Protocol – Microbiological Reference Materials

Drinking water and Food

Livsmedelsverket
Swedish Food Agency

RM micro
Since 1992

This protocol is available at: https://www.livsmedelsverket.se/en/RM-micro

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1. Introduction

Laboratories that perform analyses need to ensure that their results are reliable and accurate. Internal controls with reference materials (RM) may be used for quality controls to help identify any discrepancies in testing procedures or results. RM can also be used for validations and verifications of methods, and for training of laboratory personnel. Many laboratories require the use of RM to meet regulatory standards.

The Swedish Food Agency provides RM in the areas of food microbiology and drinking water microbiology. The RM are mainly directed to accredited laboratories within these analytical areas. They are also suitable for non-accredited laboratories, e.g. laboratories that perform controls in production lines of food or drinking water.

The purpose of this protocol is to give a description of the production and quality control of the RM. General information as well as specific information about the RM is also available on the webpage.

2. Organisation

2.1. General information

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The Swedish Food Agency is the central Swedish authority for food issues, including drinking water. Therefore, as a reference material producer (RMP), the Swedish Food Agency produces RM for food and drinking water microbiology.

Unless stated otherwise, the procedures described in this protocol applies to both food and drinking water RM.

2.2. Types of reference materials

2.2.1. RM for food microbiology

Currently, five different RM are produced, comprising a total of approximately 20 quantitative analytical parameters. The RM and parameters are presented on the webpage.

2.2.2. RM for drinking water microbiology

Currently, three different RM are produced, comprising a total of approximately 15 quantitative analytical parameters. The RM and parameters are presented on the webpage.

2.2.3. Areas of responsibility

A dedicated **coordinator** has the overall responsibility for food microbiology RM and drinking water microbiology RM, respectively. This includes planning and evaluation of RM manufacture, approving analysis results, as well as writing and approval of RM datasheets.

For all RM, dedicated **laboratory technicians** are responsible for the manufacture, quality controls and storage of the RM.

Dedicated **sales agents** are responsible for the distribution and invoicing of RM orders, as well as for maintaining the customer database.

The overall responsibility for the quality management system of the RM production and distribution is assigned to the head of the Unit for Microbiology.

2.3. Accreditation

The Swedish Food Agency is currently not accredited according to EN ISO/IEC 17034:2016 [1], but nevertheless follows the standard.

The Swedish Food Agency is accredited according to EN ISO/IEC 17043:2023 [2] and EN ISO/IEC 17025:2018 [3].

2.4. Conditions and obligations

The general conditions and obligations for customers are stated on the webpage.

Contracts with special conditions and obligations can be established between the Swedish Food Agency and an individual customer when necessary.

2.4.1. Obligations of the Swedish Food Agency

The Swedish Food Agency is responsible for keeping the information on the webpage up-to-date. This includes information regarding RM, expiry dates and prices. Datasheets for the respective RM will be maintained and updated throughout the respective RM lifecycles. Customers that have purchased an RM will be informed by e-mail when significant changes are made to the datasheets (e.g. changes in acceptance limits and expiry dates).

Current prices for the respective RM are stated on the webpage. The Swedish Food Agency reserves the right to change the prices if necessary. This is normally done annually or in response to significant changes in currency exchange rates.

2.4.2. Limited responsibility

The Swedish Food Agency has no liability regarding third party claims depending on a customer's use of the RM manufactured by the Swedish Food Agency.

The Swedish Food Agency does not in any way guarantee the continued manufacture of a specific RM.

2.4.3. Confidentiality

Customer numbers and passwords for login to the web portal are treated as confidential by the Swedish Food Agency. They are never given to a third parties, except after permission by the customer.

The customer number and/or password may be changed in order to minimise the risk of unwarranted usage, e.g. upon staff turnover. The password and/or the customer number will be changed upon written request by a customer, or when they have been used by either part in such a way that the identity of the customer has been revealed.

2.4.4. The principle of public access to information

The Swedish Food Agency is a government agency. This means that according to the principle of publicity, all communication to us is in principle considered to be public documents. Nevertheless, customer information as far as possible treated as confidential, and is not shared with third parties, unless permission to do so is given by the customer or when Swedish law requires handing out documents or information about a customer. In the latter case, the principle of publicity is tried against the customers need for confidentiality on an individual basis.

2.4.5. Complaints and deviations

Complaints and deviations are documented and investigated. If required, corrective actions and measures to avoid re-occurrence will be taken.

Definitions and procedures for managing complaints and deviations are described in internal instructions at the Swedish Food Agency. All documentation, including any corrective and preventive measures taken, are documented in a database at the Swedish Food Agency.

2.4.6. Customer obligations

Customers agree to follow all applicable local laws and regulations regarding import, transport, storage, handling, analysis and disposal of the RM. By purchasing and RM, customers guarantee that they possess all required permits for these activities.

Customers agree not to redistribute (resell, or otherwise) the RM to third-parties, without prior written approval by the Swedish Food Agency.

2.4.7. Filing

Documents derived from sale and invoicing of RM orders, e.g. correspondence related to or generated by this, are filed for at least 1 year.

All results from RM manufacture and quality control are filed for at least 4 years.

3. RM composition and manufacture

3.1. Composition

The RM consist of 0.5 ml aliquots of microorganism suspensions, freeze-dried in 2 ml glass vials. Manufacture is done according to the description by Peterz and Steneryd [4]. The final RM sample for

testing (simulated water and food homogenate, respectively) is obtained after reconstituting the material in a specific volume of suitable diluent.

3.2. Microorganisms

Freeze-dried cultures of the microorganisms used in the RM are stored at −70 °C in a collection at the Swedish Food Agency (SLV). All strains are identified by specific SLV numbers. The strains have either been isolated from food or water samples or have been bought from established culture collections.

All strains have been characterized internally by the API system, sequencing and/or biochemical tests. Alternatively, strains have been characterized by external culture collections, for example ATCC (American Type Culture Collection), CCUG (Culture Collection University of Gothenburg), the Centralbureau vor Schimmelcultures (CBS-KNAW Collection, The Netherlands), the National Veterinary Institute (SVA, Sweden) and the Public Health Agency of Sweden.

3.3. Hazards

All microorganisms used in the RM belong to hazard groups 1 and 2, as classified by the Swedish Work Environment Authority. The Swedish Food Agency's stipulation regarding handling and transport of the RM is based on a risk assessment by The Public Health Agency of Sweden on the microorganisms used in the RM. This is described in detail in the MSDS available on the webpage.

3.4. Manufacture

3.4.1. Strain purity

The purity of all strains is controlled during all stages of production by culture onto non-selective medium. If there is any doubt in the purity or identity of a strain, it is excluded from use and the production of the RM is usually aborted.

3.4.2. Monitoring of sample aliquots

Dispension of the microorganism suspension into vials is monitored by weighing. A maximum variation of 0.015 g between the dispensed amounts is allowed, corresponding to 3.0 % of the 0.5 ml target volume.

3.4.3. Storage

The RM are transferred to a freezer (−20 °C) and tested for the microorganism content. Accepted batches are checked for vacuum and are sealed with aluminium caps. The test items are thereafter stored at −55 °C. This temperature is chosen to minimize the risk of "glassing", i.e. stiffening, of the rubber stoppers causing air inlet into the vials, that might occur at a temperatures somewhere below −60 °C.

4. Quality control

RM characterization and assessment of homogeneity and stability is done in accordance with the recommendations in ISO 17034:2016 [1] and ISO 33405:2024 [5].

Three different types of quality controls are performed:

- Preliminary quality control is performed in connection with manufacture, with the aim to establish approximate property values as a *guidance for further testing*.
- Primary quality control is normally performed a few weeks after manufacture, and is aimed at determining *homogeneity, property values, and acceptance limits* of the RM.
- Continuous quality control is performed continuously and regularly until the RM expiry date. It is usually performed in the same ways as the primary quality control, but with fewer vials, and is mainly aimed at monitoring the *stability* of the RM.

4.1. Preliminary quality control

As an initial control, 1–5 vials are tested to obtain an indication on whether the RM is acceptable regarding the different included organisms. The aim is to decide which volumes and dilutions to use for further characterization and assessment of homogeneity, and therefore single replicates are usually used. Relevant confirmations on microorganisms may be carried out at this stage.

4.2. Quality control

4.2.1. Selection of vials

For the **primary** quality control, a minimum number (N_{min}) of 10 vials from the whole filling process are used (stratified sampling). The number of vials to include is given by:

$$N_{\text{min}} = \max\left(10, \sqrt[3]{N_{\text{prod}}}\right)$$

For the **continuous** quality control and stability tests, at least 3 (usually 5) randomly selected vials are used.

Homogeneity analyses may be divided into several runs, each consisting of the same number of vials (e.g. 5 vials analysed one week, and another 5 vials the week after).

4.2.2. Analytical methods

Analyses are performed with appropriate analytical methods for the property values to be tested and are done by subsequent duplicate analysis of the vials by the same person. The two duplicates from a single vial are analysed within a relatively short period of time; first all media from one series and thereafter all media from the second series.

In general, accredited analytical methods are used for characterization and assessment of homogeneity. Non-accredited methods may be used when a new parameter is tested or in certain special cases. This is noted when done.

4.2.3. Homogeneity

The aim of the homogeneity test is to establish the relative variation, both *between* and *within* vials, and to determine whether individual vials can reliably be considered to be identical subsamples of the same material.

For food analyses, results are $\log_{10}$-transformed prior to calculations of concentrations and for assessment of homogeneity. For drinking water analyses, results are square-root-transformed prior to calculations of concentrations and for assessment of homogeneity.

4.2.3.1. Regression analysis

A linear regression analysis is performed in order to determine if the order in which the vials are analysed has an impact on the results. If a statistically significant trend ($\alpha = 0.05$) is identified, it is corrected for and the corrected values are used in subsequent calculations (homogeneity, uncertainties, acceptance limits etc.)

4.2.3.2. First criterion: fulfilment of ANOVA

A one-way analysis of variance (ANOVA) is performed to determine the between-vial and within-vial variations (s_{bb} and s_{wb}, respectively). If the p value of the ANOVA fulfils the criterion $p \geq 0.05$, the results from different vials are considered comparable, i.e. no significant variation between the vials.

4.2.3.3. Second criterion: comparison with s_R

The between-vial standard deviation (s_{bb}) from the ANOVA is compared with the expected between-laboratory variation (s_R). Estimates of s_R are based on historical distribution and analysis of PT samples and are therefore considered to include the uncertainty contributions from both transport and the use of different analytical methods.

For **food microbiology**, where $\log_{10}$ transformed results are used, it is generally assumed that $s_R = 0.25$, though $s_R = 0.35$ may be used for property values where historical data imply that a higher variation may be expected. The assumption that $s_R = 0.25$ is based on historical results for participants in the Swedish Food Agency's PT, recommendations in ISO 22117 [6], and similar observations and principles used by other PT organisers.

For the **drinking water microbiology**, where square-root transformed results are used, it is instead generally assumed that $s_R = 1.25$, though $s_R = 1.50$ may be used for property values where historical data imply that a higher variation may be expected. Similar values for s_R are used by other PT organisers.

4.2.3.4. Evaluation of homogeneity

If the ANOVA yields $p \geq 0.05$, the RM batch is considered homogenous.

If the If the ANOVA yields $p < 0.05$, the RM batch is still considered homogenous, if $s_{bb} < s_R/3$. The variation is in this case considered fit-for-purpose.

If the results from the initial homogeneity study do not fulfil the homogeneity criteria, the analyses for the affected parameters are repeated. If the homogeneity criteria are still not met after a second homogeneity test – and no cause other than inhomogeneity is considered a likely explanation – the production batch will not be used as an RM.

4.2.3.5. Underestimation of within-vial variation

In some instances, the ANOVA may not give an accurate representation of the (in)homogeneity. In particular, there may sometimes be an underestimation of the variation within vials. This may for example occur when – by chance – the duplicate results *within* one or a few vials are identical or very similar. If – in such cases – artificially increasing the within-vial variation leads to an accepted ANOVA, the homogeneity criterion may still be considered to be fulfilled. This exception is used sparingly.

4.2.3.6. Exclusion of vials with deviating results and high variation

In a homogeneity test, one or both values from a duplicate analysis of a single vial may sometimes *deviate* substantially from the remaining values. In such a case, the Swedish Food Agency reserves the right to re-evaluate the homogeneity with these values excluded. This will be done if it can reasonably be assumed that the divergent results are not due to non-homogeneity of the test mixture, and instead due to e.g. a pipetting error or the analysis of the wrong dilution or volume. If the results from re-calculations with such values excluded fulfil the criteria for homogeneity, the RM will be approved. This exception is used sparingly.

Cochran's *C*-test- (*C*) is used as a *guidance* to identify if, among the set of tested vials (p), the variation within a single vial (s) is significantly larger than the variation in the set of vials. When this is the case, the homogeneity may be re-evaluated with both values from this vial excluded.

$$C = \frac{s_{max}^2}{\sum_{i=1}^{p} s_i^2}$$

4.2.4. Property values, standard deviations and uncertainties

Property values (x_{RM}) and are assigned by a single measurement procedure without direct comparison with a similar RM. The error contributions from heterogeneity (δ_{hom}) and stability (δ_{lts}) are – based on experience – assumed to be negligible. The property values thus consist of the characterised values (y_{char}), i.e. the mean value of the 10 individual mean values (each from a duplicate analysis) for each analytical parameter. Likewise, the standard deviation of the property value (s_{RM}) is the standard deviation of the 10 individual mean values.

The standard uncertainties of the property values (u_{RM}) include uncertainty contributions from characterisation (u_{char}), homogeneity (u_{hom}), and method differences (u_{lab}). The first two are determined experimentally for each RM and analytical parameter, whereas u_{lab} is assumed to be identical to s_R. The uncertainty contribution from long term stability is by experience considered to be negligible, and the uncertainty contribution from transport is considered to be included in s_R.

4.2.5. Acceptance limits

The lower/upper acceptance limits are calculated from the property values and standard uncertainties:

$x_{RM} \pm 2 * u_{RM}$ (expanded uncertainty at a 95 % confidence interval, with $k = 2$)

4.2.6. Quantitative analyses

Quality control of quantitative analyses, and analyses with a low concentration of the target organism compared to the competing background, present special challenges. For example, stress and competition may in these cases lead to a lower colony recovery than the actual concentration in the vials, and an unreasonably large dispersion of the results.

In these cases, quality control is carried out on separate vials containing a pure culture. These vials are freeze-dried *in parallel* with the vials containing the main RM (the main microorganism suspension). The volume ratios used in the manufacture of these separate vials are identical to those in the test item; the same final concentrations of microorganisms are therefore assumed in both.

4.2.7. Stability

4.2.7.1. Long term stability

The stability of many of the organisms included in the RM has been investigated for several years in different freeze-dried samples. Each RM is therefore not tested in this sense. Instead, knowledge of long-term stability of the test material is based on similar RM that have been manufactured in the same manner, stored for at least 2 years, and tested regularly. In general:

- When the RM are stored at −55 °C, the microorganisms are stable for at least 2 years, or until the stipulated expiry date.
- When the RM are stored at normal freezer temperature (−18 to −24 °C) the microorganisms are generally stable for at least 2 years, or until the stipulated expiry date. Gram-negative bacteria however, tend to decrease somewhat in colony recovery over time, while Gram-positive bacteria and fungal spores are generally unaffected. A slight decrease in the concentration of Gram-negative bacteria may thus occur.
- When stored at 25 °C, most tested bacteria and fungi are stable for 3–4 weeks, except for the bacterial genus *Campylobacter* spp. and *Pseudomonas* spp. Transport at 25 °C for 2–3 days generally only has a minor adverse effect on the stability of the microorganisms.
- Temperatures above 44 °C has an adverse effect on the recovery, especially for Gram-negative bacteria. This tendency is even more noticeable with temperatures at or above 60 °C.

4.2.7.2. Continuous quality control

Regular stability tests are carried out during the entire lifetime of each RM. This is typically done once every 6 months during the lifetime of the RM.

4.2.7.3. Action limit

The RM is considered to be stable for a given parameter as long as the following condition is met:

$$|x_{\mathrm{RM}} - x_{\mathrm{mon}}| \leq k\sqrt{u_{\mathrm{RM}}^2 + u_{\mathrm{mon}}^2}$$

Where: x_{mon} = property value in the stability test

u_{mon} = standard uncertainty of the property value in the stability test

4.2.7.4. Warning limits

These following two criteria are used for *monitoring* the stability of the RM. Non-fulfilment of these criteria do not require that an action needs to be taken.

Changes in the **concentration** is monitored by comparing the property value from stability tests (x_{mon}) with the warning limit:

$$x_{\mathrm{RM}} \pm 0.5 * u_{\mathrm{RM}}$$

Changes in the **variation** among the vials is monitored by comparing *individual results* from the stability tests with the warning limit:

$$x_{\mathrm{RM}} \pm 1 * u_{\mathrm{RM}}$$

4.2.7.5. Adjustment of property values and acceptance limits and/or retraction

When the stability tests reveal that the initial RM properties are no longer valid, the following actions are taken, in order:

1. Performing confirmatory tests and/or re-analysis of the affected parameter(s). If the criteria for stability is approved after re-analysis, the material is considered to be stable and no further action is taken in this regard.
2. Adjustment of individual or all property values (re-certification). Adjustment is done on a case-by-case basis and can be either of:
    a. Complete re-calculation of all RM properties (i.e. x_{RM}, s_{RM}, u_{RM} and acceptance limits) based in the results from the last stability test(s).
    b. Adjustment/widening of the acceptance limits. Typically done by increasing s_R in the original calculations for homogeneity, to widen the acceptance interval.
3. Retraction of the material. If the stability tests reveal that the homogeneity of the RM can no longer be ensured, the RM is retracted.

Whenever updates are made, the affected customers are informed.

4.2.8. Vacuum test

An inert environment is necessary in order to maintain the viability and concentrations of the micro-organisms in the RM. To ensure long-time stability of the RM, the freeze-dried material therefore needs to stay under vacuum after the vials are sealed and capped. Each individual vial is tested for vacuum before storage and in addition prior to performance tests. Vials without vacuum are discarded. Normally, very few of the newly produced vials need to be discarded.

4.3. Commutability

The property values (x_{RM}) in the respective datasheets are calculated with and apply for the respective methods specified. However, the standard uncertainties (u_{RM}) of the property values include uncertainty contributions from method differences (s_R), which are calculated from historical distributions of results from different methods on PT samples with a similar composition. The standard uncertainties and acceptance limits can thus be considered applicable for all comparable microbiological methods.

4.4. Subcontracting services

Manufacture and quality control of the RM is done at the Swedish Food Agency. Occasionally, various aspects of the RM production and quality control may however be subcontracted. When subcontracting occurs, it is placed with a competent subcontractor and the Swedish Food Agency is responsible for this work.

5. Distribution

5.1. Ordering

Orders of RM can be placed on the web portal, or by sending an e-mail to micro@slv.se. Processing of orders is usually 1–2 weeks, depending on the current workload. During holidays, the processing time may be longer.

5.2. Labelling of samples

Before dispatch to customer, all RM are labelled. This activity is carried out separately for each RM batch in order to avoid labelling errors.

5.3. Packaging of samples

The RM are packaged according to international regulations in a secondary packaging (a transportation tube or a safety jar containing a shock- and liquid-absorbing material) and an outer packaging for shipping (either a protective envelope or a cardboard box, respectively). A safety data sheet, as well as a delivery note, are also added to the package.

5.4. Transport

Based on the risk assessment (above), the test items are sent via ordinary postal means. In addition, a tracking number or courier service is used for certain destinations.

In general, the RM are kept at ambient temperature during packaging and transport. For some RM, or if requested by the customer, RM may also be sent with cold transport (−20 °C).

Upon arrival, the RM should be kept in darkness and in a freezer (at least −20 °C, but not lower than −55 °C) until use.

5.5. Damage during transport

In theory, the RM could be damaged during transport if it is subjected to very high temperatures or strong x-rays. So far however, this does not appear to have been a problem. RM have for example been transported for long distances to warmer countries, without problem. According to PostNord (the main Swedish postal service) only very low doses of x-rays (<1/100 of the dose for dental x-rays) are used for domestic and international goods at Arlanda (the international airport of Stockholm). Since no general negative effect from transportation has been noticed, it seems likely that neither temperature, nor the doses of x-rays utilized at airports are a problem for the freeze-dried test items.

5.6. RM Datasheets

RM Datasheets with property values and instructions for sample preparation and analysis are available on the webpage.

5.6.1. New versions of RM Datasheets

When a new or updated version of an RM Datasheet is available, customers will be informed by e-mail. The new RM Datasheet will simultaneously be published on the webpage.

5.6.2. Inaccuracies in the RM Datasheets

If a substantial error is found in RM Datasheet, customers will be informed by e-mail and a corrected RM Datasheet will be published on the webpage.

Less substantial errors or inaccuracies, or minor errors that only affect a few individual laboratories may – depending on the circumstances – be corrected directly in an e-mail without publishing a new version of the RM Datasheet.

Insignificant errors, e.g. spelling errors that do not affect the RM Datasheet in a meaningful way, do not require neither publication of a RM Datasheet nor an e-mail with information to customers.

5.6.3. Retractions of an RM

Should a substantial error be found regarding the stability or other properties of an RM, the RM will be retracted. Customers will informed about this via e-mail, and on the webpage.

Generally, the RM will be retracted if there is reason to suspect it is no longer homogenous, or if the concentration has changed in such a way that it cannot be compensated for by adjusting the property values or acceptance limits.

5.6.4. Use of RM Datasheets by customers

Copyright to all RM Datasheets remains with the Swedish Food Agency. Customers are however allowed to make copies of the RM Datasheets for their own internal use. The RM Datasheets may be cited, as long as a suitable citation is given.

5.7. Safety Datasheet (MSDS)

A safety data sheet with information regarding storage, disposal, health hazards etc. is included with the samples, and is also available on the RM webpage.

6. References

1. ISO 17034:2016. General requirements for the competence of reference material producers.
2. SS-EN ISO/IEC 17043:2023. Conformity assessment – General requirements for proficiency testing.
3. SS-EN ISO/IEC 17025:2018. General requirements for the competence of testing and calibration laboratories
4. Peterz, M. & Steneryd, A.C. 1993. Freeze-dried mixed cultures as reference samples in quantitative and qualitative microbiological examinations of food. J. Appl. Bacteriol. 74:143-148.
5. ISO 33405:2024. Reference materials — Approaches for characterization and assessment of homogeneity and stability.

6. ISO 22117:2019. Specific requirements and guidance for proficiency testing by interlaboratory testing.