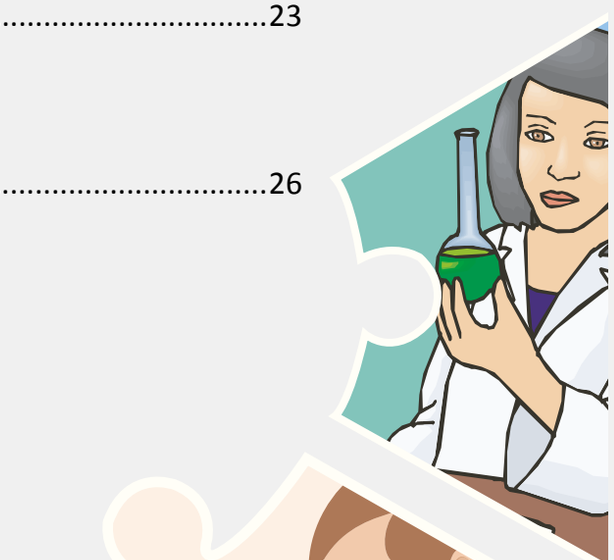
CHAPTER 7
LABORATORY



Chapter 7: Laboratory

1. Introduction

All milk processors and other manufactures of dairy products have a responsibility towards the consumer and other role players in the market to ensure that the products that they offer for sale are safe for human consumption and comply with the legal specifications that are set out in the legislation.

This chapter replaces the S*afe Milk Processing Guidelines for Laboratory Implementation* published by the DSA in 2005. It is only a guideline with work instructions that should enable processors of fresh milk to start a testing programme at reception (on farm) and to do platform testing at the factory reception as set out as a requirement in the existing legislation.

South African legislation requires that basic acceptance/rejection testing must be done on a daily basis at the collection point of raw milk on the farm and at reception at the processing facility.

R 961
R 1555

A quality control laboratory is essential in a dairy processing facility. The laboratory can assist in ensuring that there is valid information about the quality and safety of raw materials and final products. The laboratory can assist in ensuring that a product that is not fit for consumption is not processed or dispatched.

The laboratory also plays a vital role in assessing the effectiveness of cleaning processes and the efficacy of cleaning chemicals.

If a dairy processor does not test on site, no immediate corrective actions can be taken and the problem areas cannot be identified and rectified immediately. It is not the responsibility of law enforcement bodies to control the processes in the factory environment. Generally the monitoring activity of the quality and safety of a product at point of sale is done by these bodies to ensure that the products that the consumer buys and uses are safe and comply with specifications.

For safe milk processing it is essential to have an internal laboratory to do in-house quality control testing of dairy products. For the verification of results an external SANAS accredited professional laboratory must be used.

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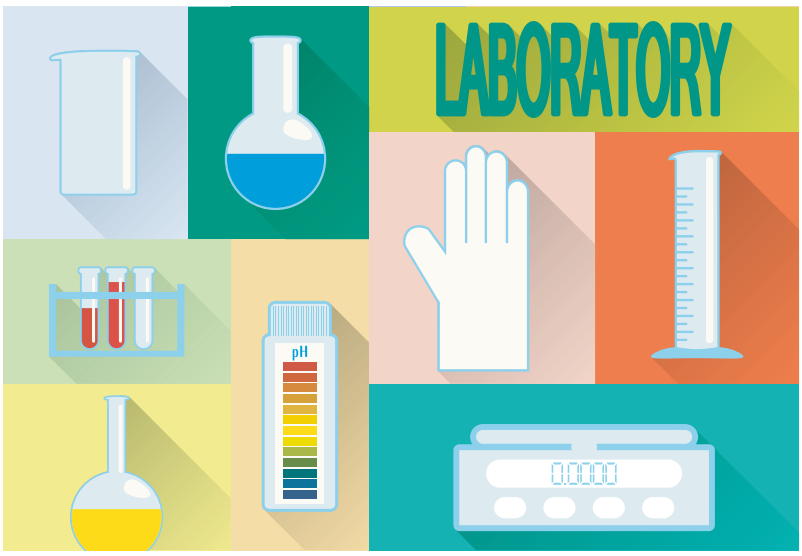
Laboratory analysis is only meaningful if comprehensive product and processing specifications are available.

It is important to perform the right tests regularly to have sufficient data to know that the process and product safety and quality are under control. A quality plan should be developed by the processing facility, which should detail the tests to be carried out and the frequency of sampling.

R 1555
R 961
Chapter 1



This quality plan must take into account the product specification, any customer specifications and the legal requirements.

SANS 10049, 7.3.3, 7.5.1, 8.2,
CGCSA-FSI GMCBP I.A.5.1, 6, 9

The internal laboratory carrying out the analyses according to the quality control plan must implement systems for the following:

- Selection and training of laboratory personnel.
- Preparation of analytical reagents.
- Design of the laboratory facility.
- Standard procedures for dealing with product samples.
- Instrumentation, calibration and instructions for use.
- Analytical methods that are traceable to nationally or internationally recognised methods.
- Interpretation, registration and storage of the results, including responsibilities for dealing with decisions relating to nonconforming products.

SANS 10049, 7.4.4, 7.4.6, 7.5.2.13
SANS ISO 22002-1, 5.5

If there is an internal laboratory, this facility must be in a separate area in order to avoid cross-contamination with the production process, unless analyses are carried out on-line. Production personnel should not enter the laboratory and protective clothing worn in a microbiological laboratory should not be worn in production areas.

Certain tests require specific environmental conditions that can invalidate results if not provided for. Suitable procedures for the safe disposal of used reagents and materials should be established.

It is important to correlate internal test results on samples taken in the factory or on the farm with other laboratories via recognised ring-testing programmes or with an accredited external laboratory.

Duplicate testing protocols should always be used and environmental control samples should always be used for microbiological testing.

R 1555
R 961, 11
R 962 Annexure D
Chapter 2

Food safety and quality is a shared responsibility. To ensure that optimal conditions are maintained for the processing facility, the factory manager should also verify the status of the official authorisation of supplying farms, collection stations and transportation trucks. He/she should also, in agreement with the farm managers and the local authorities, verify that the general production requirements are respected, namely:

- A certificate of acceptability for each milk shed that produces raw milk for collection.
- The conditions of animal housing and feeding.

- Animal sanitary conditions.
- Functional and hygienic conditions of milking and milk storage equipment.
- Handling of collected milk, milking animals and personnel.
- Cleaning and disinfection procedures.
- Milk transportation hygiene.

Records must be kept of all testing and analyses carried out. If the results are not within specification, corrective action must be taken and records must be kept of this action.

SANS 10049, 9
SANS ISO 22002-1, 9.3

If products are retested these records must also be in place.

It is important that the person responsible for releasing the product has sufficient knowledge of the testing methods and the interpretation of the results to ensure that only safe quality products are released to the consumer.

SANS 10049, 7.5.1
CGCSA-FSI GMCBP B.A.1.5

2. Raw materials other than milk

SANS 10049, 8.5
SANS ISO 22002-1, 9.3
CGCSA-FSI GMCBP I.A, 6

Any other raw material or ingredients used in the processing facility should be considered in the quality control plan. As a function of potential hazards associated with these raw materials and their use, the possibility of cross-contamination should be verified and procedures suitable for the avoidance or reduction to an acceptable level must be established.

The frequency and strictness of the quality plan will depend on the impact of the raw material on the quality and safety of the final product.

CGCSA-FSI GMCBP B.A.4.1

If the raw material testing identifies nonconforming products, the raw material should ideally be quarantined and the supplier advised. Records should be available for inspection.

If this is only identified after the product has been dispatched, the need to recall the product should be determined to ensure the consumer is not harmed in any way.

3. Final products

SANS 10049, 8.1, 8.2
SANS 1678, 8
SANS 1679, 8
SANS ISO 22000, 8.4
CGCSA-FSI GMCBP I.A.6, I.C.1.9

A quality control plan must be developed and the following defined for every product:

- Composition.
- Storage conditions.
- Physical characteristics.
- Shelf life.
- Process.
- Instructions for storage and use.
- Packaging system.
- Type of consumer.

Chapter 1, 3

Develop a process flowchart for all products manufactured.

The flowchart should provide a simple and clear description of every process.

The following parameters should be taken into account:

- Raw materials, ingredients and packaging materials used.
- The sequence of the processing phases, including the method of using raw materials.
- Time and temperature, during both processing and storage.
- How reprocessed products are used.
- Storage and distribution conditions.
- Product flows through the factory and possibilities of cross-contamination.
- Separation between areas of high and low risk of contamination (or separation between clean and dirty areas).
- Cleaning and disinfection data.
- General hygiene conditions in the factory.
- Personnel hygiene conditions and circulation of personnel.

4. Cleaning and disinfection procedures

SANS 10049, 7.4.5
SANS ISO 22002-1, 11.5
CGCSA-FSI GMCBP B.B. 3.1

Proper cleaning is essential to ensure the dairy processing facility and equipment is free of chemical and microbiological contamination which can impact on the safety and quality of the final products.

Due to the nature of dairy products, disinfection is required as the last step for effective cleaning. Given that organic matter can neutralise many disinfectants, this should only follow proper cleaning and removal of residues.

The strength of the disinfectant should be tested to ensure that there are sufficient active ingredients to be effective.

The efficacy of the disinfectant is evaluated by the microbiological monitoring of the product and product contact surfaces. This process should be carried out in accordance with SANS 5763 to ensure that valid results are obtained.

Other considerations for testing include water, air and compressed air if these are in direct contact with the product or product contact surfaces.

SANS 10049, 7.4.1, 7.4.2,
SANS 1678, 5.10, SANS 1679, 5.10
SANS ISO 22002-1, 6.2, 6.4
CGCSA-FSI GMCBP B.C 2.4

Allergen control should also include cleaning verification tests.

5. Retention samples and shelf-life testing

SANS 10049, 8.10

Retention samples are kept by the laboratory and stored in a fridge at a maximum of 7°C (UHT samples will be stored at ambient temperature). As far as possible, a unit sample of each manufactured product for each day is to be retained. A full sealed sample from the line is not required.

A small sterile sample bottle can be used to representatively sample the product, provided that it is clearly marked with the product name, date packed, expiry date and batch code.

SANS 1678, 8
SANS 1679, 8

On the expiry date of the product, the product is tested for pH, taste, texture, microbiology (total bacterial count, coliform and E. coli). For raw milk the clot-on-boiling (COB) test and Alizarol test can also be used. Retention samples can also be used as reference when customer complaints are received to assist with the investigation of the complaints. Retention samples can help the processor in times of dispute to prove that, if the cold chain had not been broken and the products had been appropriately stored at the specified temperature, the final product should have been in good condition.

6. Sampling methods for raw milk for microbiological and compositional evaluation

6.1 Sampling equipment and containers

All sampling equipment and instruments must be clean, sterile and dry prior to use. Sterilisation by hot air (170–175°C for at least 2 hours) or steaming (121°C for at least 20 minutes in an autoclave/pressure cooker) is normally recommended (see Section 9.3 for instructions). The following alternative methods can also be used:

- Direct exposure of sampler surfaces to a suitable flame.
- Immersion in at least 70% (v/v) ethanol solution.
- Ignition after immersion in 96% (v/v) ethanol.
- Exposure to sufficient gamma radiation.

After thermal sterilisation, the equipment should be allowed to cool down before using for sampling. It is essential that the sample containers and closures should be clean, sterile and dry and that they should be securely closed to prevent contamination from external sources. Any deviations from the prescribed sampling instructions, abnormal sampling conditions, or additional information concerning the samples to be tested should be noted in the sampling report to ensure the scientifically sound interpretation of the test results.

6.2 Sampling techniques

ISO 707

Samples for microbiological examination are always taken first and, whenever possible, from the same product containers as those for chemical, physical and sensory evaluation. Specific sampling techniques for milk and milk products are described in various standard methods (Grace et al., 1993; IDF, No. 50A 1995). The mixing of milk and liquid milk products, for example, can be achieved by pouring the milk from one container into another or by using a stirrer (plunger) of a suitable design, or by mechanical agitation. The milk is usually agitated for five minutes when the tank capacity is 500 to 4 000 litres, for at least 10 to 15 minutes when the volume is more than 4 000 litres, and for 30 to 60 minutes in large factory storage tanks (Lück & Gavron, 1990). If tanks are equipped with time-programmed agitation systems, samples may be taken after agitation for shorter periods.

The collection of a representative sample from large vessels, storage tanks and tankers may present problems. In a large vessel with a bottom discharge outlet, samples should preferably be taken through the manhole. If taken from the discharge outlet valve or the sampling cock, sufficient milk must be discharged to ensure that the sample is representative of the whole. Proportionate sampling is done by taking representative quantities from each container and mixing the portions in amounts that are proportional to the quantity in the container from which they were taken. With raw milk, bulk portions must be split with care and the homogeneity of the samples must be validated regularly. A useful method is to determine the butterfat levels because the distribution of microorganisms in raw milk closely follows that of the fat.

6.2.1 In-line sampling

Flow lines of modern dairy plants are complex and improper designs may cause recontamination of heat-treated products. An effective quality control programme may include sampling of milk at different sites after pasteurisation to assess the microbiological quality or

possible post pasteurisation contamination. Multiple samples from the same batch can be taken at specific time intervals. Great care should be taken when sampling unmixed milk, because significant carry-over from one sample to the next can invalidate sensitive tests.

It is also recommended that the number of samples be increased, should any drop in quality be noted. Samples could also be drawn over a period of time (for example, at the start, midway through and at the end of production) to identify time-related problems during production.

6.3 Storage and transport of samples

The most effective way to stabilise the microbial content of milk and perishable milk product samples prior to analysis, is by storage and transport of the samples in crushed ice (0 to 4°C). At these temperatures, especially between 0 and 2°C, the microbial numbers will remain virtually unchanged for up to 24 to 36 hours. Samples must reach the laboratory as quickly as possible, preferably within 24 hours. If cooling is necessary, the minimum requirements to be met are the temperature ranges that are either legally required or prescribed by the manufacturer.

Thermally insulated containers are used for the storage of cooled, frozen and quick-frozen samples during transfer to the laboratory. Crushed ice, pre-frozen ice packs, or dry ice (solid CO2) may be used as refrigerant agents. For most analyses, however, freezing should be avoided because it can cause the disruption of bacterial cells.

6.4 Preparation of samples for microbiological testing

Mostert, J.F. & Jooste. P.J. (2002). Dairy Microbiology Handbook, 3rd edition. Chapter 14, pp 655-736. *Quality control in the dairy Industry*

The preparation of samples, prior to microbiological examination, is just as important as taking representative samples. The precise procedure for the test portion varies with the nature of the product. All samples should be thoroughly mixed by shaking for the purpose of microbiological and compositional testing. Only specified diluents for general or special purposes are used for primary and further decimal dilutions. Damaging microorganisms by sudden changes in temperature, should be avoided, for example, when transferring a portion of the test sample to a diluent. The normal aseptic precautions during weighing and mixing of test portions, or transferring suspensions, should always be taken. The selection of a method for a specific test should be carefully considered and aspects such as the purpose of analysis and the required sensitivity of the method will, for example, determine which method is to be used. It is, however, recommended that procedures that are officially prescribed or generally recognised be used, such as those in Regulation No. 1555.

6.5 Records

The following records should accompany all samples to the laboratory:

- Time and date of sampling.
- Product(s), raw milk, pasteurised milk.
- Temperature of products sampled.
- Location where sampling took place.
- Transfer point control: time, date and temperature of each temperature control when samples were received or shipped.
- Sampler's name or identity.

Sampling procedure for raw milk for microbiological (sterile sampling) analysis

Take out one sample bottle, alcohol, dipper, lighter/matches

Wash hands with soap and warm water. Dry hands with paper towel

Spray hands with alcohol

Spray clean dipper with alcohol

Flame dipper using lighter/matches. DO NOT PUT DIPPER DOWN

Spray outside surface of sample bottle with alcohol

Put sample bottle into dipper holder

Spray sample bottle and dipper again with alcohol

Unscrew lid of sample bottle, holding lid in your hand. DO NOT TOUCH INSIDE LID OR IN BOTTLE

Dip into bulk tank and fill sample bottle with milk

Remove dipper and sample bottle from bulk tank

Screw lid back onto sample bottle TIGHTLY

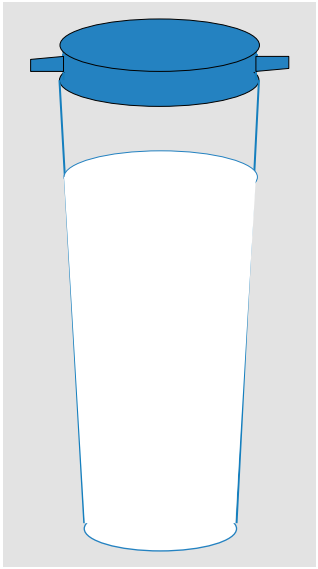
Fill sample container not more than 3/4 full (after closure) to permit proper mixing of the sample at the laboratory

Put full sample bottle onto the ice in the cooler box

Rinse dipper with water

Dry dipper with paper towel. THROW PAPER TOWEL AWAY AFTER DRYING

ONCE MICRO SAMPLE HAS BEEN TAKEN, TAKE SAMPLES FOR ALIZAROL AND COMPOSITIONAL TESTS



Sampling procedure for raw milk for Alizarol and compositional tests (essentially the same as for microbiology)

If no micro sample has been taken, then start alizarol and other sampling from the beginning

Take out one sample bottle, alcohol, dipper, lighter/matches

Wash hands with soap and warm water. Dry hands with paper towel

Spray hands with alcohol

Once micro sample has been taken, start here and take sample for alizarol and other tests

Spray clean dipper with alcohol

Flame dipper using lighter/matches. DO NOT PUT DIPPER DOWN

Spray outside surface of sample bottle with alcohol

Put sample bottle into dipper holder

Spray sample bottle and dipper again with alcohol

Unscrew lid of sample bottle, holding lid in your hand. DO NOT TOUCH INSIDE LID OR IN BOTTLE

Dip into bulk tank and fill sample bottle with milk

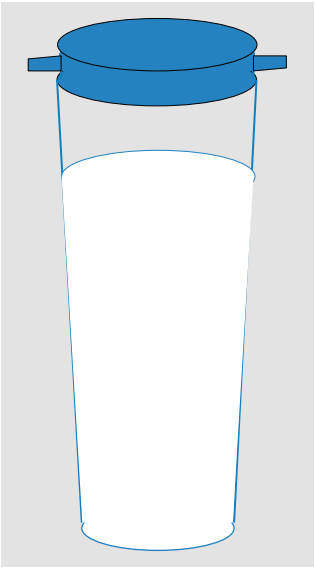
Remove dipper and sample bottle from bulk tank

Screw lid back onto sample bottle TIGHTLY

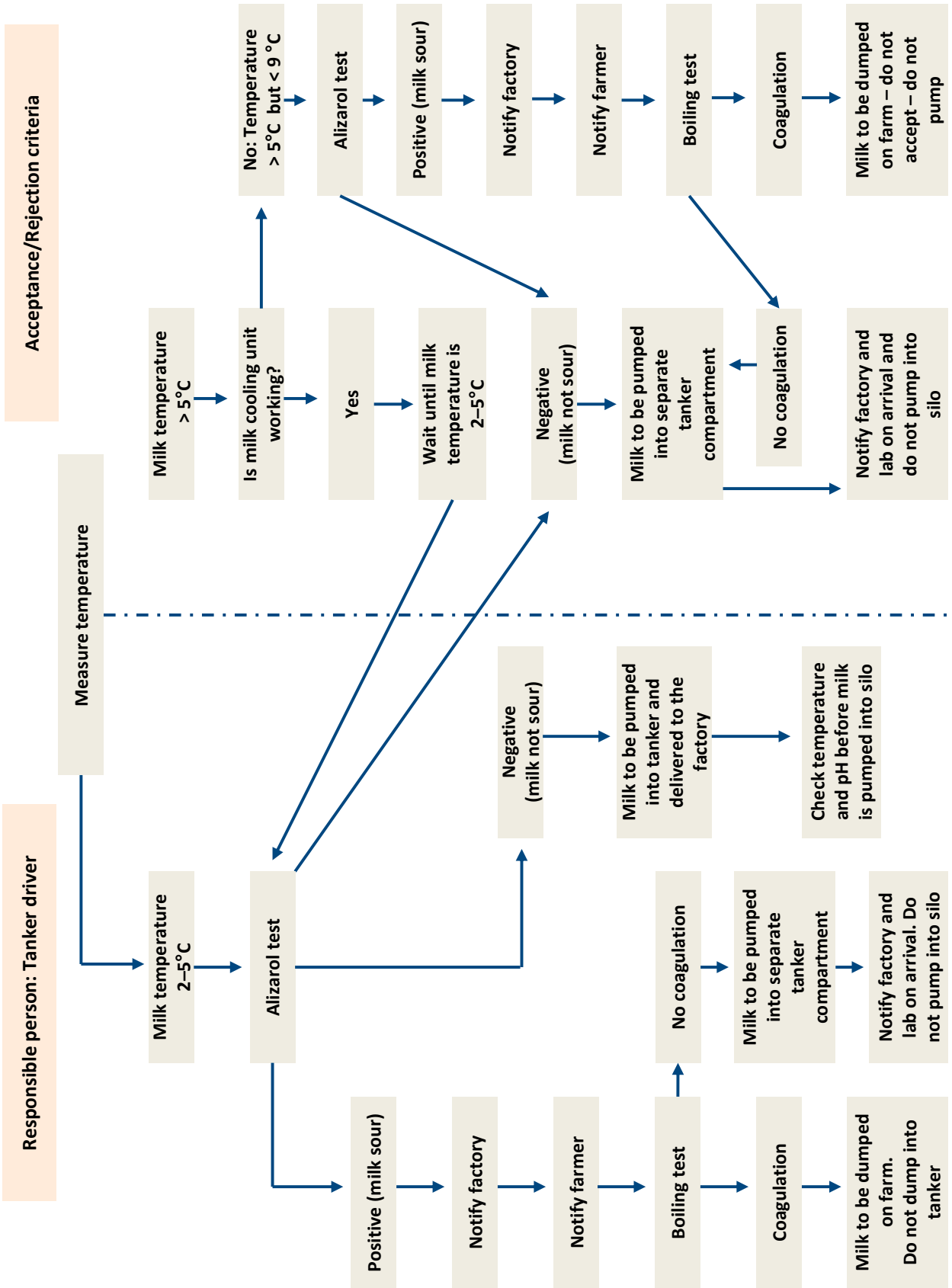
Put full sample bottle onto the ice in the cooler box

Rinse dipper with water

Dry dipper with paper towel. THROW PAPER TOWEL AWAY AFTER DRYING



Fill sample container not more than 3/4 full (after closure) to permit proper mixing of the sample at the laboratory


Acceptance/Rejection criteria
Responsible person: Tanker driver
Measure temperature
Milk temperature > 5°C
Is milk cooling unit working?
No: Temperature > 5°C but < 9 °C
Alizarol test
Positive (milk sour)
Notify factory
Notify farmer
Boiling test
Coagulation
Milk to be dumped on farm – do not accept – do not pump
Yes
Wait until milk temperature is 2–5°C
Negative (milk not sour)
Milk to be pumped into separate tanker compartment
No coagulation
Notify factory and lab on arrival and do not pump into silo
Milk temperature 2–5°C
Alizarol test
Negative (milk not sour)
Milk to be pumped into tanker and delivered to the factory
Check temperature and pH before milk is pumped into silo
No coagulation
Milk to be pumped into separate tanker compartment
Notify factory and lab on arrival. Do not pump into silo
Positive (milk sour)
Notify factory
Notify farmer
Boiling test
Coagulation
Milk to be dumped on farm. Do not dump into tanker

7. Chemical analysis

R 1555

7.1 Alizarol (68%) test

7.1.1 Purpose

This test provides an indication of the heat stability of milk and is used for testing the stability of the milk proteins before processing. The test is used as a platform (acceptance/rejection) test for raw milk for acceptability on the farm and at milk reception at the factory.

7.1.2 Background

Alcohol is a dehydrate and it destabilises protein. If milk protein is already slightly unstable due to souring, dehydration with alcohol will lead to the precipitation of protein in the form of flakes. Other factors that influence protein stability and result in a positive alcohol test are:

- Mastitis: abnormal protein content.
- Colostrum: abnormal protein content.
- Sweet coagulation: coagulation enzymes from bacterial origin present in the milk.
- Mineral imbalance: excessive calcium salts.

The alcohol test, therefore, does not only indicate acidity.

To overcome this problem an Alizarine indicator is dissolved in the alcohol to indicate the acidity of the milk.

7.1.3 Reagents

- 68% (v/v) ethanol
- Use absolute ethanol and measure 68 ml in a measuring cylinder.
- Fill to the 100 ml mark with distilled water.
- Add the Alizarine indicator (0,2 g/100 ml).

7.1.4 Apparatus

- 5–10 ml test tubes with lids.
- Measuring cylinder.
- Dark glass container/bottle for Alizarol alcohol.

7.1.5 Method

1. Place equal parts of 68% Alizarol and milk in a test tube.
2. Mix lightly.
3. Evaluate the colour:
   a. If the colour is light purple and no flocculation occurs, perform the Resazurin test. If the milk coagulates, reject it.
   b. If the milk has a brownish-pink colour with fine flocculation, reject the milk.
   c. If the milk has a brownish-pink colour but no flocculation occurs, determine the pH or percentage titratable acidity (TA). If the results are unacceptable, that is, if they do not fall within the prescribed limits, reject the milk.
   d. If the colour of the milk varies from brownish-yellow to yellow, reject the milk.
   e. If the colour of the milk is violet and flocculation occurs, reject the milk.
   f. If the colour of the milk is violet but no flocculation occurs, determine the pH or percentage TA. If the results are unacceptable, that is, if they do not fall within the prescribed limits, reject the milk.

Chapter 3.1

7.1.6 Results

In summary, colour standards indicate:

- Light purple (fresh milk)
- Yellow (sour)
- Brownish-yellow (slightly acid)
- Violet (alkaline)

Criteria for Alizarol test

Fresh milk	None	Light purple
Slightly sour	Possible small flakes	Brownish-pink
Sour	Small flakes	Brownish-yellow
Very sour	Big/large flakes	Yellow
Sweet coagulation	Big/large flakes	Light purple
Mastitis	Small flakes	Violet
Added alkaline	None	Violet

7.1.7 Caution

1. The concentration of the alcohol used in the Alizarol test is important.
2. Hence the make-up of the solution must be done precisely. The pH of the Alizarol must be the same as that of normal fresh milk, if not, it will provide incorrect results.
3. The pH and concentration of the Alizarol solution must be tested every week.

R 1555

7.2 Clot-on-boiling test (COB)

7.2.1 Purpose

This test gives an indication of the heat stability of milk. Acid development and colostrum in milk will result in a positive COB test result. The heat stability of the milk is also affected by other factors. The test is used as a platform test for the acceptability of raw milk at the factory or in the tank on the farm.

7.2.2 Background

Colostrum in milk will result in a positive COB test result. The heat stability of the milk is also affected by other factors like developed acidity.

7.2.3 Apparatus

- 5–10 ml test tubes with lids.
- Container (pot) with boiling water.
- Thermometer

7.2.4 Method

1. Thoroughly mix the milk before sampling.
2. Pour 5 ml of milk into a test tube.
3. Place the tube in boiling water.
4. Ensure that the level of the boiling water is higher than the milk level.
5. Stand the test tube with milk in the boiling water for five minutes.
6. Remove the test tube from the water and tilt the tube almost horizontally without shaking the milk inside.
7. Wait until a thin film is formed on the milk.

7.2.5 Results

Chapter 3.1

The result is positive if all the milk clots or if floccules are seen to adhere to the sides of the tube when it is returned to the vertical position.

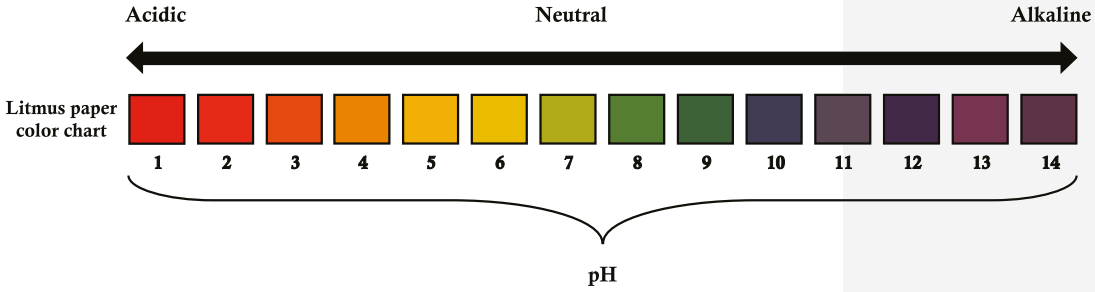
7.3 Determining the pH of milk

7.3.1 Purpose

The purpose of pH-determination is to measure the acidity or alkalinity of the product. The pH is determined by the relation between the amount of hydrogen ions (H+ or acid) and the hydroxyl ions (OH-). The pH is measured with a pH meter on a scale between 0 and 14.

An equal amount of H+ and OH- ions are present at pH 7. At pH 7,0 the product has no ionic charge and is neutral. A pH lower than 7 indicates an acidic solution with more H+ ions present than OH- ions. A pH higher than 7 indicates an alkaline solution with more OH- ions present than H+ ions. The determination of the pH value of raw milk is done to determine the amount of acid developed. This test is used as one of the acceptability tests for raw milk at the factory. The test is also used in the shelf life assessment of final products at the end of the shelf life together with a sensory evaluation.

Presentation of a pH scale



7.3.2 Background

A pH meter is an instrument which determines the concentration of hydrogen ions in a product and interprets it as a pH value. The measuring instrument consists of two sections, namely a combined electrode or sensor, which is a combination of a glass electrode and a reference electrode, and a special voltmeter which boosts the electric signal from the electrode and displays it on a scale between 0 and 14.

The glass electrode is the heart of the instrument and contains a very sensitive membrane, which is very selective for hydrogen ions. The glass electrode contains a solution with a constant pH. A metal electrode which couples the glass electrode with an electric cord to the voltmeter is placed into the solution.

When the glass electrode is placed into a solution or product, a small voltage develops between the solution on the inside and the outside of the electrode, which is equal to the concentration of hydrogen ions in the solution or product. This voltage is boosted by the voltmeter and directly displayed as a pH value. A large variety of pH meters and electrodes are used, but each one is based on the same principle as above.

The correct handling of pH meters and electrodes is important and directions from the suppliers for handling the instrument and electrode must be read thoroughly before operating the instrument.

Calibration and testing procedures must be executed according to the instructions for the pH meter.

7.3.3 Apparatus

- pH meter.
- Buffer solutions pH 4,0 and pH 7,0.
- Electrode for dairy products.
- Cleaning solution for electrodes.

7.3.4 Method

1. Switch on the pH meter.
2. Remove the electrode from the buffer solution.
3. Rinse the electrode with distilled water.
4. Dry the electrode with paper towel.
5. Immerse the electrode in the milk sample.
6. Turn the selection knob to pH or press “pH” read or measure.
7. Wait until the reading stabilises.
8. Note the pH reading.
9. Turn the selection knob to “off”.
10. Remove the electrode from the milk.
11. Rinse the electrode with distilled water.
12. Dry the electrode with paper towel. (Dab, do not wipe.)
13. Leave the electrode in pH 7,00 buffer.

7.3.5 Results

Chapter 3.1

- A pH reading of 6,6 and lower indicates the possibility of sour milk.
- Milk with a pH of 6,60 cannot be used for further processing or distribution as fresh milk.
- A pH above 6,80 is an indication of mastitis milk adulteration or the addition of bicarbonate of soda.
- Too high pH-values can also be an indication that pipe lines were not rinsed properly and that alkaline detergents were accidentally added to the milk.

7.3.6 Caution

1. Carry out the calibration and testing procedure according to the instructions for the pH meter.
2. The buffer solutions must be tightly sealed and stored in a cool and dark place.
3. Used buffer solution must never be poured back into the bottle with the stock solution.
4. Glassware, thermometers and other apparatus must be rinsed with distilled water and completely dried before use.
5. Liquid temperatures (buffer or product) must not exceed 50°C.
6. Buffer solutions and milk samples must both be at room temperature when pH readings are taken. If it is not the case a temperature adjustment must be done on the pH meter.

R 1555

7.4 Titratable acidity (TA)

7.4.1 Purpose

TA measures the natural acidity of the milk and the acid development arising from bacterial activity. The result is expressed as percentage lactic acid. The TA varies between 0,15 and 0,18% in herd’s milk. Casein and phosphate salts are part of the solid content of milk. The natural acidity increases as the solid content increases. TA is used as one of the tests to determine milk’s acceptability.

7.4.2 Background

The percentage TA is determined in order to trace the development of acidity resulting from bacterial activity. An abnormal percentage TA

can also be an indication of contamination by detergent residues or possible mastitis milk.

7.4.3 Reagents

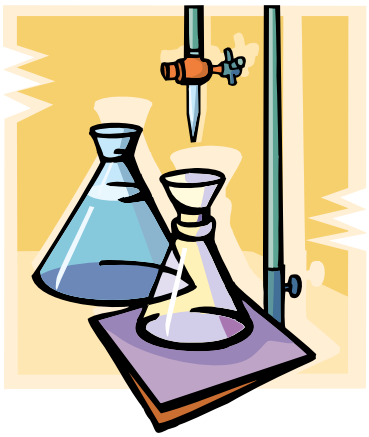
- 0,1 N NaOH (sodium hydroxide).
- Phenolphthalein – 1% (m/v) solution in ethanol.

7.4.4 Apparatus

- 10 ml or Schilling's burette.
- Bottle with dropper for phenolphthalein.
- 9 ml volumetric pipette.
- White cup or beaker with white paper.

7.4.5 Method

1. Heat the sample to 20°C by placing it in a water bath.
2. Transfer 9 ml milk with volumetric pipette into a clean beaker or cup.
3. Add 5 drops of phenolphthalein to the 9 ml of milk in the beaker.
4. Ensure that the burette is filled to the zero mark.
5. Titrate the milk sample with 0,1 N NaOH (sodium hydroxide) in a burette until it turns light pink in colour and remains so for 15 seconds.



7.4.6 Results

Read the titration figure from the burette and divide the figure by ten (10). The answer is the per cent TA.

Chapter 3.1

E.g. bottom of meniscus = 1,5

$$\% \text{ TA} = \frac{1,5}{10} = 0,15$$

7.5 Resazurin test

7.5.1 Purpose

The Resazurin test is applied to raw milk to determine the bacterial activity and, indirectly, the number of bacteria in the milk. When Resazurin, added to raw milk, is discoloured within 30 minutes at 32°C, the milk contains a bacterial count of at least 40 to 50 million CFU's per ml. The 30 minute Resazurin test is used as an intake-test, that is, the test is done at milk reception as soon as the milk is received. Every batch of raw milk must comply with the specifications.

Harrigan, W.F. and McCance, M.E. (1966). *Laboratory Methods in Microbiology*, pp 139-141

7.5.2 Background

The bacteria in milk use oxygen and produce reducing substances. The speed at which the decrease of oxygen takes place depends on the:

- Amount of bacteria.
- Growing speed of bacteria.
- Ability of bacteria to use oxygen.

Bacterial activity – the oxygen usage is observed by adding a suitable chemical indicator (colour) to the milk. The Resazurin test is based on the reduction of the indicator.

Reduction takes place when oxygen is lost and hydrogen is taken up. The indicator changes its colour as soon as the oxygen concentration is lower than a predetermined value. The speed of oxygen decrease/ usage is determined by measuring the colour change of the indicator after a specific period of time.

The colouring agent used as an indicator is Resazurin. Resazurin dye takes on a variety of colours, ranging from blue to light rose as the oxygen concentration lowers. To identify the colour of samples, a Lovibond comparator with a special Resazurin disc with seven different colours, is used. Each of the colours is numbered. When Resazurin (blue colourant) is added to raw milk, followed by incubation at 32°C, the colourant is reduced mostly by bacteria but also leucocytes. The colour change takes place in two steps, namely:

- Firstly, different shades of blue to light rose resorufene.
- Secondly, to the colourless dihydroresorufene (white).

The more bacteria and leucocytes are present in milk the more rapidly the colour changes. Four varieties of the test exist, namely:

- 30 minute test
- 1 hour test
- 2 hour test
- Three-fold reading test

7.5.3 Reagents

- Where practicable, use reagents of analytical quality.
- Preparation of the Resazurin solution:
- Add one standard Resazurin tablet to 50 ml cold, sterile, glass-distilled water. When not in use the solution must be stored in a cool dark place, preferably in a refrigerator, and should be discarded when more than two days old. Covering the container with aluminium foil helps.

7.5.4 Apparatus

- A Lovibond all-purpose comparator with a stand for work in reflected light. (The use of colour charts may be useful too.)
- Two fused-glass cells, 25 mm deep or in tubes of colourless glass; 13,5 mm internal diameter, fitted with non-p-nitrophenol-containing stoppers for use in the Lovibond all-purpose 1 000 comparator.
- A water bath capable of being maintained at 32°C ± 1°C.
- A pipette to deliver 5,0 ± 0,5 ml and an auto pipette capable of delivering 1 ± 0,1 ml.
- A one-litre volumetric flask.
- A 100 ml standard volumetric flask.
- Drying oven for drying glassware.

7.5.5 Cleaning procedure

1. After use, empty each tube, rinse in water, wash well in hot water and then in distilled water and dry, or clean using an equally effective method.
2. Pipettes should be rinsed in cold water and then cleaned and dried in a drying oven.

7.5.6 Method

1. Mix sample thoroughly by inverting it quickly at least 10 times.
2. Use a sterile 10 ml pipette to measure off a 10 ml milk sample into a sterile test tube (NB: sterile).

3. Replace the sterile stopper on the test tube.
4. Place the test tube in the rack in the water bath at 32°C.
5. As soon as the temperature of the milk in the control tube reaches 32°C (approximately three minutes), add 1 ml fresh Resazurin solution (0,005%) to the milk sample by means of a sterile pipette.
6. Replace the stopper firmly.
7. Invert the test tube twice before placing it back into the water bath and note the time. (Do not shake, as this incorporates oxygen). Remove the tubes after 20 minutes.
8. Place the control tube in the left segment below the colour slide of a Lovibond comparator.
9. Remove the tube with the milk sample from the water bath and within one minute place it in the right-hand segment of the comparator.
10. Rotate the colour slide until the colour of the control compares with the sample.
11. Note the obtained slide reading. Ensure that results are recorded.

7.5.7 Results

Interpretation of the Resazurin test results.

Chapter 3.1

Colour	Colouring matter	Lovibond slide reading	Acceptable for intake
1. Blue	Resazurin	6	Yes
2. Pink	Resorufene	3	No
3. White	Dehydroresorufene	0	No

7.5.8 Caution

1. Steps 1 to 11 must be carried out as quickly as possible to limit the effect of light.
2. All glassware and syringes must be sterilised.
3. The Resazurin solution is reusable for a maximum of two days if it is kept at a low temperature (maximum 6°C).
4. Use a different 10 ml pipette for each milk sample.

7.6 Instructions for determination of antimicrobial residues in raw milk

ISO 707

7.6.1 At farm

Take a sample according to the sample procedures and keep it as reference sample to be tested if the tanker samples are positive.

! Make sure to document which farmer's milk is in which compartment.

7.6.2 At milk reception at the factory

1. Take samples from each compartment of the tanker.
2. Analyse samples of different compartments of the tanker for antimicrobial residues.
   RED ALERT
3. If positive, fill in corrective action sheet (CAS) and send it to the manager's office.
4. If milk from a compartment tests positive, analyse individual samples from farmers to identify the problem supplier.
5. If positive, fill in the CAS and send it to the manager's office.
6. Notify the "POSITIVE milk supplier" in writing and by telephone.

7.7 Test methods for the detection of antimicrobial residues

For the detection of antimicrobial residues, a wide range of test methods are available. Dairy companies should be aware of the suitability and limitations of different test principles, should use test methods of known sensitivity and selectivity for testing of supplies and should take measures to ensure that test results are reliable. The following measures may be required:

- On-going training for technicians.
- Known positive and negative standards to confirm the claimed detection ability of the tests.
- Participation of technicians in proficiency testing programmes.

Available test methods can be grouped as below. Each group is listed with its advantages and limitations.

7.7.1 Microbiological inhibition tests

The principle is based on growth inhibition of certain test bacteria that are seeded in a specific test medium.

Advantages

- ✔ Exhibit a broad detection pattern for a wide range of antimicrobials, thereby also detecting growth inhibition by combinations of substances and/or through synergistic effects.
- ✔ Simple facilities generally suffice for a successful execution, either on-farm, at the dairy plant or in a laboratory. Required expertise is limited, although the proper reading interpretation of tests results does require some experience.
- ✔ Suitable for large-scale testing in laboratories, easy to automate and can be performed relatively cheaply.
- ✔ Can to some extent be used as group-specific tests by introducing antagonists like ß-lactamases (for the group of ß-lactam antibiotics) and para-aminobenzoic acid (for sulfonamides) in parallel tests.

Limitations

- ✘ Detectability of individual substances is widely different. Most relevant substances are detected with enough sensitivity, but some others may go undetected.
- ✘ Methods allow limited identification of growth inhibitors at the group level (see above), but are not able to identify and quantify individual substance.
- ✘ Suitable for screening, but subsequent confirmatory testing is needed in case a group identification is required or compliance against MRLs must be checked.
- ✘ Not fast; tests may take at least 2,5 hours.
- ✘ Not specific for antimicrobial agents and may be susceptible to interfering substances in raw milk such as naturally occurring inhibitors (for example, lysozyme and lactoferrin), free fatty acids and cleaning and disinfection agents.

7.7.2 Fast receptor tests

The principle is based on the binding of antimicrobials to one or more group-specific receptors that then react with a substrate to provide a quantitative reaction. Commercial tests are mostly offered as so-called strip tests for one or two groups of antimicrobial agents, for example, ß-lactams or ß-lactams+tetracyclines.

Advantages

- ✔ Group-specific and exhibit a broad detection pattern for antimicrobials belonging to the targeted group(s) of substances.
- ✔ Simple facilities generally suffice for successful execution, either on-farm, at the dairy plant or in a laboratory. Required expertise is limited, although the proper reading and interpretation of tests results does require some experience.
- ✔ Suitable for field use.
- ✔ Results are quickly available, in 2 to 15 minutes.
- ✔ Relatively inexpensive.

Limitations

- ✘ Only detects substances from targeted groups; other substances may go undetected.
- ✘ Not able to identify and quantify individual substances.
- ✘ Suitable for screening, but subsequent confirmatory testing is needed to check for compliance against MRLs.
- ✘ False negatives due to sensitivity issues may be a concern. More recently, analytical concepts with specific receptors for individual substances have been introduced in so called micro-array formats. These allow semi-quantitative detection of targeted individual antimicrobials or veterinary substances and can be applied in confirmatory testing in cases of a positive screening result.

7.7.3 Qualitative and quantitative instrumental methods

The principle is generally based on chromatographic separation of individual substances and their subsequent identification and quantification using systems such as high-performance liquid chromatography (HPLC) or liquid chromatography in combination with mass spectrometry (LC/MS-MS) or HPLC in combination with time-of-flight mass spectrometry U(H)PLC-ToF.

Advantages

- ✔ Specifically, the modern multi-component methods exhibit a broad detection pattern for antimicrobials. If required, additional tests can be targeted on non-covered individual substances.
- ✔ Sensitive; detection limits are usually far below existing MRLs.
- ✔ Suitable for checking definitive compliance against MRLs.

Limitations

- ✘ Only targeted substances are detected.
- ✘ Requires specific equipment and expertise.
- ✘ Laborious and time-consuming in execution, not suitable for large numbers of samples.
- ✘ Relatively expensive.

Due to the wide variety of test methods and kits only some are listed in the supportive CD attached to this file.

See Test Methods and Interpretation Guides on DVD

R 1555

7.8 Aschaffenburg and Mullen phosphatase test for milk

7.8.1 Purpose

The phosphatase test is applied to dairy products to determine whether pasteurisation was done properly and also to detect the possible addition of raw milk to pasteurised milk. The phosphatase test described in this method is based on the principle that the alkaline phosphatase enzyme in raw milk liberates phenol from a disodium phenyl phosphate substrate or phenolphthalein monophosphate substrate when the tests are

conducted at a suitable temperature and pH. The amount of phenol or phenolphthalein which is liberated from the substrate is proportional to the activity of the enzyme.

Pasteurisation, when done properly, inactivates alkaline phosphatase.

7.8.2 Reagents

Where practical, use reagents of analytical quality for this test. Prepare the buffer substrate solution as follows:

- Buffer solution: Dissolve 3,5 g anhydrous sodium carbonate and 1,5 g sodium bicarbonate in distilled water and fill with water to 1 litre in a volumetric flask. Keep the solid substrate disodium-p-nitrophenyl phosphate in a refrigerator.

- Buffer substrate solution: Place 150 mg of the substrate in a standard 100 ml volumetric flask and fill to the 100 ml mark with the buffer solution. Store the solution in a refrigerator and protect from light. Do not use the solution for longer than one week or reject when solution starts discolouring.

7.8.3 Apparatus

- A Lovibond all-purpose comparator with a stand for working in reflected light.
- A Lovibond comparator disc APTW 5 or APTW 7.
- Two fused-glass cells 25 mm deep or in tubes of colourless glass, 13,5 mm internal diameter, fitted with non-p-nitrophenol-containing stoppers, for use in the Lovibond all-purpose 1 000 comparator.
- A water bath capable of being maintained at 37°C ± 1°C.
- A pipette to deliver 5,0 ml ± 0,5 ml and an auto pipette capable of delivering 1 ml ± 0,1 ml.
- A one-litre volumetric flask.
- A 100 ml standard volumetric flask.
- Drying oven for drying glassware.

7.8.4 Method

1. Transfer 5 ml of the buffer substrate solution (2,1) to a test tube using a pipette.
2. Add 1 ml of milk to be tested, replace the stopper of the test tube and mix the contents well by shaking.
3. Incubate the test tube for 30 minutes; if the results are negative (no colour change) after 30 minutes, incubate for a further 30 minutes at 37°C ± 1°C.
4. With each series of samples, incubate one control sample prepared with 5 ml of buffer substrate solution and 1 ml boiled milk or cream of the same type as that undergoing the test.
5. After incubation, remove the test tubes from the water bath.
6. Place the control sample on the left-hand ramp of the Lovibond stand and the test sample on the right.
7. Revolve the disc until the colour of the test sample matches that of the control sample.
8. Record readings falling between two standards by fixing a plus or minus sign to the figure for the nearest standard.
9. A positive control using 0,1 ml raw milk in 100 ml pasteurised milk must be carried out at least once per week, preferably after a fresh buffer substrate solution has been made up. (This must give a reading of at least 14 after 30 minutes.)

7.8.5 Cleaning procedure

1. After use, empty each tube, rinse in water, wash well in hot water containing soda, rinse in hot water and then in distilled water and dry, or clean using another equally effective method.
2. Pipettes should be rinsed in cold water and then cleaned and dried in a drying oven.

Take the following precautions during, or in connection with the testing of a sample, into consideration:

- Except in the case of cultured dairy products, do not test a sample that shows signs of spoiling or souring.
- Use a clean pipette for each sample of milk or cream and ensure that no pipette is contaminated with saliva.
- Use only distilled water throughout the test.

7.8.6 Results

1. Give results in micro g p-nitrophenol or absent/present.

Disc reading (30 minutes):

0	=	Properly pasteurised
6	=	Doubtful
≥10	=	Unpasteurised

2. Samples giving a reading of 6 must be replaced in the water bath for a further 90 min incubation period. The two test results are interpreted as follows:

Disc reading after two hours:

0–10	=	Properly pasteurised
>10 to 18	=	Slightly under-pasteurised
>18 to 42	=	Under-pasteurised
>42	=	Grossly under-pasteurised

7.8.7 Alternative methods

Some processors use phosphatase strips as an indicator before using the official method.

See Test Methods and Interpretation Guides on DVD

7.9 Milk composition determination with milk analyser

7.9.1 In-house testing

Determination for protein, fat, lactose, non-fat solids and added water can be performed with a milk analyser. To calibrate equipment, reference samples must be obtained from an accredited laboratory. Calibration must be done at least every third month. Cleaning instructions for the different instruments must be followed according to the recommendations of the supplier of the equipment. See specifications for milk composition in Chapter 3.

Chapter 3

! Monthly verification of samples must be done by an accredited laboratory.

7.10 Butterfat determination – Gerber-method

7.10.1 Purpose

The Gerber-method is the legally accepted method for the determination of the percentage butterfat in milk.

The test is used to determine the butterfat content of raw milk and pasteurised milk.

7.10.2 Background

Sulphuric acid is used to destroy the fat emulsion in order to separate the fat from the water phase. The mixture is centrifuged at a high speed to separate the fat and the water phases.

The scale on the butyrometer is calibrated so that the fat can be read off directly at 65°C. The addition of amyl alcohol prevents foaming and shows the separation of the fat and water phases clearly.

7.10.3 Reagents

- Sulphuric acid (H2S04) for the milk test ("Dairy grade") with specific gravity of 1,820 to 1,823.
- Certified amyl alcohol for the milk test.

7.10.4 Apparatus

- Gerber butyrometers with stoppers and key.
- 10 ml and 1 ml safety pipettes or automatic dispenser (Kips) for dispensing.
- Centrifuge for centrifuging the Gerber butyrometers.
- Rack for butyrometers.
- Water bath (temperature 65°C).
- 10,77 ml volumetric pipette calibrated at 20°C.
- Thermometer.

7.10.5 Method

1. Heat samples to 20°C in water bath.
2. Mix thoroughly.
3. Use safety pipette to transfer 10 ml sulphuric acid to the butyrometer.
4. Add 10,77 ml milk with the volumetric pipette to the butyrometer.
5. Add 1 ml of amyl alcohol using a safety pipette.
6. Lock the butyrometer with a special stopper and key.
7. Mix the contents of the butyrometer by shaking it and inverting the butyrometer at least twice.
8. The butyrometers with their stoppers facing down (towards the outside of the centrifuge) are kept in this position until the readings are taken. Ensure that centrifuge is balanced!
9. Centrifuge for five minutes.
10. Remove the butyrometers from the centrifuge.
11. Both the butyrometers with their stoppers facing down are kept in water for five minutes at 65°C. Ensure that the water level is higher than the fat columns in the water bath.
12. Remove the butyrometer from the water bath.
13. Use the key to manipulate the fat column until it reaches a position where a convenient full calibration reading like 0,0; 2,0 can be taken.
14. Take the reading next to the lower part of the meniscus of the fat column.
15. To obtain the correct reading the lower reading must be subtracted from the higher reading. The length of the column is read and given in fat.

7.10.6 Results

To obtain the correct reading the lower reading must be subtracted from the higher reading. The length of the column is read and given as percentage fat.

8. Microbiology in food systems

8.1 Adverse and beneficial effects of microorganisms

Microorganisms are of significance in food systems because they have both adverse and beneficial effects – they can cause spoilage and illness, but they are also used to manufacture a variety of foods through fermentation.

8.1.1 Spoilage

Most foods undergo losses in desirability as a result of changes in appearance, texture, odour and taste during storage. In many foods these changes are the result of the activity of microorganisms. Many present preservation procedures are designed to either stop or at least control the growth of spoilage microorganisms.

8.1.2 Food-borne illness

Microorganisms capable of causing illness are called pathogens. *Salmonella, Staphylococcus aureus, Shigella, Clostridium botulinum* and *Escherichia coli* 0157:H7 are examples of these pathogens. Typical symptoms of food-borne disease include diarrhoea, dehydration, nausea, vomiting and sometimes fever. As new products and processing methods become available to the food industry, they need to be evaluated for microbiological safety to prevent outbreaks of food-borne illnesses.

8.1.3 Fermentation

Fermentation occurs when microorganisms grow in food and cause desirable changes. It can occur in both food originating from animal products (e.g. sausage, cheese) and plant products (e.g. pickles, bread).

8.2 Kinds of microorganisms

Microorganisms of significance in food include bacteria, yeasts and moulds. Their individual cells cannot be seen without the aid of a microscope.

8.2.1 Bacteria

Bacteria are unicellular organisms that can be differentiated on the basis of their shape. Spherical bacteria are called cocci; rod-shaped cells are called bacilli and curved and corkscrew-shaped bacteria are called spirilla. These cells are arranged in chains, clumps or organised clusters. Bacteria are important because although some types cause food-borne illnesses and food spoilage, other types can produce new food products through fermentation.

8.2.2 Yeasts

Yeasts can be distinguished from bacteria under a microscope by the yeasts' larger cell size, their oval, elongated, elliptical or spherical cell shape and the budding process of some yeasts during cell division. While yeasts in food are not considered pathogens, they are important food spoilage organisms. The growth of yeast in food can cause a film, discolouration, the formation of gas or bubbles, off-odour and/or off-flavours. In fermented dairy products like maas and yoghurts yeast contamination is a problem. Yeasts are also beneficial through their participation in food fermentation producing products such as bread, wine and beer.

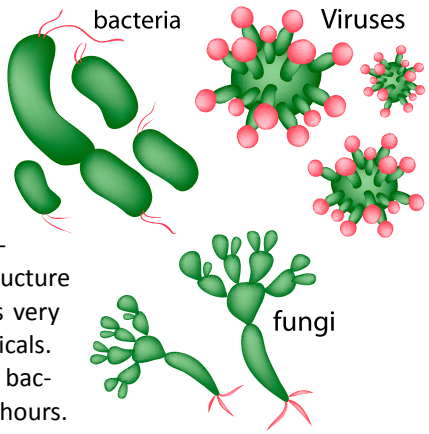
8.2.3 Moulds

Mould growth usually results in a visible fuzzy mass of filaments. Individual mould cells can be seen only with the aid of a microscope. Moulds are often associated with spoilage in food and some produce toxins that have caused cancer in test animals. Moulds may, however, also play a beneficial role in the fermentation of dairy, meat and plant products.

8.3 Reproduction

Microorganisms reproduce in different ways.

8.3.1 Bacteria



Bacteria reproduce by a process termed binary fission, a transverse (side-to-side) division across the cell which forms two cell walls. This is normally a rapid process, doubling the population every 15 to 30 minutes under optimal conditions. Certain bacteria have the ability to produce a structure called an endospore. The spore is very resistant to heat, cold and chemicals. For example, the spores of certain bacteria can survive boiling for six hours. When conditions are favourable for growth again, the spore will germinate and the bacterial cell will again divide.

8.3.2 Yeasts

Yeasts usually reproduce by budding. A small part of the cell wall swells out and a wall of cellulose soon shuts off this new growth from the parent cell. It becomes an independent cell, soon growing other buds.

8.3.3 Moulds

Moulds usually reproduce by producing asexual spores. When the spore settles on a food substance, it swells and begins to grow by producing thread-like filaments called hyphae. As the mould matures, upright hyphae are produced that contain the spores. These spores are eventually released and are carried away by air currents.

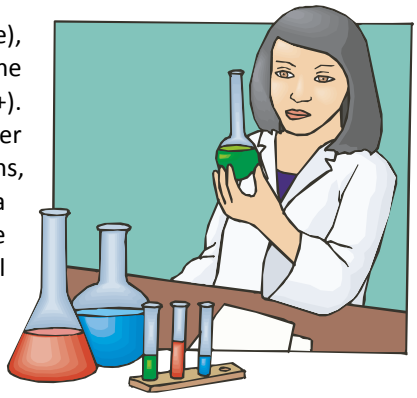
8.4 Factors affecting microbial growth

Various factors affect the growth of microorganisms. These include food (nutrients), pH, temperature, time, oxygen and moisture.

8.4.1 Food

Like humans, microorganisms need protein, carbohydrates and vitamins for energy, cell growth, maintenance and reproduction. The destruction of food by microorganisms is a natural, normal function of the ecology of all living organisms. Microorganisms capable of spoiling food are found everywhere in the soil, water and air; on the skins and hides of animals and feathers of birds and in the intestines and all other cavities of the animal body. Microorganisms generally are not found in healthy living tissue, but they are always present in the environment to invade the flesh of plants and animals if the skin is broken or weakened by disease or death.

8.4.2 Acidity



Whether a pH is basic (alkaline), acidic or neutral depends on the hydrogen ion concentration (H+). An acidic substance has a higher concentration of hydrogen ions, while an alkaline substance has a higher concentration of hydroxide ions (OH-). Pure water has an equal concentration of both ions, i.e., (H+) = (OH-). pH is measured on

a pH scale of 0 to 14, with neutral pH being 7. The pH is acidic if it is lower than 7 and alkaline if it is higher than 7. The more acidic, the lower the number on the scale. Foods with an acid pH usually have a sour taste. While sour is a generally acceptable taste, products with an alkaline pH are bitter and often rejected. Virtually all foods are either neutral or acidic.

The concentration of hydrogen ions in the food affects the growth and survival of microorganisms. Although most microorganisms can survive in the pH range of 4,6 to 9,0, most grow best near pH 7. Typically, moulds and yeasts are able to grow at pH levels lower than those tolerated by bacteria. Therefore, moulds and yeasts are the primary spoilage organisms on acidic (low pH) foods such as maas, yoghurt, cheese, pickles, tomatoes and oranges.

8.4.3 Temperature

Bacteria can grow in many different temperature ranges. However, most grow best between 16 and 44°C. Since the human body temperature (37°C) is within this range, pathogens that invade the body will find an ideal temperature for growth. Other bacteria prosper at 0 to 7°C. These bacteria are called *psychrotrophs* and are responsible for the bacterial spoilage of refrigerated foods. Although most moulds and yeasts grow best at room temperature, many grow well at refrigeration temperatures. Freezing food will stop the growth of most microorganisms but will not kill them. When the food thaws, they are again able to grow and cause spoilage or illness.

8.4.4 Oxygen

Different types of microorganisms have different oxygen requirements. Aerobic organisms must have oxygen if they are to survive. *Microaerophilic* organisms grow best when the concentration of oxygen is low. Anaerobic organisms cannot grow if oxygen is present. *Facultative anaerobes* can grow with or without oxygen. While different kinds of bacteria have different oxygen requirements, yeasts are facultative anaerobes and moulds are nearly all aerobic.

8.4.5 Time

Under favourable conditions, such as enough moisture, food and the desired temperature, bacteria reproduce by doubling every 15 to 30 minutes. The time it takes for a microbial cell to double is called the *generation time*. The longer the generation time, the slower the growth. A large number of microorganisms (more than one million) can result from one microbial cell within five hours, if the generation time is 15 minutes. If the generation time is increased to 30 minutes, the resultant population would be only 1 024 after five hours.

Time	No. of micro-organisms
0 min	1
15 min	2
30 min	4
45 min	8
1 hour	16
2 hours	256
3 hours	4 096
4 hours	65 536
5 hours	1 048 576

8.4.6 Moisture

All microorganisms need water to grow. Water activity a_w describes the degree of availability of water in different foods and a_w can be lowered by the addition of salt or sugar. These food constituents bind water, making it unavailable for use by microorganisms. Moisture requirements vary among microorganisms. Typically, bacteria require a higher a_w than yeasts, which require higher a_w than moulds.

8.5 Fermentation

The fermentation process occurs when microorganisms consume organic materials as part of their own life processes.

Today, food scientists prepare fermented food products under controlled conditions, using specific microorganisms or mixtures of organisms known as starter cultures. *Streptococcus, Lactococcus* and *Leuconostoc* species are used in milk starter cultures to produce the acid and flavour compounds in sour cream, buttermilk, fresh cheeses (such as cottage cheese and cream cheese) and ripened cheeses (such as Cheddar, Gouda and Edam). *Lactobacillus* and *Streptococcus* species are used in the production of yoghurt.

Other microorganisms may also be added along with the lactic acid bacteria. The addition of specific bacteria that produce propionic acid results in the formation of the nutty flavour and "eyes" found in Swiss cheese. The unique colour and flavour of blue cheese is due to the addition of *Penicillium roqueforti* mould. The lactic acid bacteria used for fermented sausage products are typically *Pediococcus, Micrococcus, Leuconostoc* and *Lactobacillus* species. Some naturally present mould species also assist in the fermentation of aged dry salami and country cured hams.

The production of acid and other metabolic compounds by the microorganisms provides desirable tastes and textures and improves the retention of the quality of foods. Different microorganisms can produce a wide range of desirable and undesirable breakdown products. Proteolytic organisms break down proteins and often produce putrid and rotten odours which are usually considered undesirable. Similarly, organisms that produce enzymes that attack fats (lipolytic organisms) give rise to rancid and fishy odours and are also considered spoilage organisms. The fermentative organisms of choice are those capable of converting carbohydrates into alcohols, acids and carbon dioxide. These metabolic end products contribute desirable flavour and texture to foods. In addition, the acids and alcohols produced by the microorganisms can suppress the growth of the lipolytic and proteolytic spoilage organisms.

9. Microbiological analysis

9.1 Plate count method

R 1555 Annexure B

This method is used for the microbiological testing of milk, cream and dairy products. This method includes the determination of the total bacterial count, coliforms and *E. coli.*

The tests will be conducted in appropriate cases in order to ascertain the suitability of milk, cream and dairy products for human consumption. Samples shall not be frozen but shall be kept at a temperature below 5°C and shall be tested within 48 hours of collection – provided that these requirements shall not apply to dry dairy products, sterilised milk, UHT

milk and condensed dairy products in their unopened containers. For the purpose of testing, “milk” shall include milk that has undergone pasteurisation or sterilisation or ultra-high temperature treatment and includes cream.

9.1.1 Reagents

- Plate count agar.
- Violet bile agar with MUG (4-methylumbelliferyl B-D-glucuronide) for coliforms and *E.coli.*
- Diluents: Phosphate buffer solution.
  Potassium dihydrogen orthophosphate – 5,08 g.
  Disodium Hydrogen orthophosphate in 2 l distilled water – 13,63 g.
  Sterilise for 15 minutes at 121°C.
  The final pH of buffer should be between 7,0 ± 0,1 at 25°C.

9.1.2 Method

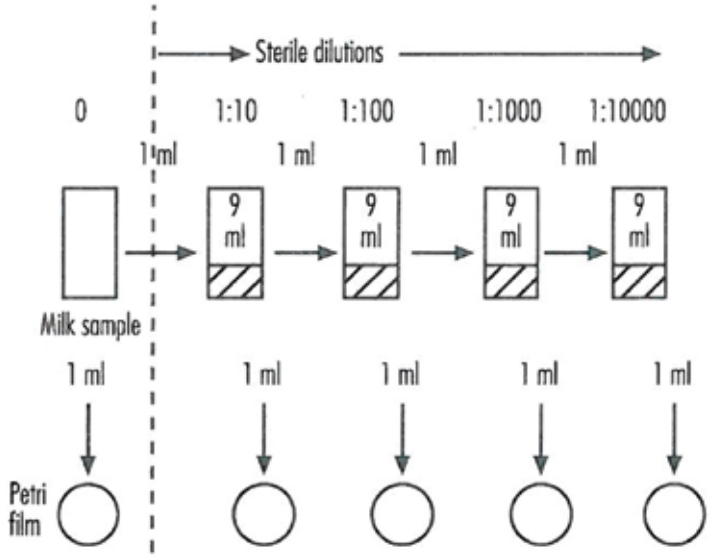
9.1.2.1 Total bacterial count (TBC)

1. Mix milk or cream thoroughly before sampling from bulk.
2. Prepare 1:10 dilution by adding 1 ml to 9 ml of sterile phosphate buffer. Mix well.
3. Prepare a 1:100 dilution by adding 1 ml of the 1:10 dilution to 9 ml of sterile phosphate buffer. Mix well.
4. Prepare a 1:1 000 dilution by adding 1 ml of the 1:100 dilution to 9 ml of sterile phosphate buffer.
5. Using a fresh pipette, transfer 1 ml of each of the dilutions at least in duplicate to sterile petri dishes, beginning with the highest concentration and ending with the lowest.

6. To each dish add 10 ml of the standard plate count agar which has been melted beforehand and cooled to 45°C ± 1°C.
7. Mix the contents of each dish thoroughly using horizontal rotational movement while the medium is still fluid.
8. Once the medium has set, invert the dishes and incubate at 30°C ± 1°C for 72 ± 2 hours.
9. At the end of the incubation period remove the dishes from the incubator and count the colony-forming units (CFU’s) with the aid of magnification under uniform artificial illumination.
10. To count the CFU’s of each dish, spreader-free dishes containing 30 to 300 CFU’s are selected; count all the CFU’s and calculate the number of CFU’s per ml or per gram.
11. If the number of CFU’s of each dish exceeds 300, count the CFU’s in portions of the dish representative of the CFU distribution and use this count to determine the total number per dish. Proceed as in (7) above, but record as an “estimated” plate count.
12. The presence of very high concentrations of colonies results in the entire growth area of the film becoming red or pink in colour and/or numerous bacteria growing on the edges of the growth zone. Report these as too numerous to count (TNTC).

See 3M Petrifilm Method and Interpretation Guide under 3M on DVD

Preparations of dilutions



9.1.2.2 *E. coli* and coliforms

1. Use a pipette to transfer 1 ml of the liquid product or the appropriate dilutions to the centre of two petri dishes. Touch a dry area in the petri dish with the tip of the pipette. Use a fresh pipette to inoculate each dilution.
2. Pour about 15 ml of the VRB MUG agar at 45°C ± 1°C into each petri dish.
3. Mix immediately after pouring by rotating the petri dish sufficiently to obtain evenly dispersed colonies after incubation. Allow to solidify on a cool horizontal surface.
4. After complete solidification, pour about 4 ml of the VRB agar at 45°C ± 1°C onto the surface of the inoculated medium and allow to solidify.
5. Prepare a control dish with 15 ml of the medium to check its sterility.
6. Incubate the plates in an adverted position. Do not stack them more than six high. Stack of plates should be separated from one another and from the sides and top of the incubator. Incubate at 30°C ± 1°C for 24°C ± 2 hours.
7. Examine the plates under a 366 nm ultraviolet light. All colonies showing a blue fluorescence in the surrounding medium are counted. Then examine the plates under normal light and count the coliform organisms.
8. Select the plates with more than 10 and fewer than 150 colonies. Count the dark red-coloured colonies with a diameter of at least 0,5 mm, characteristic of coliform organisms. These dark pink to red colonies are usually surrounded by a red zone in the medium.
9. Calculate the number of coliform organisms per gram or per milliliter, taking into account the result of the confirmatory test. Five or more fluorescent colonies are regarded as positive for *Escherichia coli.*
10. The confirmatory test is done by inoculating five colonies of each type, if available, into tubes of brilliant green lactose bile broth containing a Durham tube and incubating at 30°C ± 1°C for 24°C ± 2 hours. Consider colonies that show gas formation in the Durham tube to be coliform organisms.

Important note: all sample bottles and pipette tips (1 ml and 0,1 ml) must be sterilised for the dry rehydrated film or petri dish methods. Dilution water (phosphate buffer and Ringer's solution) can be

measured into portions in the dilution bottles or test tubes and be sterilised. For 9 ml dilutions use 10 ml to allow for evaporation. See Section 9.6 below.

Pathogen determination should be conducted at an accredited laboratory.

9.2 Dry rehydrated film method

9.2.1 Reagents

- Dry rehydrated film for coliforms and *Escherichia coli* counts.
- Dry rehydrated film for standard colony count.
- Diluents: Phosphate buffer solution.
  Potassium dihydrogen orthophosphate – 5,08 g.
  Disodium Hydrogen orthophosphate in 2 l distilled water – 13,63 g.
  Sterilise for 15 minutes at 121°C.
  The final pH of buffer should be between 6,6 and 7,0.

9.2.2 Method

9.2.2.1 Total bacterial count (TBC)

Dry rehydrated film method for standard colony count.

1. Mix milk or cream thoroughly before sampling from bulk.
2. Prepare 1:10 dilution by adding 1 ml to 9 ml of sterile phosphate buffer. Mix well.
3. Prepare a 1:100 dilution by adding 1 ml of the 1:10 dilution to 9 ml of sterile phosphate buffer. Mix well.
4. Prepare a 1:1 000 dilution by adding 1 ml of the 1:100 dilution to 9 ml of sterile phosphate buffer. Refer to Figure at end of 9.1.2.1.
5. Place the films for aerobic bacterial counting on a flat surface and label them. Lift the top film and carefully transfer 1 ml of the 1:1 000 dilution to the centre of the bottom film by holding the pipette perpendicular to the film. Release the top film to drop onto the sample. Repeat the process with the 1:100 dilution of the sample.
6. Distribute the sample evenly on the film by applying gentle downward pressure with a spreader. Remove the spreader and leave the film undisturbed for one minute to solidify. Stack the films in piles of not more han 20 and incubate the films with the clear sides up at 32°C ± 1°C for 48 ± 2 hours.
7. Remove the films from the incubator at the end of the incubation period and count the colony forming units (CFU's) under uniform artificial illumination as follows:
   a. All the red colonies, regardless of their size and intensity, should be counted. Films with 25 to 250
   b. CFU's should be counted. Calculate the number of viable bacteria per millilitre of milk.
   c. An estimated count can be made on films with the CFU's exceeding 250 by counting one and multiplying this count by 20. Calculate the number of viable bacteria per millilitre of milk and record as an "estimated" amount.
   d. The presence of very high concentrations of colonies results in the entire growth area of the film becoming red or pink in colour and/or numerous bacteria growing on the edges of the growth zone. Report these as too numerous to count (TNTC).

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9.2.2.2 *E. coli* and coliforms

1. Mix milk thoroughly before sampling from bulk.
2. Place the films for *E. coli* and coliform counting on a flat surface and label them. Lift the top film and transfer 1 ml of the milk to the centre of the bottom film by holding the pipette perpendicular to the film.

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3. Slowly roll the top film onto the sample to prevent air bubbles being trapped under the top film.
4. Distribute the sample evenly on the film by applying gentle downward pressure with a spreader. Remove the spreader and leave the film undisturbed for one minute to solidify.
5. Stock the films in piles of no more than 20 and incubate the films, with the clear sides up at 32°C ± 1°C for 24 ± 2 hours.
6. At the end of the incubation period remove the films from the incubator and count the colonies with the aid of magnification under uniform artificial illumination as follows (re-incubate films for a further 24 ± 2 hours to detect any additional *E. coli* growth):
   a. Blue colonies associated with gas are *E. coli.*
   b. Red colonies associated with gas are coliform colonies. Colonies that are not associated with gas are not counted as coliform colonies. All the red and blue colonies with gas represent the coliform colony count.
   c. Films with 15 to 150 colonies should be counted. An estimated count can be made on films where the colonies exceed 150 by counting at least one and multiplying this count by 20. Calculate the number of viable coliform colonies per millilitre of milk and report it as an "estimated" coliform colony count.
   d. The presence of a very high concentration of colonies causes the entire growth area of the film to become purple blue (E. coli) or reddish (coliforms) and/or many small colonies and/or small gas bubbles to be present. This must be recorded as TNTC.

Important note: All sample bottles, and pipette tips (1 ml and 0,1 ml) must be sterilised for the dry rehydrated film or petri dish methods. Dilution water (phosphate buffer and Ringer's solution) can be measured into portions in the dilution bottles or test tubes and be sterilised. For 9 ml dilutions use 10 ml to allow for evaporation. See Section 9.6 below.

! Pathogen determination should be conducted at an accredited laboratory.

9.3 Sterilisation of laboratory equipment

Standard Methods for the Examination of Dairy Products **(1992). 16th edition, pp 98-100.**

9.3.1 Purpose

Equipment used in the microbiology laboratory must be sterilised. Various forms of sterilisation can be used. The following methods of sterilisation are recommended:

- Steam sterilisation (autoclave/pressure cooker).
- Hot air sterilisation (oven). Standard Methods for the Examination of Dairy Products (1992). 16th edition, pp 98-100.

9.3.2 Apparatus

- Autoclave or pressure cooker.
- Dry oven.

9.3.3 Methods

- Steam sterilisation using an autoclave or pressure cooker at 121°C for 15 minutes:
  1. Place equipment to be sterilised on a supporting rack. Place glass equipment in metal (holding) containers.
  2. Ensure that enough water is in the autoclave or pressure cooker. Start heating the autoclave or pressure cooker.
  3. Before allowing steam pressure to rise, automatically or manually expel all air from the steriliser through an exhaust valve.
  4. When the desired steam pressure is reached in the autoclave, start

the timer. If using a pressure cooker, wait until the desired pressure level is reached, namely when the steam starts releasing and the weight starts to hop up and down.
5. After 15 minutes switch off the heat source of the equipment.
6. Leave the equipment to cool and wait until the safety valve indicates that there is no pressure in the equipment.
7. Open the autoclave or pressure cooker.

- Dry hot air, using a dry oven:
  1. Wash glass equipment, e.g. glass pipettes, and let them drip-dry.
  2. Wrap pipettes in aluminium foil (two layers).
  3. Sterilise in dry oven at 170°C for at least two hours.

9.3.4 Caution – important information for effective sterilisation

Dry heat

Sterilise equipment with dry heat in hot sterilisers or ovens so that the materials at the centre of the load are heated to not less than 170°C for not less than one hour. This usually requires exposure for about two hours at 170°C. When the oven is loaded to capacity, use a longer period or a slightly higher temperature.

Steam sterilisation

1. Slightly loosen stoppers to allow passage of steam into and air out of closed containers when autoclaved.
2. Ensure that the load is loosely packed.
3. For efficient sterilisation, separate containers by at least 1 cm in all directions.
4. Before allowing steam pressure to rise automatically or manually, expel all air from the steriliser through an exhaust valve.
5. If manually, ensure that all air has been expelled and that only steam is being let off before pressurisation begins. (If air remains within the chamber, a 15 pounds per square inch gauge pressure can mean a temperature as low as 100°C.)
6. Because the temperatures obtained at a constant pressure of saturated steam will vary according to atmospheric pressures, it is important to rely on a properly operating and calibrated thermometer (not a pressure gauge) to ensure effective sterilisation.
7. Avoid overloading autoclaves so that the rate of air exhaust or heating is not appreciably delayed.
8. The volume of the load generally should not exceed 30 to 40% of the volume of the autoclave.
9. The autoclave should reach 121°C slowly but within 15 minutes.
10. A steam flow that is too slow also results in decreased efficiency because air-steam mixtures form.
11. When non-liquid materials with slow heat conductance are to be sterilised or where the packing arrangement or volume of materials otherwise retards penetration of heat, allow extra time for materials to reach 121°C before beginning to time the sterilisation period and, if necessary, use longer sterilisation periods to ensure sterility.
12. After sterilisation, gradually reduce pressure in the autoclave (not less than 15 minutes is recommended) because liquids may be at temperatures above their boiling point at atmospheric pressure.
13. Liquids can be lost through boiling when the pressure is lowered too rapidly.
14. When dry materials such as sampling equipment or empty sample bottles are being sterilised, pressure may be released rapidly at the end of the 15 minutes holding period at 121°C.

15. Used petri dishes, pipettes, tubes, etc. must be routinely decontaminated in microbiological laboratories. Dairy products, especially raw milk, may harbour potential pathogens such as staphylococci and streptococci.
16. The same principles apply for loading the autoclave and sterilising used materials, e.g. used petri dishes as for sterilising media.

9.3.5 Sterility

Autoclave/pressure cooker performance should be monitored either by:

- Special autoclave tape.
- Spore strips or spore suspensions.

9.4 Hygiene verification

See Alternative Hygiene Verification Methods on DVD

a. Hand swab determination can be done using commercially available swabs.
b. Rinse water determination: plate 1 ml of sample of rinse water directly onto the dry rehydrated film and incubate.
c. Contact surface determination: use the custom-made commercial swabs and plate directly onto dry rehydrated film.