may be used as a detection media for *P. acidilactici*, which grow as distinct white colonies (approximately 2 mm diameter) after at least 3 days at 37 ± 1 °C.

Under a light-optical microscope *P. acidilactiici* produce distinctive cocci in pairs and tetrads.

Select the plates producing 20–200 cfu and count these. Results should be reported as cfu/kg or cfu/L for compound feeds and mineral feeds and as cfu/g or cfu/mL for premixtures and additives.

SACCHAROMYCES CEREVISCIAE

For detection of *Saccharomyces cerevisciae* in animal feeds the same procedure as for *E. facium* and *L. rhamnosus* may be followed but with the replacement of potassium phosphate buffer (0.08 mol/L) as a diluent for serial dilutions. Also the initial sample should have the pH adjusted to 8.1 ± 0.1 before preparation.

Use the following volumes of sample and diluents to prepare an initial suspension in Tris-Tween-Peptone solution. If required add silicone antifoaming agent.

Sample	Critical Cu content mg/kg	Weight of sample g or mL	Diluent mL	Initial suspension dilution factor
Additive	N/A	5	495	1:100
Premixture	>2000	5	495	1:100
Compound feed	>200	40	360	1:10
Milk replacer	N/A	40	360	1:10
Mineral feeds	>400	40	760	1:20

Malt extract agar should be used as a detection media for *S. cerevisciae*. Plates should be incubated for at least four days at 30 ± 1 ˚C.

Under a light-optical microscope *Saccharomyces cerevisiae* produce distinctive yeast colonies.

Select the plates producing 20–200 cfu and count these. Results should be reported as cfu/kg or cfu/L for compound feeds and mineral feeds and as cfu/g or cfu/mL for premixtures and additives.

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VDLUFA. 2007. *Methods Book III 7th supplement 2007, No 28.2.1. Enumeration of* Bacillus cereus. Speyer, Germany.

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VDLUFA. 2012. *Methods Book III 8th supplement 2012, No 28.2.3. Enumeration of* Enterococcus faecium. Speyer, Germany.

VDLUFA. 2012. *Methods Book III 8th supplement 2012, No 28.2.4. Enumeration of* Enterococcus faecium *and* Lactobacillus rhamnosus. Speyer, Germany.

VDLUFA. 2012. *Methods Book III 8th supplement 2012, No 28.2.5. Enumeration of* Pedicoccus acidilactici. Speyer, Germany.

VDLUFA. 2012. *Methods Book III 8th supplement 2012, No 28.2.6. Enumeration of* Saccharomyces cerevisiae. Speyer, Germany.

Isolation and enumeration of sulphite reducing (SR) *Clostridia* spp. from animal feed samples

PRINCIPLE AND SCOPE

Clostridia spp. are large (3–8 µm x 0.5 µm) anaerobic, Gram positive spore bearing bacilli of which there are over one hundred species. Most *Clostridia* spp. are regarded as saprophytes, normally found in soil, water and decomposing organic material. Most *Clostridia* spp. are motile although *Cl. Perfringens* (a common cause of contamination in food and feed stuffs) is not. *Cl. Perfringens* can also grow in the presence of limited amounts of oxygen.

Some species of *Clostridium* are commensals found in the intestine of animals (including humans) and play a significant role in post-mortem decomposition. A few species produce powerful exotoxins which can produce disease (e.g. *Cl. perfringens, Cl. tetani* and *Cl. botulinum*).

A significant characteristic of *Clostridium* spp. is that they produce resistant endospores which are able to survive for long periods in adverse conditions, including heat, cold and water chlorination.

This procedure applies to all samples submitted to the microbiology laboratory for detection of Sulphite Reducing (SR) *Clostridia* spp.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA and Health and Safety requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Incubator at 80 ± 1 ˚C and 37 ± 1 ˚C.
- Stomacher or similar.
- Waterbath capable of maintaining 50 ± 2 ˚C.
- Pipettor capable of measuring 100 µl.

- Anaerobic box, jar or pouch with appropriate anaerobic gas generation system and indicator (or Anaerobic incubation cabinet if available).
- Microbiological loop and straight wire.
- Fridge at 2–8 ˚C.

REAGENTS

All microbiological media used is prepared according to the manufacturer's instructions or purchased pre poured.

- Peptone buffer.
- Tryptose Sulphite Cycloserine Agar (TSCA).
- Differential Clostridial Agar (DCA) for dried feeds.
- Blood agar.
- Nitrate Motility Medium (NMM).
- Lactose Gelatin Medium (LGM).
- Sulfanilic acid.
- N,N-dimethyl-1-naphthylamine.
- Zinc powder.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

- Weigh 10 g of sample into a stomacher bag and add 90 mL of peptone buffer. Homogenise for 30 seconds.
- Add the homogenised solution to a sterile bottle or jar and incubate at 80 ± 1 ˚C for 30 min. This will kill vegetative cells of *Clostridia* spp. and any contaminating bacteria present.
- After 30 min allow the bottles to cool to approximately 50 ˚C in a waterbath and add 100 µl to sterile Petri dishes. Prepare further decimal dilutions in peptone buffer based on the expected number of colony forming units (cfu) of *Clostridia* spp.
- Add 15–25 mL of TSCA agar media, cooled to approximately 50 ˚C, to the Petri dishes and mix gently. Allow the media to solidify and incubate at 37 ± 1 ˚C for 24 ± 4 h in anaerobic conditions. An appropriate anaerobic indicator should be used in an anaerobic box, jar or pouch with an appropriate anaerobic gas generating system. Alternatively an anaerobic incubation cabinet may be used with suitable anaerobic monitoring.

After incubation *Clostridia* spp. will grow as typical black colonies on TSCA due to the reduction of sulphite. These colonies should be counted to enumerate the *Clostridia* spp. in the sample. A selection of sulphite reducing colonies should be sub cultured onto suitable medium (e.g. blood agar) for purity check and to enable confirmation tests.

Confirmation tests for sulphite reducing colonies

A Gram stain may be performed. *Clostridia* spp. are large (3–8 x 0.5 µm) Gram positive bacilli, endospores may be visible which are usually 'bulging', i.e. wider than the bacterial body. Some older cultures may stain irregularly or appear Gram negative.

Motility and nitrate testing may be done using Nitrate Motility Medium (NMM). This should be inoculated using a straight wire with half a colony of suspected *Clostridia* spp. and incubated anaerobically at 37 ± 1 ˚C for 24 ± 4 h.

If growth is observed away from the stab line and throughout the NMM (producing a cloudy appearance throughout) the culture may be considered motile. If growth is restricted to the length of the stab line the culture may be considered non-motile.

Add 10–15 drops of sulfanilic acid and N,N-dimethyl-1-naphthylamine and observe for a red colour change within five min. A red color change at this step indicates a positive nitrate reaction.

If there is no colour change it is possible that nitrate has been further reduced to ammonia or nitrogen gas so a further test is performed to detect unreduced nitrate.

Zinc can reduce nitrate to nitrite and therefore detect unreduced nitrate.

Add zinc powder to the NMM media and observe for a colour change to red (as sulfanilic acid and N,N-dimethyl-1-naphthylamine are already present). If the media turns red after the addition of zinc the result is negative, as the nitrate was unreduced before the addition of the zinc powder.

If the NMM media does not change colour there is no nitrate present, as it has been reduced to nitrite and then further ammonia or nitrogen gas and is recorded as a positive reaction.

Lactose Gelatin Medium (LGM) may be inoculated to detect liquefaction of gelatin and lactose fermentation. This should be inoculated using a straight wire with half a colony of suspected *Clostridia* spp. and incubated anaerobically at 37 ± 1 ˚C for 24 ± 4 h.

Presence of gas and a yellow colour after incubation indicates lactose fermentation.

To check for gelatin liquefaction store the LGM tube at 2–8 ˚C for one hour and observed for solidification. If the tubes have solidified they should be further incubated at 37 ± 1 ˚C for 24 ± 4 h and again checked for liquefaction after one hour at 2–8 ˚C.

An alternative medium that may be used for dried feed is Differential Clostridial Agar (DCA). If this medium is used the sample should be heated to 30 ˚C prior to inoculation to facilitate spore germination.

REPORTING OF RESULTS

- Report results as the number of confirmed sulphite reducing clostridia colonies per 100 g.
- Select the plates producing 20–200 cfu and count these.

QUALITY CONTROL

Suitable reference cultures (e.g. NCTC or ATCC) should be used to confirm the method. Participation in a suitable proficiency scheme (EQA) should be considered.

Anaerobic indicators should be used to determine the efficiency of the anaerobic condition generated during the procedure.

REFERENCES

ISO 6461-1:1986. *Detection and enumeration of the spores of sulphite-reducing anaerobes (Clostridia) – part 1. Method by enrichment in a liquid medium*. Geneva, Switzerland.

Detection of *Toxoplasma gondii* in animal feed samples

PRINCIPLE AND SCOPE

Toxoplasma gondii is an intracellular parasitic protozoon which causes one of the most common parasitic infections amongst warm blooded animals, including man. *Toxoplasma gondii* produces cysts which may lie dormant in the host for many years. Infection with *Toxoplasma gondii* may result in abortion (particularly in sheep and goats) or blindness.

The protozoon is parasitic in all warm blooded animals but felines are the definitive host and in the cat family oocysts are excreted. Contamination of soil may occur and oocysts, which are extremely resistant to the environment, may be present in vegetable matter contaminated with soil, contaminated water, or directly with definitive host faeces.

Pigs have been demonstrated to be a major source of Toxoplasma but indoor housing, where this is practiced, has reduced the incidence of infection. In countries where pigs are housed outside (and in countries where outdoor housing is seen as more animal friendly and producers move away from indoor housed systems) there may be a higher incidence of infection in animals.

Free ranging poultry, especially chickens, may be found to be infected with *Toxoplasma gondii* if the soil is contaminated with oocysts.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *Toxoplasma gondii.*

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

HEALTH AND SAFETY

Toxoplasma gondii oocysts are infectious to humans by ingestion and appropriate precautions should be taken when handling suspected material. *Toxoplasma gondii* may cause abortion in humans and laboratory workers who may be pregnant should be excluded from working with material which may be infected as should any immunocompromised workers.

EQUIPMENT

- Homogeniser or blender.
- Mesh sieves with successive decreasing sizes.
- Pipette.
- Light microscope (with optional ocular micrometer if required and with ultraviolet beam for detection of autofluorescence, if required).
- Centrifuge (capable of centrifuging a sample at 4500 x *g*).
- Centrifuge tubes.
- Glass slide and coverslip.

REAGENTS

- Sterile water.
- Saturated sucrose solution (≤1.15 specific gravity).

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure. Freezing kills *Toxoplasma* oocysts and therefore samples must not be frozen.

At least 100 g of test matrix should be tested. The sample should be homogenised in sterile water using a blender or similar. Depending on the sample matrix there will be a large amount of debris and a pre filtration stage using mesh sieves of successive decreasing sizes can be used to remove the bulk of large debris.

Once passed through the successive sieves the water should be recovered and centrifuged at 500 x *g* for 3 min and the supernatant carefully removed with a pipette and discarded, without disturbing the deposit.

The deposit is suspended in a saturated solution of sucrose (≤1.15 specific gravity) and centrifuged at 4500 x *g* for 15 min.

Additional saturated sucrose solution should be added to the centrifuge tube to fill it to the top, producing a positive meniscus. A glass coverslip is placed on the top of the tube, on the meniscus and left to stand for at least 5 min.

The coverslip should be removed and examined microscopically for *Toxoplasma gondii* oocysts using an appropriate parasitological atlas to aid diagnosis. An ocular micrometer can be used to provide accurate measurement of oocysts, which are 10–13 µm in diameter.

Examination under an ultraviolet beam (excitation filter: 330–385 nm, dichroic mirror: 400 nm, barrier filter: 420 nm) facilitates detection because both unsporulated and sporulated oocysts may exhibit a typical blue autofluorescence. It should be noted however that all oocysts in the same suspension may not exhibit autofluorescence which can lead to false negatives when oocysts are in low concentration.

It should be noted that *Toxoplasma gondii* oocysts are morphologically similar to those of other coccidia *Neospora* spp. and *Hammondia* spp.

Suspect oocysts may be carefully washed into a tube with a pipette and stored at low temperature (i.e. -80 °C) for further identification by PCR if required. Alternatively bioassay in mice, previously confirmed as seronegative for *Toxoplasma gondii*, may be performed using inoculation by either intraperitoneal or subcutaneous route. Blood is collected six weeks after inoculation for serological analysis and confirmed by seroconversion.

PCR techniques may be available and should follow a recommended protocol.

REFERENCES

Dumètre, A. & Dardé, M.-L. 2003. How to detect *Toxoplasma gondii* oocysts in environmental samples. *FEMS Microbiology Reviews* 27(5): 651–661.

Detection of *Echinococcus* spp. in animal feed samples

PRINCIPLE AND SCOPE

There are ten species of *Echinococcus* which are regarded as taxonomically valid, *E. granulosus*, *E. multilocularis, E. oligarthus, E. vogeli, E. canadensis, E. equinus, E. felidis, E. intermedius, E. ortleppi* and *E. shiquicus,* although *E. granulosa* and *E. multilocularis* occur most frequently and *E. granulosus* is associated with ungulate farm animals.

Echinococci are tapeworm species of dogs, cats and certain wild carnivores (e.g. foxes, coyotes). The definitive host animals shed eggs of *Echinococci* in their faeces which can survive for several months in the environment and can contaminate pasture used by intermediate ungulate hosts (sheep, goats, cattle etc.) or used to produce animal feed. Infection can lead to hydatid disease, also known as echinococcosis and significant economic losses in livestock.

Ingestion of *Echinococcus* eggs can produce large hydatid cysts within the internal organs of the infected (intermediate) host. Cysts can become so large over a number of years that they may contain several litres of fluid. Humans may be an intermediate host.

This procedure applies to all samples submitted to the microbiology laboratory for detection of *Echinococcus.*

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

HEALTH AND SAFETY

Echinococcus spp. is highly pathogenic to humans by ingestion and extreme care should be taken when handling suspected material within the laboratory.

EQUIPMENT

- Balance capable of weighing 5 g.
- 50 mL conical tube.
- Course sieve of up to 4 mm if required.

- Centrifuge capable of centrifuging a sample at 4500 x *g*.
- High speed vortex.
- Pipette.
- Glass slide and cover slip.
- Microscope (with optional ocular micrometre).

REAGENTS

- 5% potassium hydroxide (KOH).
- Saturated solution of sodium nitrate ($NaNO_3$) (1.35 specific gravity).

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

The procedure described may be used to detect *Echinococcus* eggs in soil and environmental samples; it may also be used for soil contaminated material including grass and cereals used for animal feed.

A 5 g sample of the material to be investigated is placed in a 50 mL conical tube and 5% KOH is added to a ratio of 1:2 and mixed using a high speed vortex. If the sample is heavily contaminated with soil or grit it can be first passed through a course sieve.

The vortexed fluid should be centrifuged at 500 x *g* for 3 min and the supernatant carefully removed with a pipette and discarded without disturbing the deposit.

The deposit should be resuspended in 5% KOH and again centrifuged at 500 x *g* for 3 min. The supernatant should be carefully removed with a pipette and discarded without disturbing the deposit.

The deposit is suspended in a saturated solution of $NaNO_3$ (1.35 specific gravity) and centrifuged at 4500 x *g* for 15 min.

Additional saturated solution of $NaNO_3$ should be added to the centrifuge tube to fill it to the top, producing a positive meniscus. A glass coverslip is placed on the top of the tube, on the meniscus and left to stand for at least 5 min.

The coverslip should be removed and examined microscopically for *Echinococcus* eggs using an appropriate parasitological atlas to aid diagnosis. An ocular micrometer can be used to provide accurate measurement of eggs. It should be noted that *Echinococcus* eggs are morphologically indistinguishable to those of other taeniid Cestoda.

Suspect eggs may be carefully washed into a tube with a pipette and stored in a low temperature freezer (e.g. -80 °C) for further identification or submission to a reference laboratory if required.

QUALITY CONTROL

Reference laboratories (e.g. Cestode Diagnostics, University of Salford, UK) may be able to assist in further identification and supply of control material.

In recent years several PCR protocols have been published on the identification of *Echinococcus granulosus* DNA from eggs or from adult parasites and new ways of diagnosing this tapeworm have been developed which can be utilised to detect *Echinococcus granulosus* eggs in environmental samples.

Eggs should firstly be recovered using the technique described above.

PCR techniques, if available, should follow a recommended protocol.

REFERENCES

Shaikenov, B.S., Rysmukhambetova, A.T., Massenov, B., Deplazes, P., Mathis, A. & Torgerson, P.R. 2004 Short Report: The use of a polymerase chain reaction to detect *Echinococcus granulosus* (G 1 strain) eggs in soil samples. Institute of parasitology, University of Zürich, Zürich, Switzerland. Institute of Zoology, Kazakh Academy of Sciences, Academogorodok, Almaty, Kazakhstan. *American Journal of Tropical Medicine and Hygiene* 7: 441–443.

Detection of *Trichinella* spp. in animal feed samples

PRINCIPLE AND SCOPE

Trichinella is a genus of parasitic nematode worms that can cause a potentially serious infection (trichinellosis or trichinosis) in animals, including humans, following consumption of infected meat. Up to ten genotypes have been described but the species of most concern to the food industry is *Trichinella spiralis*.

Trichinella species are found world wide and infect a wide variety of animal hosts, mostly carnivorous and omnivorous wild mammals, especially those that scavenge, such as foxes, bears, pigs and wild boar. Rodents are also thought to play an important role as hosts in areas where the infection is endemic. The entire life cycle normally occurs within a single host species and consists of an adult worm and two larval stages.

Pigs have frequently been a source of infection to humans from the consumption of undercooked pork containing *Trichinella* larvae. A principle source of infection to pigs has been identified as pig feed contaminated with animal waste.

This procedure applies to all feed samples submitted to the microbiology laboratory for detection of *Trichinella*.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.
- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Sealable sterile bags.
- Calibrated balance.
- Thermometer.
- Stomacher, blender or similar.
- Magnetic stirrer (with thermostatically controlled heating plate) and stirring rod.
- 3 L beaker.
- Aluminium foil.
- 11 cm sieve (180 microns).

- Separation funnel (larger than the sieve) with tap and associated stand.
- 100 mL measuring cylinder.
- Pipette.
- Plastic Petri dish.
- Stereo microscope.

REAGENTS

- Distilled or deionised water.
- 25% hydrochloride acid.
- Pepsin – strength 1:12 500 BP (British Pharmacopoeia) corresponding to 1:10 000 NF (US National Formulary) and to 2 000 FIP (Fédération Internationale de Pharmacie).
- 90% ethyl alcohol.
- Positive control material.

PROCEDURE

Sample management

Testing shall begin on day of receipt of samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 ˚C until required or in conditions which will preserve its integrity. If refrigeration is necessary, samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

Animal feed (100 g) is homogenised in a blender or stomacher in a small amount of warmed distilled or deionised water.

If raw tissue is to be tested this should be striated muscle and trimmed of fat. Raw tissue should be homogenised in a blender or stomacher with a small amount of sterile water. If an electric blender is used the blender must be operated three to four times for approximately one second each time. If a stomacher is used the tissue or feed should be pounded for up to 25 min.

Transfer the homogenised/blended meat or feed to a 3 L beaker and make up the volume of water to 2 L with sterile water preheated to 46–48 °C. Add 16 ± 0.5 mL 25% hydrochloric acid (HCl) and 10 ± 0.2 g pepsin.

Add the stirring rod and cover the beaker with aluminium foil.

Place the beaker containing the preparation on the stirring plate with the temperature set at 44–46 °C and the stirrer set at a rate which creates a deep whirl without causing any splashing.

The meat or feed should be on the heated stirring plate until the solid particles dissolve (approximately 30 min) but should not exceed 60 min.

The digest is poured through the 180 micron sieve into the separation funnel above the measuring cylinder and allowed to stand for approximately 30 min.

Remove 40 mL of liquid from the funnel into the measuring cylinder and stand for 10 min.

Carefully remove 30 mL of the supernatant with a pipette leaving not more than 10 mL and pour into a plastic Petri dish. Add 10 mL sterile water to the measuring cylinder, rinse out and add this to the Petri dish also.

Examine under a stereo microscope for *Trichinella* larvae at 15–20 x magnification. Increase magnification to 60–100 x for parasite like shapes and confirm using a veterinary, medical or microbiological atlas.

The digests should be examined immediately and not stored for later examination.

Suspect larvae may be kept in 90% ethyl alcohol for preservation and confirmation at a reference laboratory if required.

QUALITY CONTROL

Reference laboratories (e.g. Nation Reference Laboratory for *Trichinella*, AHVLA, Weybridge, UK) may be able to assist in further identification.

A *Trichinella* EQA scheme is run by VetQAS (AHVLA, Sutton Bonington, UK).

Suitable positive control tissue should be used to provide validation of both the procedure and identification of *Trichinella* by Laboratory Analysts.

REFERENCE

EN SANCO/2537/2005. *Laying down specific rules on official controls for* Trichinella *in meat*. August 2005 EU. Brussels, Belgium.

Detection of Processed Animal Protein (PAPs) in animal feed samples

PRINCIPLE AND SCOPE

Prions are small proteinaceous infectious particles (infectious agents composed of protein in a misfolded form) which cause a number of progressively degenerative neurological diseases known as Transmissible Spongiform Encephalopathy. Several specific types are found in different groups of animals, e.g. Bovine spongiform encephalopathy (BSE) in bovines, Scrapie in ovines and caprines, variant Creutzfeld Jacob disease (vCJD) in humans, Feline Spongiform Encephalopathy (FSE) in felines, and chronic wasting disease in mule, elk and deer. The incubation period for such diseases can be up to fifteen years or more.

Prions are highly resistant to heat, formalin, phenol and chloroform and contain little or no nucleic acid. Autoclaving at 136 ˚C for four min or 160 ˚C for 24 h (using dry heat) is sufficient to destroy them.

The presence of prions in farmed animals poses a risk in the animal food production chain of the disease being passed onto other farmed animals, if animal feed contains diseased animal parts, or to humans when consuming meat. The general opinion is that BSE in cattle is caused by the presence of animal proteins containing prions in the feeds.

In Europe and many other countries, Processed Animal Protein (with the exception of fish meal in pig and poultry feed) is banned from use in farmed animal feed ingredients.

Detection of Processed Animal Proteins (PAPs) in animal feed is regarded as an indication that Prions may be present in animal feed. This includes the detection of constituents of animal origin in animal feed and the detection of proteins from terrestrial animals in fish meal. Classical optical microscopy is regarded as the reference method although other methods which may be regarded as alternative or complementary include PCR, Near Infra Red Microscopy and Imaging and immunological techniques. Classical optical microscopy can, depending on the constituents of the animal feed, detect PAPs in very small amounts (<0.1%).

This procedure applies to all feed samples submitted to the microbiology laboratory for detection of PAPs.

RESPONSIBILITIES

- **Laboratory Analyst** – To ensure all samples submitted for testing are treated as stated in this SOP and that all QA requirements are adhered to and sample integrity preserved. All Laboratory Analysts must be trained and competent in the procedure being followed and have this documented in their training file.

- **Laboratory Manager/Director** – To ensure all staff have the appropriate training and competence to complete this procedure and that ongoing competence is maintained by participation in appropriate proficiency testing.
- **Quality Assurance Manager** – To ensure an appropriate SOP is available and is adhered to by way of periodic auditing of this procedure in the laboratory.

EQUIPMENT

- Analytical balance (accurate to 0.001 g).
- Material for grinding (grinding mill or a mortar, especially for feed containing > 15% fat on analysis).
- Sieve (0.5 mm maximum).
- Separation funnel or conical bottomed settling beaker.
- Stereo microscope (minimum X40 magnification).
- Compound microscope (minimum X400 magnification), transmitted light or polarised light.
- Standard laboratory glassware.
- Centrifuge.
- Pipette.
- Centrifuge tube.
- Waterbath or microwave oven (to melt fat samples).

All equipment shall be thoroughly cleaned before use. Separation funnels and glassware should be washed in a laboratory glass washing machine.

Sieves should be cleaned using a stiff brush.

REAGENTS

- Chloral hydrate (aqueous, 60% w/v).
- Lye (NaOH 2.5% w/v or KOH 2.5% w/v) for sieve fractions.
- Paraffin oil or glycerol (viscosity: 68–81) for microscopic observations in the sediment.
- Ethanol (96%).
- Acetone.
- Tetrachloroethylene (density 1.62).
- Iodine/potassium iodide solution.
- Alizarin red.
- Cystine reagent.
- Sodium hypochlorite solution.

PROCEDURE

Sample management

Testing shall begin on the day of receipt of the samples or on the first working day which allows the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored at 2–8 °C until required or in conditions which will preserve its integrity. If refrigeration is necessary, the samples should be removed from the refrigerator and stored at room temperature for a minimum of one hour prior to the start of the procedure.

Sampling

A representative sample should be obtained which has undergone suitable preparation.

The sample processed may be an incremental sample (taken from one point in the product), an aggregate (of incremental samples) or a reduced sample (a representative part of an aggregate sample obtained by reduction). Reduced and aggregate samples are homogenised to produce the final sample for processing. This should be 500 g or 500 mL.

The following guidelines should be used for obtaining final samples for processing.

Loose feed	**Minimum number of incremental samples**
< 2.5 tonnes	7
> 2.5 tonnes	√ 20 x number of tonnes (maximum of 40)*
Packaged feed (not more than 1 kg)	
1–4 packages	All
5–16 packages	4
> 16 packages	√ number of packages (maximum of 20)
Packages ≤ 1 kg	4
Liquid/semi liquid feed (containers of > 1 L)	
1–4 containers	All
5–16 containers	4
> 16 containers	√ number of containers (maximum of 20)*
Containers ≤ 1 L	4
Feed blocks and licks	1 in every 25 units

* the number obtained should be rounded up if a fraction is obtained.

For packages or containers which are less than 1 kg or 1 L and for blocks and licks which weigh less than 1 kg each an incremental sample shall be the contents of one original package, container or lickblock.

Aggregate samples should not exceed 4 kg, 4 L or the weight of four licks or blocks. If submitted samples do not exceed 1 kg or 1 L the aggregate sample should be the weight or volume of four original packages/containers. A final sample for processing should be taken from the aggregate samples and should be 500 g or 500 mL.

For large samples the following guidelines should be used:

Loose feed	**Minimum number of aggregate samples per submission**
≤ 1 tonne	1
1–10 tonnes	2
10–40 tonnes	3
> 40 tonnes	4
Packaged feed	
1–16 packages	1
17–200 packages	2
201–800 packages	3
> 800 packages	4

The following protocol is fit for handling feed with low moisture content. Feed with a moisture content higher than 14% should be dried (condensed) prior to handling.

Special feed or feed materials (e.g. fats, oils) need dedicated treatment (see additional information at end of 'Procedures').

The constituents of animal origin are identified on the basis of typical, microscopically identifiable characteristics (i.e. muscle fibres and other meat particles, cartilage, bones, horn, hair, bristles, blood, feathers, egg shells, fish bones, scales). The identification has to be done both on the sieve fraction and the concentrated sediment of the sample.

Pelleted feeds may be pre-sieved if both fractions are analysed as a separate sample.

At least 50 g of the sample shall be treated (ground with care using suitable grinding equipment if necessary in order to achieve an appropriate fine structure). From the ground material two representative portions are taken, one for the sieve fraction and one for the concentrated sediment (both at least 5 g).

Colouring with staining reagents can additionally be applied to aid identification.

In order to indicate the nature of the animal proteins and the origin of the particles, a decision support system such as ARIES can be used and reference samples can be documented.

Identification of constituents of animal origin in the sieve fractions

At least 5 g of the sample is sieved through the 0.5 mm sieve in two fractions.

The sieve fraction(s) with the large particles (or a representative part of the fraction) is applied as a thin layer to a suitable support and screened systematically under the stereomicroscope at various magnifications for constituents of animal origin.

Slides made with the sieve fraction(s) with the fine particles are screened systematically under the compound microscope at various magnifications for constituents of animal origin.

Identification of constituents of animal origin from concentrated sediment

The sample (5 ± 0.1 g) shall be transferred into a separation funnel or conical bottomed settling beaker. Tetrachloroethylene (50 mL) is added and the mixture is shaken or stirred repeatedly.

If a closed separation funnel is used the mixture is left to stand for at least 3 min before the sediment is separated off. Shaking is repeated and the sediment is left to stand again for at least 3 min. The sediment is separated off again.

If an open beaker is used, the sediment is left to stand for at least 5 min before the sediment is separated off.

The total sediment shall be dried and subsequently weighed (accurate to 0.001 g). The weighing is only necessary in case estimation is required. If the dried sediment consists of many large particles it may be sieved through a 0.5 mm sieve in two fractions. The dried sediment shall be examined for bone constituents under a stereo microscope and a compound microscope as described previously.

Differentiation and clarity can be improved and the identification of the constituents of animal origin can be supported by using various embedding and staining reagents.

Chloral hydrate

By carefully heating, cell structures can be seen more clearly because starch grains gelatinise and unwanted cell contents are removed.

Lye (either sodium hydroxide or potassium hydroxide)
Clears the material of the feed, assisting in the detection of muscle fibres, hairs and other keratin structures.

Paraffin oil and glycerol
Bone constituents can be well identified in this embedding agent because most lacunae remain filled with air and appear as black holes about 5–15 µm .

Iodine/potassium Iodide solution
(Dissolve 2 g potassium iodide in 100 mL water and add 1 g iodine while frequently shaking).

This used for the detection of starch (blue-violet colour) and protein (yellow-orange colour). Solutions may be diluted if required.

Alizarin red solution
(Dilute 2.5 mL 1M hydrochloric acid in 100 mL water and add 200 mg alizarine red to this solution).

Red/pink colouring of bones, fish-bones and scales. Before drying the sediment the total sediment shall be transferred into a glass test tube and rinsed twice with approximately 5 mL alcohol (add 5 mL alcohol, vortex, allow to settle for 1 minute and remove the supernatant).

Before using this staining reagent, the sediment should be bleached by adding at least 1 mL sodium hypochlorite solution and leaving for 10 min after which the tube is filled with water and the sediment allowed to settle. After 2–3 min the water and the suspended particles are be poured off. The sediment is rinsed twice more with approximately 10 mL of water (add 10 mL water, vortex, allow to settle for 1 minute and remove the supernatant).

Up to 10 drops (or more, depending on the amount of residue) of the alizarine red solution is added and the mixture is shaken. The coloured sediment should be rinsed twice with approximately 5 mL alcohol followed by one rinse with acetone. For each rinse the solution should be placed on the vortex, allowed to settle about one minute and poured off.

The sediment is now ready to be dried.

Cystin reagent
(2 g lead acetate, 10 g NaOH/100 mL H_2O).

By carefully heating, cystin-containing constituents (hair, feathers, etc.) become black-brown.

Examination in feed possibly containing fishmeal

At least one slide shall be examined from the fine sieve fraction and from the fine fraction of the sediment under the compound microscope.

Where the label indicates that the ingredients include fishmeal, or if the presence of fishmeal is suspected or detected in the initial examination, at least two additional slides of the fine sieve fraction from the original sample and the total sediment fraction shall be examined.

The following protocol may be used for the analysis of fat or oil:

- If the fat is solid, it is warmed until it is liquid. This may be done in a water bath or by using a microwave oven.
- Using a pipette, 40 mL of fat is transferred from the bottom of the sample to a centrifugation tube.
- Centrifuge for 10 min at 2700 x *g*.
- If the fat is solid after centrifugation warm it as before until it is liquid.
- Centrifuge for 5 min at 2700 x *g*.
- By using a small spoon or a spatula one half of the decanted impurities are transferred to a small Petri dish or a microscopic slide for microscopic identification of a possible content of animal constituents (meat fibres, feathers, bone fragments etc.).
- As an embedding agent for microscopy, paraffin oil or glycerol is recommended.
- The remaining impurities are used for sedimentation as described above.

CALCULATION AND EVALUATION

The calculation can only be made if the constituents of animal origin contain bone fragments.

Bone fragments of terrestrial warm-blooded species (i.e. mammals and birds) can be distinguished from the different types of fish bone on the microscopic slide by means of the typical lacunae.

The proportion of constituents of animal origin in the sample material is estimated taking into consideration:

The estimated proportion (weight %) of bone fragments in the concentrated sediment and the proportion (weight %) of bone in the constituents of animal origin.

The estimate has to be based on at least three (if possible) slides and at least five fields per slide. In compound feed the concentrated sediment generally contains not only terrestrial animal bone and fish bone fragments, but also other particles of high specific weight, e.g. minerals, sand, lignified plant fragments etc.

Estimated value of the percentage of bone fragments:

- % terrestrial bone fragments = (S × c) / W
- % fish bone and scale fragments = (S × d) / W

Where

- S = sediment weight (mg);
- c = correction factor (%) for the estimated portion of terrestrial animal bones in the sediment. d = correction factor (%) for the estimated portion of fish bones and scale fragments in the sediment; and
- W = weight of the sample material for the sedimentation (mg).

Estimated value of constituents of animal origin

The proportion of bone in animal products can vary greatly. The percentage of bone in bone meals is usually 50–60% and in the case of meat meals is usually 20–30%. In fish meal bone and scale contents vary according the category and origin of the fish meal but are usually 10–20%.

If the type of animal meal present in the sample is known, it is possible to estimate the content:

- Estimated content of constituents of terrestrial animal products (%) = (S × c) / (W × f) × 100
- Estimated content of constituents of fish products (%) = (S × d) / (W × f) × 100

Where

- S = sediment weight (mg);
- c = correction factor (%) for the estimated portion of terrestrial animal bone constituents in the sediment;
- d = correction factor (%) for the estimated portion of fish bones and scale fragments in the sediment;
- f = correction factor for the proportion of bone in the constituents of animal origin in the sample examined; and
- W = weight of the sample material for the sedimentation (mg).

CALCULATION

The laboratory report should at least contain information on the presence of constituents derived from terrestrial animals and from fishmeal. To comply with European Commission Regulation (EC) No 152/2009 – 'Laying down the methods of sampling and analysis for the official control of feed' the different cases should be reported in the following way:

With regard to the presence of constituents derived from terrestrial animals:

- as far as was discernible using a microscope, no constituents derived from terrestrial animals were found in the submitted sample; or
- as far as was discernible using a microscope, constituents derived from terrestrial animals were found in the submitted sample.

Where bone constituents from terrestrial animals are identified, the report should also contain the additional clause: 'The possibility that the above constituents are derived from mammals cannot be excluded.'

This additional clause is not necessary in cases where the bone fragments from terrestrial animals have been specified as bone fragments from poultry or mammals.

With regard to the presence of fishmeal:

- as far as was discernible using a microscope, no constituents derived from fish were found in the submitted sample; or
- as far as was discernible using a microscope, constituents derived from fish were found in the submitted sample.

In the event that constituents derived from fish or terrestrial animals are found, the report of the examination result may, if required, further indicate an estimation of the amount of constituents detected as a percentage (< 0.1%, 0.1–0.5%, 0.5–5% or > 5%).

If required further specification of the type of terrestrial animal and the animal constituents identified may be made (muscle fibres, cartilage, bones, horn, hair, bristles, feathers, blood, egg shells, fish bones, scales).

For the case where the amount of animal ingredients is estimated the correction factor

f used shall be stated.

REFERENCE

European Commission Regulation (EC) No 152/2009. – *Laying down the methods of sampling and analysis for the official control of feed*. January 2009. EU, Brussels, Belgium.

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Andreas Adler
Österreichische Agentur für Gesundheit
und Ernährungssicherheit GmbH
Institut für Tierernährung und Futtermittel Abt. Kartoffelprüfung,
Mikro- & Molekularbiologie
A-4020 Linz, Wieningerstraße 8,
Austria
e-mail: andreas.adler@ages.at

Jim Balthrop
Office of the Texas State Chemist
Quality Assurance Manager
P.O. Box 3160
College Station, Texas 77841, USA
e-mail: jeb@otsc.tamu.edu

Harinder P.S. Makkar
Animal Production Officer
Animal Production and Health Division
Food and Agriculture Organization of the United Nations
Viale delle Terme di Caracalla
00153, Rome, Italy
e-mail: harinder.makkar@fao.org

Alicia Nájera Molina
International QA Manager
Masterlab BV,
The Netherlands
e-mail: a.najera@nutreco.com

Michael Runyon
Office of the Texas State Chemist
Quality Assurance Manager
P.O. Box 3160
College Station, Texas 77841, USA
e-mail: mmr@otsc.tamu.edu

Alfred Thalmann
Elbinger Str. 10c
D 76139 Karlsruhe
Germany
e-mail: alfred-thalmann@t-online.de
(Formerly: Staatliche Landwirtschaftliche Untersuchungs- und Forschungsanstalt Augustenberg or Landwirtschaftliches Technologiezentrum Augustenberg)

Stijn Van Quekelberghe
Technical Responsible Food Chemistry - R&D
Chemiphar n.v.
Lieven Bauwensstraat 4, B-8200 Brugge
Belgium
e-mail: stijn.vanquekelberghe@chemiphar.com

Wolfgang Wagner
Sachgebietsleiter Mikrobiologie und Molekularbiologie
Landwirtschaftliches Technologiezentrum Augustenberg
Neßlerstraße 23-31
76227 Karlsruhe
Germany
e-mail: wolfgang.wagner@ltz.bwl.de

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Animal feeding influences every sector of the livestock industry. High quality animal feed with the correct nutritional content and free from harmful contaminants, such as microbiological agents or their toxins, ensures that animals will be in the best possible condition, content and healthy. As a result animals will produce increased yields of quality products (meat, milk, eggs or wool) resulting in improved health and wellbeing of the human population.

This publication provides comprehensive guidance on the requirements of a microbiology laboratory performing animal feed analysis and includes examples of standard operating procedures, prepared by experts from around the world. These procedures will assist laboratories in attaining the competence required, will enhance the quality of data reported and ensure the safety of laboratory workers. The procedures contained in this publication are compliant with ISO/IEC 17025:2005 (General requirements for the competence of testing and calibration laboratories) and take into account ISO 14001:2004 (Environmental management systems) and BS OHSAS 18001:2007 (Occupational health and safety management systems) and will assist laboratories to gain accreditation or certification.

This publication will be useful for laboratory analysts, laboratory managers, students and teachers and will enable workers in the livestock industry to appreciate the importance of proven reliable data and quality assurance. Implementing the procedures will strengthen the research and education capabilities of students and promote a better trading environment between developing and developed economies. This will have long-term benefits and promote investment in feed industries and R&D.

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