

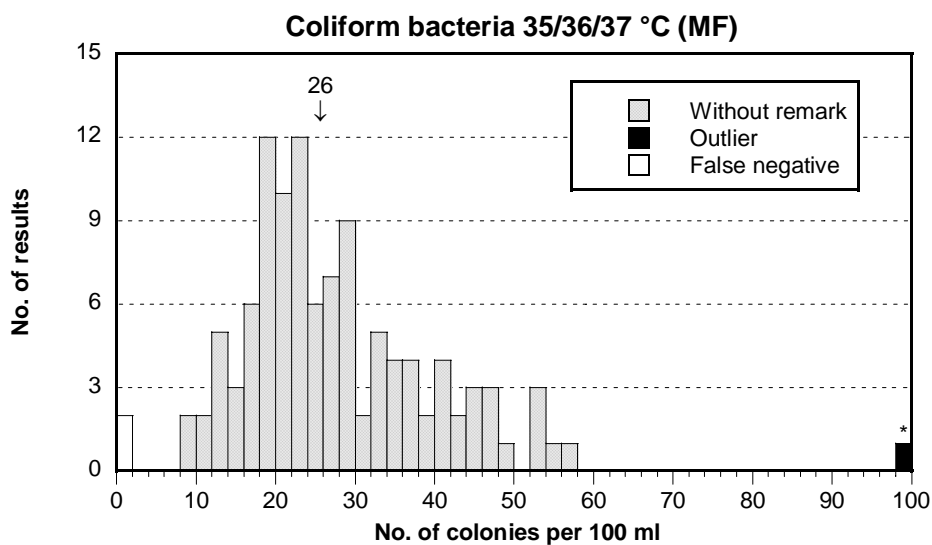
## PROTOCOL

## Microbiology

*Drinking water*

&

*Food*



A resolution on accreditation of the activity was made 2004-12-10



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**Proficiency Testing**  
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## Contents

1. Introduction.....	1
2. Organisation.....	1
2.1. Design of the activity.....	2
2.2. Scheme information present on the Internet ( <a href="http://www.slv.se/absint">www.slv.se/absint</a> ).....	2
2.2.1. The public part.....	2
2.2.2. Participant part accessible by login.....	2
2.3. Areas of responsibility.....	3
2.4. Advisory group.....	3
2.4.1. Duties.....	3
2.4.2. Members.....	3
2.5. Accreditation.....	3
3. The two microbiological schemes.....	4
3.1. The Drinking water scheme.....	4
3.2. The Food scheme.....	4
3.3. Target dates and analytical parameters for a testing round.....	4
4. The test material.....	5
4.1. General.....	5
4.1.1. Type of material.....	5
4.1.1.1. Advantages.....	5
4.1.1.2. Disadvantages.....	5
4.2. Production and control.....	6
4.2.1. Composition.....	6
4.2.2. Manufacture.....	6
4.2.3. Quality controls.....	7
4.2.3.1. Amount of test material.....	7
4.2.3.2. Concentration determination.....	7
4.2.3.3. Homogeneity.....	7
4.2.3.4. Vacuum test.....	7
4.2.3.5. Stability.....	8
4.3. Storage and usage.....	8
4.3.1. How should the material be stored.....	8
4.3.2. What should the material not be used for.....	8
4.4. Hazards.....	8
4.4.1. Risk of infection.....	8
4.4.2. Environmental danger.....	9
4.5. Before a proficiency test.....	9
4.5.1. Randomisation of test items to laboratories.....	9
4.5.1.1. Numbering of vial labels.....	9
4.5.1.2. Selection of vials for a laboratory.....	9
4.5.2. Package of test items.....	9
4.5.3. Transport of test items.....	9
4.6. Handling of the test material.....	10
4.6.1. Storage.....	10

4.6.2. Preparation of samples .....	10
4.6.3. Destruction of test material .....	10
4.6.3.1. Unopened vials containing test material.....	10
4.6.3.2. Opened and used vials .....	10
4.6.3.3. Remains of prepared sample .....	10
5. Instructions at delivery of test material .....	11
5.1. Target dates .....	11
5.2. Analyses .....	11
5.3. Other information in the instructions .....	11
6. Participant activities in a testing round .....	11
6.1. Instructions and analyses.....	11
6.2. Reporting analytical results .....	12
6.3. Reporting method information .....	12
6.4. Adjusting a reported result .....	12
6.4.1. Responsibilities of participating laboratories .....	12
6.4.2. Accepted adjustments.....	13
7. Follow-up of analyses.....	13
8. Statistics.....	14
8.1. General .....	14
8.2. Determination of concentration and homogeneity .....	14
8.2.1. The Food scheme.....	15
8.2.1.1. Initial check of concentrations.....	15
8.2.1.2. Concentration determination and homogeneity test, quantitative analysis .....	15
8.2.1.3. Determination of concentration and homogeneity test, qualitative analysis .....	15
8.2.1.4. Criteria for homogeneity .....	16
8.2.2. The Drinking water scheme.....	16
8.2.2.1. Initial check of concentration .....	16
8.2.2.2. Determination of concentration and homogeneity test.....	16
8.2.2.3. ANOVA.....	17
8.2.2.4. “Index of dispersion” – check of randomness .....	17
8.2.2.5. Criteria for homogeneity .....	17
8.3. Outliers .....	17
8.4. False positive and false negative results.....	18
8.4.1. False positives – definition .....	18
8.4.2. False negatives – definition .....	18
8.5. Statistics for an analysis .....	18
8.5.1. Median .....	18
8.5.2. Mean value / Assigned value .....	19
8.5.3. Standard deviation .....	19
8.5.4. Coefficient of variation (CV) .....	19
8.5.5. Standard values (z-scores).....	19
8.5.6. Interpretation of z-scores .....	20
8.6. Histograms.....	20

8.7. Box plots .....	20
9. Confidentiality and user identity .....	22
9.1. Confidential laboratory number .....	22
9.2. Confidential password.....	22
9.3. Usage of laboratory number and password.....	22
9.4. Changing the laboratory number and password.....	22
10. Filing .....	22
11. Complaints and other contacts with clients.....	23
11.1. Policy .....	23
11.2. Definition .....	23
11.3. Handling of remarks that are not complaints .....	23
11.4. Points of view made by participants .....	24
12. Conditions and obligations.....	24
12.1. General conditions for participation .....	24
12.1.1. Who can participate.....	24
12.1.2. Which methods may be used.....	24
12.1.2.1. Food .....	24
12.1.2.2. Drinking water .....	24
12.1.3. Fee .....	25
12.2. Other obligations of participating laboratories .....	25
12.3. Obligations of the National Food Administration.....	25
12.4. Limited responsibility .....	25
13. Participation costs .....	25
14. This protocol .....	26
15. References.....	26



## 1. Introduction

Laboratories that perform analyses need to know that they obtain reasonable results. They also need to be able to prove this to their clients in order to be trustworthy. They may obtain this knowledge by performing different kinds of controls. Internal controls may be used in order to see that no unexpected changes have occurred within the laboratory. Since microbiological analytical results to some part are dependent on which analytical method was used, it is also important to be able to compare ones analytical results with those of other laboratories. One way is to participate in some kind of interlaboratory comparisons. Such comparative tests are compulsory for laboratories that aspire for or are accredited for their analyses, if they are available. This is e.g. the case according to the standard EN ISO/IEC 17025 (1) where the name Proficiency Testing is used. These interlaboratory comparisons are named differently in different languages.

Proficiency testing is normally arranged by a third, independent party in relation to the laboratories and their clients. The evaluations are made by this third part and the process is an external assessment of the analytical competence of the laboratory.

The National Food Administration in Sweden provides such tests, in among others, the areas Food microbiology and Drinking water microbiology.

The purpose with this protocol is to give participants, other laboratories and other interested a description on how this microbiological proficiency testing activities are organised, and how the basic actions are performed. In particular are those parts described, of which corresponding information is not available by other means. This applies, for example, to production and handling of test material and statistical processing.

General information regarding the proficiency testing, as well as specific information about testing rounds is available on the website for the schemes.

## 2. Organisation

The National Food Administration is the central Swedish authority for food issues, including drinking water.

**Address:** The National Food Administration  
Box 622  
SE-751 26 Uppsala  
Sweden  
Telephone: +46 (0)18 17 55 00

*The Microbiological Division* within the department of Research and Development organises the microbiological proficiency testing.

## **2.1. Design of the activity**

The proficiency testing activity is divided into one scheme for Food and one scheme for Drinking water.

E-mail address for questions and opinions concerning the schemes:  
***PT-micro@slv.se***

## **2.2. Scheme information present on the Internet ([www.slv.se/absint](http://www.slv.se/absint))**

The schemes are described in general terms on the website [www.slv.se/absint](http://www.slv.se/absint).

The website is composed of two parts each with a number of pages, one public part and one participant part, which has to be logged into with a user identity.

### **2.2.1. The public part**

This part contains pages that deal with:

- the proficiency testing activity in a general perspective, including the most recent edition of this document as a pdf-file
- basic information regarding the drinking water and food scheme, respectively, e.g. which analytical parameters they comprise
- what is included, along with target dates for each testing round
- conditions for participation
- current prices
- addresses for contact with the National Food Administration as provider, including a web form for registering participation in a scheme
- login to the pages that are available to participants only

### **2.2.2. Participant part accessible by login**

This part comprises:

- one page for reporting of analytical results from the current round
- one page with a web form, by which information for each method is registered directly in our data base
- one page where reported results and calculated results from previous rounds are presented in one of three possible ways: 1) as preliminary results shortly after finished rerecording of results (mainly used for check of reported results and comparison with preliminary statistics), 2) as preliminary results during processing of results, when reported results and preliminary median values are presented only, 3) as final results when a proficiency test has been fully evaluated and the laboratory results and the statistics correspond to what is presented in the final report.
- one page for electronic cancellation of participation in coming rounds

- one page with reports and information to the participants (e.g. information letters) available as printable pdf-files.

### ***2.3. Areas of responsibility***

For each scheme, there is a *person responsible for the scheme*, who has the overall responsibility but also specific responsibility for e.g. planning, correspondence and reporting. For each scheme, there is also a *person doing the laboratory work*, who is responsible for manufacture and quality checks of samples, and packaging before dispatch. There is also an *administrator* to support the activity. The administrator handles the register of addresses, contacts regarding invoices, and laboratory contacts regarding e.g. participation.

The person responsible for respective scheme is also responsible for the statistical processing.

### ***2.4. Advisory group***

For each scheme, there is an advisory group composed of representatives from countries with the largest numbers of participating laboratories, presently the Nordic countries. The advisory group usually assembles once a year in Uppsala. They congregate as one group and use the first half of the day to discuss issues that the two schemes have in common. The other half of the day is used for issues specific for the two individual schemes.

#### **2.4.1. Duties**

The members should first and foremost represent their countries and to a lesser extent those organisations they represent. Their role is mainly advisory, but with influence on included analytical parameters, frequency, costs, accepted methods and the content of the reports. Major changes within a scheme should be sanctioned by the corresponding advisory group.

#### **2.4.2. Members**

Current rolls of the group members are enclosed with the annual letter that is published as information on the page Info & Reports on the participant part of the website.

### ***2.5. Accreditation***

The Microbiological Division is accredited for arranging microbiological proficiency testing since December 2004. The accreditation was executed by Swedac, and performed according to the documents ISO/IEC Guide 43-1, 1997 (2) and relevant sections of EN ISO/IEC 17025 (1; current edition). In practise, the accreditation may be considered to be subordinated to the guide ILAC G:13,

(3; current edition). The overall responsibility to keep up the quality and the quality system on a high level lies on the head of the division through the appointed quality coordinator.

### **3. The two microbiological schemes**

#### ***3.1. The Drinking water scheme***

The Drinking water scheme has been ongoing with participants from several countries since 1992.

The scheme comprises 2-4 mixtures 2 times a year. It includes ca 10 quantitative analytical parameters – bacteria, mould and yeast – with concentration on indicative organisms, including some that may be pathogenic.

Which the parameters are is presented on the page General Information, Drinking water scheme, on the website [www.slv.se/absint](http://www.slv.se/absint).

#### ***3.2. The Food scheme***

The Food scheme has been ongoing with participants from several countries since 1988.

The scheme comprises 3-4 samples 3 times a year. It contains ca 25 different quantitative and qualitative analytical parameters – bacteria, mould and yeast – including the analyses of pathogenic bacteria normally searched for in food.

Which the parameters are is presented on the page General Information, Food scheme, on the website [www.slv.se/absint](http://www.slv.se/absint).

#### ***3.3. Target dates and analytical parameters for a testing round***

As concerns target dates and analytical parameters for current testing rounds, we refer to the page PT rounds on the website [www.slv.se/absint](http://www.slv.se/absint).

Being providers, we will make an effort to stay with the time schedules. In case of unforeseen events, we reserve ourselves the right to postpone a particular round. The participants will be informed about such actions before the original date for that round.

## **4. The test material**

### ***4.1. General***

#### **4.1.1. Type of material**

Natural samples or for the purpose manufactured samples may be used in proficiency testing. A variant in between, where the test organism is added to a natural test material ("spiking"), may also be used at times.

The National Food Administration has chosen to use manufactured samples for the microbiological proficiency testing. They are composed of simulated food or drinking water samples containing mixtures of organisms, and there is a certain purpose with each test material. Depending on the parameters of the round, they may include both bacteria and fungi (mould and/or yeast). Some mixtures include pathogenic bacteria, while others contain organisms possessing specific indicative properties only. No test material containing protozoa or virus is manufactured.

The ready-to-use test material consists of freeze-dried serum broth with different microbial mixtures. The test item consists of 0.5 ml freeze-dried material in a 2 ml glass vial. The material is manufactured according the description by Peterz and Steneryd (4). When the content is dissolved in a specific volume of suitable liquid, the sample, or homogenate concerning food, for testing is obtained.

Freeze-dried artificial test material in vials has a number of advantages compared to natural samples, but also some disadvantages.

##### ***4.1.1.1. Advantages***

- + The vials do not require much space, which facilitates storage, package and transport.
- + The vials are pretty tough and are therefore easy to transport.
- + Several organism groups (analytic parameters) may be tested by the same test item.
- + The test material has good stability; the microbial concentrations do not change appreciably for a long time.
- + It is possible to manufacture a large number of identical sub samples, which have organism concentrations that are Poisson distributed or, at higher concentrations, are log-normal distributed.
- + Expenses are kept low by having a standardised and relatively simple procedure of manufacture.
- + Dissolved test material may be used for "spiking" of natural samples.

##### ***4.1.1.2. Disadvantages***

- The freeze-drying requires a good freeze-drier in which the process can be reasonably controlled.

- The organisms require a protective substance, “cryoprotector”, which helps them survive the freeze-drying.
- The material has to be dissolved in liquid, which takes a certain amount of work where it is possible to make mistakes.
- When dissolving the material, some foam is sometimes formed. Due to this, the sample is likely to be distinguished from real samples, which means that the analyst may know it is a control sample.
- As regards food analyses, natural matrices with preparation of the test material are absent, only analyses of samples similar to a ready-to-use homogenates are included.

## ***4.2. Production and control***

### **4.2.1. Composition**

Microorganisms that have been isolated from foods and water, or been bought from an established culture collection, are in general used in the test material. Cultures of the organisms are kept in a collection at the Microbiology Division, freeze-dried and frozen (–70 °C).

ISB (Inositol Serum Broth) is used as “cryoprotector” in order to protect the organisms when being freeze-dried. ISB is composed of sterile filtrated horse serum mixed with inositol, and a certain amount of nutrient broth. After washing and diluting the nutritious cultures, the component SPG (Saccharose Phosphate Glutamate), which also possesses organism protective characteristics in the freeze-drying process, is added. Peptone water or a potassium phosphate buffer containing MgSO<sub>4</sub> (see e.g. ISO 8199:2005; 5) is used when diluting and washing. The same solutions are best used when dissolving ready-to-use material before analysis.

### **4.2.2. Manufacture**

Each organism is cultured individually in suitable nutrient medium, usually TSB (Tryptone Soy Broth) or BHI (Brain Heart Infusion) Broth/Agar for bacteria and MEA (Malt Extract Agar) for fungi. After the stated period of culturing time the obtained cultures are used. From a specific volume of each culture (or for mould, spore suspension) the nutritious culturing medium is washed or diluted off. Specific volumes of the washed or diluted cultures are transferred to a specific volume of ice-cold ISB, and a mixture of organisms is obtained.

The test mixture is kept stirred on ice while portions (0.5 ml) of the mixture are transferred into sterile glass vials on special racks. The vials are half-way sealed with sterile rubber stoppers before placed on cooled shelves in the freeze-drier. The freeze-drying procedure itself is started when all vials that are to be filled have been placed on the shelves. The procedure takes roughly 20 hours and proceeds according to a fixed program. When it is completed all vials are fully

sealed in the freeze-drier while under vacuum. A pressure equilibration follows by the inlet of air, for the door to be opened and the vials taken out. The vials may be filled with nitrogen gas before sealed, as an alternative to vacuum.

The produced testing material is placed cool (refrigerator or freezer) and tested with respect to its organism content. Vials with satisfactory content are tested for vacuum and sealed with aluminium caps. The test material is thereafter stored in a freezer (–24 alternatively –65 to –70 °C) until dispatch. Labelling is made in connection with the dispatch.

### **4.2.3. Quality controls**

#### *4.2.3.1. Amount of test material*

While dispensing a testing mixture into vials, dispensing is also made into a number of pre-weighed vials. Initially, the dispensed volume from 5 vials is weighed in order to make sure that the dispensing may start. Thereafter, the volumes from at least one vial in the beginning and one at the end of each rack with 144 vials are weighed. The total range and measure of dispersion (coefficient of variation) are calculated. The range may be at most 0.015 g between the dispensed volumes, which corresponds to ca 3.0% of the dispensed volume in average (the target volume 0.5 ml). The weighing is made parallel to the dispensing in order to be able to adjust or interrupt it, if the volume differences would become too large.

#### *4.2.3.2. Concentration determination*

Concentrations of the organisms included, by use of one or several vials of the test material, is determined after the freeze-drying. As regards new strains, the concentration is often determined before the freeze-drying as well. When analyses are made both before and after the freeze-drying, the killing of the individual strains by the freeze-drying process can be determined (reduction factor).

#### *4.2.3.3. Homogeneity*

Before the test material is delivered to the users, a determination of homogeneity is made. As a rule, duplicate analyses are made, of 10 randomly picked vials, with methods intended for the parameters that are to be tested. In order for the material to be regarded as homogenous, certain criteria must be fulfilled as regards dispersion within and between vials (see below the heading Statistics, Homogeneity).

#### *4.2.3.4. Vacuum test*

In order to be long lasting, the freeze-dried material need to stay under vacuum after the vials are sealed and capped. Each individual vial is vacuum tested before storage or delivery. Vials without vacuum are discarded, since vacuum is essential. Normally, no vial needs to be disposed of.

#### *4.2.3.5. Stability*

The stability of many organisms has been investigated in many different freeze-dried mixtures. Each mixture is however not tested in this sense. General knowledge is available from similar material (reference material), which has been manufactured in the same manner. It has been stored for at least 2 years and has been tested regularly. That material is stored frozen at a low temperature, normally  $-70\text{ }^{\circ}\text{C}$ . Most tested bacteria and fungi have shown stability for at least this period of time. Gram negative bacteria however, often have a tendency to decrease somewhat in colony recovery with time, while Gram positive bacteria and fungal spores are unaffected, in general.

If stored at normal freezer temperature ( $-18$  till  $-24\text{ }^{\circ}\text{C}$ ) the content is stable for at least one year, with the same observation for Gram negative bacteria as above.

If stored in refrigerator ( $5\pm 3\text{ }^{\circ}\text{C}$ ), the content is stable for at least a few months, usually considerably longer.

If stored in room temperature, the content is usually stable for at least a month, usually longer.

### ***4.3. Storage and usage***

#### **4.3.1. How should the material be stored**

When the test material is to be used within a short period of time after delivery, as in proficiency testing, the need for long stability is little. Storage in refrigerator after delivery is therefore quite enough. Storage in room temperature is generally not critical either in those cases. The vials should always be stored in darkness.

#### **4.3.2. What should the material not be used for**

A liquid sample prepared from the freeze-dried material cannot be guaranteed stability for more than an hour or so. A prepared sample should therefore not, not even after cooling, be used during a longer period of time than that. Bacterial spores may however survive longer periods.

### ***4.4. Hazards***

#### **4.4.1. Risk of infection**

Microorganisms in risk group 1 and 2 according to the classification of the Swedish Work Environment Authority (6) are used in the schemes. A risk assessment has been made by the Swedish Institute for Infectious Disease Control concerning risks of infection by the organisms we use according to how they are handled (7). The conclusion is that it is unlikely that the test material would leak during transport. Besides, the risk for disease after consumption of the contents of one vial is extremely small. The National Food Administration has, based on the

statements, made a stipulation regarding handling and transport (8; see further under 4.5.3.).

#### **4.4.2. Environmental danger**

The material itself consists of horse serum, inositol and microorganisms, and usually also nutrient broth, SPG, peptone, potassium phosphate, and magnesium sulphate. The container consists of glass, rubber and aluminium. Since these do not include any specifically classed or in any other way potentially dangerous chemical compound, it may be disposed of in the common waste management after the microorganisms have been rendered harmless by killing. See below under Destruction of test material.

### ***4.5. Before a proficiency test***

#### **4.5.1. Randomisation of test items to laboratories**

##### *4.5.1.1. Numbering of vial labels*

Before dispatch to participating laboratories of a proficiency test, the labels are numbered. The numbering is made automatically and is saved in our data base. The numbers are made up by the specific and confidential laboratory number, a hyphen and a singular figure which makes a connection to a mixture. This singular figure is randomly picked for the laboratories for one of the different mixtures A, B, C etc., and the connection is available in the data base.

##### *4.5.1.2. Selection of vials for a laboratory*

Before package, vials from each mixture are carefully shuffled in a container. For each laboratory, a randomly picked vial is taken from the container and labelled with the label specific for the particular laboratory and the mixture. This activity is carried out on a separate work bench for each mixture in order to avoid errors.

#### **4.5.2. Package of test items**

The individual labelled vials are placed in shock-absorbing cases, which in turn are put in a plastic bag. This bag is put in a safety jar which is aimed for use when transporting infectious material. The jar and instructions for the proficiency testing are put in a protective cardboard box along with information stating that the samples may be regarded as freeze-dried artificial food samples, and what should be done if one, of some reason, would get in contact with the test material. A label and, when necessary, a customs declaration is attached on the box. Other markings necessary for transport are also put on the box.

#### **4.5.3. Transport of test items**

Based on the stipulation (8) made by the National Food Administration, according to the risk assessment of the Swedish Institute for Infectious Disease Control (7),

the test items are sent, after packaging with enclosed instructions, via ordinary postal means, that is by car, train, boat and plane, depending on destination. The deliveries are sent as registered letters in order to, as far as possible, enable tracking.

## ***4.6. Handling of the test material***

### **4.6.1. Storage**

The material is kept cooled before package and transport (see Manufacture above).

During packaging and transport, the material is kept at room temperature or other ambient temperature. This has under normal circumstances not shown to have had any observable negative effects on the test materials.

The laboratories are requested to keep the received material in darkness and in refrigerator or freezer until used.

### **4.6.2. Preparation of samples**

The preparation is clear to the participating laboratories from the enclosed instructions. These comprise pictures with explanatory text. The procedure implies that the test material is transferred to an already measured volume (e.g. 250 or 800 ml) of a solvent (e.g. a diluent). That liquid with the material is then carefully blended in order to obtain the prepared sample for analysis.

The prepared sample should be analysed within one hour.

### **4.6.3. Destruction of test material**

#### *4.6.3.1. Unopened vials containing test material*

Before the material can be discarded in the bin aimed for regular waste, the microorganisms have to be killed by e.g. autoclaving at 121 °C long enough (e.g. 50 minutes) for all material to obtain the right temperature. One alternative is to hand in the vials containing the test material to a special destruction establishment for infectious material.

#### *4.6.3.2. Opened and used vials*

A glass vial that contains/has contained test material, and the rubber stopper may suitably be disposed of in containers for infectious material which are to be destroyed in a special establishment. The aluminium cap can be discarded along with regular waste.

#### *4.6.3.3. Remains of prepared sample*

Remains of the prepared sample should be killed by autoclaving at 121 °C for at least 15 minutes, or in corresponding fashion, before it is disposed of.

## **5. Instructions at delivery of test material**

### ***5.1. Target dates***

Dispatch of test items and instructions is made 1–2 weeks ahead of the start date for analyses in a testing round. The instructions do among other things state the start date of the round and the final day to report the analytical results. These target dates, as well as other dates for respective round, are stated on the website [www.slv.se/absint](http://www.slv.se/absint).

### ***5.2. Analyses***

Which analytic parameters that is included in each round can be gathered from the website [www.slv.se/absint](http://www.slv.se/absint), but are also stated in the instructions for the round.

### ***5.3. Other information in the instructions***

The instructions that are enclosed with the test material also contain information regarding:

- preparation of samples
- particular conditions concerning the different analyses, such as which dilutions or volumes that should be tested or which methods that can be used
- reporting of results and method information
- that the analytical results are to be reported through a page of the website that requires login, as well as how they should be entered
- that method information for the respective analysis can be stated on the website only
- that the method information may be used in order to group analytical results per method in final reports and to enable discussion of particular circumstances.

## **6. Participant activities in a testing round**

### ***6.1. Instructions and analyses***

The participating laboratories are presumed to handle the test material according to the recommendations in the enclosed instructions. They are presumed to take part of the entire instructions and perform the analyses in the way recommended. They should however to as large extent as possible perform the analyses as routine samples, but with consideration to the limitations or addenda stated in the instructions.

## ***6.2. Reporting analytical results***

In what way the analytical results should be reported differs between the schemes, but is thoroughly described in the instructions enclosed with the test material. The form in which the results should be reported differs between the scheme for Drinking water and that for Food.

In the case of Drinking water, the real colony number or MPN index rounded to integer is stated before or after confirmation, for the volume that is specified for respective analysis.

For the quantitative analyses in the food scheme, a  $\log_{10}$  result for the specified sample volume is stated. As regards the qualitative analyses, Pos (positive; detected) or Neg (negative; not detected) is stated.

In order for the results from a participating laboratory to be accepted and assessed in the final report of a proficiency test, these have to be reported on time. Corrections, if any, must also have reached the provider within the time period stated for corrections (see below under 6.4.).

## ***6.3. Reporting method information***

Method information can only be reported on the website and applies until further notice. It can be entered and adjusted whenever after logging in, which means also after last report date for analytical results and between testing rounds. The address is: [www.slv.se/absint](http://www.slv.se/absint). The method variations that are used to obtain the reported analytical results should be the ones reported.

Whenever there is a need for accounting for possible method differences in the reports, the method information will be used to group the results. They will normally be used in the way they are stated in the data base just after the last day for correction of analytical results. Although differences may be difficult to prove statistically, trends and possible differences will be discussed, as help to interpret the varying results obtained by the laboratories. Method differences underlying the results will not be discussed each time a certain analytical parameter is included, rather, different parameters will be discussed on different occasions.

## ***6.4. Adjusting a reported result***

### **6.4.1. Responsibilities of participating laboratories**

It is the responsibility of the participating laboratory to check its own results in the data base, via the website, to see that they are stated correctly. Normally, no differences should exist nowadays when the results are reported digitally directly to the data base via Internet. If the laboratory has had to send the results in some other manner, for instance by mail, e-mail or fax, it is extra important to make a thorough check. The organiser has in that case entered the results to the data base.

The checks and corrections of results must be done and reported within the period of time set. After the deadline, corrections are not guaranteed to be made, since the final processing of data may have started. The last date is stated for the particular round on the web page that describes the various testing rounds. As long as we find it justified, we also send a reminder to the participants, in shape of a card, that the preliminary processed results are available and that the laboratory ought to check their reported results. On this card, the final date for corrections is also given.

#### **6.4.2. Accepted adjustments**

Normally, only adjustments due to technical reasons (computer errors) are accepted, or if we as organiser have made a faulty recording of the results. If the error has been caused by us having instructions that are indistinct or difficult to understand, corrections may be accepted after individual consideration.

Errors in the results depending on mistakes made by the participating laboratory are normally not accepted and hence not corrected. Such errors are e.g. mistakes made when entering results, results reported for wrong sample, results reported for wrong analysis, errors caused by calculation mistakes, results stated for wrong dilution and results stated in any other way than what the instruction described.

Method information can, however, be corrected anytime by the participant. It can even be done in between rounds. In a particular report is reflected the method information available when the evaluation of that round starts. It is usually right after the final day for corrections of analytical results.

## **7. Follow-up of analyses**

The National Food Administration does not require from the participating laboratories to follow up obtained results and take suitable actions. Such demands can only be made by the laboratory itself or by a third party to which the laboratory has subordinated, e.g. an accreditation body. They can demand that the laboratory keeps a certain quality level and take actions when the quality may be questioned. How the follow-up should be done is therefore designed by the laboratory itself or in cooperation with the third party.

The National Food Administration does not take any responsibility for how or if the follow-up is made.

Data and guidelines for how the laboratories can calculate z-scores for their analyses is however provided (see below under Statistics) in the appendix of the reports, containing all analytical results. The z-scores may be a tool to evaluate an analytical parameter over time.

As provider, we also facilitate the follow-up by delivering extra vials of the test material free of charge, while stocks last, to laboratories that ask for it. Each

laboratory may receive one extra vial per test mixture. In order to receive a vial from a certain mixture, the laboratory has to be able to state which analytical parameter they failed to analyse in the sample from that mixture.

## **8. Statistics**

### ***8.1. General***

The statistical processing in the proficiency testing of microbiological Drinking water and Food laboratories includes the following.

- Determination of amount, concentrations and homogeneity of the test material.
- Transformations of the colony counts (CFU) of the analytical results before calculations are made, in order to obtain a normal distribution and a uniform variance within the range of results for the respective analysis. As regards the Food scheme, *log<sub>10</sub> transformation* is made and as regards the Drinking water scheme, *square root transformation* is made.
- Identification of deviating analytical results as false positive and false negative results, and low and high outliers.
- Accountancy for all results stated by participating laboratories in a table, along with summary statistics (deviating results excluded) and the number of deviating results per analytical parameter.
- Visualizing the results for each relevant quantitative analysis in a histogram for respective test mixture.
- Describing the standardised analytical results (z-scores) of a laboratory in an individual box plot.
- Pointing out outliers and false results in the table containing all analytical results and stating the number of these for each laboratory below the respective box plot.

Results that are obviously erroneous (e.g. undoubtedly false) based on the knowledge of the test material, are excluded without any statistical test before the identification of outliers is made.

### ***8.2. Determination of concentration and homogeneity***

Concentrations of the various organisms in a test material are determined in part to check that the material possesses all the qualities wanted, and in part to be used as providers reference when evaluating the laboratory results. The determination of homogeneity is made based on the organism concentrations in several randomly picked vials.

The homogeneity of the freeze-dried test material is normally checked before dispatch to the participating laboratories. Several samples are examined parallel by the same person. How much they differ depends on which parameter is analysed but also on at which concentration the organism is counted.

The criteria for homogeneity differ between the two schemes but are in both cases to some extent based on what is stated in international protocols (9, 10). These protocols are mainly worked out for quantitative chemical analyses and can therefore in some aspects not be strictly followed within microbiology. In the most recent editions of these protocols (11, 12), determination of homogeneity is treated somewhat differently. These newer ideas have not yet been applied onto the microbiological activity described here.

### **8.2.1. The Food scheme**

#### *8.2.1.1. Initial check of concentrations*

In direct connection to the freeze-drying of a mixture, one vial is checked with respect to content and concentrations. The content of each vial is dissolved in a fixed volume of diluent, making what is regarded as the zero dilution. Single analyses are made from the different steps in a dilution series. This concentration check is used in order to receive an indication on whether the test mixture seems acceptable as regards the different included organisms. This initial concentration check is also used to decide which dilution should be used for a certain analysis in the final determination of concentration and the homogeneity tests.

#### *8.2.1.2. Concentration determination and homogeneity test, quantitative analysis*

Before dispatch of testing material, the same person analyses 10 vials on the same occasion for a final determination of concentrations and test of the homogeneity in the test items. Preparation of test material is done as for the initial concentration check. Inoculation is either made by hand or by a spiral spreading instrument. A dilution series is made from each vial and two petri dishes are used for each analytical parameter from the dilution most suitable for the parameter based on the initial concentration check. An average result is calculated for each vial, derived from the two petri dishes. The final concentration is calculated as the mean value of these 10 average results, *log<sub>10</sub> transformed*, after having been converted to the original concentration with respect to the dilutions made. The standard deviation, which is also calculated as a *log<sub>10</sub>* result for these 10 results, is compared with the criteria for homogeneity.

#### *8.2.1.3. Determination of concentration and homogeneity test, qualitative analysis*

For determination of concentrations in qualitative examinations (e.g. Salmonella), separate vials containing pure cultures, so called special vials, are freeze-dried at the same time as the vials of the mixed culture. Such amounts of the specific organism are used that the same concentration is expected in the pure culture as in the mixed culture. The concentration is measured by analysing 10 special vials, from which duplicate inoculations are made. Calculation of mean value and

standard deviation is made as for the quantitative analyses, and gives the primary assessment of homogeneity. The mean value is considered to reflect upon the concentration in the mixed culture.

Sometimes semiquantitative analysis of mixed cultures is used, with inoculations of various test volumes of broth, and determination of concentration based on MPN tables.

Listeria and Campylobacter may also at times be quantified from mixed cultures by direct inoculation on selective agar media. This may however, due to stress and competition, lead to a somewhat lower colony count than the actual concentration in the vials. The variation will in that case also usually become larger.

#### *8.2.1.4. Criteria for homogeneity*

In a quantitative analysis, according to Peterz, 1992 (13), the range for the  $\log_{10}$  transformed values of the mean result of the 10 vials should not exceed 0.5  $\log_{10}$  units. The standard deviation should at the same time be  $< 0.15 \log_{10}$  units.

The same criteria go for a qualitative analysis, with the addition of a secondary criterion that the target organism should be detected in all 10 vials used.

### **8.2.2. The Drinking water scheme**

#### *8.2.2.1. Initial check of concentration*

In direct connection to the freeze-drying of a mixture, 5 randomly picked vials from different stages of the process of filling the vials (beginning, middle and end) are analysed. The content of each vial is dissolved in a fixed volume of diluent. Single analyses only are performed from various normally used volumes. This concentration check is used in order to receive an early indication on whether the mixture seems acceptable and may be *assumed* to be homogeneous with respect to the various organisms included. This initial concentration check is also used to decide which sample volume should be used in the final determination of concentration and the homogeneity tests.

#### *8.2.2.2. Determination of concentration and homogeneity test*

Before dispatch of the samples, 10 randomly picked vials per mixture are tested with respect to homogeneity and concentrations (these are the reference mean values reported by the provider). Preparation of test material is done as for the initial concentration check. Duplicate analyses are performed on the 10 vials for respective mixture during one and the same day. Only one specified sample volume, obtained from the initial concentration check, is used for each parameter. The analyses are performed under conditions of repeatability. The coefficients of variation (CV; see 8.5.4.) are calculated from the 10 mean values (each from 2 determinations) per analysis.

#### 8.2.2.3. ANOVA

One-way analysis of variance is performed on the results from the 10 vials with duplicate determinations. The analysis is carried out with the results *square root transformed*. An F-test is made in order to see that the dispersion between vials is not markedly larger than within the vials.

Besides, the standard deviation between vials is determined and compared to a fictive target standard deviation fit for the purpose, according to the protocol from IUPAC (9). This determination is used as a preliminary measure on whether homogeneity exists or not.

#### 8.2.2.4. “Index of dispersion” – check of randomness

As a complement to the analysis of variation, tests of “Index of dispersion” are used to check that obtained analytical results both within vials (10 duplicate analyses) and between vials (10 vials) do not differ markedly from what may be expected, based on the appropriate Poisson distributions (14, 15). In these tests the original, *non-transformed colony counts* are used from the specific sample volume that was chosen for analysis of the parameter. The test is somewhat dependant on concentration in the sense that it is easier to obtain acceptance for randomness, i.e. does not contradict Poisson distribution, at low colony counts compared to high.

#### 8.2.2.5. Criteria for homogeneity

As a guideline for the homogeneity to be acceptable, the coefficient of variation (CV, see 8.5.4.) may not exceed 25% when the average content is at least 10 CFU per analysed sample volume. With an average of less colonies (< 10; often less good normal distribution also after transformation), a higher CV than 25% is accepted if the distribution of the colony counts is as expected. That means that a test with “Index of dispersion” (with 95% confidence) does not contradict that there is a Poisson distribution, and that the analysis of variation does not indicate a significant F-value, if there is not a considerable “under-dispersion” within vials in the “Index of dispersion” test.

Calculations according to ANOVA and “Index of dispersion” are used as guidance and are not decisive whether a set of test material is homogenous or not.

### 8.3. Outliers

Outliers are results that differ that much from the other results that they cannot be explained by the ordinary variation. Outliers can be objectively identified in different ways. In both the Food and Drinking water schemes, Grubbs test (16) is used, modified by Kelly (17). The level of 1% is used as risk to erroneously identify a result as an outlier. A prerequisite for a correct test is that the results are normally distributed.

In order to start from an as good normal distribution as possible, the results are always transformed before being processed. Square root transformation is used for

Drinking water results and  $\log_{10}$  transformation is used for the Food results. The test is used as an “objective instrument” to identify deviating results also when the results are not normal distributed. The assumption of normal distribution is in those cases violated.

Outliers are excluded before the final calculations of medians, mean values and measures of dispersion are made for the various analyses. Z-scores (see 8.5.4) are, however, calculated also for the outliers, using the same mean value and standard deviation for a parameter as for not outlying results.

#### ***8.4. False positive and false negative results***

The number of reported false results varies strongly depending on which analysis was performed, the composition of the sample and the degree of difficulty, e.g. concentration and/or background flora.

##### **8.4.1. False positives – definition**

A false positive result is an analytical result where an organism is considered detected even though it was not present in the sample.

##### **8.4.2. False negatives – definition**

A false negative result is an analytical result where the target organism has not been detected in a proper volume even though it was present in the sample.

From time to time in the drinking water analyses, the colony counts are so low that the result zero may be obtained by random. Such results are not false negative.

When mean concentrations are higher than ca 10 colonies per unit of volume, false negative results for drinking water analyses are defined as zero results that are identified as outliers when tested (also when there is not a perfect normal distribution).

No outlier test is used when the concentration of the analysed organism is so high that it is obvious that a zero result is false.

#### ***8.5. Statistics for an analysis***

Calculations of mean results, standard deviations and z-scores for the result are made *log<sub>10</sub> transformed* for Food or *square root transformed* for Drinking water.

##### **8.5.1. Median**

Medians are stated in the preliminary results of the laboratories instead of mean values. They are also stated parallel to the mean values in the final report. The median is more robust than the mean value, which means it is less affected by outliers and the distribution.

### **8.5.2. Mean value / Assigned value**

The assigned value is made up of the mean value based on the results of the participating laboratories when outliers and false results have been excluded. It is regarded as the true value.

Regarding Drinking water, the mean value, and the median, is accounted for in the normal CFU scale (retransformed), while  $\log_{10}$  values are kept for Food.

### **8.5.3. Standard deviation**

The variation around the mean for an analysis is estimated based on the actual variation in the analysis and is made up by the standard deviation, when outliers and false results have been excluded. It is stated as a measure of dispersion in the Food scheme and works as a relative measure since logarithms are used.

### **8.5.4. Coefficient of variation (CV)**

The coefficient of variation (CV) is a relative measure and is made up by the standard deviation in percent of the mean value. It is stated as the measure for dispersion in the Drinking water scheme.

### **8.5.5. Standard values (z-scores)**

All results (outliers included) but the false results are transformed into standard values (z-scores) according to the formula:

$$z = \frac{x - m}{s}$$

*x = the result of the individual laboratory (in transformed form)*

*m = the mean of the participating laboratories ("the true result")*

*s = standard deviation around m*

After this transformation, the standard values have, apart from those based on outliers, a mean value equal to zero (0) and a standard deviation equal to one (1), and makes up a distribution that can be compared to a standardised normal distribution. The z-scores make it possible to compare the various analyses with each other since they are stated according to the same scale.

After the transformation, an individual z -score, apart from those based on outliers, will to 95% of the cases be found in the interval [-2; +2]. The probability to fall outside these limits is less than 5%. The probability to fall outside the interval [-3; +3] is less than 0.3%.

The z -scores that are calculated based on the outliers can be regarded as artificial, not fully true, since the outliers are not included in the calculations of the common mean value and the common standard deviation for an analysis. These "artificial" z-scores exist in addition to the ordinary ones and are in general found outside of the interval [-3; +3].

The z -scores form the base for the box plots (see 8.7.).

### 8.5.6. Interpretation of z-scores

When performing a follow-up of the own analytical results, the following guidelines may be used:

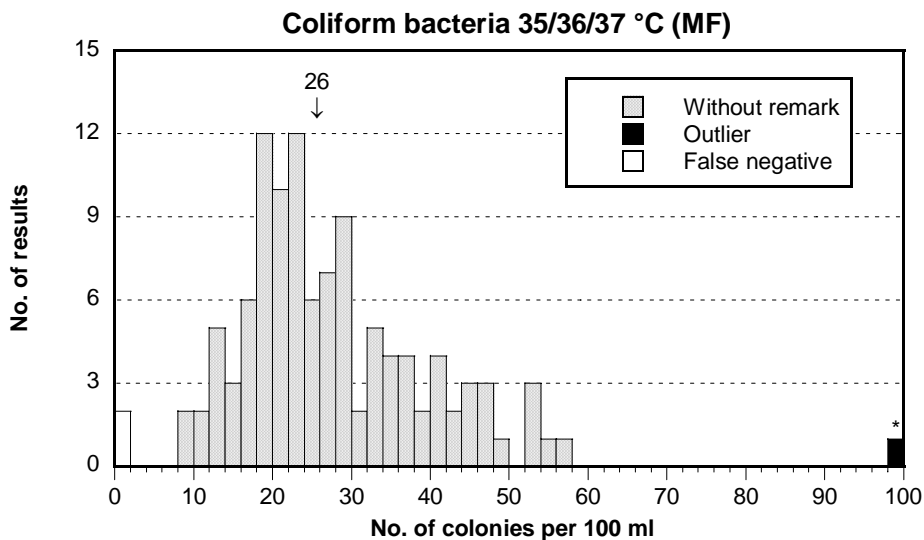
- $|z| \leq 2$  means that (the original) result is acceptable
- $2 < |z| \leq 3$  means that there is a warning that the result may be deviating, and might possibly motivate an action in a follow-up
- $|z| > 3$  means that the results is regarded as deviating and should lead to an action in a follow-up.

### 8.6. Histograms

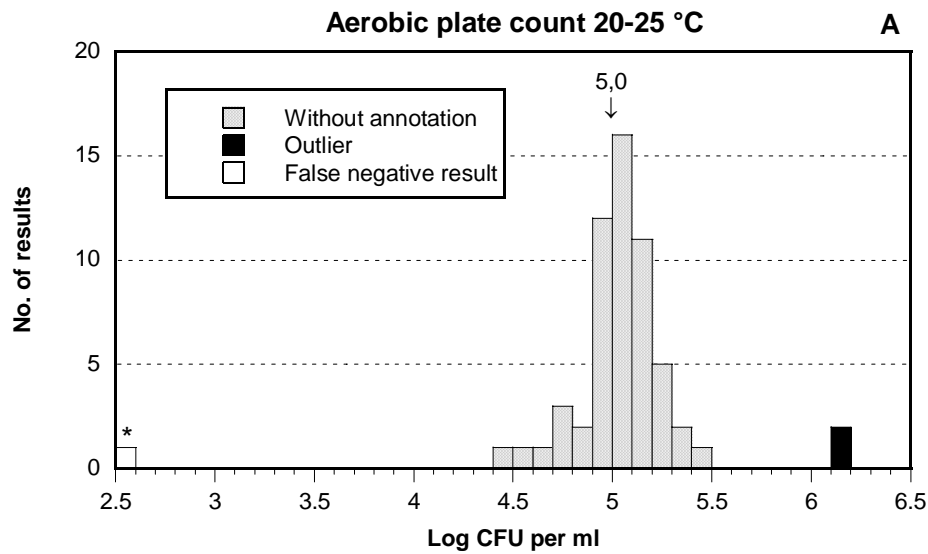
A histogram is made for each analysis and illustrates the distribution of the analytical results. The histograms for Drinking water are based on original colony counts and the histograms for Food are based on  $\log_{10}$  results. Examples are given in figure 1 and 2. An asterisk indicates that the results are actually placed to the left or right of the interval on the colony count axis.

### 8.7. Box plots

Each box plot is based on the z-scores of an individual laboratory and illustrates how these standardised results as a group and as median are placed in relation to the common, “true”, mean value zero. The box is made up by the 50% middle



**Figure 1** Example of a histogram for a drinking water analysis

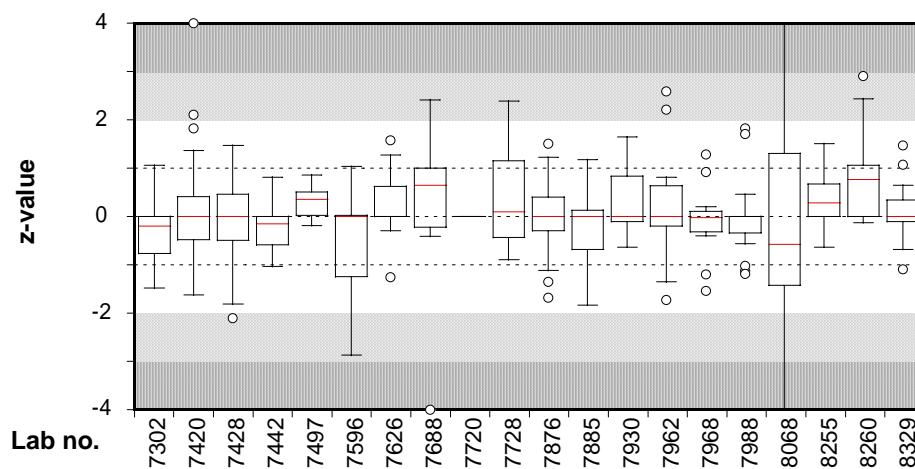


**Figure 2** Example of a histogram for a food analysis

results. The upper respectively lower 25% of the results are stated as vertical lines together with, if there are any, in the box plot deviating results. These deviating results are marked with a circle and are made up by values:

$$\begin{aligned}
 &< [( \text{Smallest value of the box} ) - 1.5 ( \text{Largest value of the box} - \text{Smallest value of the box} )] \\
 &\text{or} \\
 &> [( \text{Largest value of the box} ) + 1.5 ( \text{Largest value of the box} - \text{Smallest value of the box} )]
 \end{aligned}$$

Z-scores greater than +4 or lower than -4 are in the box plots presented as +4 and -4, respectively. An example of a box plot is presented in figure 3.



**Figure 3** Example of box plot

## **9. Confidentiality and user identity**

### ***9.1. Confidential laboratory number***

Each laboratory is given a unique laboratory number when registering. The laboratory receives a separate number for each scheme it participates in. The obtained number is confidential. It means that the provider reveals the number to no one but the laboratory in question. The provider never states the laboratory number to a third party.

### ***9.2. Confidential password***

In addition to the laboratory number, the laboratory is also given a password when registering. The password is confidential, just as the laboratory number.

### ***9.3. Usage of laboratory number and password***

The laboratory should state its laboratory number when communicating with the provider of respective scheme. This number is also used by the provider in order for the laboratory to be identified on the website when logged in, and in compilations and final reports.

The password should be used together with the laboratory number when logging into the part of the website that is available to participants only.

The laboratory number and password is stated on a label on a document that is enclosed with the test material in each round, together with the instructions.

### ***9.4. Changing the laboratory number and password***

The laboratory number may be changed on a regular basis in order to minimise the risk of unwarranted usage, e.g. when staff has left one employer for another. The password and an individual laboratory number may also be changed when a participant so asks in writing.

## **10. Filing**

All results that are registered in the test rounds of the two schemes are filed in the data base Absint for as long as the laboratory is a registered participant. Documents derived from correspondence or that have been generated by the results are filed for at least 4 years.

## **11. Complaints and other contacts with clients**

### ***11.1. Policy***

Complaints and discrepancies on work performed at the Microbiological Division should be documented and investigated. Actions should, if they are called for, be taken in order to prevent repetition. How this should be done is stated in a separate instruction processed at the department of Research & Development (18).

### ***11.2. Definition***

*Complaint* from customer or internal discrepancy *exists* if remarks are made when work that is performed at the Microbiological Division in any sense has failed in a way that agreements or routines have not been followed. The remark of a participant regarding how its results or other matters have been treated within a finished round should be handled as a complaint.

During an ongoing round of proficiency testing, *it is not regarded as a complaint* when specific remarks are made concerning deliveries, results and method information etc. whilst being processed.

### ***11.3. Handling of remarks that are not complaints***

A remark that cannot be defined as a complaint is handled accordingly:

- Should the remark be considering a mistake regarding analytical results or method information, caused by the organiser in a testing round, it is treated within the limits of accepted adjustments (see 6.4.2.) in the current round.
- Should the remark be considering a mistake regarding analytical results or method information caused by the laboratory itself in a testing round, no actions outside the limits for accepted adjustments are made (see 6.4.2.).
- Should the remark be opinions on something that is not promised or expected, it is regarded as a contribution to a discussion. If the remark may be looked upon as general, it is saved among questions that may be brought up in future advisory group meetings and/or user meetings.
- If the remark can be regarded as a specific point of view that should be answered in detail, or is a question of principal interest, it is brought into the records and answered within the frame of handling of matters.
- Could the remark be regarded as a specific point of view of little principal interest, it is received and put aside without further actions.

#### ***11.4. Points of view made by participants***

Participants may gladly contact us as providers and discuss matters that concern the proficiency testing. We do however not possess the possibility to undertake specific investigations or be a consultant concerning an individual laboratory.

As regards matters that concern which methods that should or may be used, and how they should be used, we refer to the authorities concerned in the respective countries.

### **12. Conditions and obligations**

Conditions for participation and obligations for participating laboratories as well as the organiser are stated here and on the website [www.slv.se/absint](http://www.slv.se/absint).

#### ***12.1. General conditions for participation***

##### **12.1.1. Who can participate**

- Laboratories that perform analyses within the frames of the schemes and that are using relevant methods.
- Laboratories dealing with microorganisms in risk group 1 and 2 according to the classification of the Swedish Work Environment Authority (6).
- Laboratories to which consignments arrive on time with the ordinary postal system, and that are able to report results and pay invoices within the stated time limits.
- Laboratories that have access to the Internet and are prepared to use this website.

##### **12.1.2. Which methods may be used**

###### *12.1.2.1. Food*

- Methods aimed for the analysis under consideration. The methods should, preferentially, be used as the routine methods.

###### *12.1.2.2. Drinking water*

- Methods aimed for the analysis under consideration, at first hand the ISO and EN methods used within the European Union. The methods should, preferentially, be used as the routine methods.
- There is no guarantee about evaluation of strongly deviating results from other methods. In that case the laboratory has to compare their results with other laboratories in the final report.

### **12.1.3. Fee**

- An invoice will be sent for a testing round for which the laboratory has not made an active cancellation.
- The fee has to be paid, for each round separately, within the time period stipulated on the invoice (normally 30 days after print-out).

### ***12.2. Other obligations of participating laboratories***

- To visit this website and actively make cancellation of participation if necessary.
- Analyse samples according to instructions within stated period of time.
- Report results according to given instructions.
- Check and report erroneous preliminary results within stated period of time.
- Follow-up of the outcome of the proficiency testing – if required by a third party (e.g. accreditation body).

### ***12.3. Obligations of the National Food Administration***

- Keep the laboratory number and password confidential.
- Keep the information on the website up-to-date regarding testing rounds, analyses, dates and prices.
- Safe delivery of relevant and homogenous testing material.
- Providence of original and preliminary processed results on this website for checking within stated period of time.
- Alerting, by e-mail or ordinary letter, that new preliminary results are available on the website.
- Final report as a pdf-document on this website within stated period of time.
- Delivery of extra test material to laboratories for their own follow-up.
- Maintain the accreditation for running the proficiency testing schemes.

### ***12.4. Limited responsibility***

- The provider has no liability regarding third party claims depending on a laboratory's participation and performance in any of the schemes run by the National Food Administration.

## **13. Participation costs**

Current prices for respective scheme are stated on the website [www.slv.se/absint](http://www.slv.se/absint). We keep the rights to change the prices if it should be necessary, in order to be able to continue the activity based on requirements set.

The fee for participation is currently paid by invoice in connection with each testing round. Payment with credit cards may be possible in the future. The prices are stated in Swedish crowns (SEK) but payment can also be made in the currencies US\$ and Euro (€).

## 14. This protocol

The latest version of this protocol is available as a pdf-file on the website [www.slv.se/absint](http://www.slv.se/absint). The protocol will be revised when necessary and participating laboratories may order a printed copy if they want one.

## 15. References

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# Proficiency Testing Schemes